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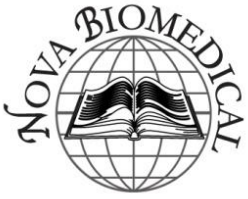
CURRENT IMMUNOSUPPRESSIVE THERAPY IN ORGAN TRANSPLANTATION

HUIFANG CHEN
AND
SHIGUANG QIAN
EDITORS



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Contents

Foreword

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Organ transplantation has revolutionized the treatment for end-stage organ failure. Over the past decades, improvements in HLA histocompatibility, organ procurement and preservation, surgical technique and clinical care, the discovery of many new potent immunosuppressants, and multiple immunosuppressive strategies have reduced short-term morbidity and lowered acute rejection rates. In the absence of attainment of successful immunological tolerance, immunosuppression is still the major approach currently used for prevention and management of rejection. More specifically calcineurin inhibitors (CNIs) cyclosporin A (CsA) and tacrolimus (FK506) have opened a new era in organ transplantation since the 1980s. Both of them have contributed to the decreased rate of acute rejection and reduced short-term morbidity. However in past 25 years, long-term graft survival has not improved to the same degree, owing to the negative impact of CNIs-induced progression of chronic nephropathy, accelerating the long- term allograft decline. That is why, in the past 10 years, scientists have tried to minimize, withdraw, or avoid CNIs and steroids, and use biological agents based immunosuppressive regimens which will satisfy the ideal goal of transplantation to improve long-term recipient and graft survival without fatal complications. At present, only a few potent CNI-free biological agents are in clinical trial, mostly costimulation blockers such as belatacept and another anti-CD40 mAB, ASKP1240. Preclinical studies however remain active.

Both editors of the book Drs. Huifang Chen and Shiguang Qian and their colleagues have been contributing to immunosuppressant preclinical research since the 1990s in the University of Montreal, Canada, and the University of Pittsburgh, and Cleveland Clinic, U.S.A. They evaluated CsA, tacrolimus, MMF, rapamycin, FK778, FK779, ASKP1240, CTLA4-Ig etc. Many of these agents are currently involved in clinical organ transplantation. It is my pleasure to write this foreword for the inestimable book of *Current Immunosuppressive Therapy in Organ Transplantation* that Drs. Chen and Qian provide as a reference book for graduate students, residents, surgeons, physicians, immunologists, pharmacologists and nurses in organ transplantation. I am sure this volume will be a valuable resource for these individuals and their patients.

Preface

Huifang Chen and Shiguang Qian

The success of modern organ transplantation requires the talent of multifaceted teams working together; these teams of skilled individuals include transplant surgeons, physicians, nurses, coordinators, social workers, nutritionists, physical therapists, immunologists, pharmacologists, microbiologists, virologists, pharmacists and administrators. Immunosuppressive therapy is still a major approach currently used for the prevention and treatment of rejection. The editors realized that a reference book was needed that would include basic immunology in transplantation, various immunosuppressive agents used in clinic, and the new development of cell therapy in the induction of transplant tolerance. We sincerely hope that this volume will be helpful for all of these professionals, including medical students, residents and trainees in transplantation as well as their patients.

The editors are most grateful to all authors and contributors. We are grateful to Dr. Pierre Daloze, who is a pioneer of transplantation surgery in Canada, for writing the foreword and reviewing the chapters of this book. We also appreciate Dr. Muhammad Zafarullah for contributing his valuable time in proofreading the contents of this book.

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Part I: Immunologic Basis

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Chapter 1

Immunology Principles in Organ Transplantation

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Abstract

This chapter outlines the basic machinery of the human immune system in the context of solid organ transplantation. The various cells and organs that form the alloimmune response are discussed followed by an integrated discussion to create the entire picture of the alloimmune response. The most pertinent aspects of the innate immune system are discussed including the role of complement and ischemia reperfusion injury. The adaptive immune system is discussed with a broad emphasis on the currently employed solid organ allografts. We describe the post-transplant immunological milieu from the extreme of acute rejection to graft versus host disease. We conclude the chapter by discussing the innovative areas of composite tissue allograft transplantation and xenotransplantation.

Keywords: transplantation, xenotransplantation, rejection, antibody, immunosuppression, ischemia/reperfusion injury, regulatory T cells

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Abbreviations

AICD: Activation-induced cell death
AMR: Antibody-mediated rejection
APC: Antigen-presenting cell
BO: Bronchiolitis obliterans
CDC: Complement-dependent cytotoxicity
CNI: Calcineurin inhibitor
CR: Chronic rejection
CTA: Composite tissue allograft
CTD: Chronic transplant dysfunction
CTL: Cytotoxic T lymphocyte
DC: Dendritic cell
DSA: Donor specific antibody
GvHD: Graft versus host disease
HAR: Hyperacute rejection
HLA: Human leucocyte antigen
HSC: Hematopoeitic stem cell
IDO: Indoleamine-pyrrole 2, 3-dioxygenase
iNOS: inducible nitric oxide synthase
ISP: Immunosuppression
IRI: Ischemia reperfusion injury
MDSC: Myeloid-derived suppressor cell
MHC: Major histocompatibility complex
NK: Natural killer cell
OPTN: Organ procurement and transplant network
PAMP: Pathogen-associated molecular pattern
PMN: Polymorphonuclear neutrophil
PRA: Panel-reactive antibody
SPI: Solid phase immunoassay
TLR: Toll like receptor
Tregs: Regulatory T cells.

Introduction

Transplantation is the gold standard treatment for life-threatening end-stage organ failure and is increasingly being utilized to improve quality of life as well as extending life e.g., intestinal transplantation and composite tissue allograft (CTA) transplantation. There have been immense technical advances in surgical technique and critical care that allow transplantation to be performed successfully even in the sickest of patients. However, the alloimmune response of the human immune system to the allograft remains a significant challenge in terms of long-term allograft survival.

While immunosuppressive regimens work well in preventing acute rejection, the majority of organs develop chronic rejection. Moreover, there are numerous side effects associated

with current immunosuppressive regimens not least of which are opportunistic infections and de novo malignancies. For example 85% of liver allograft recipients develop infections (bacterial, viral, fungal) and it accounted for 28.4% of deaths in one series [1]. Malignancy is a major cause of post-transplant postoperative morbidity and mortality with more than one-third of recipients developing a malignancy in the ten years following transplantation [2]. Immunosuppression also constitutes a major cost to healthcare systems and patients, with some patients being unable to afford their medications leading to graft loss. Many contemporary agents require multiple daily dosing and pose challenges to patients and their doctors in terms of compliance with adolescents having inferior outcomes due to medication non-compliance [3].

The alloimmune response to an organ allograft is a complex event in which the graft and the recipient are active participants. For example, cardiac allografts are almost universally rejected without immunosuppression whereas up to 25% of liver allografts are accepted without immunosuppression [4]. In rare circumstances, the passenger leucocytes contained within the allograft can proliferate and lead to GvHD in the recipient. It is imperative that the transplantation physician or surgeon seeking immunological homeostasis has a clear understanding of the basic machinery of the immune response so as to avoid transplant rejection at one end of the spectrum and overwhelming infection and de novo malignancies at the other end of the spectrum.

The immune response to a transplanted organ may be crudely divided into the innate and adaptive response. This is an overly simplified view since the ability to develop a highly specific high-affinity immune response or indeed the ability to induce tolerance is reliant on an integrated response between the innate and adaptive arms of the immune system. Allograft rejection has been historically classified based on histopathological and chronological features rather than on the basis of the immune effector mechanisms. This chapter will outline the basic mechanisms of allograft rejection with a focus on the role of the innate immune response in particular since chapter 2 elucidates the specifics of the humoral and cellular responses.

The 'Anatomy' of the Immune System

It is useful to have a rudimentary understanding of the cells, tissues and organs that constitute the immune system. The net immune response whether it is to a microbe, an allograft or indeed self in the case of autoimmunity, is as a result of the interaction between all these individual components. We only address the cells that have a known sizeable impact on the process of allotransplantation.

Mononuclear Phagocytes

Monocytes are incompletely differentiated circulating phagocytes that originate from the bone marrow; they give rise to tissue-resident dendritic cells or macrophages. Macrophages serve as active phagocytes engulfing microbes and the body's own dead or apoptotic cells. They express receptors for non-self molecules (e.g., mannose), antibody and complement.

The engulfed cells are killed by generation of reactive oxygen species, nitrogen species and various enzymes. In addition to the rudimentary phagocytic function, macrophages also serve as antigen-presenting cells (APCs) to T cells. They are also metabolically active in terms of cytokine production and can modulate the immune response.

The role of monocytes and macrophages has not been extensively studied in organ transplantation. There is evidence that these cells play a role in rejection. One study demonstrated an association between C-C ligand-positive (T cell chemoattractant) macrophage infiltration at the time of reperfusion and the onset of acute cellular rejection in intestinal grafts at a later time point [5].

Neutrophils

Neutrophils (PMNs) are the most populous of leucocytes; arising in the bone marrow, they are among the first cells to respond to infection. PMNs play a large role in ischemia reperfusion injury with large numbers infiltrating grafts within hours of reperfusion [6]. Upon activation, neutrophils release a variety of factors such as lysozyme, collagenase and elastase that can be deleterious to the organ allograft. In vivo, murine CXCR2 (chemokine receptor for neutrophil chemokines) deficient cardiac allografts were notable for reduced T cell infiltration and exhibited prolonged allograft survival with this being attributed to a reduced production of proinflammaotry cytokines such as TNF-alpha, IFNγ and IL-6 [7]. Kreisel et al., eloquently describe the interaction between the innate and adaptive arms of the immune system where neutrophil-derived TNFα stimulates IL-12 production by dendritic cells, which in turn skews T-cell differentiation towards the TH-1 phenotype resulting in graft rejection [8].

Natural Killer Cells

Natural killer cells are a distinct population of lymphocytes ($CD3^-$, $CD\ 56^+$) that destroy infected and malignant cells [9]; in particular they form an important part of the body's antiviral and anti-tumor immunity. Natural killer (NK) cells express IgG-binding Fc receptors and are thus capable of antibody-dependent cellular cytotoxicity.

NK cells also directly interact with a series of both activation and inhibitory ligands expressed on cell surfaces, for example NK cells recognize self major histocompatibility complex (MHC) class I as an inhibitory signal, with the loss of MHC class I indicating that the cell is stressed or damaged. NK cell-mediated cytotoxicity is primarily affected by perforin and granzyme similar to $CD8^+$ T cells. NK cells also secrete IFNγ, which activates macrophages and skews naïve T cell differentiation towards the TH1 phenotype. In terms of organ transplantation, there is evidence to show that NK cells are able to both enhance alloimmunity by recognizing the absence of 'self' MHC class I on the graft, yet they may attenuate T cell activation by eliminating donor-derived dendritic cells [10].

Dendritic Cells

Dendritic cells (DC) are without doubt the most important antigen presenting cells for activating naïve T cells. Initially described by Steinman et al., dendritic cells are derived from myeloid precursors and have characteristic membranous projections and are widely distributed throughout the body including in lymphoid tissues, mucosal epithelia and organ parenchyma [11]. Circulating monocytes also have the capacity to differentiate into DCs in an inflammatory microenvironment. The principal role of DCs is to capture and present antigen in the context of MHC class II to naïve and memory T cells. DCs are highly mobile and migrate to secondary lymphoid organs following antigen capture and activation. Dendritic cells upregulate costimulatory molecules (e.g., B7 family – CD80/86) upon activation providing the second signal that is crucial for full T cell activation. There are several subpopulations of DCs including conventional DCs, plasmacytoid DCs (involved in viral immunity) and follicular DCs, which present antigen to B cells.

Conventional DCs are central to the orchestration of the alloimmune response and are the highway that links the innate and adaptive immune systems. In the setting of organ transplantation, dendritic cells from both the donor (direct pathway) and recipient (indirect and semi-direct pathway) play a role in antigen presentation and T cell priming. Donor dendritic cells compose an important part of the passenger leucocyte population that accompanies the organ allograft. The process of brain death, organ harvesting/preservation and reperfusion provides donor DCs with multiple maturation signals e.g., HMG-B1 (High-mobility group protein B1) so that they are primed to activate alloreactive T cells upon migration to the draining lymph nodes in the recipient.

Despite being one of the most potent immunogens, dendritic cells also play a key role in maintaining peripheral self-tolerance. Tissue dendritic cells that reside in a non-inflammatory microenvironment are “immature” expressing only modest levels of MHC class II and few or no co-stimulatory molecules, nonetheless they continue to present antigen to T cells and render them anergic given the lack of the co-stimulation. These immature cells also attenuate T cell reactivity by the production of regulatory factors (e.g., arginase-1, iNOS and IDO) and the induction of Treg cells [12]. There has been much interest in harnessing these properties to induce donor-specific tolerance in the transplant recipient. Indeed, this concept of negative vaccination has proven effective in prolonging graft survival and down regulating allospecific responses in animal models [13].

B Cells

B cells are produced in the bone marrow and circulate as immature cells until they encounter antigen in the secondary lymphoid organs (spleen, lymph nodes). Upon activation, B cells become proficient antigen-presenting cells and upregulate MHC class II molecules and activate the T cell receptor. They also produce cytokines such as IL-2 that are critical for T cell activation, maturation and development of memory [14]. Activated B cells may also differentiate into memory B cells or long-lived plasma cells, which can reside in the bone marrow and are a rich source of IgG antibodies.

B cells and their plasma cell progeny are traditionally thought to play an important role in organ transplantation by the production of antibody which leads to acute and chronic antibody-mediated rejection [15]. In addition to the deleterious role of human leucocyte antigen (HLA) antibodies on transplant outcome (especially in kidney, lung and heart allografts), the shortage of donor organs has necessitated the exploration of HLA-incompatible organ transplantation, which requires specific measures to deplete and suppress antibody production. It is in more recent times that the role of B cells in presenting antigen to CD 4^+ T cells has been recognized as a key pathway in T cell activation and subsequent acute allograft rejection [16]. A subset of B cells may also play a role in graft acceptance, better known as Bregs, this population of B cells is thought to inhibit effector T cells and induce Treg cells, partly by the production of IL-10 [17]. This is an emerging science but it would seem to hold merit since operationally tolerant kidney transplant recipients appear to exhibit a strong B cell signature [18].

T Cells

We will discuss later in the chapter that the most common and clinically important form of organ allograft rejection is mediated by the activation of host T cells in response to donor MHC molecules presented by donor or recipient antigen-presenting cells most notably dendritic cells as described above. Activated B cells, endothelial cells, macrophages and some epithelial cells (e.g., tubular epithelial cells) can also act as APCs [19]. T cell activation will be discussed in the context of acute cellular rejection later in the chapter.

Naïve T cells consist of functionally distinct populations that can be classified as helper T cells ($CD4^+$) or cytotoxic T cells ($CD8^+$). Following TCR receptor activation, $CD4^+$ cells differentiate into different cell types with distinct cytokine profiles, these subtypes of helper T cells include type I (Th1) helper cells, Th2 cells, Th9 cells, Th17 cells, Th22 cells and Treg cells. Th1 cell differentiation is supported by dendritic cell and macrophage-derived IL-12. Th1 cells produce large amounts of inflammatory cytokines that drive the cellular immune response e.g., IFN-gamma, IL-2. Th2 cells are driven by IL-4 and produce IL-4, IL-5 and IL-13, promoting the humoral immune response in addition to driving eosinophil activation [20]. Th17 cells are a more recently discovered subset of $CD4^+$ T cells whose differentiation is driven by IL-6, TGF-beta and IL-1 beta (they also require IL-23 for maturation), they produce large amounts of IL-17 and have been associated with acute allograft rejection [21]. Regulatory T cells (Tregs) are a unique population of tolerance-promoting cells and are discussed later in the chapter.

Platelets

Platelets are frequently ignored outside of their important role in hemostasis. In fact, in the activated state, they express a variety of proinflammatory cytokines and surface ligands. Platelets are the first to respond to endothelial damage and react with the exposed extracellular matrix through glycoprotein Ia. The allograft endothelium is the first site at which the recipient immune system and allograft cells interact. Platelets interact with both the

innate and adaptive immune system, for example they release platelet factor 4 and RANTES, which recruit neutrophils and monocytes respectively [22]. In addition, they express P-selectin, which arrests leucocytes at the site of platelet activation [23]. They are also involved in antibody-mediated rejection as a source of complement storage and C3 activation. As well as recruiting monocytes, RANTES recruits T cells, which in turn further activate platelets via the CD40/CD154 pathway setting up a feedback loop [22]. These among other mechanisms implicate platelets in all types of rejection but it remains an area of ongoing research.

Lymphoid Tissue

The adaptive immune response is the net result of a complex interplay of antigen, antigen-presenting cells and lymphocytes. Specialized tissues facilitate this process and play an important role in the immune response to organ allografts. Lymphoid tissues are classified as primary organs, where lymphocytes differentiate and attain functional maturity, and as peripheral or secondary organs, where lymphocytes encounter and respond to antigen.

The bone marrow harbors the common hematopoietic stem cell (HSC) which gives rise to red cells, granulocytes, monocytes, dendritic cells, B and T lymphocytes, NK cells and platelets. HSCs give rise to two multipotent progenitor cells, namely the common lymphoid progenitor and the common myeloid progenitor. The common lymphoid progenitor gives rise to T, B and NK cells. The common myeloid progenitor gives rise to erythroid (RBCs), megakaryocytic (platelets), granulocytic (neutrophils, basophils, eosinophils) and monocytic lineages. The majority of dendritic cells arise from the monocytic lineage. The bone marrow is also home to antibody-secreting plasma cells; these plasma cells repopulate the bone marrow after peripheral education in the secondary lymphoid tissues. These plasma cells are long-lived and produce antibody even in the face of heavy immunosuppression, therefore they present a therapeutic challenge in highly sensitized patients requiring organ transplantation.

The thymus is the site of T cell maturation, indeed its absence leads to severe defects in cell-mediated immunity as is seen in DiGeorge syndrome. The thymus is composed of an outer cortex containing immature T cells and an inner medulla. The medulla is populated by bone marrow-derived macrophages, dendritic cells and thymic medullary epithelial cells, which present self antigens to developing T cells inducing deletion of self-reactive T cells.

The lymphatic system is central to the development of the adaptive immune response. The lymphatic network collects interstitial fluid from the tissues and delivers it to lymph nodes and eventually to the circulation via the thoracic duct and right lymphatic duct in the case of the right head, arm and upper trunk. This network collects microbial, self and alloantigens and delivers them to lymph nodes where the adaptive immune system is primed. Lymph nodes are secondary lymphoid organs that facilitate the interaction of antigen-presenting cells and lymphocytes. Lymph nodes are composed of medullary cords, sinuses, cortex and paracortex [24]. The centrally located medulla receives lymphatic fluid from the afferent lymphatics and contains lymphocytes, plasma cells and macrophages; it is also the site by which lymph leaves the node via the efferent lymphatic vessels [25]. Lymphocytes may also enter from the blood stream via high endothelial venules. The surrounding paracortex is composed of the T cell zone and contains activated and naïve T cells and DCs [25]. The outermost cortex is the composed of the B cell area, which contains primary and

secondary follicles, macrophages, follicular DCs and scattered $CD4^+$ T helper cells [25]. The morphology is of course variable depending on the state of the local immune system.

The spleen is the largest secondary lymphoid organ and lies at the center of the mononuclear phagocyte system (or reticuloendothelial system). The red pulp acts to scavenge senescent red blood cells from the circulation. It is intimately involved in both the innate and adaptive immune system. The immunologically active white pulp of the spleen is composed of periarteriolar lymphoid sheaths containing T lymphocytes, a follicular zone containing B cells and finally the marginal zone, which is rich in APCs. Healthy individuals who have undergone splenectomy have impaired cellular and humoral immunity and are at an elevated risk of overwhelming infection with encapsulated bacteria such as *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Haemophilus influenzae* type B [26]. In transplantation, the spleen is a source of antibody production and more importantly a site of generation and maintenance of immunoglobulin M memory B cells that can mount a robust immune response with little stimulation [27]. Splenectomy has been reported to be an effective treatment of antibody-mediated rejection refractory to medical management with conventional immunosuppression [28].

Innate Immune System

The innate immune system is typically described as the first line of defense to microbes. It reacts to pathogens in a non-specific manner and does not confer immunological memory. Innate immune cells respond to a theoretically fixed number of unique pathogenic sequences that include carbohydrate and lipid moieties present on microorganisms e.g., LPS, so called pathogen associated molecular patterns (PAMPs) [29]. The toll-like receptor (TLR) family is the most commonly studied example [30]. Activated innate immune cells recruit cells from the adaptive immune system by producing chemotactic factors and cytokines. It targets foreign or malignant cells and either removes them directly or through priming of adaptive immunity. Most importantly, many innate immune cells act as professional antigen-presenting cells that efficiently activate the T and B cell response.

The innate immune system is composed of both cellular (e.g., dendritic cells, NK cells) and soluble components (complement, acute phase proteins & cytokines). The innate immune system is not merely limited to reacting to "non-self" moieties but is also intrinsically involved in the ischemia reperfusion process that is of course germane to the transplantation process. Ischemic reperfusion-induced allograft injury (IRI) leads to the production of damage-associated molecules, such as high mobility group box 1 and is recognized by dendritic cells expressing TLR4 leading to their activation. Furthermore, the process of brain death results in a cytokine storm and activates the passenger leucocytes in the donor organ resulting in magnified IRI [31]. The activation of the innate immune system by the ischemia reperfusion process can lead to primary non function, delayed graft function and a greatly amplified adaptive immune response. This is evidenced by the superior outcomes seen in recipients of living organs in which ischemic injury is minimized [32]. The innate immune system plays a key role in priming the adaptive immune response by presenting antigen and providing necessary signals (cell-cell signaling, soluble factors e.g., cytokines, chemokines) to induce a robust alloimmune response.

The cellular effectors of the innate immune response include monocytes, macrophages, dendritic cells, neutrophils, NK cells and platelets. Dendritic cells (DC) reside in tissues and survey for pathogens by constantly endocytozing extracellular antigens. They become activated and behave as professional antigen-presenting cells when activated by PAMPs that include danger signals from damaged cells. Activation leads to the upregulation of MHC Class II and costimulatory molecules (e.g., CD80, CD86) on their cell surface, the activated DCs then migrate to secondary lymphoid organs where they present antigen to T cells. This is probably the most important interface between the innate and immune arms of the immune system.

Complement

The complement system is composed of over forty soluble and cell surface proteins that recognize pathogens or damaged cells and subsequently activate the complement cascade leading to cell lysis or opsonization. The complement system also interacts with adaptive immunity by modulating antigen presentation by dendritic cells, increasing the alloreactivity of T cells and perhaps most notably its role in antibody-mediated rejection.

The liver is the main producer of circulating complement components (e.g., C2, C3, C4, Factor B) whereas local complement components are manufactured by tissue resident cells or migratory cells. The complement cascade can be activated by three separate pathways [33]. The classical pathway (dependent on C1) involves activation by the antigen-antibody complex whereas the lectin pathway utilizes mannose-binding lectins and ficolins to identify carbohydrate ligands present on microorganisms. Both the classical and lectin pathway result in the generation of the C4b, C2a, C3 convertase complex. The alternative pathway undergoes spontaneous activation on cell surfaces forming the C3bB C3 convertase complex. C3 convertase is the final common pathway for all three complement activation pathways and leads to the cleavage of C3 to C3a and C3b. C3b is required for the formation of C5 convertase and the subsequent membrane attack complex (C5b-9), which lyses the phospholipid membrane of cells, this pathway being responsible for the majority of complement-mediated IRI [36]. Other split products function as opsonins (C3b, iC3b, C3d) and chemoattractants (C3a, C5a). The complement cascade is tightly regulated by both membrane-bound and soluble proteins to prevent collateral damage to host cells. Decay-accelerating factor (DAF/CD55) is a membrane bound complement regulator that expedites the decay of C3 convertase. Other examples of complement regulators include membrane cofactor protein (MCF/CD46) that inactivates C3b to iC3b and CD59, which inhibits formation of the MAC at the C9 step [34].

The reperfusion of relatively hypoxic and ischemic organs is inherent to the organ procurement and preservation process and leads to complement activation, for example, deceased donor kidneys expressed higher amounts of complement genes than live donor kidneys [35]. The complement cascade is a key mediator of reperfusion injury. This is evidenced by a reduction in IR injury in complement component (C3/Factor B/C5) deficient mice [36−38], whereas animals lacking regulatory proteins (e.g., DAF, Factor H) exhibit more severe IR injury [34]. It is thought that the lectin pathway plays a significant role in complement-mediated IR injury [39]. As well as playing a key role in IRI and antibody

mediated rejection there is evidence that complement influences the long-term immunological milieu. It has been demonstrated than certain C3 gene polymorphisms in kidney allografts are associated with long-term survival [40].

Adaptive Immunity and Allograft Rejection

Herein, we describe the most important immunological mechanisms by which organ allografts are rejected. The discussion focuses on adaptive immunity and the mechanisms are described in terms of the clinico-pathological classification of allograft rejection.

Hyperacute Rejection (HAR)

Hyperacute rejection is the most dramatic and devastating form of allograft rejection. It occurs in recipients who have high titers of pre-existing donor-specific antibodies (either anti-donor ABO or HLA). Hyperacute rejection results in immediate allograft dysfunction that is often visible to the naked eye due to widespread small vessel thrombosis following antibody binding and complement activation on the vascular endothelium [41]. Histological findings include neutrophil infiltration, hemorrhage, platelet thrombi, endothelial necrosis and positive staining for C4d [42]. Fortunately, hyperacute rejection is an uncommon entity nowadays with the careful selection of donor-recipient pairs based on blood type, antibody screening and clinical cross matching. There are however instances where organ allografts can be transplanted between incompatible (including ABO) donor-recipient pairs. This has been made possible by depleting antibodies by plasmapheresis or immunoadsorption along with targeting the antibody-producing B and plasma cell population. It is worth noting that different organs vary in their susceptibility to antibody-mediated rejection with liver allografts rarely succumbing to HAR whereas renal allografts are much more susceptible [43].

Acute Rejection

Acute rejection is not immediately apparent upon implantation of the allograft, rather it manifests after several days to weeks after transplantation. It was initially observed following allogeneic skin transplantation where transplanted skin would initially engraft only to undergo necrosis and slough off after one to two weeks. Acute rejection is orchestrated by the adaptive arm of the immune system with effector T lymphocytes and antibodies being the mediators of immune attack. The adaptive immune response is characterized by the properties of memory and specificity. It takes several days for acute rejection to manifest clinically in the naïve host since it takes time for effector T cells and antibodies to be generated from naïve T and B cells respectively. However, if the host has been exposed to the specific alloantigen in the past (e.g., blood transfusion, pregnancy), acute rejection manifests must much faster since there are already memory T cells and antibody-producing plasma cells present. Moreover, humans and indeed any mammal outside the confines of a pathogen-free

facility harbor a significant number of endogenous memory T cells that recognize foreign MHC as a result of cross reactivity between microbial and MHC antigens.

Acute cellular rejection is primarily mediated by cytotoxic T lymphocytes. The transplanted graft contains a large number of viable donor-derived antigen-presenting cells that have immediate access to the recipient's circulation upon organ implantation. These donor APCs migrate to secondary lymphoid organs (lymph nodes, spleen, mucosa associated lymphoid tissue) and activate recipient T cells, this is known as the 'direct pathway' of antigen presentation and is largely responsible for the acute cellular alloimmune response in the immediate post-transplantation period [44]. There is indeed evidence that depleting grafts of donor APCs leads to prolonged graft survival in the absence of immunosuppression [45]. The direct pathway of antigen presentation is unique to transplantation with the indirect pathway being the default mechanism by which T cells normally recognize foreign antigens. In this way, recipient APCs process shed donor antigen and activate T cells in the usual fashion. The indirect pathway is thought to be mainly responsible for chronic rejection since donor-derived APCs are eliminated by the host immune system. It is well recognized that activation of the T cell receptor (signal 1) alone is not sufficient to generate a strong T cell alloimmune response. T cells require a second "co-stimulatory" signal to proliferate and differentiate, the absence of which leads to anergy or cell death and potential immune tolerance [46]. There are multiple costimulatory pathways described with the B7/CD28 and CD40/CD40L pathways being the best described. Co-stimulatory molecules are not constitutively expressed by APCs including those of the donor, however, danger signals (e.g., HMG-B1) released during process of organ retrieval and preservation induces these molecules making the APCs highly activated and immunogenic.

Histologically, acute cellular rejection is characterized by a mononuclear cell infiltrate primarily composed of T lymphocytes, granular cells may also be observed. The pattern is graft-specific with rejecting liver grafts showing portal tract mononuclear cell infiltrates, non suppurative cholangitis and endothelitis [47]. Renal allografts are notable for a similar interstitial infiltrate, tubulitits and intimal arteritits [50].

Acute Antibody Mediated Rejection (AMR)

Acute AMR is most common in presensitized patients in the immediate post-transplant period, but can also develop later following the formation of de-novo donor-specific antibodies (DSA). De-novo DSA are frequently provoked by a decrease in immune-suppression, often as a result of noncompliance. While antibodies originate from the B cell progeny, it is important to note that activated T cells are required to 'help' B cells differentiate into antibody-producing plasma and memory cells.

Antibody-mediated rejection is mediated through a number of pathways. Firstly, antibody ligation results in complement activation resulting in the production of chemo attractants (e.g., C3a, C5a) that recruit additional immune cells (PMNs, T cells) and most importantly, endothelial cell lysis orchestrated by the membrane attack complex (C5b-9). The Fc portion of tissue-ligated IgG antibodies acts as an agonist to innate immune cells, which triggers the activation of PMNs, mononuclear phagocytes and NK cells and subsequent antibody-dependent cellular cytotoxicity in addition to the inflammatory cytokine storm released from

these infiltrating leucocytes. Furthermore, the antibody response synergizes with alloreactive T cells and there is often associated cellular rejection.

Acute AMR is best described in the kidney but it can affect any transplanted organ including the liver but to a lesser extent. As in the case of hyperacute rejection, microvascular endothelial injury is the hallmark of acute AMR in all transplanted grafts [48]: peritubular capillaritis and glomerulitis in kidney transplants, myocardial capillaritis in heart transplants, interacinar capillaritis in pancreas transplants and alveolar capillaritis in lung transplants [49]. C4d deposition (a marker of complement activation) in the vasculature has been a cornerstone in the diagnosis of AMR although the entity of C4d-negative AMR has recently been formally recognized [50]. This is an important development since up to 50% of renal grafts with AMR do not stain positive for C4d. Fortunately, there are new assays in development that analyze the molecular signature of endothelial cells that can point the finger towards antibody-mediated attack [51]. It is crucial to be capable of making the correct diagnosis of AMR since anti-T-cell treatment (e.g., steroids, anti thymocyte globulin) does not directly target the antibody-producing plasma cells.

Chronic Rejection

Improvements in immunosuppression, antimicrobial therapies and critical care mean that acute rejection is no longer the major barrier to the success of organ transplantation. However, chronic rejection remains one of the major factors limiting long-term graft survival. For example, the long-term outcomes of lung transplantation remain disappointing with a five-year recipient survival of 50% [52]; the high attrition rate being largely as a result of bronchiolitis obliterans syndrome, which is the leading manifestation of chronic rejection in of lung allografts. While the long-term outcome of heart, liver and kidney grafts is much better than that of the lung, the survival of these grafts has remained stagnant for almost two decades despite monumental improvements in one-year graft survival [53].

Chronic transplant dysfunction (CTD) is associated with distinct pathologic changes. In the kidney and heart, the hallmark of chronic rejection is a fibroproliferative endarteritis leading to vascular occlusion and interstitial fibrosis [54]. This results in poor parenchymal blood flow, tissue damage and eventual replacement with fibrous tissue. Liver grafts are seen to have a paucity of bile ducts (vanishing bile duct syndrome) with fibrosis of the remaining ducts in addition to obliterative arteriopathy in the most severe of cases [55]. Perhaps the most troublesome form of chronic transplant dysfunction is that of the lung, better known as bronchiolitis obliterans (BO). This irreversible phenomenon affects 49% of lung recipients at five years, increasing to 76% at ten years, making it the leading cause of death after the first post-transplant year [52].

The pathogenesis of chronic transplant dysfunction is poorly understood but is likely a result of both immunologic and non-immunologic factors. The term chronic rejection is reserved to describe the immune-mediated chronic changes found in late allograft dysfunction. It is thought that all arms of the immune system are involved in the process of chronic rejection including innate immunity, cellular and humoral alloimmunity and indeed cellular and humoral autoimmunity.

Antibody-mediated rejection is one of the major mediators of chronic rejection. This was initially seen in animal studies where infusion of donor-specific MHC Class I antibodies accelerated the development of vasculopathy in cardiac allografts [56]. Antibodies are also implicated in the chronic rejection of lung, kidney and heart allografts, for example de novo DSAs have been shown to predict the onset of BO and decrease survival following lung transplantation [57, 58]. The liver is relatively resistant to AMR for numerous reasons including an ability to 'dilute' antibody and antigen-antibody complexes, dual blood supply and secretion of soluble MHC Class I [59]. Nonetheless, AMR does occasionally occur in liver grafts particularly in those patients with high titers of IgG3 DSAs [50], it is critical to make the correct diagnosis in these cases since traditional immunosuppression does not target antibody production.

The process of CTD resulting from AMR is best described in the case of kidney transplantation. Criteria for the diagnosis of chronic AMR in renal allografts include classical signs of antibody-associated tissue injury such as pertitubular capillary basement membrane multilayering, arterial intimal fibrosis and transplant glomerulopathy [50]. The diagnosis of chronic rejection is not always clear since there is overlap in the histological findings with other entities e.g., calcineurin inhibitor toxicity in the kidney, recurrence of the primary disease, infections such as hepatitis C recurrence in the liver or polyoma virus in the kidney.

Human Leucocyte Antigens and Calculating Immunological Risk Prior to Solid Organ Transplantation

The HLA system is the name given to the human MHC; this consists of a group of cell-surface antigen-presenting proteins encoded by a region on the short arm of chromosome 6. There are two classes of HLA molecules, class I and II. Class I HLA (A, B, C) are present on all nucleated cells whereas class II HLA (DR, DP, DQ) expression is limited to antigen-presenting cells, lymphocytes and endothelial cells. HLA is highly polymorphic with almost 6,000 HLA Class I and over 1,500 HLA Class II having been identified [60]. HLA molecules distinguish self from non-self and therefore are the prime target for host immune system.

Before the era of calcineurin inhibitors much attention was given to HLA matching since outcomes were dramatically improved with fewer HLA allele mismatches. The majority of data originates from kidney transplantation since donor kidneys can be stored for up to 72 hours allowing detailed HLA analyses unlike heart, lung and liver grafts, which are more susceptible to cold ischemia. The Organ Procurement and Transplantation Network (OPTN) require typing for HLA-A, B and DR antigens, many laboratories also type for HLA-Cw, DQ and DP antigens. Nonetheless, the typing results are only used to give priority in renal allocation in the case of a zero HLA-A, B and DR mismatch [61].

Despite the seemingly infrequent use of HLA matching data, there is robust evidence to show that outcomes are superior when HLA mismatches are minimized. HLA matching is well recognized to have a positive impact in the case of renal transplantation even when one accounts for the improvement in immunosuppression protocols with candidates receiving a graft with one HLA allele mismatch faring poorer than those with none [62]. There is also evidence for poorer outcomes in heart and lung transplantation with increasing HLA

mismatch especially that of the HLA-DR allele [63]. There does not appear to be a benefit to performing HLA matching in the case of liver transplantation [64]. The reality of clinical practice limits the extent to which HLA matching can be implemented, for example the minimization of cold ischemia time takes precedence over HLA matching in the case of heart and liver transplantation. Also HLA matching was seen to have an unintended negative consequence on non-Caucasian potential kidney recipients since most donors are white, resulting in organs being allocated to white recipients with similar HLA profiles [65].

As well as typing donors and recipient for their respective HLA haplotypes, potential recipients are screened for the presence of preformed antibodies against donor HLA molecules that could lead to accelerated acute rejection or even hyperacute rejection. The panel reactive antibody (PRA) test screens for antibodies reactive with allogeneic HLA molecules prevalent in the donor population; these antibodies may arise as a result of prior pregnancies, blood transfusion or organ transplantation. Historically panel reactive antibody was determined by complement dependent cytotoxicity assay, however nowadays it is largely performed using solid phase immunoassays (SPI). SPI are composed of soluble HLA molecules bound to beads or other another matrix, patient serum in is then incubated with the immobilized HLA targets and antigen-antibody binding is then detected using enzyme linked or fluorochrome-conjugated anti-human IgG [66]. The results are reported as the percentage of the MHC allele pool with which the patient's serum reacts. The data can then be used to perform a "virtual cross match" when a donor becomes available allowing for the identification of unacceptable HLA antigen mismatches without the need for a formal in vitro cross match. The solid phase assays are highly sensitive and specific; each individual tissue-typing laboratory determines their cut offs at which a donor recipient pair is considered incompatible. In general a positive complement dependent cytotoxicity (CDC) crossmatch is a contraindication to transplantation whereas low-level alloantibodies detected by SPI are not always clinically significant [67].

Once a donor is identified, a specific donor-recipient cell based crossmatch assay can be performed to test the level of reactivity between a patient's serum and a potential donor's lymphocytes. Cell based cross matching was traditionally performed using a complement-dependent cytotoxicity method. This approach is limited by a low level of sensitivity but is an effective safeguard against hyperacute rejection, it does not rule out the possibility of the development of acute AMR in the early post-transplantation period [68]. Flow cytometry-based methods are far more sensitive in the detection of alloantibodies. They also can distinguish between lymphocyte subtypes i.e., B cells and T cells. It is worth-noting that neither method is specific for HLA alloantibodies alone and that the result should be interpreted in the context of the specificity and titer of donor-specific antibodies. There are multiple factors that can interfere with the above assays including therapeutic agents (e.g., thymoglobulin, high dose IV Ig, bortezomib), immune complexes and high levels of IgM [66].

Tolerance and Immune Regulation

Contemporary organ transplantation has been made possible by the development of potent immunosuppressive medications with a particular emphasis on targeting T cells.

While acute rejection is now relatively easy to prevent and treat, this comes with significant morbidity and mortality risks for the recipient. Long-term complications in many transplant recipients are related to infection, post-transplant malignancy, nephrotoxicity, cardiovascular disease and diabetes mellitus; all of these are direct consequence of commonly used immunosuppressive medications [69−72]. Moreover, current immunosuppressive are excellent at targeting acute rejection, however chronic rejection continues to account for a significant proportion of late graft loss [73]. It is with this data in mind that physicians and scientists alike are in hasty pursuit of identifying mechanisms to induce donor-specific tolerance. The ability to selectively block the immune responses against the graft without interfering with the other functions of the immune system would undoubtedly revolutionize transplantation. It is important to have a fundamental understanding of tolerance mechanisms and how current therapies impact on the emergence of tolerance.

Immunologic tolerance is defined as unresponsiveness to a specific antigen in the absence of exogenous immunosuppression; in addition, the physiologic response to other third party antigens is maintained. Clinical (operational) tolerance is defined as normal graft function and histology in the absence of immunosuppression in an immunocompetent host. Healthy individuals are tolerant of their own "self" antigens and defects in this process results in autoimmunity. Self-tolerance arises either centrally (thymus) or via peripheral tolerance involving multiple mechanisms including clonal deletion, anergy, immunologic ignorance, clonal exhaustion and active regulation of effector cells.

Central tolerance occurs in the generative lymphoid organs (thymus for T cells, bone marrow for B cells) and ensures that an individual's lymphocyte repertoire does not respond to self-antigen. The process of central tolerance takes place during the development of immature lymphocytes; precursor B and T cells that strongly recognize self-antigen undergo apoptosis in a process known as clonal deletion (negative selection), other self-reactive lymphocytes undergo receptor editing and a small population of CD4+ T cells differentiate into Treg cells which in turn play a role in maintaining peripheral tolerance. Central tolerance does not result in complete unresponsiveness to self-antigen since not all self-antigens are expressed in the thymus and bone marrow.

Peripheral tolerance is a multimodal mechanism by which auto-reactive lymphocytes are eliminated by apoptosis, rendered anergic or suppressed by regulatory cells (e.g., Tregs). Anergy is an important mechanism of immune regulation in which T cells become unresponsive or refractory to antigen stimulation. T cells are rendered anergic following T cell receptor ligation without concomitant costimulatory signaling via the B7-CD28 and other costimulatory pathways [74]. Self-reactive T cells in the periphery may also be eliminated by apoptosis in response to ligands expressed by the peripheral tissues especially mesenchymal cells, the effects of tissue expressed PD-L1 is one such example [75]. In addition to inducing anergy, TCR stimulation without costimulatory signaling (costimulatory molecule ligation upregulates antiapoptotic factors e.g., bcl-2) can induce apoptosis by activating Bim (pro-apoptotic factor) [76]. Activation-induced cell death (AICD) is another mechanism by which T cells are eliminated; repetitive TCR stimulation leads to the engagement of the Fas/FasL pathway which activates intracellular caspases and induces cell death [77].

Tregs

In the past decade, there has been an explosion in research concerning active regulation of effector cells. This is not a new concept with Billingham having described the phenomenon of regulatory cells in 1953 [78]. Treg cells are one of the most studied populations of regulatory cells; they can arise in the thymus and are known as natural Tregs (nTregs) or arise in the periphery (iTregs) in response to antigen stimulation in the presence of certain conditions. Tregs are $CD4^+$ T cells characterized by the expression of the IL-2 receptor alpha chain (CD25) and most notably Foxp3, which when absent in humans leads to a devastating autoimmune disease namely IPEX syndrome [79]. The generation of iTregs is largely dependent on the presence of IL-2 and TGF-beta, which drives the expression of Foxp3. It is also worth noting that the requirement of IL-2 for Treg survival since CNIs, the mainstay of contemporary immunosuppression block IL-2 signaling and may abrogate the tolerogenic effects of Tregs [80]. CD25 (IL2-RA) blockade (e.g., basaliximab) may hinder the development of donor-specific Tregs in the long term [81]. Other agents such as rapamycin and mycophenolate mofetil may in fact enhance Tregs [82]. Of course the complexity of the generation and stable proliferation goes far beyond the interaction of $CD4^+$ T cells with IL-2 and TGF-beta, particularly in the typical inflammatory microenvironment of the allograft. Retinoic acid and PD-1 signaling are examples of molecules that have been implicated as important factors in stabilizing Tregs in the inflammatory setting [83, 84]. Tregs interact with the effector arm of the immune response at multiple levels. They produce large amount of the regulatory cytokines IL-10 and TGF-beta. They express CTLA-4 which blocks the interaction of the B7 molecules with CD28 preventing the second signal required for T cell activation [82].

It is also thought that Tregs act as competitors for IL-2 with effector T cells. The interaction between Tregs and their alloimmune effector counterparts takes place principally in the lymphoid tissue draining the graft, however, in long surviving grafts there is evidence that Tregs accumulate in the graft itself [85]. Tregs may be expanded in vivo and there is much hope that they may become a therapeutic tool in inducing tolerance. Finally it is worth noting that T cells with a regulatory function are not exclusively $CD4^+CD25^+Foxp3^+$ T cells, there is evidence that $CD8^+CD28^-$T cells also play a role in regulating the immune response [86].

Myeloid Derived Suppressor Cells

In addition to regulatory T cells there are other immune-suppressive populations including IL-10-producing regulatory B cells, immature dendritic cells (discussed above) and myeloid-derived suppressor cells (MDSC). MDSC are a heterogeneous population of innate immune cells that were initially identified in cancer patients as key contributors to tumor immune evasion [87]. The MDSC family includes dendritic cells, monocytes, macrophages and granulocytes. Despite the diversity of MDSC they tend to share the following characteristics: immature phenotype, resistance to maturation, expression of arginase-1 and iNOS and an ability to suppress the adaptive immune response [88]. In humans, MDSC are characterized by low expression of MHC Class II and expression of CD33, CD11b and

CD34. MDSC are induced by factors that arise in the inflammatory microenvironment (e.g., GM-CSF, IL-6, iC3b), for example in liver transplantation the non-parenchymal cells of the liver (e.g., stellate cells) are thought to be potent inducers of MDSC which in turn enhance the emergence of alloantigen-specific Treg cells [89]. Activated MDSC suppress T, B and NK cell proliferation and cytokine production via a number of pathways including arginase-1, iNOS, indoleamine 2, 3-dioxygenase and NADPH oxidase production [87]. In a mouse model of allogeneic islet cell transplantation, MDSC were able to induce long-term islet graft survival [89].

Mixed Chimerism – A Spectrum from Tolerance to Graft versus Host Disease

While the overwhelming challenge for transplant immunologists remains control of the host alloimmune response, the phenomenon of Graft versus Host Disease (GvHD) is not unknown in solid organ transplantation. GvHD arises in two forms, the first being humoral GvHD where a blood type O graft produces anti A/B antibody resulting in a mild self-limiting hemolytic anemia in the case of an ABO mismatched transplant [90]. The second and more clinically-relevant form of GvHD is the cellular form in which passenger donor lymphocytes become activated and undergo clonal expansion in the recipient leading to widespread host tissue damage. GvHD is most frequently reported in the case of intestinal (~5%) and liver (1%) transplantation with rare case reports in pancreas and kidney transplantation [91]. The incidence is a function of the donor lymphoid inoculum at the time of transplantation with the average liver graft harboring 10^9-10^{10} donor lymphoid cells with intestine grafts containing several-fold more [92]. Clinically GvHD is most commonly manifested as a pruritic skin rash, gastrointestinal involvement with diarrhea being the prominent symptom and perhaps most importantly myelosuppression. The disease typically comes to light in the first few weeks after transplantation although later cases have been described. The diagnosis is centered on a high index of suspicion and can be confirmed by tissue biopsy (skin, gastrointestinal mucosal biopsy) and by peripheral chimerism (short tandem repeat analysis). Patients are at enormous risk of bacterial and fungal sepsis due to profound myelosuppression. The mortality rate approaches 75% with overwhelming sepsis and gastrointestinal bleeding being the leading causes of death [93]. Purported risk factors include HLA matching (with a positive cross match being a protective factor against GvHD), increasing age (although GvHD is more common in pediatric intestinal transplant recipients) and profound host immunosuppression (e.g., redo transplant patients) [94]. It is clear that the treatment of GvHD is far from clear-cut with three in every four patients succumbing to the disease. Most centers begin with a steroid bolus as the first-line treatment, followed by thymoglobulin as a second-line treatment. Other approaches include anti-CD25 (IL-2RA) and extracorporeal phototherapy but the reports are mere anecdotes. Our center has had good success with alefacept (no longer on market), which selectively targets memory T cells by disrupting the interaction between LFA3 and CD2. Following the successful employment of increased immunosuppression, in particular with CNIs in GvHD following HSCT, the traditional approach has been to intensify immunosuppression in patients who develop GvHD following solid organ transplantation; the outcome however has not been as successful with in fact some evidence to pointing towards

reducing immunosuppression to allow resaturation of the host immune system in an effort to garner a "host versus graft" alloimmune response.

The engraftment of donor hematopoietic cells is not always detrimental for the recipient and is in fact associated with tolerance. There are well-described reports of children who received HLA-matched allogeneic bone marrow transplants going on to receive renal allografts from the same donor without the need for immunosuppression [95]. Of course these are examples of full chimerism and have little day-to-day relevance due to the risks of complete myeloablation and GvHD, however, there is evidence that even low levels of chimerism may support operational tolerance [96]. For example, patients undergoing non-myeloablative chemotherapy and allogeneic HSCT for multiple myeloma accept allogeneic kidney allografts from the bone marrow donor without the need for pharmacological immunosuppression [97]. It is thought that donor stem cell engraftment allows for the continuous presentation on donor antigens, in particular the presentation of donor antigen in the thymus by dendritic cells leading to negative selection and clonal deletion, it may also provide the antigen stimulation required for Treg development in the periphery. In mixed chimeras, this allows for the elimination of both host-reactive and donor-reactive T cells in a sense both eliminating rejection and graft versus host disease in the perfect balance i.e., the newly developing T cells in a mixed chimera are both tolerant of the donor and the host [98]. While it may seem like a straightforward solution to the woes of conventional immunosuppression, it has proven difficult to maintain long-term chimerism in human studies to date, although the group at MGH was able to induce long-term tolerance with transient chimerism and attribute the tolerance to the formation of regulatory pathways such as Tregs [99].

Infection, Malignancy and Monitoring Alloimmunity

The success of contemporary transplantation is largely as a result of increasingly potent immunosuppressive medications. While this has slashed the morbidity and mortality associated with acute rejection, there has been a shift in morbidity due to the rise of opportunistic infections and post-transplant malignancy. The diagnosis and management of infections in the transplant patient can be challenging for several reasons, for example there are many etiologies of fever including infection, rejection, GvHD and drug fevers. There are then the competing interests of treating infection versus fears of rejection associated with tempering immunosuppression. Furthermore, the field is a moving target; opportunistic infections (OI) such as CMV and Pneumocystis jiroveci pneumonia are less common with prudent prophylaxis whereas multidrug resistant bacteria pose a major challenge to the transplant physician and patient.

Infections following transplantation may be classified into donor-derived, recipient-derived, nosocomial and community acquired. OIs are unusual in the first month following transplantation with the majority of infectious disease being related to technical factors surrounding the transplant operation. *C. difficile* is a common infection and can be a devastating event with some evidence demonstrating a link to hypogammaglobulinemia [100]. One also must remain vigilant for the possibility of donor-derived infections. Viral

infections are an important source of morbidity for the transplant patient given the emphasis on suppressing cell-mediated immunity especially in the immediate post-transplant period. CMV is an important cause of viral infection and appropriate donor and recipient serological studies are crucial in identifying recipients at high risk of developing invasive infection. Epstein-Barr Virus can lead to post transplant lymphoproliferative disorder in 3−10% of adult transplant recipients, with the incidence being higher in children; risk factors include primary EBV infection, allograft rejection, CMV infection and exposure to antilymphocyte serum [101]. It can vary from an indolent polyclonal mononucleosis-like syndrome to a malignant monoclonal lymphoma [102]. The polyclonal form may respond to a reduction in immunosuppression, highlighting the interplay between antiviral immunity and immunosuppression. Polyomaviruses have been identified in transplant recipients in association with nephropathy (BK virus) and progressive multifocal leucoencephalopathy (JC virus); the mainstay of therapy involves reducing immunosuppression to allow reconstitution of the host's endogenous antiviral immunity [103]. The defects in cellular immunity also result in a susceptibility to invasive fungal infection, especially non-albicans *Candida* and *Aspergillus* species.

Current immunosuppression regimens also impact on anti-oncogenic immune activity with attenuated immunosurveillance against oncogenic viruses, DNA damage and neoplastic cells. There is evidence that links the magnitude of immunosuppression to cancer risk with heart and lung transplant recipients having an increased risk of malignancy compared to kidney recipients who require lighter doses of immunosuppression [104]. Non-melanoma skin cancer is the most common post-transplant malignancy, other common cancers include Kaposi's sarcoma (related to HHV-8), lymphomas, renal cell carcinoma and cervical/vulvovaginal cancer (related to HPV) [71]. Many of these are related to oncogenic viruses but this alone does not account for the increased risk.

Contemporary management of immunosuppression remains guided by relatively crude objective measurements (e.g., biochemistry for liver and kidney recipients), therapeutic drug monitoring and tissue biopsy to identify histological signs of rejection. These measurements do not provide any information on what is happening at a cellular or molecular level. Objective measurements that could assess functional immunity would be of great benefit to prevent rejection and minimize infectious complications post transplantation. The only FDA approved assay is the ImmuKnow (Cylex Ltd, USA); the test measures ATP produced after stimulation of T-cells with plant lectin phytohemagglutinin mitogen [105]. There are varying reports of its usefulness but most of the studies are of limited sample size and many are retrospective in nature. A recent meta-analysis by Ling et al., reported disappointing results with sensitivity and specificity of 0.58 and 0.69 respectively for infection, while the sensitivity and specificity for rejection was 0.43 and 0.75 respectively [106]. There is much in interest in donor-specific assays including limiting dilution assays which quantify cytokine production in recipient PBMC and donor stimulator cell co-cultures; mixed lymphocyte reaction assays operate on a similar principal but instead quantify recipient T cell proliferation. There has been recent interest in enzyme-linked immunosorbent spots (ELISPOT); this assay quantifies the frequency of previously activated (memory) T cells that respond to donor antigens by producing a selected cytokine in vitro. Recipient T cells are cultured with donor cells on plates coated with a cytokine-specific antibody that is detected using labeled secondary antibodies. Each spot detected represents a reactive effector or memory T cell [107]. There is some evidence to support the use of an IFNγ ELISPOT in

identifying T-cell pre sensitization in renal transplant recipients [108]. While the concept of objectifying donor-specific responsiveness is attractive, the labor-intensive and time-consuming nature of these assays, the requirement for donor cells and conflicting data available have prevented the wholesale use of this technology. There are many other areas of research ongoing including gene expression studies searching for genetic fingerprints of tolerance, Treg analysis and so on.

Composite Tissue Transplantation

The transplantation of composite tissue allografts (CTAs) is an increasingly used option in the rehabilitation of patients with devastating soft tissue and bone loss. There are at least 27 face transplants, 89 upper extremity transplants and 18 abdominal wall transplants described in the literature but undoubtedly there have been more cases performed but not reported. In the United States, the Department of Health and Human Services classified CTAs as organs in 2013 and as such the OPTN now governs the use of these 'organs' (109). CTA provides a new level of immunological challenge given the intense immunological barrier posed by the skin with skin tissue itself being more antigenic than the entire organ that is a CTA [110], frequently the skin demonstrates histological signs of rejection while the remainder of the graft is in immunological quiescence. It is unsurprising that the skin is highly immunologically active since it provides a barrier to environmental pathogens; the skin of a human contains twice the amount of T cells that are present in the circulation, the majority of which are of an effector memory phenotype [111]. The skin is also resident to specialized dendritic cells called Langerhans cells, which have been traditionally thought to serve as the first line of defense against exogenous pathogens. In transplantation the role of Langerhans cells is not entirely clear with some studies suggesting that they induce $CD4^+$ proliferation towards TH2, TH17 and TH22 phenotypes with resulting cross priming of $CD8^+$ cytotoxic T lymphocytes (CTLs); while others suggest a more regulatory role particularly in the resting state where they may induce the emergence of Treg cells [112].

In earlier years the depth of immunosuppression required to induce acceptance of CTAs carried an unacceptable risk of side effects not least of which was infection. The advent of T cell-depleting antibodies and tacrolimus has improved the risk versus benefit ratio nonetheless the side-effects of CNIs (e.g., diabetes mellitus, nephrotoxicity) are not benign particularly for an operation that serves to improve quality of life rather than extending life. Despite the immunological challenges posed by the CTA, short-term outcomes are excellent with 100% and 96% one-year patient and graft survival in the case of upper extremity transplantation; this is despite an 85% incidence of some form of acute rejection in the first year post transplantation [113]. There is one reported loss of a facial allograft due to acute rejection in a patient who was non-compliant with immunosuppression, thus highlighting the importance of social and psychological considerations in all transplant candidates [114].

The side-effect profile of conventional immunosuppression has led physicians and scientists interested in CTA to pursue tolerance induction protocols with particular fervor; animal studies have demonstrated that donor bone marrow infusion prolongs graft survival and allows for a reduction in the dose of traditional multidrug immunosuppression regimens [115]. The fact that the skin is the prime site of alloimmune attack has led to some groups

using topical tacrolimus and steroids as a means to reduce the systemic dose, there are no randomized controlled trials but it is certainly of interest. The ease by which a skin biopsy can be performed means that surveillance for rejection is much easier in CTAs allowing for a rapid diagnosis and treatment of acute rejection [116]. There is a paucity of long-term data to assess the prevalence and significance of chronic rejection although one upper extremity CTA had to be removed due to a process consistent with chronic rejection i.e., allograft vasculopathy.

Xenotransplantation

Despite the advances in the use of marginal donors, deceased after cardiac death donors and live donors a shortage of donor organs remains one of the principal challenges in transplantation. In the US, the average wait time for a kidney is now five years while 1,500 patients die a year while waiting for a liver transplant. Xenotransplantation may represent an alternative method of transplantation especially in the case of acute liver failure where there is still no 'bridge' to transplant as in the case of dialysis for renal failure or indeed ventricular assist devices and ECMO (Extracorporeal membrane oxygenation) for terminal heart and lung failure.

There has been was intermittent enthusiasm for xenogeneic transplantation over the years, in the early 90's Starzl et al., performed a series of two baboon to human liver transplants at the University of Pittsburgh with the grafts surviving 70 and 26 days respectively, neither of the grafts actually succumbed to rejection [117]. The use of concordant species as a source of donor organs is problematic since many of the species are endangered (e.g., chimpanzee) and due to their small size and slow rate of breeding.

The limitations of using nonhuman primates as a source of organs have led researchers to consider pigs as a potential source of organs. Their organs are a good size match for humans and there are many physiological similarities between pigs and humans. However, pig organs provide an entirely different level of immunological barrier to graft acceptance. Wild type pig organs are rejected within minutes due the presence of preformed antibodies to antigens on the porcine endothelium, most notably to galactose- α1, 3-galactose (αGal). Antibody binding to the endothelium leads to activation of the classical complement cascade and the coagulation cascade resulting in vascular congestion, edema, interstitial hemorrhage and thrombosis with eventual graft necrosis i.e., classical hyperacute rejection. This initial barrier has been addressed by the development of pigs deficient in αGal; porcine renal xenografts deficient in αGal are resistant to HAR [118]. While αGal knockout pigs have solved the problem of HAR, *de novo* antibody production leads to acute humoral xenograft rejection (AHXR) (also known as delayed xenograft rejection or acute vascular rejection) within 7−10 days [119]. The addition of the complement regulatory transgenes (e.g., CD46, CD55/DAF, CD59) to the porcine donors (i.e., transgenic pigs) also attenuates HAR in both kidney and liver transplantation (120). The innate immune system, in particular NK cells and macrophages mount an attack on porcine cells; it is thought that porcine MHC Class I does not send an inhibitory signal to human NK cells [121]. In addition to the robust antibody and innate immune cell response, pig allografts are susceptible to dysregulated coagulation when implanted in primates. Grafts are also notable for a thrombotic microangiopathy and

recipients develop a severe coagulopathy, while the exact cause is unclear it is undoubtedly related to widespread endothelial cell activation and damage [122]. Xenografts are of course susceptible to classical cellular rejection but this is less of a concern given the currently available immunosuppressive agents that target T cells.

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Chapter 2

Cellular and Humoral Responses in Organ Transplantation

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Abstract

Organ transplantation remains the mainstay of treatment for patients with end-stage organ failures. Since the supply of transplantable organs is extremely limited, it is critical to avoid graft loss due to immune rejection. Currently, transplanted patients must take many immunosuppressive drugs daily (i.e., cyclosporine or tacrolimus combined with other drugs) to prevent graft rejection. These drugs are associated with significant nephrotoxicity and increased risks for opportunistic infections and malignancy. Transplant biologists need to find better therapies that can induce transplantation tolerance, a state in which the donor graft is accepted without chronic immunosuppression while the remainder of the immune system is left intact. Herein, we will discuss the immune mechanisms that underlie transplant rejection, in particular, the T cell-mediated cellular response and B cell-mediated humoral responses in transplantation. We will also summarize the current information on immunosuppressants that aim at modifying T and B cell response to transplanted organs. In order to develop an effective therapy that induces transplant tolerance, there must be a continued effort on defining the molecular basis that underlies T and B cell responses to transplanted organs.

Keywords: transplantation, T cells, B cells, costimulation, cytokine

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Abbreviations

ACR: Acute cellular rejection
ADCC: Antibody-dependent cell-mediated cytotoxicity
AMR: Antibody-mediated rejection
APCs: Antigen-presenting cells
BCR: B cell receptor
CNIs: Calcineurin inhibitors
CTL: Cytotoxic T lymphocyte
DSAs: Donor-specific antibodies
iTreg: Induced Treg
IVIG: Intravenous immunoglobulin
MHC: Major histocompatibility complex
MMF: Mycophenolate mofetil
TCR: T cell receptor
T_{FH}: T Follicular helper
TGF-β: Transforming growth factor-β
Th: T helper
Tregs: Regulatory T cells

Introduction

Seven decades ago Sir Peter Medawar's work showed that the body's immune system rejects allogeneic transplants [1]. In 1953, Medawar and colleagues further indicated that immunological tolerance to alloantigens can be acquired in neonatal mice [2]. A year later, Joseph Murray performed the first successful kidney transplantation between identical twins, whose immune systems naturally did not reject each other's tissues. Once the calcineurin inhibitor cyclosporine was used to suppress immune system in the 80s, allogeneic organ transplantation quickly became a major treatment for patients with end-stage organ failures. Today the survival of transplanted organs still depends on continual use of *calcineurin inhibitors* (CNIs) and other immunosuppressive drugs that non-selectively impair immune cell function. This chapter reviews the immunological mechanisms of transplant rejection, in particular, the central role of allogeneic T and B cell responses in transplantation. The T and B cell-targeted therapeutic approaches will also be discussed. We hope that the in depth characterization of T and B cell responses in transplantation may eventually lead to the creation of transplantation tolerance in human.

T Cell Biology in Transplantation

T cells are a major type of lymphocytes that are developed and matured in the thymus. A majority of T cells express an alpha/beta (α/β) T cell receptor (TCR) that recognizes a peptide antigen when it is bound to a major histocompatibility complex (MHC) molecule on antigen-presenting cells (APCs). MHC-restricted αβ T cells play a central role in rejecting a

transplanted allograft. The biology of these T cells in transplantation will be discussed in the following sections [3].

Direct and Indirect Alloantigen Recognition

The genes encoding MHC are among the most polymorphic genes between different members of the same species. For instance, the human MHC, HLA, contains at least 350 alleles for HLA-A genes, 620 alleles for HLA-B, 400 alleles for HLA-DR, and 90 alleles for HLA-DQ. An individual's TCR repertoire is pre-selected in the thymus so that they are capable of binding self MHC but such binding is not very strong [4]. In the context of transplantation, the recipient's TCR repertoire is not pre-selected using donor MHCs. Strong TCR-donor MHC/peptide binding thus occurs and triggers potent allogeneic T cell responses. It is believed that a very high proportion (~0.1−10%) of a recipient's T cells can directly recognize donor MHC molecules in an allogeneic transplantation. Crystal structure analysis verified that a self-MHC-restricted TCR is able to bind with allogeneic MHC/peptide [5]. The binding of intact donor MHC on donor cells by the TCRs on the recipient T cells is termed the direct allo-recognition [6, 7]. On the other hand, peptides derived from allogeneic MHCs can also be presented by self MHC on recipient APCs, which is termed indirect allo-recognition [6, 7]. Minor histocompatibility antigens derived from non-MHC polymorphic proteins can only be recognized via the indirect pathway [8]. After transplantation, allogenic T cell response triggered by the direct allo-recognition pathway is potent but also transient due to the gradual loss of donor APCs. By contrast, the indirect pathway is permanently active as the donor antigens are persistently present, unless T cell tolerance to allografts is induced [6, 7]. Because hundreds of thousands or even millions of T cell clones can respond against alloantigens, the T cell response to allograft is extremely intensive and destroys an entire transplanted organ in days in the absence of immune suppression.

TCR Signal Transduction

Mature naïve T cells initially recognize transplant antigens in lymphoid organs, and depend on APCs to pick up and present cognate antigens to them. Peripheral mature T cells mainly include $CD4^+$ T helper (Th) and $CD8^+$ cytotoxic T lymphocytes (CTL). $CD4^+$ T cells provide help to other immune cells through direct cell-cell interaction and through the release of cytokines. $CD8^+$ T cells are furnished to destroy virally infected cells, tumor cells and allogeneic cells. Intriguingly, about 10% $CD4^+$ T cells constitutively express CD25 (IL-2Rα) on cell surface and transcription factor Foxp3. These Foxp3-expressing $CD4^+$ T cells are called regulatory T cells (Tregs). While $CD4^+$ Th cells play a central role in establishing and maximizing various immune responses, Treg cells suppress virtually all immune responses [9].

Naïve T cells must receive TCR signals to exert their effector function. In the secondary lymphoid organs, naïve T cells receive activation signals upon specific engagement of the TCR by the cognate peptide-MHC complex on APCs. Such TCR recognition of its cognate antigen triggers phosphorylation of the ITAM motifs of CD3 chains, which are parts of the

TCR complex expressed on T cells. Phosphorylation of CD3 chains recruits ZAP-70 and aggregates signaling complexes (i.e., PLC-γ, VAV, ItK, PI3K) around LAT and SLP-76. These signaling complexes act on PtdIns(4,5)P2 to create the active intermediaries including DAG, IP3, and PIP3, which in turn activate various downstream pathways, such as PKC/NF-κB, AP-1, and calcium/calcineurin/NFAT. Transcription factors NF-κB, AP-1, and NFAT induce the expression of various genes to fulfill the T cell needs of proliferation and effector function [10]. For instance, the production of IL-2 is critical for the expansion of activated T cell clone, whereas the induced expression of Batf and IRF4 control the production of other cytokines (i.e., IL-21) as well as further program the effector differentiation [11] (Figure 2.1).

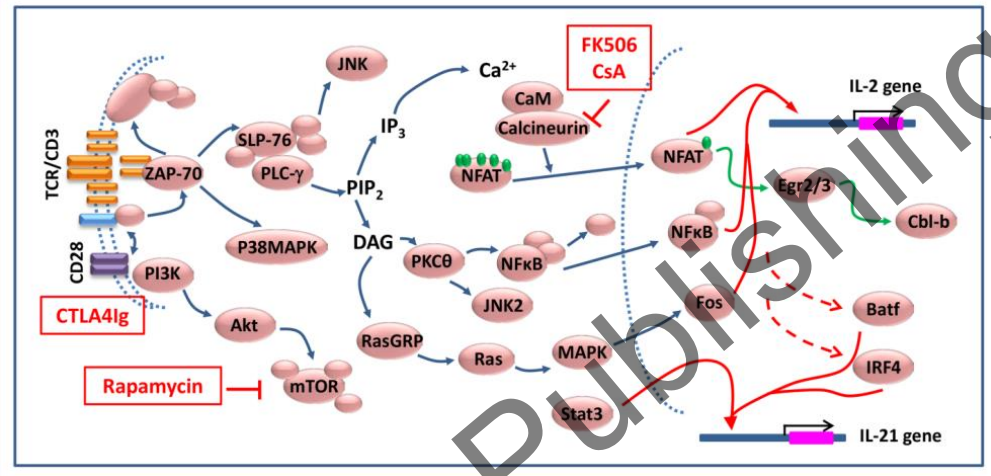

Figure 2.1. TCR and costimulatory signaling pathway. TCR recognition of cognate antigens on naïve T cells triggers phosphorylation of the ITAM motifs of CD3 chains, recruits ZAP-70, and aggregates signaling complexes around LAT and SLP-76. These signaling complexes act on PI(4,5)P2 to activate DAG and IP3, which in turn activate PKC/NF-κB, AP-1, and calcium/calcineurin/NFAT transcription pathways to fulfill the T cell needs of proliferation and effector function, such as IL-2 production and induced expression of transcription factors Batf and IRF4 (red lines). Batf and IRF4 control T cell differentiation and production of cytokines (e.g., IL-21). CD28 costimulation acts through PI-3K/Akt/mTOR, Lck and Grb-2/ITK to provide its signal for T cell activation. In the absence of costimulation, TCR stimulation leads to calcineurin-mediated dephosphorylation and nuclear translocation of NFAT without full activation of AP-1 complexes. This induces T cell anergy by up-regulating Egr2/3 and Cbl-b (green lines). FK506, cyclosporine, rapamycin, and CTLA4-Ig (red fonts) prevent T cell activation by targeting the indicated pathways.

Costimulatory and Inhibitory Signals

T cell function is regulated by a variety of inducible cell surface molecules that induce stimulatory (ICOS, OX40, and 4-1BB) or inhibitory (CTLA-4, PD-1, BTLA) signals. Most costimulatory and inhibitory molecules are belonged to two families. The Ig superfamily includes the costimulatory receptor (CD28 and ICOS) and the inhibitory receptors (CD152, PD-1, CD272 and CD160). The TNF superfamily includes the co-stimulatory receptors CD40, OX40, HVEM [12].

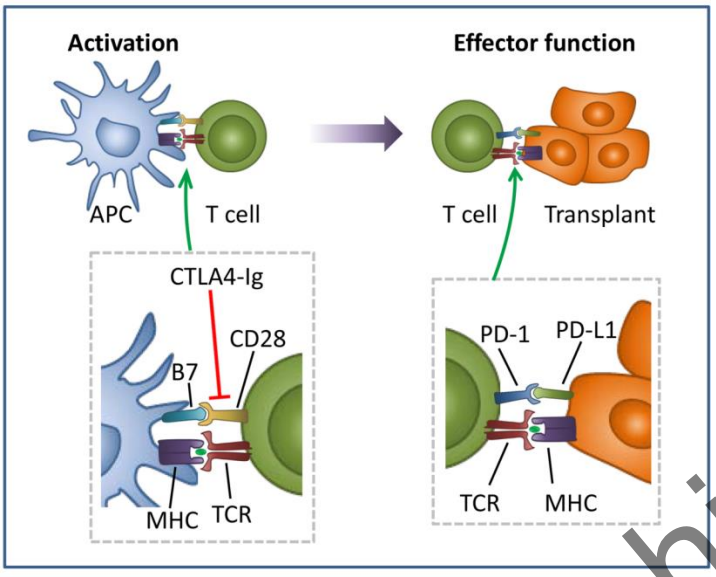

Figure 2.2. Immune checkpoints for T cell activation and effector function. Naive T cells are primed in the secondary lymphoid organs, where they receive activation signals upon engagement of the TCR by the antigen-MHC complex on APCs and interaction of CD28-B7 co-stimulatory molecules. Activated T effector cells then infiltrate transplanted organ and kill targeted cells in a MHC-restricted manner. CTLA4 expressed on the surface of activated T cells binds B7 molecules on APCs to restrain CD28/B7 signaling and T cell activation. CTLA4-Ig fusion protein has been used to inhibit T cell responses in transplantation. PD1 expression is up-regulated on activated T cells and maintained on tissue-infiltrating T cells. Binding of PD1 with PDL1 on tissue cells down-regulates the activity of T cells.

In addition to TCR engagement, T cell activation also requires interaction of CD28 on T cells with the B7 co-stimulatory molecules on APCs. CD28 signal is critical for the recruitment of PI3K, Grb2 and Gads, the induced expression of anti-apoptotic gene Bcl-xL via mTOR, as well as the sufficient production of IL-2. In the absence of CD28-B7 interaction, TCR stimulation leads to a state of functional inactivation, known as T cell anergy. TCR anergizing stimuli induce the calcineurin-mediated dephosphorylation and nuclear translocation of NFAT in the absence of full activation of AP-1 complexes, which up-regulates the expression of several E3-ubiquitin ligases, including Cbl-b, Itch, and GRAIL. These E3-ubiquitin ligases downregulate TCR signaling by degradation of signaling molecules such as phospholipase C-γ and protein kinase C-θ [13].

T cell activation leads to increased expression of an inhibitory molecule CTLA4, which also binds to B7 molecules on APCs but transmits an inhibitory signal to T cells. Mice deficient in CTLA4 result in severe autoimmunity and die within weeks after birth. Thus CTLA4 is a critical checkpoint regulator that turns off T cell response. Activated T cells also express another critical checkpoint regulator, PD-1. Mice deficient in PD-1 develop lupus-like glomerulonephritis and dilated cardiomyopathy. In the context of tumor immunology, interaction of PD-L1 on tumor cells with PD-1 on infiltrating T cells inhibits anti-tumor activity [14]. It would be interesting to investigate the role of PD-1 signaling in transplantation (Figure 2.2). Unlike other solid organs, liver allografts may be accepted without or with minimal immunosuppression. Hepatic stellate cells (HSCs) express PD-L1, which binds PD-1 on T cells and prevents liver transplant rejection [15−18]. In addition, co-

culturing HSCs with DCs leads to the generation of PD-L1 expressing myeloid-derived suppressor cells (MDSCs). Co-transplantation of islet allografts with these MDSCs results in long-term islet allograft survival in a PD-L1 dependent manner [19].

Cytokine Regulation of T Cell Response

The homeostasis and function of T cells are highly dynamic and regulated significantly by the cytokine environment. Dysregulation of cytokine production may breach immune tolerance and cause autoimmune diseases. For instance, IL-7, IL-12, IL-21, and IL-23 are essential cytokines that promote T cell differentiation and survival. Recent genetic studies have revealed the stimulus role of these cytokines or their cognate receptors in susceptibility to autoimmune diseases. By contrast, anti-inflammatory cytokines [e.g., transforming growth factor-β (TGF-β), interleukin-10 (IL-10), IL-27, and IL-37] exert direct inhibitory effects on immune cells to enforce immune tolerance. Interestingly, IL-2 was initially identified as a T cell growth factor. Recent findings indicated that IL-2 is the most potent cytokine in regard to promoting the development and survival of Treg cells, thereby mediating immune tolerance. Polymorphisms in IL-2 and IL-2Rα genes are common genetic factors for various autoimmune diseases [20]. In certain transplantation models, pro-inflammatory cytokine IFN-γ also plays an important role in mediating transplant tolerance, as it promotes PD-L1 expression on HSCs [17, 21, 22].

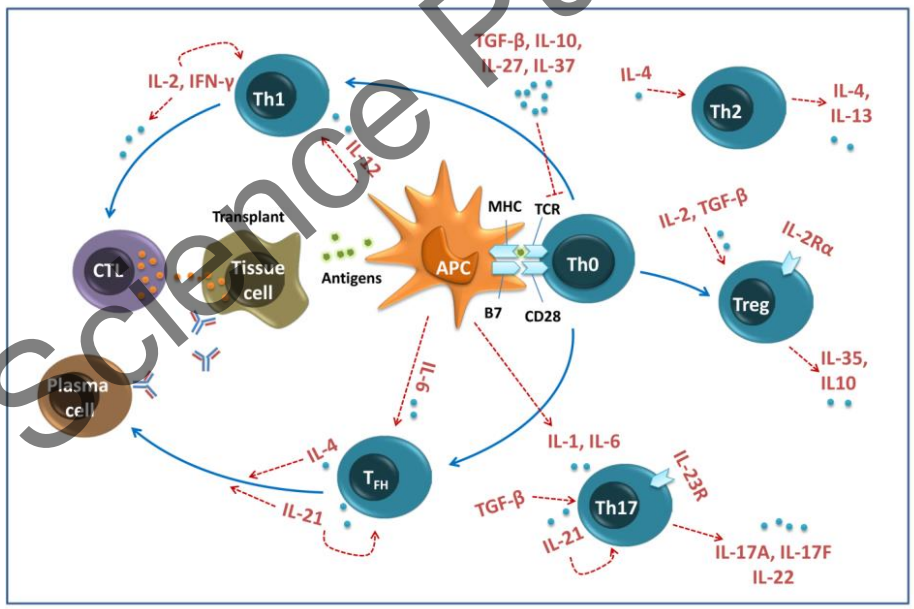

Figure 2.3. Cytokines regulate T cell activation and differentiation. TGF-β1 and IL-2 facilitate the generation and survival of inducible Treg cells, which further produce suppressive cytokines, IL-10 and IL-35. TGF-β1, IL-6, autocrine IL-21, and IL-1β direct Th17 differentiation, whereas IL-23 maintains Th17 cell differentiation. IL-6 and autocrine IL-21 also promote the differentiation of T_{FH} cells. IL-12/IFNγ and IL-4 initiate Th1 and Th2 cell differentiation, respectively. TGF-β1, IL-10, IL-27, and IL-37 are suppressive cytokines that inhibit APCs and T cell activation.

A tremendous progress has been made in determining the role of cytokines in the differentiation and function of effector T cells. Currently, the CD4 T helper cells are subdivided into following subsets: 1) Th1 cells that produce IFN-γ and TNF-α; 2) Th2 cells that produce IL-4, IL-5, and IL-13; 3) Th17 cells that produce IL-17a, IL-17f, IL-21, and IL-22; and 4) T follicular helper (T_{FH}) cells that produce IL-4 and IL-21. On other hand, cytokine signals also play the decisive role in specifying the differentiation of these Th subsets from naive CD4 T cells upon activation. For instance, IL-12/IFN-γ specifies Tbet-expressing Th1 cells, whereas IL-4 directs differentiation of Gata3-expressing Th2 cells. RORγt-expressing Th17 and Bcl6-expressing T_{FH} are newly identified subsets, and their differentiation is controlled by TGF-β/IL-6/IL-1β and IL-6 /IL-21, respectively (Figure 2.3) [23].

The expression of Foxp3 is required for establishing and maintaining the $CD4^+$ Treg lineage. Foxp3 mutations result in lethal autoimmunity both in mice and humans. $CD4^+Foxp3^+$ Treg cells developed in the thymus are called naturally occurring Treg (nTreg) cells. $CD4^+Foxp3^+$ Treg cells can also be derived from peripheral naive $CD4^+$ T cells and are named adaptive or induced Treg (iTreg) cells. TGF-β and IL-2 are polarizing cytokines for iTreg cell differentiation [24]. We found that neonatal naïve T cells can default to Foxp3-expressing Treg cells in the absence of exogenous polarizing cytokines, which may explain the ease of tolerance induction in neonatal mice [25]. Moreover, in the absence of exogenous TGF-β, IL-2 plays a dynamic dual role in inducing adult iTreg cells [26]. Overall, our understanding of the cytokine biology has dramatically advanced during the last three decades, but the applications of such knowledge in transplantation are rare.

T Cell Response to Allograft

T cell-deficient rodents were incapable of rejecting allogenic grafts, indicating that T cells are requisite factors of transplant rejection. We have previously shown that mice deficient in $CD4^+$ T cells, but not $CD8^+$ T cells, were capable of preventing transplant rejection [27]. Hence, $CD4^+$ T cells play a central role in coordinating immune response to transplanted organs. Alloreactive T cells are mainly primed in the secondary lymphoid organs. Lakkis et al., have shown that cardiac allografts are accepted indefinitely in recipient mice that do not have secondary lymphoid organs [28]. Immediately after transplantation, donor passenger leukocytes within the grafts may migrate to the secondary lymphoid organs, and trigger the direct recognition pathway by presenting intact donor MHC: peptide to alloreactive T cells [29, 30]. Alloreactive T cells can also recognize donor antigens via the indirect recognition pathway. To this end, donor antigens are engulfed by the recipient's APCs and presented on self MHC molecules. Recipient DCs may infiltrate into the grafts and then bring donor antigens to secondary lymphoid organs [6, 7]. The role of graft-infiltrating DCs in transplantation remains largely unknown. We have shown that the majority of DCs remained in an immature state within the tolerated cardiac grafts in contrast to those in the rejecting grafts [31]. Immature infiltrating DCs may impair anti-donor T cell function in allografts and thus contribute to graft acceptance.

The maturation process of APCs is critical for inducing sufficient activation signals to T cells. Transplanted organ produces proinflammatory cytokines, TNF and IL-1, which stimulate DC maturation and up-regulate the cell surface expression of MHC class II, CD40,

B7-1, and B7-2 molecules. These MHC: peptide and costimulatory signals are essential for DC-mediated T activation. Primed $CD4^+$ T cells complete the DC maturation process via the CD40L "licensing" signaling, and in turn increase the expression of costimulatory molecules (e.g., B7-1, B7-2, OX40L, and 4-1BB) and production of cytokines (e.g., IL-1, TNF, chemokines, IL-6, and IL-12). Primed $CD4^+$ T cells differentiate into several Th cell subsets, which support the function of $CD8^+$ CTLs, B cells, macrophages, NK cells, and eosinophils [32].

T cells attack an allograft through various effector pathways. For instance, $CD4^+$ Th cells provide help to $CD8^+$ CTLs, which in turn secrete and deliver granzyme B to kill allograft cells. $CD4^+$ T cells can also directly destroy allograft cells through Fas-dependent or –independent pathways. Moreover, $CD4^+$ Th1 cells-secreted IFN-γ and TNF activate monocytes/macrophages and trigger a delayed-type hypersensitivity (DTH) response. Either the DTH or the CTL response alone is sufficient to reject skin allografts [33−35]. $CD4^+$ T cells also provide help to produce alloantibodies and initiate humoral response to allografts [36], as well as promote eosinophil-mediated graft damage by secreting IL-4 and IL-5. Hence, $CD4^+$ T cells play a central role in transplant rejection via providing help in multiple effector pathways [37].

T Cell–Targeted Therapies for Transplantation

Current immunosuppressive drugs for transplantation mainly target T cell activation and expansion. Cyclosporine is a cyclic nonribosomal peptide isolated from the fungus *Tolypocladium inflatum*. Cyclosporine was approved for clinical use in 1983. The immunosuppressive effect of cyclosporine is to lower the activity of T cells by binding to the cytosolic protein, cyclophilin. The complex of cyclosporine and cyclophilin inhibits calcineurin-mediated dephosphorylation of NFAT and prevents T cell activation. Tacrolimus (FK506) is also a CNI, and was approved for use in transplanted patients in 1994. Tacrolimus binds to FKBP12, a FK506 binding protein. The FKBP12-FK506 complex interacts with calcineurin, and in turn prevents the dephosphorylation of NFAT. The use of calcineurin inhibitors is associated with various severe side-effects including infection, nephrotoxicity, cardiac damage, hypertension, diabetes mellitus, and increased risk of malignancy [38].

Mycophenolate mofetil (MMF) and sirolimus (Rapamycin) have also been widely used as immunosuppressive drugs in organ transplantation. MMF suppresses proliferation of T and B cells by inhibiting inosine monophosphate dehydrogenase, an enzyme that controls the synthesis of guanine monophosphate. Sirolimus binds FKBP12. Unlike the FKBP12-FK506 complex that inhibits calcineurin, the FKBP12-sirolimus complex inhibits the mammalian target of rapamycin (mTOR) by directly binding to mTOR Complex 1 (mTORC1). MMF, sirolimus, and other immunosuppressive drugs have been used for calcineurin inhibitor minimization to reduce nephrotoxicity after transplantation. MMF maintained allograft survival upon late withdrawal of calcineurin inhibitor but exhibited an increased risk of acute rejection and infection. Further studies are needed to determine whether MMF combined with sirolimus can be an effective calcineurin inhibitor sparing strategy [39].

The comparatively higher binding affinity of CTLA4 to B7 has been applied to block CD28-B7 interaction. Fusion protein CTLA4-Ig (Figure 2.2), known as belatacept, was

approved for renal transplantation in patients that are sensitized to EBV. Targeting co-stimulatory pathways remains an attractive research field in transplantation [40]. Nevertheless, caution should be exercised when designing immune interventions to modulate T cell responses. For instance, six healthy young male volunteers were enrolled in a phase 1 clinical trial of TGN1412, a novel superagonist anti-CD28 mAb. Within hours of receiving a single intravenous dose of this mAb, all six volunteers had a systemic inflammatory response and became critically ill [41]. Moreover, Th1 cells were previously considered as the major mediators of transplant rejection. A recent study found that T-bet-deficient mice that lack Th1 cells develop markedly accelerated allograft rejection rather than prolonged survival [42]. Hence, transplant immunologists need to further define the molecular mechanism underlying T cell-mediated transplantation.

Tolerance in Transplantation

Long-term immunosuppression is associated with increased susceptibility to infection and risk of malignancy, which increase morbidity and mortality. Hence, the optimal therapeutic option for a transplant patient is to induce transplantation tolerance, a state in which the allograft is indefinitely accepted without the need for life-long immunosuppression. Six decades ago Medawar and colleagues induced transplantation tolerance in mice during ontogeny of the immune system. They showed neonatal recipient mice injected with donor splenocytes did not reject skin grafts from the same donor strain when they became adults [2]. Neonatal mice do not have mature peripheral T cells, which may provide a critical time period for donor antigens to be tolerated. Neonatal T cells are also intrinsically different from adult T cells [25]. The ease of tolerance induction in neonatal mice cannot be replicated in adult mice and in human.

Adult recipients contain high frequency of alloreactive T cells, which cause potent acute immune response upon transplantation and represent a major challenge for the induction of transplantation tolerance. CNIs are the most potent immunosuppressive drugs that impair TCR-mediated activation signals by preventing the nuclear translocation of NFAT. However, CNIs may also interrupt the induction of T cell tolerance. Indeed, NFAT is also required for transcription of T-cell tolerogenic genes, such as Egr2, Egr3, Cbl-b, itch, and GRAIL [13]. A question arises here is whether TCR signals can be modulated to prevent acute anti-graft response but spare the tolerogenic mechanisms. We have recently shown that transient targeting TCR with an anti-TCR mAb H57-597 not only arrested ongoing allogeneic T cell responses, but also exerted a tolerogenic effect that promotes long-term allograft survival in mice [43, 44]. The molecular mechanisms underlying this long-lasting tolerogenic effect remain unknown.

Blocking costimulatory signals with anti-CD154 mAb and CTLA4Ig has been proven as an effective approach to induce transplantation tolerance in mice. Costimulatory blockade may induce transplantation tolerance by promoting peripheral deletion, anergy induction, and Treg cell-mediated active suppression of alloreactive T cells [45]. CTLA4Ig has been successfully translated to the clinic [46]. Compared to calcineurin inhibitors, a higher incidence of acute rejection was observed in CTLA4Ig-treated patients. Clinical trials of anti-

CD154 mAb were terminated due to coagulation, as activated platelets also express CD154 [47]. Costimulation blockade remains as an attractive strategy for tolerance induction.

Over the past 30 years, much has been learned about TCR, costimulatory, and cytokine signals in T cell activation, differentiation, and effector function. The survival of transplanted organs still depends on the long-term use of CNIs. Possibly a successful tolerogenic therapy for transplantation should abrogate the use of CNIs that non-selectively block both immunity and tolerance. Selective targets need to be identified to eliminate alloreactive T cell response but spare the Treg suppression and the expression of tolerogenic molecules (suppressive cytokines, CTLA4, PD1, Egr2, Cbl-b *etc*.). Much still needs to be learned about the molecular mechanisms of T cell tolerance before successful induction of transplantation tolerance can be achieved.

Memory T Cells in Transplantation

Most activated T cells die but some of their offspring become long-lived memory T cells, including $CD62L^{hi}CCR7^{+}$ central memory T cells, $CD62L^{lo}CCR7^{-}$ effector memory T cells, and $CD69^{+}CD103^{+}$ tissue-resident memory T cells [48]. When memory T cells re-encounter their cognate antigens, they provide potent and rapid immune responses [49]. In murine transplantation models, a naïve mouse rejects a transplanted cardiac allograft in about 10 days. By contrast, if a mouse has previously received a skin allograft, it rejects a cardiac allograft from the same donor strain within only 3−4 days due to memory response. In clinical transplantation, it remains unclear how memory T cells are generated prior to transplantation. Blood transfusion and pregnancy may lead to pre-exposure of some donor antigens in recipients. Moreover, heterologous immunity of a T cell to different antigens may occur. Indeed, alloreactive T cells recognizing intact allo-MHC are all restricted by self-MHC. Thus, viral infections may generate memory T cells that can also respond to alloantigens [50]. Memory T cells can also be generated in the absence of cognate antigens, in particular, in a lymphopenic environment. This has been proven in an animal model. Transferring a small number of syngenic T cells into lymphopenic mice leads to rapid homeostatic proliferation and generation of memory T cells, some of which can response to alloantigens [51−53].

Costimulation blockade induces transplantation tolerance in mice but not in human. One possible explanation for this phenomenon is the presence of alloreactive memory T cells in humans, as memory T cells are resistant to costimulation blockade. Indeed, about 50% of T cells in an adult human have a memory cell phenotype. Some of these memory T cells should be able to recognize alloantigens due to heterologous immunity. By contrast, a naïve mouse contains less than 10% memory cells among T cell population. Naive T cells and antigen-experienced memory T cells represent different developmental stages and exhibit different propensities in survival, activation, and tolerance. TCR signaling in naive T cells in the absence of co-stimulatory signals induce an anergic state of these cells, which is a major self-tolerant mechanism. By contrast, TCR signaling alone in memory T cells triggers rapid recall responses [54, 55]. Moreover, mTOR and AMP-activated protein kinase (AMPK) distinctly direct metabolic programmes in naïve and memory T cells, respectively [56]. Further

delineation of the molecular mechanisms underlying memory T cell response will facilitate the induction of transplantation tolerance in clinic.

B Cell Biology in Transplantation

B cells controls the humoral immunity of the adaptive immune system. B cells express B cell receptor (BCR) that specifically binds to cognate antigen. In addition to making antibodies against antigens, B cells are also APCs that can regulate T cell response. Memory B cells and long-lived plasma cells can be generated after activation by antigen interaction and provide long-term protection. The following sections discuss the role of B cells in transplant rejection and tolerance.

B Cells and Antibody Production

B cells are derived from bone marrow. B cell development occurs through several stages that correlate to the changes in the genome content at the BCR/antibody loci. Each mature B cell expresses a BCR, which is a membrane-bound immunoglobulin composed of light and heavy chain segments [57]. When BCRs recognize cognate antigens, B cells proliferate and develop into plasma cells that produce antigen-specific antibodies. Antibody is a soluble form of the BCR with identical antigen-binding sites [58]. Some antigen-encountered B cells develop into long-lived memory B cells, which provide quick response if they are re-exposed to the same antigen later [59]. Intriguingly, two groups of transcription factors antagonistically affect late B cell differentiation. B cells develop into plasma cells in the presence of Irf4, Blimp1 and Xbp1. By contrast, B cell program is maintained in the presence of Pax5, Bach2 and Bcl6 [60].

T_{FH} cells play a crucial role for humoral immunity. T_{FH} cell differentiation is regulated by BCL-6, IRF4, Batf, c-Maf, and STAT3/5 [61]. Crosstalk between B cells and T_{FH} cells in the follicle leads to class switch recombination and affinity maturation in B cells. T_{FH} cells also control the generation of memory B cells and long-lived plasma cells in germinal center (GC). Thus, T cell help is required for maximal antibody production [62].

Upon antibodies binding to pathogens or target cells, the Fc region of antibodies initiates activation of the classical complement system, facilitates phagocytosis, or mediates antibody-dependent cell-mediated cytotoxicity (ADCC), all of which can kill the antibody-bound target cells [63]. Different from conventional B cells, CD5-expressing B1 cells produce low-affinity antibodies independent of T cell help. B1 cells reside predominantly in the peritoneal and pleural cavities, and few B1 cells can be found in the secondary lymphoid organs. The physiological function of B1 cells remains unclear [64].

Alloreactive Antibodies

Alloreactive antibodies may be pre-existing in recipients prior to transplantation. Transplant patients with obvious preformed antibodies against HLA are called sensitized

patients, who are associated with greatest risk for antibody-mediated rejection (AMR). Previous transplantation, pregnancy and transfusions are the risk factors inducing preformed alloantibodies. To avoid severe AMR or hyperacute rejection in sensitized patients, it is critical to assess the serum levels of anti-HLA antibodies. To this end, patient's serum is mixed with complement and a panel of cells harboring diverse HLA antigens. The percentage of cells killed grossly represents the presence of anti-HLA antibodies. To assess the specific identity and intensity of anti-HLA antibodies, patient's serum can be mixed with latex beads bound with different HLA molecules, followed by flow cytometric analysis [65].

Donor-specific antibodies (DSAs) can be developed at any time after transplantation. Transplanted patients with de novo generated DSAs have lower 10-year graft survival compared with patients without obvious DSAs [66]. DSAs to HLA I appear earlier, whereas anti-HLA II DSAs develop late after transplantation. Non-HLA DSAs can also be found in transplant patients, but the pathogenic potential of non-HLA DSAs remains unclear [67]. Many patients with DSAs can exhibit good graft function, which promotes the studies to identify deleterious DSAs and their function. Recent studies indicated that the presence of DSAs to HLA-DQ, in particular the IgG1 and IgG3 subclasses, increases the risk of allograft rejection [68]. IgG1 and IgG3 are the most efficient IgG subclasses in C1q binding and activation of the classic complement pathway. In a recent study that enrolled 1016 individuals, patients with C1q-binding DSAs after kidney transplantation exhibited increased deposition of C4d within graft capillaries and the lowest 5-year rate of graft survival (54%), as compared with patients with non–complement-binding DSAs (93%) and without detectable DSAs (94%) [69]. To further understand the origin and identity of deleterious and beneficial DSAs, the molecular regulation of plasma and B cell differentiation needs to be defined in transplant setting.

Antibody-Mediated Rejection and Treatments

Alloreactive antibody contributes to hyperacute, acute and chronic allograft rejections. Hyperacute rejection is a complement-mediated response and could occur within minutes in recipients with pre-existing anti-donor antibodies, such as ABO blood type antibodies and anti-HLA DSAs. The incidence of hyperacute rejection is rare due to the requirements for blood and tissue typing prior to transplantation, as well as the availability of therapies. If a sensitized patient will be transplanted, pre-existing antibodies may be removed by plasmapheresis and inactivated by intravenous immunoglobulin (IVIG). IVIG is highly purified polyvalent IgG derived from pooled human plasma. IVIG suppresses inflammatory and immune response through unidentified mechanisms. Moreover, rituximab, an anti-CD20 mAb depleting CD20–expressing B cells, may be used to suppress antibody production [70].

Early episode of acute rejection begins days after transplantation, whereas the recurrent episodes of acute rejection occur in months to years. Recurrence of acute rejection is a serious problem and causes long-term damage to the grafts. Acute rejection involves acute cellular rejection (ACR) and AMR. AMR was identified in 12–37% of biopsies taken for grafts with acute rejection. The biopsy features of AMR are diffuse deposition of complement C4d and morphologic indications of acute tissue injury. Evidence of graft dysfunction and the

serologic presence of DSAs are also requisite diagnostic elements for AMR [66]. The presence of AMR is associated with a poorer prognosis [65, 66].

Current immunosuppressive drugs prevent T cell-mediated ACR but may be less effective to control B cell-mediated AMR. Theoretically, desensitization agents (e.g., IVIG, plasmapheresis, and rituximab) may be used to treat AMR. However, none of these approaches selectively target memory B cells or antibody-producing plasma cells. Recently, bortezomib, a proteasome inhibitor, has been shown to induce a transient decrease of bone marrow plasma cells and reverse acute AMR in kidney recipients [71, 72]. Moreover, eculizumab, an anti-C5 mAb that inhibits terminal complement cascade, significantly decreased the incidence of AMR in kidney recipients who had a positive crossmatch against their living donor [73, 74].

Chronic rejection may occur due to a poorly defined chronic immune attack to the allograft, which triggers the repair processes within the transplanted tissues. Although all components of the immune system may contribute to chronic rejection, transplant immunologists currently focus on antibody-mediated chronic damage. Endothelial cells may be the primary target of DSAs. Binding of antibodies to endothelial cells of the grafts induces complement activation. In the absence of immunoglobulin, C4d deposition is absent in allografts. Complement activation leads to destruction of endothelial cells by the membrane attack complex. Moreover, complement components in grafts facilitate leukocyte infiltration. Importantly, DSAs can induce vasculopathy in the absence of complement activation, which is elegantly shown by using C3-deficient mice [75]. Currently, there is no therapy to treat chronic rejection [68]. In addition to antibodies and complement, we should also study the mechanisms of action of T cells and macrophages in mediating chronic rejection.

Antigen-Presenting B Cells in Transplant Tolerance

B cells are capable of presenting antigens to T cells and releasing immune cytokines to regulate immune responses. Recent clinical studies found a significant increase of B cell numbers in operationally tolerant transplant patients [76, 77], who had enhanced expression of B cell differentiation genes *TCL1A* and *VH4-34*. Hence, B cells may be involved in maintaining transplant tolerance. Results from murine studies suggested that antigen-presentation by B cells diminishes antigen-specific T cell responses in some settings, and facilitates the generation and expansion of Treg cells [77, 78]. Moreover, B cells produce some suppressive cytokines under inflammatory conditions. In particular, both $CD5^+$ B1 cells and regulatory B cells can produce IL-10 [79], which inhibits and reverses the progression of inflammation. Our understanding of B cells in transplantation is still in its infancy, therefore, both immunity and regulatory properties of B cells need to be further clarified.

Conclusion

Current immunosuppressive drugs are associated with significant side effects and increased risks for opportunistic infections. Thus, transplant research needs to seek novel

immune interventions that selectively abrogate allogeneic immune responses but leave the remainder of the immune system intact. Resolving this issue requires in-depth understanding of cellular and humoral responses in transplantation, in particular, the molecular mechanisms underlying T and B cell function. In this chapter, we discussed T and B cell biology as well as their roles in transplantation, and hope that such knowledge will be effectively applied in the near future for the induction of transplant tolerance.

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Chapter 3

Historical Remarks of Immunosuppressive Therapy in Organ Transplantation

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Abstract

Immunosuppression is still a major approach currently used for the prevention and treatment of allograft rejection. In this chapter, historical milestones of immunosuppressants in organ transplantation were reviewed. From 1960 to 1980, azathioprine, steroids, and cyclophosphamide were the main treatment options for the prevention of solid allograft rejection. Induction therapies were used quite early with polyclonal antilymphocytic serum (ALS), to be followed by more specific and potent agents up to date. There were some notable and good long-term allograft survivals in patients transplanted with kidney, heart, liver and pancreas in this period, but the high transplant failure rates and serious side effects of the drugs did not provide confidence to clinicians. The discovery of cyclosporine A (CsA), a calcineurin inhibitor (CNI), opened a new era in immunosuppressive therapy since 1980s. CsA treatment significantly improved 1-year allograft survival rates. In 1987, another powerful CNI, tacrolimus (FK506, Prograf), was discovered and was later used in organ transplantation. Both CNIs reduce the short-term morbidity of transplant patients by lowering the incidence of acute allograft rejection. Nevertheless, long-term allograft survival has not improved significantly over the past 25 years, and CNIs induce chronic allograft nephropathy. From 1990s, sirolimus and mycophenolate mofetil (MMF) were also discovered and used for transplantation. MMF, an inhibitor of inosine monophosphate dehydrogenase (IMPDH),

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is a powerful purine analogue replacing azathioprine, while sirolimus inhibits mammalian target of rapamycin (mTOR) function. Since then, scientists have been trying to use these non-nephrotoxic regimens and some biological agents for CNIs reduction/withdrawal/avoidance and steroid avoidance, while maintaining low acute rejection rates, maximizing long-term allograft survival, modulating the risk of cardio-metabolic side effects, and avoiding opportunistic infection and malignancy. Results from preclinical and clinical studies suggest that the use of some selected combinations of immunosuppressants are more effective for preventing allograft rejection and have considerably less side effects.

Keywords: azathioprine, ASKP1240, belatacept, calcineurin inihibitor, cyclophosphamide, cyclosprine A, immunosuppression, mycophenolate mofetil, sirolimus, steroid, transplantation, tacrolimus

Abbreviations

ALG: Antilymphocyte globulin
ATG: Antithymocyte globulin
AZA: Azathioprine
BPAR: Biopsy-proven acute rejection
CNIs: Calcineurin inhibitors
CsA: Cyclosporine A
CTLA-4: Cytotoxic T-lymphocyte-associated antigen 4
CMV: Cytomegalovirus
CPP: Cyclophosphamide
FDA: Food and drug administration
GFR: Glomerular filtration rate
GVHD: Graft-versus-free diseases
HSCT: Hematopoietic stem cell transplantation
IMPDH: Inosine monophosphate dehydrogenase
IVIG: Intravenous immunoglobulin
IL-2RAs: Interleukin 2 receptor antagonists
MMF: Mycophenolate mofetil
MPA: Mycophenolic acid
mTOR: Mammalian target of rapamycine
NODAT: New onset diabetes after transplantation
PTLD: Post-transplant lymphoproliferative disorder
SRL: Sirolimus
α-SMA: α-smooth muscle actin
TAC: Tacrolimus
TGF-β: Transforming growth factor β

Introduction

Organ transplantation is one of the best choices of treatment for end-stage organ failures [1, 2]. Over the past two decades, improvements in clinical care, the discovery of new and potent immunosuppressants, and the use of multiple immunosuppressive strategies have reduced short-term morbidity and lowered the rates of acute rejection. Immunosuppression is still the major approach currently used for the prevention and management of transplant rejection. Calcineurin inhibitors (CNIs), cyclosporin A (CsA) and tacrolimus (FK506, Prograf), have made great contributions to the prevention of acute rejection in human organ transplantation [1, 2]. In the past 25 years, 1-year renal graft survival rates increased significantly from 50% to nearly 90% in cadaver-donor transplantation, and to 95% in living-donor transplantation [3−5]. This is mainly contributed by CNIs-based immunosuppression [4]. However, long-term graft survival has not improved dramatically and 10-year survival rates fall down to 51% in cadaver-donor transplantation and to 68% in living-donor transplantation, which are mainly due to chronic nephropathy [3, 6−8]. The main culprits are the CNIs which, like a double-edged sword, have the negative effect to induce the progression of chronic allograft nephropathy and accelerate long-term allograft decline [9−11]. As a consequence, long-term graft survival has not improved significantly over the past 25 years [8].

In the past 10 years, scientists have aimed to minimize the use of CNIs for avoiding nephrotoxicity and to develop new biological immunosuppressants, which will: 1) maintain low acute rejection rates; 2) maximize long-term allograft survival with preserved allograft function; 3) reduce cardio-metabolic side effects seen with CNIs; and 4) avoid opportunistic infection and malignancy. In this chapter, we will review the milestones of immunosuppressive therapy and the history of the development of major immunosuppressants since 1960s. We will also discuss the recent discoveries that may lead to the development of more selective, potent and less toxic immunosuppressants for the treatment of organ transplantation and autoimmune diseases.

Azathioprine (1960s – 1980s)

The use of azathioprine in organ transplantation was a cornerstone in the development of immunosuppressants. Azathioprine (AZA) is a thiopurine drug that affects cell proliferation by inhibiting the synthesis of nucleic acid. Originally, scientists developed the thiopurine drugs for the treatment of cancer [12], and one of the thiopurine drugs, 6-mercaptopurine (6-MP), is effective in the treatment of certain leukemia.

Roy Calne is a pioneer in the development of azathioprine as an immunosuppressant. He initially found that 6-MP was effective in the prevention of renal allograft rejection in dogs in 1960 [13]. In the same year, Zukoski and Hume [14] reported the efficacy of 6-MP in preventing canine kidney allograft rejection. Roy Calne further tested the immunosuppressive function of various thiopurine drugs in Joseph Murray's laboratory in Boston and found that an imidazole-linked 6-MP (later named azathioprine) was slightly more potent than the other tested compounds [15].

In 1963, Goodwin [16] reported that a high-dose of corticosteroids could reverse an acute rejection episode in clinical renal transplant, and that combination of corticosteroids and azathioprine achieved better results in prevention of renal allograft rejection than either as a single agent. Meanwhile, Starzl and his colleagues [17] in Denver used azathioprine and corticosteroids as their immunosuppressive protocol for liver transplantation in clinic.

The combination of azathioprine and steroids had been used to prevent acute rejection in the kidney, heart, liver and pancreas transplantation for around two decades prior to the 1980s. Although long-term allograft survival can be achieved in some transplant patients, the high failure rate and serious side effects became major obstacles. The main adverse effect of azathioprine is bone marrow suppression, which can be life-threatening. This encouraged scientists to find more potent and less toxic immunosuppressant. The introduction of CsA in the early 1980s represents a "watershed" in the history of immunosuppressive therapy [18]. Since then, triple therapy with CsA, azathioprine, and steroid had been the most common regimen for transplant patients worldwide until the new potent purine analogue mycophenolate mofetil (MMF) was introduced in clinic in 1990s to replace azathioprine.

Azathioprine inhibits both DNA and RNA synthesis by interfering with the precursors of purine synthesis and suppressing *de novo* purine synthesis. It is thus effective to inhibit the proliferation of T and B cells, as well as suppress both primary and secondary antibody responses. Azathioprine is less effective to prevent the production of cytokines. The main side effects of azathioprine are marrow suppression, hepatic dysfunction and, in some cases, gastrointestinal disturbance.

In summary, azathioprine prevents lymphocyte proliferation by inhibiting an enzyme required for the nucleic acid synthesis. Before the introduction of CsA in the early 1980s, and for two decades, azathioprine had been a key immunosuppressant for organ transplantation. Today, however, it has been replaced by another purine analogue, MMF.

Cyclophosphamide (1960s – 1980s)

Cyclophosphamide (CPP) is an anticancer drug developed in 1958 in Germany [19], and since then it has served as a cornerstone for cancer therapy, in particular for lymphoma and leukemia. CPP also non-specifically supresses the immune system and was used to treat autoimmune diseases and prevent graft rejection. In the 1960s, CPP was used to treat renal allograft rejection [20, 21], but the results were not so encouraging. Starzl reported in 1971 [22, 23] that CPP combined with prednisone and horse antilymphocyte globulin (ALG) prevented acute renal allograft rejection in patients. In addition, in some hepatic and renal recipients who had been treated with azathioprine for months or even years, CPP was substituted for azathioprine to reduce azathioprine-mediated liver injury. Graft function was maintained in those patients after replacing the drug [22]. Other authors reported the results of a triple therapy consisting of CPP, azathioprine and prednisone in the first post-transplant month in kidney transplantation recipients. This CPP-containing regimen displayed no significant differences in patient or graft survival, graft function, or infectious complications when compared with those of a non-CPP regimen [24]. After MMF was used in transplantation patients to replace azathioprine [25], the application of CPP in transplantation became even less common. CPP has serious and even fatal side effects, which are varied

among individual cases. One of the major side effects of CPP is bone marrow suppression [26−31].

B-cells- and antibody-mediated allograft rejection are detrimental for long-term allograft survival [32]. Because CPP is more toxic to B cells than to T cells [33], scientists regain interests in the use of CPP in some special cases of transplantation. For instance, combinations that include CPP, plasmapheresis, intravenous immunoglobulin (IVIG), and rituximab (a chimeric monoclonal antibody against the protein CD20) are utilized to treat transplant patients who are highly sensitized to HLA alloantigens [34]. A triple chemoimmunotherapy (CPP, prednisone, and rituximab) has also been used to treat patients with post-transplant lymphoproliferative disorder (PTLD) after organ transplantation [35]. Moreover, CPP remains a possible treatment for autoimmune diseases and has shown efficacy in abrogating autoimmunity [36, 37].

Induction Therapies (1960s – Present)

In order to minimize the triggering of rejection processes after solid organ transplantation, short-term treatments were developed to be used immediately before and/or after transplantation. The idea was to prevent early acute rejection, allowing also a reduction of long-term immunosuppression [38, 39].

The first clinically used agent was antilymphocytic globulin (ALG). Initially developed in Boston [40−42], its first clinical use occurred in 1966 by Thomas Starzl in Denver [43, 44]. Lymphocytes extracted from the spleens of cadaveric donors were used to immunize horses. Their sera were then harvested, purified, and administered to kidney recipients at the time of transplantation and for about two weeks. This agent was thus rich in polyclonal antibodies to lymphocytes, and was quite potent. It had two major disadvantages: a compulsory intra-muscular administration that was very painful, and the occasional development of serum sickness. Ultimately, this approach was refined by the use of human thymocytes administered to rabbits, a much less immunogenic species [45−50]. This anti-thymocyte globulin (ATG) proved very efficacious, with only very rare occurrences of intolerance, and could be administered intravenously. It is still in wide use today [51, 52].

The next step was the development of monoclonal antibodies that target specific molecules responsible for triggering T-cell activation after transplantation. OKT3 was developed as an anti-CD3 agent, and was the first monoclonal antibody to be approved for clinical use in human renal, heart and liver transplantation [53−55]. It was potent, but its allogeneic property–evokes severe allergy. Furthermore, the animals used to develop this agent were mice, and frequently organ recipients developed anti-murine antibodies that inactivated the active agent. It is uncommonly used now [56].

One of the latest and most successful family of monoclonal antibodies targets IL-2 receptor alpha (IL-2Rα, CD25). The use of these monoclonal antibodies does not lead to lymphocyte depletion, but rather prevention of early T cell activation. Basiliximab [57−61], daclizumab [62−67] and alemtuzumab [68−73] are IL-2R antagonists that have been proved efficacious and had very rare side-effects [57−73]. Administered for a short duration as an induction agent at transplantation, they are still widely used currently [60−62, 67, 72, 73].

Cyclosporine (1980s – 1990s)

Cyclosporine (cyclosporin A, CsA) has revolutionized the immunosuppressive therapy for organ transplantation since 1980s. The use of CsA has dramatically improved the 1-year survival rates of kidney transplantation. With the treatment of CsA, 1-year patient survival rates were improved significantly in liver, heart, lung, and small bowel transplantation [74−83]. Although the newer and powerful CNI, tacrolimus, was introduced in 1990s, CsA still continues to play an important role in some immunosuppressive protocols. A CsA analog, voclosporin, has recently been shown to be as efficacious as tacrolimus in the prevention of acute rejection, and is associated with a reduced incidence of new onset diabetes after transplantation (NODAT) [84, 243].

CsA was initially developed as an antifungal agent with limited efficacy. It was isolated from two strains of imperfect fungi from soil samples by a microbiology lab at Sandoz (Basel, Switzerland) [85]. There were 25 natural lipophilic cyclic undecapeptides and more than 2,000 derivatives originally obtained from fermentation products of the fungal species *Tolypocladium inflatum Gams* [86−88]. Only cyclosporin A, cyclosporin C, cyclosporin G, and cyclosporin M exhibited immunosuppressive function *in vivo*.

Soon after the discovery of immunosuppressive properties of CsA, it was shown to effectively prevent the rejection of vascularized organ allografts in various preclinical models, such as transplantations in rats, rabbits and dogs [89−93]. Roy Calne's group at Cambridge University then conducted the clinical trials of CsA in organ-transplant patients [94]. In the early 1980s, CsA was approved for use in kidney-transplant patients, first in Europe and then in the United States.

Neoral (Sandimmune Neoral; Novartis Basal, Switzerland), a microemulsion formulation of CsA, was approved by the Food and Drug Administration (FDA) in 1995. This modified formulation improved bioavailability with more rapid absorption, and showed less variability in individual transplant patients [95, 96]. Since its introduction, a number of randomized and nonrandomized clinical trials have been conducted to determine whether the use of this new formulation improved outcome of transplant patients. Goel and his colleagues [97] reported that when compared to the previous formulation, the use of Neoral lowered the acute graft rejection rates in transplant patients, but exhibited no difference in patient and graft survival, renal function, and progression to chronic allograft nephropathy. Moreover, the long-term use of Neoral increased the risk of Kaposi's sarcoma (a tumor caused by infection with human herpes virus 8) in transplant patients, which may be attributed to the enhanced bioavailability of this microemulsion formulation [98].

In conclusion, in the early 1980s, two decades after the advent of azathioprine, the discovery and use of the first CNI, CsA, significantly improved the graft and patient survival. Thus, CsA represented a major advance in immunosuppressive therapy. In the past decades, because of various side effects (in particular nephrotoxicity) associated with CsA therapy, a number of efforts have been attempted to develop new regimen to allow CsA sparing or withdrawal. Nevertheless, CsA currently remains as one of the major immunosuppressants for organ transplantation.

Tacrolimus, Rapamycin and Mycophenolate Mofetil (1990s−2010s)

In this period, many new immunosuppressants were discovered and developed for clinical use in transplantation. The wide array of these new drugs facilitates the design of combined immunotherapies that simultaneously block different immune activation pathways and in turn optimally prevent allograft rejection. Meanwhile, deleterious drug side effects may be avoided by selecting drug combination with non-overlapping toxicity or by reducing the use of toxic drugs.

Tacrolimus (FK506, Prograf)

Tacrolimus (FK506, Prograf) is a macrolide compound isolated from *Streptomyces tsukubaensis*, a soil fungus found at the base of Mount Tsukuba near Tokyo [99]. Its immunosuppressive properties were first identified in 1984 during a screening program aimed at the discovery of new immunosuppressants. Further studies in Japan and at the University of Pittsburgh defined its mechanism of action as well as its potency in preventing allograft rejection in animals [100−104]. In the spring of 1990, a clinical trial of tacrolimus as a primary immunosuppressive drug was initiated at the University of Pittsburgh. Results from this trial showed that tacrolimus has an advantageous therapeutic index in liver transplantation [105]. Because of these encouraging results, multicenter randomized trials of tacrolimus in transplantation have subsequently been conducted [106, 107]. The use of tacrolimus led to significantly fewer and less severe acute rejection episodes in transplant patients compared with the CsA treatment [108−112].

Immunosuppression is still the major approach currently used for the prevention and management of transplant rejection. CNIs, CsA and tacrolimus, have been available for clinical use for decades. Though the mechanism of action is similar to CsA, tacrolimus is 10- to 100-fold stronger to inhibit lymphocyte proliferation and more potent to prevent allograft rejection in both experimental and clinical transplantations [113–115]. At present, tacrolimus is the mainstay for immunosuppressive therapy after transplantation and are important components of most immunosuppressive protocols [116–118].

Both CsA and tacrolimus inhibit lymphocyte activation and proliferation, as well as decrease the cytokine production in antigen-stimulated helper T cells [99, 117, 119]. Mechanistically, CsA and tacrolimus bind to different intracellular proteins, cyclophilin and FK506 binding protein (FKBP), respectively. However, both cyclophilin/CsA and FKBP/FK506 complexes inhibit the phosphatase activity of calcineurin, and in turn inhibit the activation of NFAT transcription factors to suppress lymphocyte activation and cytokine (e.g., IL-2) production [120]. Because CsA and tacrolimus affect the calcineurin/NFAT pathway in a similar manner, they show quite similar side effects, such as nephrotoxicity, neurotoxicity, and hyperglycemia [2, 18, 120, 121]. To mitigate those side effects, CNI-free and CNI-reduced regimens (e.g., sirolimus and MMF) are under investigation in transplant patients [122–125], which will be discussed in detail in Chapter 8.

Sirolimus and Everolimus

Sirolimus (AY-22989, rapamycin, SRL) is a microbial product isolated from the actinomycete *Streptomyces hygrocopicus*. It was initially discovered as an antifungal agent in 1975 by Sehgal and colleagues [126, 127], but later its immunosuppressive properties were discovered. After the introduction of tacrolimus in 1990s, the chemical structures of sirolimus and tacrolimus were found to be similar. This led two independent research groups to investigate the immunosuppressive function of sirolimus in preclinical transplantation models [128, 129]. In addition, Novartis synthesized a derivative of sirolimus, SDZ-Rad (Everolimus, RAD001, Certican), which has a 2-hydroxyethyl chain substitution at position 40 of sirolimus structure. Everolimus is more water-soluble than sirolimus and thus improves its oral bioavailability [130]. In renal transplant recipients who received single doses of everolimus capsules, everolimus exhibited a much shorter half-life than sirolimus (16−19 hours) and a rapid absorption (maximal concentration reached within 3 hours) [131].

Sirolimus and everolimus impair T cell proliferation by inhibiting the mammalian target of rapamycin (mTOR). The efficacy of these mTOR inhibitors in preventing acute rejection and their side effects has been extensively investigated. For instance, clinical studies conducted in Europe have examined the efficacy of concentration-controlled sirolimus in patients who received the first cadaveric renal allograft. The control group was treated with CsA, and all patients received azathioprine and prednisolone. The incidence of acute rejection in the sirolimus group was similar to that in the CsA group, but these two drugs exhibited different toxicity profiles [132]. In a subsequent study in which azathioprine was replaced by MMF, there was also no significant difference in the incidence of acute rejection and in patient and graft survival rates between the sirolimus- and CsA-treated groups [133]. Further follow up of the above two studies showed significantly higher glomerular filtration rates (GFR) and lower serum uric acid in sirolimus-treated patients than in CsA-treated patients at 2 years post-transplantation. Thus, regarding the renal function, sirolimus has a favorable safety profile compared to CsA [134]. However, results from large-scale trials [135, 136] suggested that the combination of sirolimus and MMF produced inferior renal transplant outcomes compared to the commonly used regimens, and that in combination with daclizumab/MMF/corticosteroids, low-dose tacrolimus may be more advantageous than low-dose sirolimus for maintaining renal function and allograft survival. Webster et al., have analyzed the results from 33 trials. They found that, when mTOR inhibitors (sirolimus or everolimus) replaced CNIs, there was no significant difference in the incidence of acute rejection at one year post-transplantation, but the levels of serum creatinine was lower and bone marrow was more suppressed [137].

In organ transplantation, the strategy of multiple immunosuppressive drug therapy (namely, the use of a selected combination of drugs with distinct immunosuppressive properties) is aimed to enhance therapeutic efficacy while minimizing the toxicity of individual drugs used in the regimen. Sirolimus inhibits mTOR activity, an immunosuppressive mechanism distinct from those of CNIs. Therefore, efficacy of sirolimus combined with full-dose or reduced-dose of CsA was investigated in *de novo* renal recipients [138]. All patients also received steroids. The results showed that the combination of sirolimus and CsA was more potent than CsA alone in reducing the incidence of acute rejection episodes within the first 6 months post-transplantation. Among non-African-Americans, half-dose CsA and sirolimus was as efficacious as full-dose CsA and sirolimus in

preventing acute rejection. Subsequently, two large-scale Phase III studies, conducted in the United States [139] and in centers worldwide [140], have investigated the efficacy of a fixed dose of sirolimus (2.0 mg/day or 5.0 mg/day) combined with concentration-controlled CsA. Both studies showed that including sirolimus (in particular the higher dose sirolimus) in the regimen significantly reduced the rates of acute rejection in transplant patients. Furthermore, synergistic interactions of CsA and sirolimus were assessed by the median effect analysis of data collected from multiple clinical studies [141]. Without impairing the therapeutic efficacy (90% of patients were rejection-free), the use of sirolimus permitted a 2.2-fold reduction in CsA dose, whereas the use of CsA enabled a 5-fold reduction in sirolimus dose [141].

Sirolimus and tacrolimus are classified as an mTOR inhibitor and a CNI, respectively, but these two agents are structurally-related macrolides. Results from *in vitro* studies suggested that sirolimus and tacrolimus antagonize each other's biologic activity, such as suppression of T cell proliferation and IL-2 production [142−144]. This raises the question that whether sirolimus can be combined with tacrolimus to optimize graft and patient survival. Our previous preclinical studies indicated that sirolimus and tacrolimus in combination are not antagonistic *in vivo* but produce a synergistic effect in prolonging small bowel allograft survival in mice, heart allograft survival in rats, and kidney allograft survival in nonhuman primates [145−148]. Furthermore, low-dose sirolimus-tacrolimus combination has been shown to lower the incidence of acute rejection and maintain excellent graft function in clinical kidney, liver, pancreas and islet transplantations [149−152]. Several large clinical studies reported that the combination of tacrolimus with sirolimus has achieved acute rejection rates of less than 10% [153−155]. Potential side effects associated with the sirolimus-tacrolimus combination include hyperlipidemia, hypertension and lymphoceles [153, 156−158].

The combination of sirolimus and tacrolimus also improved the outcome of unrelated donor hematopoietic stem cell transplantation (HSCT). When compared with CsA plus MMF, the sirolimus-tacrolimus combination reduced the risk of acute and chronic graft-versus-host diseases (GVHD), lowered the non-relapse mortality, and improved the event-free and overall survivals. Moreover, the sirolimus-tacrolimus combination was associated with a significant reduction in cytomegalovirus (CMV) reactivation and minimal transplant-related toxicity [159−162].

It would be interesting to understand why sirolimus and tacrolimus act as antagonists to each other *in vitro*, but exert synergistic effect *in vivo* to prevent allograft rejection. Both sirolimus and tacrolimus interact with FKBP12, an abundant and ubiquitous protein. The abundance of FKBP12 and relatively low levels of sirolimus and tacrolimus *in vivo* could prevent the competitive inhibition of binding as seen in the *in vitro* assays [163, 164], and allow the simultaneous formation of sirolimus-FKBP12 and tacrolimus-FKBP12 complexes. Indeed, only a small fraction of the cellular pool of FKBP12 needs to be occupied by the drugs in order to achieve maximal immunosuppression [165]. Binding of FKBP12 with sirolimus or tacrolimus is not sufficient to inhibit T-cell function [164, 166] and mediate immunosuppression [143]. The tacrolimus-FKBP12 and sirolimus-FKBP12 need to further bind with calcineurin and the FKBP12/rapamycin binding (FRB) domain of mTOR, respectively, and in turn impede T-cell responses by blocking the calcineurin/NFAT and mTOR pathways. The synergistic *in vivo* effects of tacrolimus and sirolimus may be attributed to the inhibition of both pathways.

Mycophenolate Mofetil

Mycophenolic acid (MPA), a fermentation product of *Penicillium brevicompactum* and related fungi, was first isolated by Gosio more than a century ago and was identified as an inhibitor of nucleic acid synthesis in the 1960s [167, 168]. MPA is a highly selective, non-competitive and reversible inhibitor of inosine monophosphate dehydrogenase (IMPDH), a crucial enzyme in the *de novo* biosynthesis of guanosine. Thus, inhibition of IMPDH by MPA leads to a depletion of guanine nucleotides [169]. Proliferating lymphocytes are different from most other cells in the body in that they are dependent on both the *de novo* pathway and the salvage pathway of purine synthesis. Other cells mainly utilize the salvage pathway for purine synthesis. Hence, MPA can be a specific inhibitor for B and T lymphocyte proliferation by abrogating the IMPDH-controlled *de novo* pathway of purine synthesis [170]. Antibody formation is also inhibited by blocking this pathway [171−173].

Because of its poor absorption, high doses of MPA were used and were poorly tolerated. To improve the oral bioavailability, the mofetil analogue MMF was developed through MPA ester derivatization. MMF is rapidly hydrolyzed to the active MPA after oral administration, and thus is a prodrug of MPA [174].

The first clinical application of MPA was in the treatment of severe psoriasis. Two studies reported in the 1970s showed attenuation of psoriatic lesions following MPA administration [175, 176]. Once MMF had been developed, its immunosuppressive efficacy was promptly demonstrated by preclinical transplantation studies. MMF potently prolongs the survival of islet allografts in mice, heart allografts in rats, kidney allografts in dogs, and heart allografts in nonhuman primates [177−182]. Based on the strong preclinical evidence that MMF prevented allograft rejection with mild side effect, a phase I clinical trial was conducted in the early 1990s [183−185] to determine the safety and proper dosing of MMF in kidney transplantation. Patients were randomized into eight dosing groups and received MMF doses between 100 mg/day and 3.5 g/day. MMF was well tolerated in all dose groups. There was only one gastrointestinal adverse event and no overt hepatotoxicity, nephrotoxicity and neurotoxicity. A significant correlation between doses higher than 2.0 g/day and lower rates of rejection was observed. Later in 1996, an open label and multicenter trial reported the efficacy of MMF for the treatment of refractory, acute cellular rejection in kidney recipients [186]. The trial included 150 patients who were randomized to receive MMF or high-dose intravenous steroids. Six months after treatment started, graft loss and death was 45% lower in the MMF group compared to the high-dose steroid group. This significant reduction was maintained at the 12 months follow-up.

In the mid-1990s, the efficacy of MMF in renal transplant patients was reported in three large phase III, double-blind, multi-center trials [187−190]. The US and the Tricontinental study groups enrolled 499 and 503 patients, respectively, and both study groups compared MMF with azathioprine. The European study group included 491 recipients and compared MMF with placebo. In all these trials, MMF (2.0 g/day or 3.0 g/day) was combined with CsA and corticosteroids. Antithymocyte globulin (ATG) was used as an induction therapy in the US study group, but not in the other two study groups. The primary endpoint was biopsy-proven acute rejection (BPAR) or treatment failure during the first 6 months after transplantation. Results from these three studies indicated that the frequency of acute rejection in MMF-treated patients was approximately half of that in patients who received azathioprine or placebo. The Tricontinental study group further performed an analysis of the

results of MMF-based therapy at the end of 3 years post-transplantation, and indicated that MMF significantly reduced graft losses (due to acute rejection) from 9.9% in azathioprine-treated patients to 5.8% and 3% in patients treated with MMF 2.0 g/day and 3.0 g/day, respectively [189].

Importantly, patients who had acute rejection within the first 6 months exhibited high incidence of graft loss at later time points [191]. MMF significantly reduced the rates of early acute rejection, and should have a beneficial effect on long-term graft survival. Indeed, an analysis of outcome of 66,774 renal recipients in US Renal Transplant Registry demonstrated that the MMF-based therapy produced significantly better 4-year patient and graft survival. The risk of late acute rejection episodes was reduced by 65% [181], and the risk of chronic allograft nephropathy was reduced by 27% [191, 193].

Based on the above-mentioned three large international trials in renal transplant patients, MMF was approved by FDA for clinical application in 1995. As of today, mycophenolate is commercially available as prodrug MMF (Cellcept, Roche) and EC-MPS (Myfortic, Novartis). Generic forms of MMF are also available. After two decades of clinical experience, MMF has now gained wide acceptance in transplantation based on its relatively low and tolerable side-effects, as well as its potent efficacy in improving patient and graft survival.

Current Mainstay Immunosuppressants

CNIs (CsA and tacrolimus) remain a mainstay therapy for transplantation. The use of CsA has decreased from approximately 79% in renal transplant patients in 1996 to only 15% in 2005, while the use of tacrolimus has increased from 13% to 76% in the same time period. This trend reflects mainly the potency of tacrolimus in preventing graft rejection [194−198]. CNI-based combination therapies (e.g., tacrolimus and azathioprine, CsA and MMF, and tacrolimus and MMF) showed a low risk of acute renal allograft rejection. However, in patients who experienced delayed allograft function, tacrolimus in combination with either MMF or azathioprine exhibited superior outcomes at 3 years post-transplantation as compared to CsA in combination with MMF [199−201]. In general, CsA is currently only used in selected renal transplant recipients who have potential high risk of post-transplant diabetic mellitus to minimize the risk of complications.

In liver and other organ transplantations, tacrolimus also have superior efficacy in promoting patient and graft survival, as well as in reducing the rejection risk and severity compared with CsA [202]. Randomized comparative studies demonstrated that 62% of patients randomized to tacrolimus after liver transplantation were alive at the 3-year follow-up compared to 42% in the CsA group [203, 204]. Nevertheless, these differences were not observed in hepatitis C virus (HCV) positive patients with liver transplants [205, 206]. Because the potential benefits of CsA in reducing HCV reactivation and in minimizing the risk of post-transplant diabetes are not fully confirmed, the preferred agent (CsA *vs.* tacrolimus) in liver transplantation remains unclear [207].

MMF strongly inhibits lymphocyte proliferation and impairs both cellular and humoral immunity. Sirolimus inhibits T cell proliferation, differentiation, and cytokine production. Partial replacement of CNIs with MMF and sirolimus have presumed benefits to alleviate the deleterious side effects related to the long-term use of CNIs, such as chronic allograft

nephropathy, neurotoxicity, and hyperglycemia. As of today, multiple immunosuppressive drug therapy (the selected combinations of CNIs, MMF, and sirolimus) remains the optimal strategy for organ transplantation in terms of enhancing therapeutic efficacy while minimizing the toxicity of individual drugs.

Replacement of CNIs and Application of Biological Agents (2010s – Future)

The use of MMF to achieve CNI dosage reduction has been generally accepted as a way to moderate immunosuppression and decrease CNI-related side-effects, such as nephrotoxicity, hypertension, hyperlipidemia, hyperuricemia and gingival hyperplasia that are associated with the CsA treatment, as well as nephrotoxicity and diabetes mellitus that are commonly associated with the tacrolimus treatment [208−210]. Results from a controlled trial proved that in patients who have already developed chronic allograft nephropathy, addition of MMF followed by withdrawal of CsA led to a significant improvement in graft function in terms of improved creatinine clearance [211].

Results obtained from DeKAF clinical trials [212−214] and other studies [215, 216] suggested that the majority of late graft losses attribute to the anti-donor antibody-mediated graft injury. Because CNIs remain as the most potent immunosuppressants, the use of CNIs is still required to prevent immunologic graft injury during the early phase post-transplantation. In theory, at later time points after transplantation, CNIs may be converted to less nephrotoxic agents to further control anti-graft humoral response and reduce CNI nephrotoxicity [217, 218]. Hence, efforts are underway to identify optimal maintenance regimens in a CNI-free setting.

CNIs Nephrotoxicity

Nankivell et al., [219] evaluated the natural history of allograft nephropathy using biopsy samples from simultaneous pancreas-kidney recipients. They characterized allograft nephropathy by two distinct phases of injury. The initial phase was represented by early tubulointerstitial damage, which was presented in about 94% of patients and was induced by ischemic injury, prior severe rejection, and subclinical rejection. The late phase of allograft nephropathy occurred more than one year post-transplantation and was represented by microvascular and glomerular injury. The use of CNIs resulted in progressive high-grade arteriolar hyalinosis with luminal narrowing, increased glomerulosclerosis, and additional tubulointerstitial damage. Nephrotoxicity was almost universal at 10 years, irrespective of early histologic findings. Further studies [220] demonstrated that CNIs also induced fibrogenic responses in one-year renal transplant biopsies, as evident by the increase in mRNA levels of transforming growth factor β (TGF-β), collagens α-1(I), and α-1(III), as well as the elevated protein expression of TGF-β and α-smooth muscle actin (α-SMA). Taken together, CNI nephrotoxicity in kidney transplantation is a significant concern and appears to be progressive over time when CNI exposure is maintained [219, 221].

CNIs Replacement with MMF and Sirolimus

In recent years, scientists have tried to improve transplantation outcome by avoiding CNI exposure and using non-nephrotoxic immunosuppressants (e.g., sirolimus and MMF) [222−230]. MMF, a reversible IMPDH inhibitor, potently blocks lymphocyte proliferation and reduces inflammation, and was shown to significantly reduce the risk of chronic renal failure in transplant patients [231, 232]. Sirolimus is a potent inhibitor of mTOR, which is a key kinase involved in T-lymphocyte activation and proliferation [233]. The complementary properties of these two agents may provide a combined immunosuppressive strategy with the potential for delaying or preventing progressive renal allograft dysfunction [234]. For instance, Guba and colleagues have recently reported [225] the results of the SMART trial, and indicated that early conversion to a CNI-free immunosuppression with sirolimus, MMF and steroids was associated with a sustained improvement of renal function up to 36 months after transplantation as compared with a CsA-based regimen.

At 36 months' follow-up, patient and graft survival were excellent in both treatment groups, and there was no difference in late rejection episodes. Late infections and adverse events were similar in both groups except higher rate of hyperlipidemia in the CNI-free maintenance group and higher incidence of malignancy in the CsA-based group. Moreover, the Spare-the-Nephron study from USA [222] also evaluated the combination of MMF and sirolimus as a CNI-free regimen for maintaining renal allograft survival and function. In this open-label multicenter trial, 299 transplant patients were randomized to receive either MMF/CNI or MMF/sirolimus maintenance regimen. At 2 years' follow-up, compared with MMF/CNI treatment, the MMF/sirolimus treatment resulted in similar measures of renal function but was associated with fewer deaths and less graft loss.

In contrast to the above mentioned studies, the ELITE-Symphony and ORION Studies [235, 236] reported that inclusion of tacrolimus in the maintenance regimen may be advantageous for renal allograft survival and function, and sirolimus-based maintenance regimens were not associated with improved transplantation outcomes. In addition, the ZEUS study [237] showed that conversion to the everolimus-based maintenance regimen resulted in a higher GFR with similar graft and patient outcomes, but led to higher rejection rates at 36 months' follow-up compared with continuous use of CsA-based regimen.

Application of Biological Agents

The new strategies using costimulation blockade agents may help to minimize or withdraw the use of CNIs, or even facilitate the development of safe CNI-free regimens [238, 239]. Belatacept is a fusion protein composed of the extracellular portion of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and the Fc fragment of a human IgG1 immunoglobulin. It is the first biological costimulation blocker approved by FDA for the prevention of renal allograft rejection. Phase II and III trials proved that the use of belatacept was associated with better preservation of GFR and improved metabolic end points when compared with CNIs. Recent studies showed that belatacept can be used as a first-line immunosuppressive agent, and that combination of belatacept and sirolimus successfully maintained kidney allograft survival without CNIs or steroids [240, 241]. These results need to be further validated in large, multicenter, randomized trials.

Costimulation blockade seems to be a very promising strategy for developing new immunosuppressive agents. In addition to belatacept, another costimulatory blocker, anti-CD40 monoclonal antibody (ASKP1240) has successfully completed a phase I trial, and phase II development in renal transplant patients is underway [242, 243].

Summary

The success of organ transplantation heavily depends on the development of immunosuppressants. The patient and allograft survival have been progressively improved following the historical discovery of azathioprine, glucocorticoids, cyclophosphamide, CsA, tacrolimus, MMF, sirolimus and belatacept. Scientists are constantly searching for more potent and selective immunosuppressive agents with the purpose of avoiding CNI-related nephrotoxicity and reducing metabolic and cardiovascular side-effects. Recently, clinical trials of sotrastaurin and tofacitinib as maintenance agents have been discontinued because of the side-effects after long-term use [242−247]. For the treatment of antibody-mediated humoral rejection, in addition to the available therapies (e.g., plasmapheresis, IVIG, and rituximab), clinical trials of novel agents such as bortezomib and eculizumab are underway [248−252]. At present, although there are very few potent immunosuppressive agents available for clinical trials, preclinical studies are being in progress to identify new candidates for drug development [253−257].

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Chapter 4

Animal Models in Immunosuppression

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Abstract

Cells of the immune system interact in a complex integrated network. Although *in vitro* the result of stimulating a single type of cell may be stereotyped and reproducible, *in vivo* this is often not the case. This phenomenon often necessitates the use of animal models in predicting the impact of immunosuppressant drugs. A heavy burden of responsibility lies on the shoulders of the investigator using animal models to study immunosuppressive agents. The principles of the three R's: refine (less suffering,), reduce (lower animal numbers) and replace (alternative *in vitro* assays) must be applied. Well-designed animal model experiments have allowed us to develop all the immunosuppressive agents currently available for treating transplant recipients. In this chapter, we examine the common animal models used in developing immunosuppressive agents, focusing on drugs used in transplant surgery. We look at the utility and limitations of small and large animal models in measuring potency and toxicity of immunosuppressive therapies.

Keywords: immunosuppression, animal model, transplant, immunology, drug

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Abbreviations

ALT: Alanine transaminase
AP: Alkaline phosphatase
APCs: Antigen presenting cells
AST: Aspartate transaminase
ATG: Anti-thymocyte globulin
AZA: Azathioprine
CNIs: Calcineurin inhibitors
CsA: Cyclosporine
CTLA-4: Cytotoxic lymphocyte antigen-4
HLA: Human lymphocyte antigen
MHC: Major histocompatibility complex
MMF: Mycophenolate mofetil
RAG: Recombinase activating gene

Introduction

The development of immunosuppressive drugs largely parallels the development of organ transplantation, as it was only with the availability of these agents that successful human therapeutic transplantation became possible. Prior to development of potent immunosuppressive drugs, only transplantation between identical twins was possible, as in the first successful renal transplant between humans in 1954 by the Nobel laureate Joseph Murray [1]. In the 1950s, experiments in dogs facilitated the development of 6-mercaptopurine and later its derivative azathioprine, important for short-term survival of renal allografts. These were introduced clinically for human renal transplantation during the 1960s. Both of these are purine analogues, acting as competitive inhibitors of DNA synthesis. Co-administration with cortisone and other steroids gave better outcomes. In the 1980s, cyclosporine was licensed as the first calcineurin inhibitor, and improved transplant survival dramatically. In the 1990s, this was largely superseded by the calcineurin inhibitor (CNI) tacrolimus, also known as FK506. Subsequently, azathioprine (AZA) was largely replaced by mycophenolate mofetil (MMF), an inhibitor of the enzyme inosine monophosphate dehydrogenase, important for purine synthesis. Modern transplant maintenance immunosuppressive regimens largely use triple therapy with tacrolimus, MMF and prednisone.

Immune responses are generally split into adaptive and innate responses [2−4]. In practise, the two systems interact a great deal. Cells of the innate family include dendritic cells, monocyte/macrophages and natural killer (NK) cells. They have conserved receptors which typically bind similarly conserved epitopes on alloantigens e.g., lipopolysaccharide which enable a response to so-called “danger signals” [5]. In evolution terms, the innate system is older and important for many taxa including plants and invertebrates. The adaptive system is thought to have evolved more recently, in jawed fish 500 million years ago [6]. T cell receptors and antibody are only present in jawed vertebrates. This naturally has implications for choice of animal model in simulating immunosuppression for humans.

Adaptive responses, driven by B and T lymphocytes, are specific to the particular proteins of the foreign substance, or alloantigen [7]. T cells mature in the thymus (hence their name), where they learn to distinguish between self and non-self, mediated through recognition of epitopes by the T cell receptor; formed by genetic rearrangements among V, D and J segments, making it the most heterogeneous protein in the body [8−10]. T cells govern the immune response, providing activation signals to other cells and directly lysing cells with perforin and granzymes. B cells on the other hand are responsible for the production of antibodies and typically require T cell help for initial activation. Similar to T cells, B cells undergo V, D, J recombination of their immunoglobulin receptor during somatic hypermutation to increase the affinity of the antibody they will ultimately produce for cognate antigen.

Animal models have been used extensively in the development of immunosuppressive drugs. Some models mirror specific autoimmune diseases, such as the extrinsic allergic encephalomyelitis mouse model for multiple sclerosis [11]. Animal models have been used to simulate all types of human transplant, common examples being heart and skin transplants [12]. Complementing the *in vivo* models, various *in vitro* techniques are available to elucidate the contribution individual cell types make to the overall immune response. These include routine analysis of blood levels of antibodies and antigens using enzyme-linked immunosorbent assay (ELISA). More specific investigations can include the analysis of mixed lymphocyte reaction (MLR), which examines the proliferation rates of T cells to alloantigen. Alternatively, flow cytometry can be employed to measure cell surface or internal expression levels of various markers of immune cell activation and maturation.

Rodent Models for Immunosuppression

In the early days of transplantation, the dog and pig model were most commonly used because it was technically easier to perform the larger vascular anastomoses. With the development of microsurgical techniques in the 1960s, rodents became the preferred model because of simplicity, favourable public and animal protective agencies opinion, and reduced costs [12]. The Rat genome was sequenced in 2004 [13]. Results revealed that the rat genome contains 2.75 billion base pairs. This compares with 2.9 billion in the human genome and 2.6 billion in the mouse. Humans have 23 pairs of chromosomes, compared with 21 in rats and 20 in mice. In spite of this the three species contain overall a very similar number of genes, and most disease-associated genes are highly conserved between the species.

Mice have proven invaluable in the assessment of immunosuppressive agents [12]. The widespread availability of genetically modified mice, both transgenic and knockout, has made them invaluable assets in experimental models. Unlike rats, embryonic stem cells have been isolated and genetically manipulated in mice. In rats, eggs are sensitive to activation and do not tolerate genetic modification well, although this has been resolved within the last decade with cloning techniques allowing production of genetically modified rats [14]. Interestingly, studies on the rat genome sequencing have demonstrated that immune system-related genes have the highest rate of evolutionary change. This diversification of lymphocyte genes may mean that it is more difficult to extrapolate results of animal models such as the rat to humans. Interestingly, genes involved in detoxification show important differences between

humans and rodents. Cytochrome P450 (CYP450) is important in metabolism of calcineurin inhibitors like cyclosporine, among other immunosuppressive drugs. The CYP450 subfamily member CYP2J has a single gene in humans, but four in rats and eight in mice [15]. It can be seen that although rodents bear similarities to humans and are vital to examine and test agents, there are important constraints in applicability due to these pharmacodynamic and pharmacokinetic differences.

The use of *in vivo* models facilitates the utilisation of various measurements to determine immunosuppressive efficacy of drug and others agents. Take alcohol for example – if administered to a mouse orally for a week, numbers of T and B cells in the thymus and spleen diminish [16−17]. If these cells are isolated and stimulated *in vitro* with a MLR, cellular proliferation is reduced. Steroids, when given to rabbits, give similar results to humans. If dexamethasone is administered, neutrophilia is seen within a day. This is accompanied with lymphopenia. Lymphocyte numbers in the bone marrow are increased. Both lymphocyte and neutrophil function is suppressed, as measured by concanavalin-A proliferation and reactive oxygen species production respectively [18]. Understanding of the complex effects that these agents have on the body could not be easily understood with *in vitro* models alone.

Organ-Specific Rodent Transplant Models

Transplant models in rodents have been invaluable in understanding the human immune response, as well as helping development of immunosuppressive agents. Various models are available each with their own utility [19]. Lung and intestine transplants are more readily rejected than heart, kidney and liver [20]. Skin transplants, the most commonly used non-vascularised organ, are very readily rejected. Liver transplants are often spontaneously accepted and become tolerant even in the absence of exogenous immunosuppression [1, 21, 22].

The skin transplant was the first model developed and used by Sir Peter Medawar in the 1940s and 50s in seminal experiments which led to the understanding of self and non-self [23]. This was the only model to study transplant immunology until the 1960s. The skin transplant model is technically straightforward. It involves excising a small square of full-thickness skin from the donor animal and deep fat is dissected off. This is placed on a similar-sized defect on the recipient abdominal wall and secured with glue or sutures. The graft is protected for 3−5 days with a dressing. This procedure will induce a rapid tempo of T cell mediated rejection within 7−12 days for mice different at the major histocompatibility complex (MHC) locus [24].

The first rodent vascularized organ transplants were performed in the rat but now virtually all organ transplants performed in the rat are also performed in mice. Although technically more challenging (due to very small diameter of vessels), mouse models of transplantation have several advantages. First, many congenic, transgenic, and knockout strains are available (Table 1). There are specific mouse strains available to study specific cell populations. Second, there are more reagents including monoclonal antibodies available for mice. Third, due to small body weight, mice need only about one tenth the quantity of drug that rats consume. The disadvantage of the mouse models is mainly technical, but it has been

shown that spontaneous acceptance of both liver and kidney graft occurs more frequently in all mouse strains compared to rats [25].

Table 1. Mice strains useful in Immunologic Experiments

Scid	Absence of functional T cells and B cells
RAG-1	Absence of functional T cells and B cells
Nude	Deficiency of T cell function
129/Sv	Deficiency of B cell function
Xid	Deficiency of B cell function
Beige	Decreased NK activity
DC-less	Absence of dendritic cells
MHC II deficient	lack of MHC class II
MHC I / II deficient	lack of MHC class I and II

Adapted from Cosgrove et al. [26].

Heart transplants are the most popular vascularised allograft performed. The heart is transplanted in a heterotopic position, with the veins of the heart tied off. The donor aorta is anastomosed to recipient aorta and donor pulmonary artery anastomosed to recipient inferior vena cava. This makes a non-physiological model of circulation, and the heart is subject to a moderate level of chronic ischemia, so histological changes do not exactly mirror those seen in humans [12]. Graft function is monitored by palpation of heart beating, or by using electrocardiogram [27].

Kidney transplants in the mouse are generally performed in nephrectomised animals so that it is a life-sustaining procedure. The kidney is revascularised and ureter connected to bladder. Graft function is monitored by analysis of serum creatinine, however this is technically difficult. In some MHC mismatched strain combinations e.g., BALB/c to C57BL/6, up to 40% of recipients become tolerant to the renal allograft in the absence of immunosuppression, in marked contrast to humans where this never occurs [28]. Again, this highlights the important differences between rodents and humans, limiting the translational reliability of these models.

Liver transplant in rats and mice are technically very challenging and success is directly related to very short anhepatic phase (< 20min). There are different techniques, with cuff and without cuff to perform the vascular anastomoses. The graft can be arterialized or not to simplify technique, however, arterialized grafts have better outcomes and long-term survival rates. The biliary reconstruction is always performed with stenting. Graft function is monitored with aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (AP), bilirubin and albumin [25].

Rodent Models of Rejection

The advent of DNA recombination technologies has allowed us to produce genetically modified murine models of antibody-mediated rejection. Recombinase activating gene (RAG) knockout mice lack the gene necessary for production of T cell and B cell receptors, so that they are B/T cell deficient. Anti-donor MHC antibodies injected into RAG knockout mice can

precipitate hyperacute (at time of transplant) or acute rejection of allografts. This is a weak model for hyperacute rejection, with the tempo and magnitude of rejection much less than that seen in humans. This is thought to be the result of excess complement regulatory proteins in mice, and can be overcome by the addition of exogenous complement. Anti-donor MHC antibody has also been injected to allograft recipient mice in models of chronic rejection [29]. In these models, the degree of human lymphocyte antigen (HLA) mis-match between donor and recipient is less. These studies have shown that complement fixation is not always necessary, and that other effector functions such as NK cell binding to antibody is adequate to drive chronic rejection [30].

Some mouse heart transplant models have shown the importance of regulatory B cells, which can down-regulate an immune response in an IL-10 dependent fashion [31]. These models have used induction with anti-T cell Ig and mucin 1 (TIM-1) in order to boost numbers of regulatory B cells. The process of accommodation has also been better understood through murine models. Accommodation occurs in human ABO blood group incompatible kidney transplants where the kidney survives in spite of alloantibody against it. The graft ameliorates antibody damage through the up-regulation of a number of protective regulatory proteins such as Bcl, haemoxygenase-1 (HO-1) and decay accelerating factor (DAF). Complement is activated to some extent on the graft, as evidenced by C4d deposition. This is seen in mouse xenotransplant models, for example in rat to mouse heart transplants, where cobra venom factor is used in induction to prevent early complement activation [32].

Mouse models have been used in developing tolerance strategies, where the allograft survives in the absence of ongoing immunosuppression [23, 28]. In some strain combinations, kidney transplants are accepted spontaneously, in the absence of any medication, for example transplantation from A/J (H-2a) to C57BL/6 (H-2b) murine genetic backgrounds. This is in contrast to the scenario in humans, where spontaneous tolerance has never been seen. Two other approaches to tolerance in murine models are mixed chimerism or co-stimulation blockade [33]. Mixed chimerism is the only approach to date which has been extrapolated to humans. Myeloablation is followed by donor bone marrow infusion around the time of organ transplant, and can reliably lead to donor-specific tolerance. Co-stimulation with cytotoxic lymphocyte antigen-4 (CTLA-4) or anti-CD154 also gives tolerance in the mouse model [34−36]. This has not been applied to humans. Indeed, anti-CD154 gives thromboembolic side-effects in humans, because of expression of CD154 on platelets in humans unlike mice [37].

Large Animal Models

The first recorded experimental large animal transplants took place from 1900−1910. Alexis Carrel who pioneered vascular anastomosis and performed renal and heart transplants on dogs in the absence of immunosuppression, experiments that led him to win the Nobel prize in Medicine in 1912. Kidneys were transplanted from rabbits to humans, but failed within a couple of days [1].

Although rodent models of immunosuppression have provided useful data, large animal models mirror much more closely the events in humans. Laboratory mice are typically bred in specific pathogen-free conditions, so their immune systems are relatively naive and have not

had alloantigen exposure sufficient to develop significant immunologic memory [12]. Rodents are also often transplanted very young, in the first few months of life, which further limits how well experiments can be extrapolated to humans, where recipients are mostly middle-aged. This contrasts with research primates, for example, which are captured in the wild, having had exposure to environmental pathogens which allow generation of immunological memory. Although experiments on outbred rodents bred in non-specific pathogen free circumstances are useful, large animal models bear more similarities to humans [38].

Almost all immunosuppressive agents are tested in large animals prior to phase one human trial for both safety and efficacy. In general, mechanistic studies for proof of concept are carried out *in vitro* and in rodents. Because of ethical concerns and expense, large animal studies are usually limited to proving effectiveness and lack of toxicity. Much important information about pharmacologic agents is gleaned from rodent studies, but there remain important constraints in the applicability to humans. Drug absorption is greatly different between large animals and humans [38−39]. Drug distribution also differs between species. Differences in target protein tertiary structure between species have implications for drug action, in particular for biologic antibody-based therapies. Although large animals are more immunologically similar to humans than rodents, there have been several examples of important differences, again chiefly where tolerance is more easily achieved in large animals than in humans. Large animal studies are used for final confirmation of safety and efficacy after extensive drug development studies in rodent models [38−39].

Studies of tolerance aim to have animals accept allogeneic organs / tissues long term in the absence of ongoing drugs. Duration of studies for immunosuppressive agents in animal models varies considerably. In rodent models, tolerance is often defined as survival beyond 100 days, whereas in large animals and humans, this usually is defined as several years. Tolerance studies are particularly prone to effects from the age of the animal. Younger animals are easier to tolerate as they have a more naive immune phenotype with less memory cells [28, 40].

Common Large Animal Models

Pigs, dogs and non-human primates are the large animal species generally used in testing immunosuppressive agents. They all share the same components of the immune system that humans have. They are also anatomically very similar to humans. The tempo and histological findings of organ rejection are also very similar to humans [38].

Early immunosuppressive experiments in the 1960s−1970s mostly used dogs, for example in the development of the anti-metabolites 6-mercaptopurine and azathioprine [1]. Over time, ethical concerns over the use of domesticated animals for research have gradually reduced the use of dogs for research. Dogs are still used for some research on islet and bone marrow transplant. Pigs have largely superseded dogs due to fewer ethical concerns for an industrially farmed animal. Their fecundity also makes them much more practical and has enabled the development of inbred major histocompatibility complex characterized mini-pigs [40]. Non-human primates are certainly the most similar to humans, and are most often used for immediate pre-clinical research. For work with biologic immunosuppressive agents such

as monoclonal antibodies, primates are essential, as dogs and pigs are often too antigenically different from humans for reagents to bind effectively [39]. However, although the use of primates is considered more appropriate because of the ability of antibodies to cross-react between species, it is not an exact science and there are exceptions such as a human anti-CD3 antibody which did not cross-react with macaque CD3 [42]. Furthermore, primates are very expensive to work with and raise much ethical concern from the public and scientists alike. Baboons, macaques (*Rhesus*, *cynomolgus* and *pigtail*) and chimpanzees are most commonly used. Baboons, being larger, are often used for xenotransplant experiments as there is space to fit pig organs into them. Chimpanzees are most similar to humans, as our closest living relatives (estimated 1−2% in nucleotide differences), but their endangered status makes their use ethically questionable [39].

As with smaller rodent species, a key aspect of variance in these large animals is differences in drug absorption. For example, although sirolimus is fairly well absorbed in humans, it is poorly absorbed in monkeys. In monkeys, unabsorbed sirolimus passes to the distal ileum, where it causes ulcerative lesions and severe diarrhea. Dogs also poorly absorb sirolimus, and are much more prone to tacrolimus toxicity. Pigs, in contrast, tolerate very high levels of CNIs, even to the extent of inducing tolerance in some transplant models [38].

Dogs have been used in the development of most small-molecule immunosuppressive agents, particularly AZA, cyclosporine (CsA), MMF and tacrolimus. Tacrolimus was also extensively tested in macaques prior to human use because of toxicity concerns. Interestingly, tolerance has never been achieved in dogs with small-molecule immunosuppressives alone, unlike the situation for pigs and primates. This may be because of dogs being used older when they have more immunological memory, and dogs being perhaps more outbred than pigs/primates. Thus dogs are often regarded as more immunologically stringent than pigs and primates because it is difficult to achieve tolerance [38].

Interestingly, cyclosporine or tacrolimus administration for a week at very high doses not tolerated in humans (trough level 60−80 ng/mL) can lead to tolerance of kidneys or livers in pigs. Indeed in around one fifth of pig transplants, a liver will become spontaneously tolerant in the absence of any immunosuppressive agent, albeit after a severe rejection episode [51, 41]. In primates, like pigs, tolerance can result sometimes from calcineurin inhibitor monotherapy [37–38]. Again, this points out the marked differences from humans, where there has never been tolerance following CNI monotherapy in an allogeneic transplant.

Rapamycin is well tolerated in swine, with side effects of hyperlipidaemia and pneumonia. Monotherapy prolongs allograft survival to a similar extent to standard triple therapy. Primates also tolerate rapamycin well, and monotherapy prolongs renal transplant survival to 30 days [43]. In humans, rapamycin is poorly tolerated at the time of transplant because of wound dehiscence and seromas. This complication was not predicted in animal models.

Costimulation Blockade

In an adaptive immune response, initiation involves binding of a T cell receptor to its cognate specific alloantigen in the context of MHC. In humans, major histocompatibility complex is known as HLA. Activation also requires the presence of costimulation from other

accessory molecules on the surface of the T cell and antigen presenting cell. This requirement for costimulation is less stringent for memory cells. This partly explains why young naïve specific pathogen-free rodents are more permissible to tolerance than older large animals which have many memory cells independent of costimulation [44].

There are many costimulation molecules. The best characterised is CD28 on T cells which binds B7.1 (CD80) and B7.2 (CD86) on antigen presenting cells. This CD28 provides the so-called signal two for T cell activation; with signal one being the T cell receptor – MHC interaction with antigen presenting cells. CD28 ligation activates downstream GTPases and causes IL-2 production. For the B cell-T cell interaction, the best characterised co-stimulation interaction is between CD40 on B cells and CD40L on T cells [35].

CTLA4 immunoglobulin, (CTLA-4-Ig) has been developed with activity against B7, as well as other immunosuppressive agents against CD40, CD40L and other costimulatory molecules [34]. These agents have been tested in rodents and non-human primates. Costimulation blockade has strong efficacy in rodent models, giving rise to tolerance for example in heterotopic heart transplant. In primates however, efficacy is less marked with prolongation of survival seen rather than tolerance. In non-human primate models, administration of CTLA4-Ig prolongs kidney transplant survival from 5-8 days to 20−30 days [34]. CTLA4-Ig prevents the binding of B7.1 (CD80) and B7. 2 (CD86) to CD28 and CTLA4. This is normally a stimulatory interaction, although inhibitory signals are also transduced by CTLA4. The reasons for reduced efficacy in primates are unclear, but it may be that the antibody has less affinity, or the need for costimulation is less with the more pronounced memory response. Efforts to improve the binding efficacy of CTLA4-Ig or to use separate antibodies to B7.1 and B7.2 have shown some albeit modest improvement in survival. Combining the antibody with other therapies including basiliximab (anti-CD25) and giving the antibody repeatedly as a maintenance rather than induction agent, have likewise shown some improvement in survival [34, 40].

The CD40-CD40L (CD154) costimulation interaction has also been the target for blocking strategies [37]. CD40L on T cells binds CD40 on antigen presenting cells (APCs), and seems to function more in activating the APCs rather than the T cell. Humanised antibody against CD154 has shown reasonable efficacy in primate models [46]. The dose used is generally higher than that for other antibody therapies, and may relate to the expression of CD40L on cells other than T cells, including platelets. Anti-CD154 has prolonged survival in renal, islet, heart and skin transplants in primates. When used as monotherapy with monthly injections for one year, transplant survival as long as five years has been seen, although there are typically changes of chronic rejection. There has been some debate about whether conventional immunosuppressive drugs are antagonistic with anti-CD154. Studies targeting CD40 itself have not shown as much efficacy as antibody against CD40L, suggesting the antibody has less avidity, or perhaps that CD40L has other functions than simply binding to CD40 [36].

The limitations of animal studies were highlighted very tragically in the development of the biological therapeutic agent TGN1412 [47, 48]. This is a humanized monoclonal antibody targeting the costimulation molecule CD28. It was aimed for use in rheumatoid arthritis and some forms of leukaemia. Initial studies in mouse and subsequent work in cynomolgus macaques gave promising results. Although an agonist anti-CD28 antibody, it caused significant immunosuppression. This was purported to be the result of preferential activation of regulatory T cells versus effector cells. Phase one clinical trials in humans were carried out

in London on eight healthy male volunteers. Two were given placebo. The remaining six were given TGN1412 at 1/500th the dose given to macaques. In spite of the low dose, all six rapidly developed severe cytokine release syndrome and became profoundly unwell. All were admitted to intensive care and treated with steroids. One volunteer had all fingers and toes amputated. All survived, but studies showed a persistent leucopaenia and particularly a deficiency of regulatory T cells. One volunteer has early signs of a lymphoid malignancy.

Predictably, the company developing TGN1412, TeGenero, became bankrupt shortly afterward. There was much criticism for the trial design, where all six volunteers received the drug within 20 minutes of each other, meaning it was too late to prevent drug administration to the other five when the first volunteer became unwell.

Reasons for the idiosyncratic human reaction to the drug are unclear, in spite of the dose being 1/500th that used in the primate study. Speculation is that the antibody was raised to human CD28, and therefore has a much higher avidity and potency in humans versus the macaques. Some suggested that effects on humans are more pronounced because of the abundance of memory cells when compared to laboratory animals which are raised in a more pathogen-free environment.

Strategies using CTLA4-Ig together with anti-CD40L have shown synergism in some circumstances. Results have been much more impressive in rodents than in primates. In primates, survival of renal allografts up to 300 days has been seen, with only a short induction course of CTLA4-Ig and anti-CD40L. Lower levels of alloantibody have been seen with combination therapy. This is somewhat contentious, and some say though that if the agents are administered singly in optimal doses that little synergism exists [35].

Cell Depletion

Cell depletional strategies began with the use of anti-CD4 and anti-CD8 antibodies. In dogs, these had a modest effect on survival of renal allografts, up to 35 days. When combined with total body irradiation to further deplete T cells, these antibodies could result in operational tolerance in baboons, prolonging survival beyond one year [40].

Depletional studies have used the anti-CD3 diphtheria immunotoxin chimera [42]. This provides very potent T cell depletion, and kills the T cells unlike the human anti-CD3 OKT3 which simply causes internalization of CD3. Results using this immunotoxin have been very impressive, with operational tolerance in many primate transplant models. Survival over two years without histologic changes of chronic rejection has been observed in primate renal transplants.

In humans, T cell depletion strategies are potent as in primates, although tolerance has not been seen in the absence of mixed chimerism. Depletion at the time of transplant reduces the need for long-term maintenance immunosuppression. Antibodies used in primates such as the anti-CD3 immunotoxin are not usable in humans because of the different protein structure, so that antibodies against the same epitope do not cross-react between species. In humans, alemtuzumab (anti-CD52) and anti-thymocyte globulin (ATG) have been the clinically used depletional antibody therapy [49].

Tolerance Strategies

Many attempts to derive tolerance to allotransplants have aimed to provide haemopoietic chimerism. This relates to studies in the 1950s of the importance of having donor and recipient haemopoietic cells mixed for achieving tolerance [23, 12]. Twin cattle were observed to have a mix of each other's red cells, and also to be tolerant of skin transplants from each other. This relates to a shared placenta in utero. Experiments in rodents demonstrated that injecting alloantigenic haemopoietic cells to a foetus results in subsequent tolerance to that alloantigen.

In large animal models, efforts to induce tolerance involve initial T cell depletion with anti-thymocyte globulin, followed by allogeneic bone marrow transplant around the time of organ transplant [40]. Some protocols have used myeloablative conditioning to make space prior to bone marrow infusion. This has used total body irradiation or cyclophosphamide. Results have been encouraging with evidence of tolerance in primate models and dogs. Interestingly the haemopoietic chimerism tends to be transient, lasting around a month, but the organ tolerance persists in spite of loss of donor haemopoietic cells. Even using polymerase chain reaction (PCR) to detect donor blood cells at the DNA level generally fails to demonstrate microchimerism beyond a month. These strategies have been applied clinically to humans with success. Trials have shown that myeloablative or non-myeloablative conditioning followed by bone marrow and renal transplant can lead to tolerance up to ten years, again in the absence of ongoing microchimerism [33, 49].

Studies of Toxicity: Cyclosporine As Paradigm

Cyclosporine was first used clinically in the early 1980s [41, 51]. It gave a massive improvement in survival of transplanted organs, and brought organ transplantation from experimental therapy to mainstream practise. Cyclosporine acts by binding cyclophilin in the cytoplasm of T cells. This complex acts to inhibit the phosphatase calcineurin, which in turn inhibits T cell proliferation [52]. For the purposes of this article, it will be used as a prototype in describing the use of animal models in toxicity studies for immunosuppressive agents.

Perhaps the most important side-effect of cyclosporine is deterioration in renal function [52]. This has been shown in rat single nephron puncture studies to be driven by increased vascular resistance, and reduced blood flow, in the glomerulus. The reduced blood flow is paralleled with a proportional or greater drop in glomerular filtration rate. This indicates that the vascular resistance is principally governed by constriction of the afferent arteriole which is the inflow vessel to the nephron, more so than constriction of the efferent arteriole. Urine volume, concentrating ability and osmolality are unaffected by cyclosporine. Studies in rats have revealed a series of electrolyte abnormalities precipitated by cyclosporine administration. These include low magnesium and bicarbonate, and high potassium. It seems cyclosporine inhibits loop of Henle reabsorption of magnesium and bicarbonate, as well as inhibiting distal tubule potassium and acid secretion. These electrolyte abnormalities resemble pre-renal azotaemia (dehydration) [52].

The histological changes or damage to the kidney following cyclosporine administration have been extensively studied in the rat [52]. Changes are seen in both the vasculature and the

tubules. Tubular ballooning (vacuolisation) and inclusion bodies are seen, and are reversible on discontinuation of cyclosporine. This represents dilated endoplasmic reticulum and lysosomes respectively, and is generally seen with high serum levels of cyclosporine. Interestingly, changes to the vasculature are only seen in humans and not in rats. In humans, cyclosporine leads to vacuolisation of the smooth muscle cells and endothelium of arterioles. This can lead to endothelial necrosis, exposing the basement membrane to activate the clotting cascade with platelet adhesion. Ischaemia of the tubules ensues, which is typically seen as striped interstitial fibrosis on biopsy [52].

In humans, cyclosporine administration is associated with hypertension [41]. The precise mechanism is unclear. Certainly constriction of renal afferent arterioles activates the renin-angiotensin axis to cause peripheral vasoconstriction. There also seems to be a direct effect of cyclosporine making endothelium and smooth muscle cells more sensitive to vasopressin and angiotensin II, again leading to systemic hypertension. Interestingly, this peripheral vasoconstriction is not seen in rats given cyclosporine, although rabbits have a similar response to humans.

The effect of cyclosporine on the renin-angiotensin system varies between species. In rats, there is a clear activation, but this seems more transient in humans with prolonged administration. In both species, there is a hypertrophy of the juxtaglomerular apparatus where renin is produced. The relative importance of the renin secretion driving the systemic hypertension or the other way around is not entirely clear [52].

Cyclosporine leads to impaired glucose tolerance and diabetes in humans [43]. This is more common in co-therapy with steroids. The cause is unclear, but in rats cyclosporine causes vacuolisation of pancreatic islets and leads to reduced insulin content and release from the pancreas. In humans this is less clear, and there is more evidence for insulin resistance rather than reduced insulin output.

On the liver, cyclosporine causes a cholestatic picture in both humans and rats. This seems to be mediated by inhibition of hepatocyte membrane bile salt transport proteins. There is no corresponding toxicity to hepatocytes, as evidenced by the serum biochemical picture of raised bilirubin without corresponding change in transaminases.

In summary, it can be seen that animal models provide a useful means of testing immunosuppressive agents prior to use in humans. Limitations of these studies are an important constraint, and not all animal data can be extrapolated to humans. In particular, differences in the immune system between humans and rodents relate to the older age of human transplant recipients and greater exposure to pathogens.

This creates more memory cells in humans, and therefore less dependence on co-stimulation and greater resistance to tolerance. Large animals more realistically model memory cells of humans as they are exposed to pathogens in the wild, unlike rodents raised in clean laboratories. However, important differences exist between humans and large animals, particularly in drug absorption and in the effects of biologic agents such as antibody where target protein epitopes may differ. Ethical concerns are naturally greater for large animal use over rodent use. In spite of limitations, animal models remain the most useful predictor of immunosuppressive agent utility, and currently seem a pre-requisite for human use of new agents.

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Chapter 5

Ethical Issues of Immunosuppressive Drugs Use in Organ Transplantation

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Abstract

Immunosuppressive drugs are essential for transplant recipients in preventing and treating graft rejection. Their discovery and new advances allow many transplant recipients to live longer and healthier lives despite associated adverse effects such as tumors and infections. At this point, more research studies are recommended in an attempt to minimize or avoid complications related to adverse effects, and to improve grafts survival time, along with induction of tolerance in patients. Balancing scientific validation with ethical acceptability during these immunosuppressive drugs research studies by carefully considering ethical issues will greatly benefit humanity. Ethical principles such as autonomy, beneficence, non-malficence, justice, independence and dignity are needed to be explored, updated and respected as medical advances with a goal for controlling immunosuppressive therapy continue to develop. In this chapter, ethical issues related to the application of immunosuppressive drugs in biomedical research and clinical trials such as challenges related to the use of generic immunosuppressive drugs, the individualization of immunosuppressive drugs, application of immunosuppressive agents during gestation, childhood and in ageing periods, use of immunosuppressive agents in tolerance transplant recipients, use of stem cells as immunosuppressive therapy,

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use of immunosuppressive agents during immunodeficiency status and its long-term usage are highlighted as pertinent assignments which need accurate regulations with respect to human dignity as priority.

Keywords: Ethics principles, immunosuppressive drugs, biomedical research, clinical research, immuno-pharmacogenetic, immune-pharmacogenomics, tolerance

Abbreviations

CIOMS: Council for international organizations of medical sciences
CsA: Cyclosporine A
EC: Ethics committee
FDA: Food and drug administration
GCP: Good clinical practice
GLP: Good laboratory practice
GISD: Generic immunosuppressive drugs
GCs: Glucocorticoids
HIV: Human immuno-deficiency Virus
ICH: International conference on harmonization
IEC: Independent ethics committee
IUGR: Intra-uterine growth retardation
IRB: Institutional review board
ISD: Immunosuppressive drugs
WHO: World health organization
WMA: World medical association

Introduction

The ethical conduct in medical care for human subject protection has its origins in the ancient Hippocratic Oath, which specified a prime duty of a physician to avoid harming the patient. This oath appeared to be often ignored in medical research implicating human beings. Medical research history has reported a very little effort in informing human subject participants about the nature of experiments, and many unethical experimentation using invasive medical interventions with remarkably adverse effects on vulnerable human groups have been processed in full sight of the whole world. In recent decades, these medical experimentations and research on human beings without their consent, and their exposure to serious risks of death or permanent impairment of their faculties have raised major concern about subjecting human beings to medical research. In 1946, the universal declaration of human rights stipulated that "All human beings are born free and equal in dignity and rights. They are endowed with reason and conscience and should act towards one another in a spirit of brotherhood" [1]; and expressed throughout this article the choice of dignity, rights and equality that should be made by society in the distribution of resources and access to health care.

Late in 1947, the first International Statement on the ethics of medical research using human subjects was formulated, and named "the Nuremberg Code" [2]. This code highlighted the quintessence of voluntariness of informed consent for participation in any research and served as a landmark document in medical and research ethics [2]. In 1948, the General Assembly of the United Nations adopted the Universal Declaration of Human Rights which expressed concern about rights of human beings being subjected to involuntary maltreatment [1]. In 1966, the International Covenant on Civil and Political Rights specifically stated, "*No one shall be subjected to torture or to cruel, inhuman or degrading treatment or punishment. In particular, no one shall be subjected without his consent to medical or scientific treatment"* [3]. Realizing the need of ethical implementation on the use of human subjects, the World Medical Association (WMA) established the Declaration of Helsinki in 1964, guiding medical doctors involved in research using human subjects. This declaration stated that all research with humans should be based on the results from laboratory and animal experimentation, research protocols should be reviewed by an independent committee prior to initiation, informed consent from research participants should be compulsory, research should be conducted by qualified individuals and the risks of research should not exceed the benefits [4]. To date, the Declaration of Helsinki did not completely barricade the unethical medical research studies using human subjects. The case of Tuskegee Syphilis Study conducted by the U.S. Public Health Service from 1932 to 1972 illustrated phenomenally the absolute necessity of the application of ethics principles in medical research. In this study, six hundred low-income African-American males, 400 of whom were infected with syphilis, were monitored for 40 years.Free medical examinations were given; however, subjects were not told about their disease. Even though a proven cure (penicillin) became available in the 1950s, the study continued until 1972 with participants being denied treatment. Many subjects died of syphilis during the study. The study was stopped in 1973 by the U.S. Department of Health, Education, and Welfare only after its existence was publicized and when it became a political embarrassment. In response to this, a foundational document for the ethics of research with human subjects in the U. S., The Belmont report stated in 1979 the basic ethical principles that should assist in resolving the ethical problems that surround the conduct of medical research: respect for persons, beneficence and justice. These three basic principles were used to enforce the need for informed consent, the need to assess the risks and benefits and the need for proper selection of subjects [5].

In 1982, the World Health Organization (WHO) and the Council for International Organizations of Medical Sciences (CIOMS) emitted the "Proposed International Guidelines for Biomedical Research involving Human Subjects" which subsequently stated out the "International Ethical Guidelines for Biomedical Research involving Human Subjects" in 1993[6]. Recent international studies reported that the majority of biomedical research has been predominantly motivated by concern for the benefit of already privileged communities, and the WHO estimates that 90% of the resources devoted to medical research and development on medical problems are applied to diseases causing less than 10% of the present global suffering. The regulation of international guidelines that assist in strengthening the capacity for the ethical review of biomedical research in all countries is very important to redress this inequality observed in global health [7]. The Helsinki Declaration has been updated several times; nonetheless, it remains most common form of independent ethical review. Most of developed and developing countries have national institutional processes to protect the rights of participants in medical research that are enforced through obligatory

compliance with the WMA's Declaration of Helsinki, or local ethical frameworks that are consistent with the principles set out in the Helsinki Declaration. In organ Transplantation, the breakthroughs made in the fields of immunology and pharmacology unlocked the mysteries of organs rejection by discovery of immunosuppressive drugs (ISD), and allowed transplant surgeons to use organs from donors who were not precise biological matches by suppressing the rejection phenomena for a determined time in recipients.

The one-year graft survival is approaching 90% after use of standard immunosuppressive in kidney transplants patients. However, long-term graft survival has not improved appreciably since the early 1980s and only about 45% of cadaveric kidneys survive ten years after transplantation. For other organs (e.g., liver, lung and pancreas), graft survival does not approach this level [8].

The great need to develop new ISD that are more effective and safer, or the development of innovative immunosuppressive treatments for currently untreated conditions in organs transplantation involving invasive medical interventions that may directly harm or benefit study participants, seems imperatively necessary. On average, only one out of dozen new molecule drugs that enter clinical testing programmes is eventually introduced for clinical use. The main reasons for this high failure rate are unpredicted side-effects or inadequate treatment effects. As new molecules are commonly discovered by means of laboratory research through animal studies to clinical trials before being used in medical care, new immunosuppressive agents research testing can only forecast their clinical efficiency and side-effects in laboratory animals and humans in clinical trials [9]. Immunosuppressive clinical research is very important as its biomedical research part can only partially indicate effects in humans. But lack of conducting quality of life measurement with appropriate number of participating patients, target of specific organ transplanted and high-risk age groups including children and other associated systemic disease make it often difficult to predict the outcome. ISD treatment has significant implications for transplanted patients or patients with auto-immune diseases, leading to topics as important as life and death, pain and suffering, privacy, racial discrimination and equality. The risks and benefits associated with decisions to initiate human studies related to immunosuppressive treatment and the design and monitoring of human studies on immunosuppressive therapy should be reviewed on a case-by-case basis by experts in bioethics, law and basic and clinical research. This chapter underlines significant ethical issues linked with use of ISD in clinical research and its complex future implications.

Basic Ethical Principles in ISD Treatment

The application of ethical principles in ISD use is necessary. These principles which include autonomy, beneficence, non-maleficence, justice, independence and dignity, are a starting point for discussion and a guide for a convincing debate and discussion that might produce decisions about appropriate action for identified ethical challenges [10, 11].

Autonomy

In the contexts of biomedical and clinical research with ISD, the principle of respect for autonomy guarantees the respect of rational persons to decide freely on a course of action without being coerced or forced. This requires following points:

1. Research participants should give an informed and voluntary consent to participate in ISD projects.
2. Confidentiality and privacy of any personal information from research participants should be guaranteed.
3. Researchers should be truthful with participants about the risks that may arise from their study participation.

Non-Maleficence

Full information about the application of ISD should be given to research participants to minimize the risks of participation. Failing to do so violates the principles of respecting autonomy as well as that of non-maleficence.

Beneficence

This principle requires that the potential benefits of participation in ISD research outweigh the risk to society and the risks for study participants.

Distributive Justice

This principle requires that ISD research policy is headed to ensure that the risks of research participation are not unfairly distributed (i.e., to the poor and indigent), and that any benefits of research participation (i.e., access to promising new treatments) are fairly shared between all potential beneficiaries.

Ethical Requirements in ISD Research

Medical ethic codes have set up some ethical requirements for researchers or investigators, which must be observed during progression of research study to be ethically and scientifically authorized (Table 1). These ethical requirements are accepted in most national and international research ethic guidelines [10, 11], and are decrypted in following points:

Table 1. The Principles of International Committee of Harmonisation-Good Clinical Practice

Clinical trials should be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki, and that are consistent with GCP and the applicable regulatory requirement(s).
Before a trial is initiated, foreseeable risks and inconveniences should be weighed against the anticipated benefit for the individual trial subject and society. A trial should be initiated and continued only if the anticipated benefits justify the risks.
The rights, safety, and well-being of the trial subjects are the most important considerations and should prevail over interests of science and society.
The available nonclinical and clinical information on an investigational product should be adequate to support the proposed clinical trial.
Clinical trials should be scientifically sound, and described in a clear, detailed protocol.
A trial should be conducted in compliance with the protocol that has received prior IRB/IEC approval/favourable opinion.
The medical care given to, and medical decisions made on behalf of, subjects should always be the responsibility of a qualified physician or, when appropriate, of a qualified dentist.
Each individual involved in conducting a trial should be qualified by education, training, and experience to perform his or her respective task(s).
Freely given informed consent should be obtained from every subject prior to clinical trial participation.
All clinical trial information should be recorded, handled, and stored in a way that allows its accurate reporting, interpretation and verification.
The confidentiality of records that could identify subjects should be protected, respecting the privacy and confidentiality rules in accordance with the applicable regulatory requirement(s).
Investigational products should be manufactured, handled, and stored in accordance with applicable good manufacturing practice (GMP). They should be used in accordance with the approved protocol.
Systems with procedures that assure the quality of every aspect of the trial should be implemented."

Source: Adapted from Karlberg JPE and Speers MA 2010, USA.

Independent Ethical Review of Research Proposals

An independent assessment testifies whether the benefits of research involving ISD research outweigh its risks to participants; need to be provided after an external review by an independent institutional or committee of ethical review. A prior approval from the ethics committee (EC) for research investigators is required to conduct any ISD research with human beings.

Free and Informed Consent

This is an essential condition of ethical research. In ISD research, this requirement does not present any special problems for autonomous adults who can understand the nature of their participation and can freely decide to be involved in research study or not. Contrarily, in those research studies which would be particularly carried out for long time, this ethical issue presents a major problem for children and adolescents.

Privacy, Confidentiality and Legal Hazard

This requirement is a necessary component of an ethical trial in research involving human beings. In ISD research, it is essential that personal information concerning a research participant be protected. Any divulgation to any individual or group of individuals should require a direct consent of the participant. Also, the identity of participants should not be noticeable from the published results of the study. Not respecting these rules may create legal problems for the implicated investigators.

Vulnerable Research Participants

In immunosuppressive drug research, following three important elements related to the ethical framework when recruiting vulnerable participants should be taken in consideration:

1. Vulnerable participants must usually benefit from the research.
2. Vulnerable participants must not usually be exposed to more than a minimal risk of harm.
3. ISD treatment proposed in research study must be more effective than any available treatment.

Following other ethical issues should also be taken in consideration, debated and discussed: participants cognitively or physically impaired or in a dependent relationship with investigators, such as clients, students or patients, children, ethnic minorities, prisoners, capability of participants to understand the rationale for a research study; capability of participants to give the free and informed consent to participate in the study; capability of participants to understand exactly what is required for a study and why.

Payment to Research Participants

This issue is controversial and remains unresolved in drug research in general, and in ISD particularly. A key consideration is the potential for payments to increase the risks to participants. Payment to participants needs to be approached on a case-by-case basis in accordance with local circumstances and ethical values.

Ethical Issues in Preclinical and Clinical ISD Research

In organ transplantation there is a persistent demand, in addition to a great need, to develop new immunosuppressive agents that are as effective and safe as, or more effective or safer for transplant patients than immunosuppressive treatments already in the market.

The development of any ISD requires a well-reasoned, well-planned, judiciously designed and ethically approved research plan prior to be undertaken in research process; by means of laboratory research and animal studies before being tested in humans through clinical trials [12].

It is stated in the Declaration of Helsinki: "The research protocol must be submitted for consideration, comment, guidance and approval to a research EC before the study begins." It is also stated by Good clinical practice (GCP) that "A trial should be conducted in compliance with the protocol that has received prior institutional review board (IRB)/independent ethics committee (IEC) approval/favourable opinion." All pre-clinical and clinical studies of ISD should be carefully monitored under strict legal rules and ethical principles to ensure that all aspects of the research including science, ethical and data quality are considered in a proper manner and research should be conducted without putting participants at risk without any valid reason (Tables 1-2).

Table 2. Frame-work for evaluating the ethics of clinical research

Requirement	Explanation	Ethical value
Social or scientific value	Treatment or intervention that will improve health, well being or increase knowledge	Scarce resources and non exploitation
Scientific validity	Use of accepted scientific principles and methods to produce reliable and valid data	Scarce resources and non exploitation
Fair subject selection	No target of stigmatized or vulnerable subjects in risky research, no favoritism for potential beneficial research	Justice
Favorable risk-benefit Ratio	Minimization of risks, enhancement of potential benefits	Nonmaleficense, beneficense and non exploitation
Independent review	Review of research study design	Public accountability, minimisation of potential conflicts of interest
Informed consent	Information to the subjects (risks, benefits and alternatives	Respect for autonomy of subjects
Respect for potential and enrolled subjects	Protection of privacy, confidentiality, information about the risk-benefits, research results	Respect of autonomy and welfare of subjects

Source: Adapted from JAMA. 2000; 283(20):2701−2711.

Ethical principles and legal rules are not always consistent, and both differ greatly over jurisdictions. There is not any single human research ethics guide that can provide universal answers to all the ethical issues of research involving humans. Therefore researchers and ethic committee members implicated in ISD research project should be aware of both the institutional requirements and the applicable laws during ethics review.

In ISD research, most approved drugs were developed and tested by the pharmaceutical industries and not by academic institutions or non-profit organizations. This means that the

network between pre-clinical and clinical research is consequently more obvious in for-profit rather than non-profit. Thus, an assessment of independent scientific and ethical review of the research study prior to an approval from institutional EC or IRB of the establishment, where related research may be performed, must be imperative [2, 4, 13].

Ethical Issues in Pre-Clinical Research

At this stage, research studies of immunosuppressive agents involve animals such as rodents and primates because of similarity in anatomy and physiology which is required for drugs development. Laboratory animal uses at this stage of research have been reduced in recent years for ethical reasons and in others cases, for cost reasons. Furthermore, the use of these laboratory animals needs an authorization from the animal EC. Standard criteria adopted internationally from a good laboratory practice (GLP) code have to be fulfilled and also abided by researchers performing the study at this stage [13].

Ethical role of researchers involved in pre-formulation phase of immunosuppressive agents, as at other pre-clinic research phases, have to be considered because the immunosuppressive drug dosage determination is pertinent, as the new compound will be given later to humans for the clinical trials purpose. All information related to ISD collected at any pre-clinical research phases should be kept and taken in consideration to ensure that the follow-up clinical trial on humans is safe and there are no unexpected adverse effects [6, 13].

Ethical Issues in Clinical Trials

At this stage of research, the well-being and interest of human subjects involved in the trials have to always predominate over the interest of science, community and business. Trials with ISD can only be carried out if they can be scientifically and medically justified, and if their potential benefits exceed their potential risks. Trials with ISD have to be conducted similarly all the time, no matter where in the world and by whom they are carried out (Table 2). Human subjects participating in ISD clinical trial have to be offered the best possible treatment, after the study is finished, at the discretion of the investigator. All trials results with ISD have to be publicly available and for the good of humanity [14, 15].

It seems to be very unethical to use a placebo control group, particularly in organ transplantation. In such cases, it is appropriate to carry out a research using the immunosuppressive standard treatment as an active control. Placebo can only be used if ethically acceptable. Children participants should only be included in a trial if there is no other research alternative [16, 17].

Transnational Research of ISD

More ISD research projects, especially in phase II and III trials are often carried out multinationally, to help accrue adequate number of participants to fulfill the trial goal within a reasonable time frame and to gain more general findings, with participants recruited from a

large diverse population and an extensive range of clinical status. To be meaningful, multinational ISD research projects need to be conducted according to standardized procedures and evaluation criteria.

ISD research project should be carried out only at research sites or states in which ISD research activities are envisaged and where the ethical committee has given a favourable opinion after submission for ethical review. Other ethical concern for immunosuppressive multinational research is the possibility that the different countries involved in research might have different standards of protection for research participants. Ethical committee involved in reviewing research that is to be conducted internationally has to ensure an appropriate mechanism in which research has to be undertaken according to a common set of ethical standards. Cultural differences particularly regarding informed consent and an independent nature of each ethic committee involved in ISD multinational research projects should be also taken in consideration [18, 19].

Issues related to ISD research carried out in developing societies have raised concern and have been debated, especially when the research is externally funded. In this case, EC 's review should take place in the host countries as well as the country of the sponsor. Guidelines for ethical research should minimize the risk of exploitation of human participants. It is recommended to the ethic committee members, as well as researchers and research sponsors to resort to developing countries national ethic guidance that takes account of specific local needs and cultural context.

ISD research project should be not supported by organizations from developed countries which are pursuing their own goals and if that research can be carried out reasonably well in a developed community or country. Reason to carry out research should be its relevance to the health or healthcare needs of the society in which it is to be carried out, either in the short-term or in the long-term. Responsibility should be taken to guarantee that the social and economic circumstances do not improperly influence people to participate in research, especially where participation in a research project may be the only way to access to ISD therapies. Particular attention should be needed to obtain valid informed consent from participants to ensure that the implications of participation are fully understood [20, 21].

Ethical Challenges in ISD Use

Common medical ethical principles usually fail to guarantee a specific direction in the management of the complexities and ambiguities of ethical challenges that emerge in quotidian medical practice [22]. Major concern about the applicability of standard ethical principles and guidelines in the different contexts which may have either very different or no research traditions, and may not have established a form of institutional ethics review; the relevance of issues such as participant vulnerability, levels of awareness and expectations about rights, communication difficulties, documentation issues, literacy and the rules of obtaining consent in hierarchical societies are still contested and deserve further attention in ISD research trials. In addition, issues of race, culture and gender also have an impact upon the safety of ISD research trials, specifically in developing countries [23].

Standardized structure of actual ISD development protocols studies require an increased number of participating subjects to conclude the outcome for wider population with specific

conditions. Although, there is still concern about the increase in the number of participants, specifically in the study with chronic allograft dysfunction, this matter provides the opportunities to study the safety and efficacy of ISD and their refinement.

Matters related to individualization of ISD , application of ISD during pregnancy, in children, in immunodeficiency patients, long-term use of ISD , use of ISD for tolerance and use of generic immunosuppressive agents and associated challenges need to be seriously examined as medical advances with a need for controlling immunosuppressive therapy continue to occur.

Ethical Issues in Individualization of ISD

Immuno-pharmacogenetic or individualizing immunosuppressive therapy holds enormous promise by increasing the safety and efficacy of ISD development while decreasing their adverse reactions, and may allow clinicians to make most appropriate prescription and accurate dosage decisions in therapies. Its full application will require to characterize variation between DNA of patients to predict the responses to specific drugs, and subsequently change the practice and economics of healthcare. Its introduction into the clinical setting will allow a wider usage of genetic tests with a genotypic stratification of patients, and as a consequence, in clinical trials. This stratification of patients in clinical trials might raise a number of questions which researchers do not normally take into consideration in a conventional trial involving genotypically diverse patient population tested with a single medicine. Addition of pharmacogenetic tests in organ transplantation would also increase minimal additional financial costs to existing required tests such as genetic tissue typing of potential organ recipients needed as a routine part of the medical assessment prior to organ transplantation [24, 25].

Although there is discord about the advancement and timescale to which this individualizing immunosuppressive treatment approach might become a standard immunosuppression therapy in organ transplantation, ethical issues arising with its application need to be carefully highlighted and resolved. Major ethical issues associated to the use of this therapy include the potential for misuse of genetic information collected for immunotherapy purpose and the unwillingness of pharmaceutical industry to pursue research because of potentially prohibitive costs associated with developing products, [26, 27]. In regard to its complexity compared to the existing healthcare system, individualizing ISD use might eventually induce consequences in various areas of law, such as patent law, medical liability, insurance decisions, individual privacy and discrimination law.

The applicability of individualizing immunosuppressive would impact the current insurance system by increasing the cost of health insurance due to a potentially higher cost of targeted medicines. Also, this individualizing treatment might change who is considered insurable. Patients who are expected to have a high likelihood to respond positively to the treatment, would be classified in a favourable insurance risk group. Thus, a genetic profile would make an important financial risk-assessment parameter than primary treatment susceptibility [28, 29].

Rigorous assessment of research on individualisation of immunosuppression procedures and the management of genetic information is needed. A clear distinction have to be made

between genetic testing for disease susceptibility and genetic testing for immuno-pharmacogenetic profiles, and some caution will be needed over the relationship between genotype and phenotype [30, 31].

The applicability of the individualizing immunosuppressive treatment should consider all social factors such as ethnicity, race, language and geography when collecting genetic information in order to avoid exclusion or a neglect of certain ethnic minorities from both ISD research and treatment [27, 29, 30].

Education of healthcare professional and the public regarding the promises and consequences of this treatment is essential. Genetic experts should be included in IEC or in IRB, and all members of these ethics institutions should be well-informed in issues related to consent, identification and genetic sample storage to fulfill ethics requirements prior to any clinical trials. Ethics guidelines related to the individualization of ISD research trials should be formulated by experts in research and clinical transplantation, genetics, bioethics, law, pharmacy, economics and would serve as the starting point for the expansion of this field of organ transplantation research.

ISD Application during Gestation Period

The capacity of ISD and their metabolites to cross the placental barrier and enter into the fetal circulation has raised questions about the fetal development and the long-term outcome of children exposed to these agents in uterus [32, 33]. In autoimmune diseases treatment research, clinical randomized trials to evaluate the safety of immunosuppressive medications during pregnancy also seem difficult to be designed, for both ethical and practical reasons. In most of trials, observational studies guide clinical decisions and the low prevalence of adverse outcomes makes such trials difficult to interpret. Also, an estimation of the relative effects of immunosuppressive agents might be difficult during gestation period by the fact that in most of cases, the diagnosis of underlying maternal diseases as well as the concomitant use of other medications unavoidably could confound interpretation of pregnancy outcome.

Results compiled from studies of transplanted women treated with ISD have shown no consistent pattern of congenital malformations in the offspring and reported prevalence of major structure malformations has been found to be 4–5%, similar to the 3% documented in healthy women [34, 35]. Moreover, immunosuppressants given during pregnancy for autoimmune diseases did not impair significantly the development of immunity in babies. These data were in contrast with the results obtained in infants born to mothers who received organ transplants, which showed an impaired immunity in offspring [35, 36]. This may be explained by the fact that in autoimmune diseases, the immunosuppressive therapy is usually characterized by the use of a single drug in a lower dose range, while in organ transplantation, immunosuppressive treatment may require higher doses or combination of different immunosuppressive agents. Ethics principles in this issue recommend that health care professionals and patients consider carefully the potential risks of pregnancy complications as well as the risks for the offspring when immunosuppressive medications are required to treat maternal disease despite a possibility of successful pregnancy outcomes in autoimmune diseases.

Previous studies reported that selected ISD use does not apparently increase the risk of birth defects. Others trials reported that maternal use of immunosuppressive medications could increase the risk of gestational complications; Cyclosporine A (CsA) and Glucocorticoids (GCs) were reported to induce hypertension, diabetes mellitus, lower mean birth weight and intra-uterine growth retardation (IUGR) [35, 36]. Infants born to mothers with autoimmune diseases, taking immunosuppressants during gestation, are more likely to be pre-term, low birth weight (<2500 g) and small for gestational age when compared with the general population [37]. Data on long-term follow-up of children exposed in utero to immunosuppressive medications is obviously needed, particularly for the outcome of ISD related side effects. These data would be necessary to improve the current state of knowledge for an ethics guideline update in this field.

ISD Application in Children

Medical literature supported that graft survival for young adults is significantly worse compared with older adults while young adults are supposed to have a more vigorous immune system. Previous studies reported also that medication in children and young adolescents is still frequently prescribed off-label, and dosages of drugs for children are calculated on the same principles as that of adults while young children have poor bioavailability and high rate of drug clearance [38]. Many obstacles make application of ISD in children and young adolescents a huge challenge issue in clinical research. These issues include: confidentiality for young participants, less interest for pharmaceutical companies or medical institutions involved in initiating a research study because of lack of financial profit and fear of a complex study design. Most of the children or young adults are not very accurate in taking medication on time, especially when it does not fit in with their life-style and with important social activities such as going out with friends. About 40% of young adults on immunosuppressive medications think nothing bad might happen when they forget their ISD [38, 39]. Encouragement to pharmaceutical companies should be initiated to promote ISD research projects in children and young adults to increase the current safety of immunosuppressive treatment and allow this group of population to benefit from therapeutic advancements. Accurate and clarified research concepts should be explained to children and adolescent participants in trials in a comprehensive manner. Assent and consent forms should be clear and understandable. EC s should play an important and major role in protecting children and young adults from harm [38, 39].

Ageing and Immunosuppressive Therapy

In recent decades, the numbers of elderly transplant recipients and the demand for organs from older donors have increased remarkably that have raised an important challenge issue in transplantation medicine. Ageing was reported as a highly complex process involving multiple mechanisms at different levels leading to a continuous and complex modification of the immune system, with direct impact on transplant outcome and organ quality. Previous studies reported a greater effect of ageing on adaptive immunity which results in part from

thymic involution, replicative senescence, and atrophy of secondary lymphoid organs, leading to a decrement in circulating lymphocytes and altered cytokine profiles [40, 41].

Previous studies reported that in older kidney recipients, the state of immunosenescence allows application of nonaggressive immunosuppressive protocols, particularly in the course of maintenance treatment. In older cardiac recipients, immunosuppressive protocols with lower doses and fewer ISD adopted as standard showed impressive results [42, 43].

The altered alloimmune responses and increased immunogenicity of organs were reported to present risk factors for inferior patient- and graft survival. Advanced knowledge on age-dependent modifications of allorecognition and alloimmune responses necessitates an age-adapted immunosuppressive treatment. An ethics guideline in this field of transplantation research should take a major consideration of both advanced medical theories on ageing and nature of immune responses during ageing in the application of ISD.

ISD Application in Immunodeficiency Patients

The management of human immuno-deficiency Virus (HIV)/AIDS which includes a combination of nucleoside reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors and/or a protease inhibitors, has changed the natural history of HIV infection by improving immune function in HIV-infected individuals. Actually, more deaths from end-stage organ disease rather than acquired immunodeficiency syndrome (AIDS)-associated opportunistic infections and neoplasms are recorded in clinic. Thus, the use of therapies such as solid organ transplantation and stem cell transplantation are considered in patients with HIV infection [44, 45]. Protease inhibitors are reported to strongly inhibit the cytochrome P450 3A (CYP3A) enzyme system that is responsible for the metabolism of ISD, such as tacrolimus, sirolimus, and cyclosporine [46, 47]. Pharmacodynamics of drug-drug interactions was also reported in studies using concomitantly highly active antiretroviral therapy and antimetabolites, such as mycophenolate mofetil. Based on previous clinical and pharmcological research findings, reducing the HIV viral load and ensuring an adequate immunosuppression to avoid any graft rejection should be considered equally [47, 48]. Ethical guidelines in this field should take account of accurate monitoring for complexities of interactions between immunosuppressant drugs and anti-HIV medication, in order to ensure that effective therapy for both HIV and rejection is guaranteed. Healthcare by multidisciplinary team evaluating and caring for these patients must include specialists with expertise in HIV, transplantation, and pharmacology.

Long-Term Exposure to ISD

In standard immunosuppressive treatment, the patient receives a massive dose of antirejection drugs immediately after transplantation in hope of avoiding acute rejection, which commonly develops in the first year after transplantation. After a while, patient is put on a life-long course of relatively high doses of ISD to prevent long-term rejection despite associated side effects. Previous studies reported several issues that positively ameliorate the onset of side effects, in particular timing of steroid withdrawal, advantages and risks

associated with total steroid avoidance, optimal concomitant immunosuppression, requirement for biologic induction therapy and the role of immune monitoring [49, 50]. Besides progress reported in long-term use of ISD, clinical efficacy studies of these drugs are still concerned about confusion on trial outcome interpretation (such as morbid complications) due to the complexity of immunosuppressive regimens. Inadequacy of scientific and clinical experience data for a reduction or an addition of these drugs during life-long treatment have raised ethics issues [51, 52]. Pharmacokinetics and pharmacodynamics knowledge of ISD is a crucial contribution to the long-term well-being of the transplant recipients; therefore it should be required for all scientists involved in research trials. During treatment, the need to be aware of the long-term adverse effects to avoid a delay in detecting problems, to be vigilant and enterprising in preventing, monitoring and managing adverse effects should be imperatively required to all healthcare professionals involved in the patient's management [50, 51, 52]. Full respect of ethical principles should be applied in life-long immunosuppression treatment research study. Ethic guidelines related to this field of research should cautiously recommend a close monitoring of research participants and a continued follow-up long after the drugs have been stopped as adverse effects associated to long-term use of these drugs may have atypical clinical presentations.

Stem Cells As Immunosuppressive Therapy

Stem cells hold tremendous therapeutic potential. Their ability to generate a limitless number of cells with many different characteristics renders them to be a miracle treatment when it comes to replacing cells in medical conditions such as diabetes, spinal injury, Alzheimer etc [53]. Most opinions consider that their use violates the principle of respect for human life and respect for human embryos, as an embryo is totally destroyed during the process of stem cell line derivation. Also, the establishment of embryos for research purposes and the derivation of stem cell lines from them may lead to the de-sensitization of human life and to potentially uncontrolled commercialization or instrumentalization of the human body. These concerns highlighted moral dilemmas and raised pertinent ethical issues associated to the use of the stem cells in clinical research [54, 55]. Many scientific techniques have been inspired to resolve some pertinent ethical issues. An accurate evaluation of the procedure used to collect, to study, to protect and to preserve the stem cells; a full evaluation and long-term follow up of risk associated to surgical processes such as inadequate migration, transplant rejection, management of adverse effects such as tumors, infections related to the use of stem cells in clinical research; serious evaluation of public health impact and potential deviations of the stem cells therapy in society should be recommended as basic requirements of ethical guidelines in the use of stem cells in biomedical and clinical research.

Generic Immunosuppressive Drugs (GISD)

In organ transplantation, most of the ISD are “critical dose drugs” which are described as drugs where comparatively small differences in dose or concentration lead to dose- and concentration-dependent serious therapeutic failures and/or serious adverse drug effects [56,

57]. The use of GISD in transplantation raise pertinent ethical issues associated to their dosages. Referring to medical literature, there is a small theoretical risk of a variation in the drug concentration between generic and brand-name drugs, and even though this risk is unquantifiable, the consequences could be disastrous for both patients and the community who is already in shortage of organs for transplantation, as it has been proven that under-immunosuppression could lead to graft loss while over−immunosuppression could lead to neoplastic complications or life-threatening infections [58, 59]. Ethical issue related to a distributive justice has been also questioned in the use of GISD in organ transplantation. It has been demonstrated that from an individual perspective, a prescription use of GISD provide no obvious benefits and may incur some risk. However, in wider societal perspective, prescribing generics might procure through potential cost savings, an additional available resource that benefits the other patients and to the healthcare system [58, 59]. Others concerns consist of a possibility of uncontrolled substitutions (i.e., substitutions that are made without informing the prescriber) of GISD on the market, which could lead to under- or over-immunosuppression caused by generic drift (Table 3). Ethics studies reported that majority of pharmaceutical manufacturers usually offer grants and invest in long-term relationships with clinicians. Issue of conflict of interest with pharmaceutical companies involved in manufacturing of GISD should be seriously taken in account.

Table 3. Statement of related to use of GISD

American Society of Transplantation (2003)	- Supports the availability of efficacious, less expensive immunosuppressive medications and endorses efforts to introduce generic alternatives. Medication costs may contribute to non-compliance with prescribed medical regimens. - Food and Drug Administration (FDA) approved generic immunosuppressive agents appear to provide adequate immunosuppressive to low-risk patients. - Insufficient data to make recommendations for at-risk populations (African-Americans or paediatric)
Canadian Society of Transplantation (2012)	- Insufficient literature regarding efficacy and safety - Close monitoring with any change - Not recommended in paediatric patients - The intended drug formulation must be explicitly stated on all prescriptions to avoid substitutions. - Educate patients about formulations and substitutions. - Prescriber and patient should be involved in any decision to change formulation. Mandatory notification of the prescriber should be a legal requirement - Licensing requirements for critical dose drugs must be re-assessed. Bioequivalence in solid organ transplant recipients. Requirement for generic manufacturers to provide clinical outcome data in solid organ transplant recipients. - Transplant centres should be funded according to the increased costs associated with managing solid organ transplant recipients arising from the introduction of generic immunosuppression
European Society of Transplantation (2011)	- Generic formulations that do not meet the stricter criteria should not be used. - Substitution should only be initiated by the transplant physician. Pharmacists or insurance providers should refrain from forcing substitution. - Repetitive substitution should be avoided. - Patients should be informed about substitution and taught how to identify different or formulations of the same drug so they can alert their physician if an uncontrolled substitution is made. - The simultaneous use of different formulations in the same patients should be avoided

Source: Adapted from *Can J Kidney Health Dis* 2014; 1:23.

Pertinent ethical guidelines should be accurately defined with care in minimizing the risks that could be associated to use of GISD in transplantation. A comprehensive cost-effectiveness to ascertain the benefits of these drugs compared to a brand-name drugs have to be adopted, and new policies such as a law prohibiting non-physicians from authorizing substitutions of brand-name with GISD, substitutions between GISD should strictly be regulated to allow the use of these drugs ethically [60, 61] (Table 3).

ISD Application in Tolerance Transplant

Achieving substantial improvements in long-term graft survival remains a burden in transplantation as long-term outcome is currently limited by several factors, such as chronic graft rejection and the substantial side effects of life-long immunosuppression. The issue of ISD application in tolerance research requires firstly, a design of clinical protocol that is scientifically valid and ethically acceptable despite limitations in understanding mechanisms of action, and insufficient data on safety and toxicity [62−65]. Ethic committee or /and IRB representatives should be involved in designing research trials and assisting in the development of ethically acceptable consent forms and processes. Safety of patients should be a priority. The risks and benefits linked to initiation, the design and the monitoring of these research trials should be reviewed on a case-by-case basis by experts in bioethics, law, and basic and clinical research in transplantation. Considering all consequences such as side effects, toxicities, financial burden, poor long-term survival rates, the risk and benefit ratio in pursuing these research trials should be at least neutral.

Participants involved in these research trials should be able to fully understand the complex issues of the study and weigh risks and benefits of the outcome. Selection of patients in these research trials should balance the potential risks and benefits in terms of expected clinical outcome for different types of transplant recipients. Medical literature reported that the first-time transplant recipient, i.e., the patient with a "naïve" immune system, would be an appropriate candidate for initial tolerance induction studies versus the patient with severe chronic rejection who may be sensitized. Children should be ethically enrolled in these research trials only after proof of research concept has been demonstrated in adults [66−68].

These research trials should incorporate the most available methods for detecting and/or predicting rejection prior to severe organ damage, including both pathology and intragraft gene expression. Standard immunosuppressive therapy was proven to block the intracellular signals necessary to induce at least some types of tolerance. Therefore, safety and efficacy of tolerogenic agents while withholding standard immunosuppressive therapy or altering immunosuppressive regimens should be evaluated. Research design of tolerance induction trials should incorporate specific rules for rescue of the rejecting graft and use of conventional immunosuppressive therapy [69−71].

Conclusion

Advances in immunosuppressive treatment have evidently reduced the incidence of acute rejection. More research on specific ISD and protocols is needed to avoid complications

related to adverse effects and to improve further extending the life of the grafts with induction of tolerance in patients. However, use of ISD in biomedical research and clinical trials as treatment is unquestionably associated with pertinent ethical issues which need to be taken in consideration. The risks and benefits of ISD research in humans should be evaluated with respect to ethic principles such as autonomy, beneficence, non-maleficence, justice, independence and dignity of patients. Challenge issues related to individualization of ISD, use of GISD, application of immunosuppressive agents during gestation, childhood and in ageing periods, use of immunosuppressive agents for tolerance in transplant recipients, use of stem cells as immunosuppressive therapy, use of immunosuppressive agents during immunodeficiency status and their long-term exposure need accurate regulations in order to have proper balance between what can be done and what should be done, according to a morally and socially accepted specific context.

Appendix

Important International Documents Related to the Ethical Conduct of Medical Research
The Nuremberg Code **www.hhs.gov/ohrp/archive/nurcode.html**
World Medical Association's Declaration of Helsinki **www.wma.net**
United Nations International Covenant on Civil and Political Rights **www.cirp.org**
Belmont Report **www.hhs.gov**
Int'l Ethical Guidelines for Biomedical Research Involving Human Subjects **www.cioms.ch**
Guidelines for Good Clinical Practice (GCP) **www.who.int/medicines**
ICH: International Conference on Harmonisation **www.ich.org**
Ethical Considerations in HIV Preventive Vaccine Research **www.unaids.org**
European Directive on clinical trials **www. europa.eu.int**
Convention on Human Rights and Biomedicine **www.coe.int**
National Bioethics Advisory Commission Report **www.georgetown.edu**

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Part II: Therapeutic Agents

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Chapter 6

Glucocorticoids

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Abstract

Glucocorticoids (GCs) were the essential components of induction and maintenance immunosuppressive regimens in solid organ transplantation in past decades. The pharmacokinetics of these agents is extremely complex. Unbound GC binds and activates its cytosolic receptors. Activated receptor dimers interact with GC response elements and then inhibit cytokine production, lymphocyte proliferation, acid metabolites, and influence the immune cell trafficking. For renal transplant recipients, conventional immunosuppressive regimen generally consists of a calcineurin inhibitor in combination with an antiproliferative agent and a GC. It has effectively reduced acute rejection rates and has improved short-term allograft survival but there is no clear evidence for a beneficial effect on long-term allograft survival. However, long-term use of GCs has been associated with well-described adverse effects, such as hypertension, hyperlipidemia, diabetogenesis, osteoporosis, cataracts, and a myriad of other side effects. Withdrawal or avoidance of GCs following organ transplantation without impairing graft function has been attempted over the past decade but the results remain a controversial issue due to the sampling bias of "selected" populations that are composed of low-risk patients. The choice of steroid off regimen, in low-risk patients, must take the risk of shortening graft survival into account.

Keywords: glucocorticoids, transplantation, glucocorticoid receptor, adverse effects, steroid withdrawal/avoidance

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Abbreviations

AP-1: Activator protein-1
ARE: Acute rejection episodes
APCs: Antigen-presenting cells
AS: Adrenal suppression
AZA: Azathioprine
BMD: Bone mineral density
CNS: Central nervous system
CNIs: Calcineurin inhibitors
CRH: Corticotropin-releasing hormone
CsA: Cyclosporine A
GR: Glucocorticoid receptor
GREs: Glucocorticoid response elements
GC: Glucocorticoid
GLUT-2: Glucose transporter-2
GM-CSF: Granulocyte-macrophage-colony stimulating factor
HSP90: 90-kDa heat shock protein
HPA-axis: Hypothalamic-pituitary-adrenal-axis
IFN-γ: Interferon gamma
MAPK: Mitogen-activated protein kinase
MMF: Mycophenolate mofetil
MPA: Mycophenolic acid
NODM: New-onset diabetes mellitus
NSAIDs: Non-steroidal anti-inflammatory drugs
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
SLPI: Secretory leukoprotease inhibitor
TAC: Tacrolimus
TNF: Tumor necrosis factor

Introduction

Since synthetic cortisone was developed for clinical application in the 1940s, glucocorticoids (GCs) exhibit a wide range of effects on anti-inflammation and immunosuppression in animals and humans. They have also played a major role in the treatment of graft rejection post-transplantation for more than 6 decades. In the 1950s, during the initial stage of organ transplantation, GCs had been used for immunosuppression and as an adjunct to irradiation. Goodwin et al., [1] described the effectiveness of high-dose of GCs in suppressing and reversing rejection episodes in one of the first technically successful human renal allograft transplantation. In the early 1960s, azathioprine (AZA) was introduced for maintenance immunosuppression in renal transplantation. Subsequently a most commonly cited improvement regimen was established with combination of AZA and prednisone, a regimen with high-dose steroids anti-rejection therapy and lower dose steroids adjunctive therapy. From the 1960s to the early 1980s, kidney transplant recipients were primarily

maintained on the combination of AZA and steroids [2, 3]. This regimen provided 50% one-year allograft survival and 80–90% patient survival.

Throughout the decades, it has been demonstrated that GCs inhibit cytokine production, circulation of lymphocytes, acid metabolites, and microvascular permeability. They also block T cell activation and proliferation and thus have synergism to combine with other immunosuppressants to induce a state of immune hyporesponsiveness. However, GCs are associated with many well-known side effects, such as hypertension, hyperlipidemia, glucose intolerance, osteoporosis, cataracts, and growth retardation. Consequently, investigators have attempted to lower steroid dosage, but with an increased rate of acute rejection episodes, late graft dysfunction, and graft loss.

The emergence of cyclosporine A (CsA) has had a revolutionary effect on the overall success of renal transplantation. It is also the backbone of maintenance immunosuppressive regimens post-liver and post-heart transplant. With the introduction of calcineurin inhibitors (CNIs) including subsequently developed tacrolimus (TAC), post-transplant immunosuppressive protocols have undergone a major change. CsA/AZA/Prednisone became the classic triple-therapy combination used in virtually all renal allograft recipients in the early 1990s. This potent immunosuppressive regimen effectively reduced early immunologic injury and acute rejection rates but there has not been a proportionate corresponding improvement in extending long-term graft survival. The steroid sparing effect of CNIs and the use of either polyclonal or monoclonal antilymphocyte antibody induction therapy appear to facilitate avoidance or withdrawal of GCs which has been aimed to eliminate steroid-related side effects while not increasing the rates of acute rejection episodes (ARE) or chronic graft loss.

Minimizing GC use following organ transplantation has been a goal for many years [4–6] but it still remains a controversial issue. By summing up growing clinical data in the modern era, the current consensus is that a large majority of patients can safely undergo early steroid withdrawal; however GCs still play an important role as immunosuppressants in selected patients [7].

Structures and Metabolism

Corticosteroids are produced in the adrenal cortex from their common biochemical precursor, cholesterol. The main physiological corticosteroids are GCs and mineralocorticoids, classified by their specific receptors, target cells, and effects (Figure 1). GCs are so named from their early discovery for their effect on carbohydrate metabolism, and that these steroid hormones increase and maintain normal concentrations of glucose in blood by stimulating gluconeogenesis.

However, the term “corticosteroids” is generally used as a synonym of GCs. Cortisol is the major natural (endogenous) GC in humans (also called hydrocortisone when it is used pharmacologically). The release of cortisol is initially triggered by corticotropin-releasing hormone (CRH) secreted by the hypothalamus. CRH triggers cells in the neighboring anterior lobe of the pituitary gland to release corticotropin which stimulates the synthesis and the release of cortisol from the adrenal cortex. The synthesis and secretion are very sensitive to negative feedback by the circulating cortisol and exogenous (synthetic) GCs.

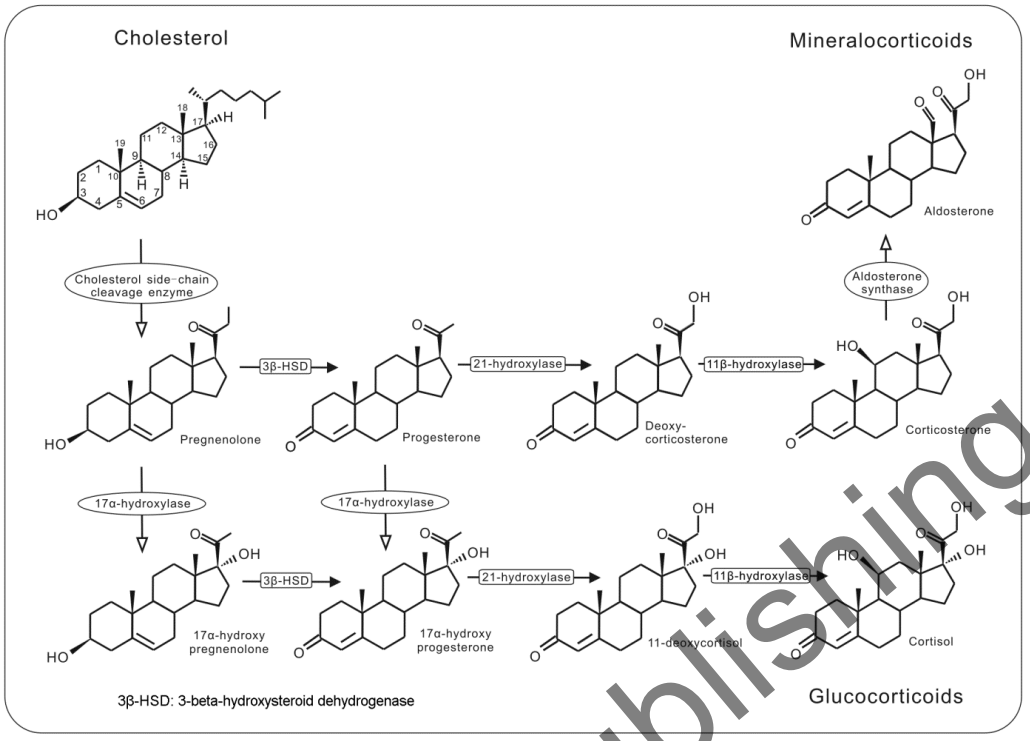

Figure 1. Major pathways in corticosteroid biosynthesis. All adrenal steroids are derived from cholesterol by various modifications in its structure. The cells of zona glomerulosa synthesize and secrete aldosterone, the principal mineralocorticoid; whereas those of zonae fasciculata and reticularis follow the glucocorticoid pathway and produce cortisol.

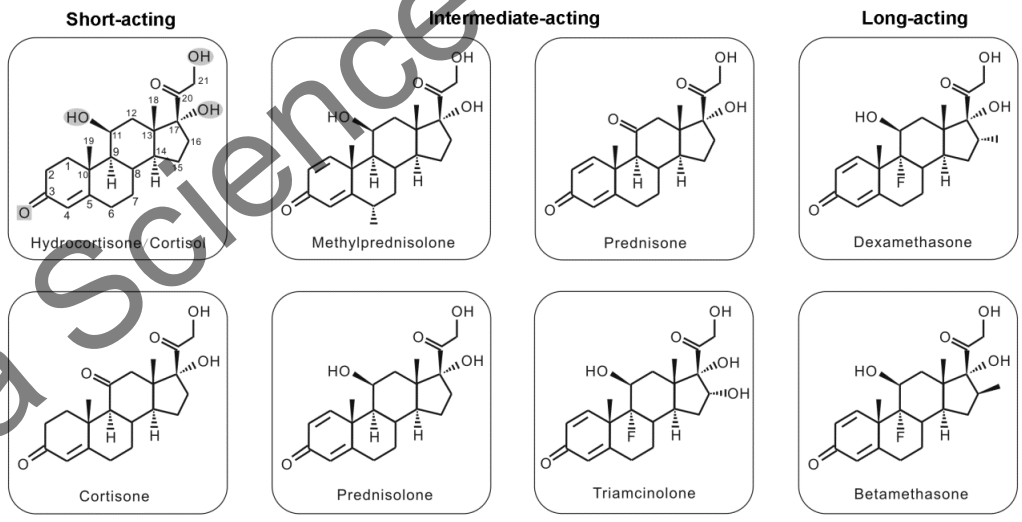

Figure 2. Pharmacologic group of commonly used synthetic glucocorticoids. The chemical modifications introduced to basic glucocorticoid structure can increase glucocorticoid and/or mineralocorticoid activity. The delta-4, 3-keto-11-beta, 17-alpha, 21-trihydroxyl configuration (the groups with gray-background) is required for glucocorticoid activity and is present in all natural and synthetic glucocorticoids.

Many synthetic GCs have been developed for pharmaceutical purposes since last 60 years. They have effects similar to the natural hormones produced by the adrenal cortex. The commonly used synthetic (exogenous) therapeutic GCs include hydrocortisone, cortisone, methylprednisolone, prednisone, prednisolone, triamcinolone, dexamethasone, betamethasone, and paramethasone. When compared to a natural form, the synthetic GCs appeared to have higher ratio of GC activity to mineralocorticoid activity [7, 8], better absorption rate through lipid barriers, and longer half-life (long-acting) [9, 10].

Table 1. Properties and dosing equivalents of systemic glucocorticoids

	Equivalent glucocorticoid dose(mg)	Relative glucocorticoid activity	Relative mineralocorticoid activity	Duration of action (hours)
Short-acting				
Cortisone	*25*	*0.8*	*0.8*	*8–12*
Cortisol	*20*	*1*	*1*	*8–12*
Intermediate-acting				
Methylprednisolone	*4*	*5*	*0.5*	*18–36*
Prednisolone	*5*	*4*	*0.6*	*18–36*
Prednisone	*5*	*4*	*0.6*	*18–36*
Triamcinolone	*4*	*5*	*0*	*18–36*
Long-acting				
Dexamethasone	*0.75*	*20–30*	*0*	*36–54*
Betamethasone	*0.6*	*20–30*	*0*	*36–54*

All glucocorticoids have a common sterane (steroid) core but each derivative carries different chemical modifications designed to increase its immunosuppressive/anti-inflammatory effects and decrease its mineralocorticoid properties. Methylprednisolone and prednisone are intermediate-acting glucocorticoids used frequently as part of the immunosuppressive regimen in organ transplantation. They are potent drugs with weak mineralocorticoids.

Data from:

1. National Institute for Health and Clinical Excellence (NICE) [Internet]. London: Clinical Knowledge Summaries: Corticosteroids - Oral. NICE; 2012 [cited 2015 Jan 30]. Available from: http://www.cks.nhs.uk/corticosteroids_oral.
2. Furst DE, Saag KG. Determinants of glucocorticoid dosing. UpToDate 2012 [Internet]. Waltham (MA): Wolters Kluwer Health; 2013 [updated 2014 Sep 26; cited 2015 Jan 30]. Available from: http://www.uptodate.com/contents/ determinants-of-glucocorticoid-dosing? source=search_ result&searh=glucocorticoid&selectedTitle=4~150.

Figure 2 and Table 1 summarize the differences of the commonly used systemic GCs in their structures, potency, duration of action, and ratio of mineralocorticoid to GC properties, which determine the GC's efficacy and therapeutic use, in comparison with mineralocorticoids.

In the current era of transplant immunosuppression, the most commonly prescribed GCs are prednisone and prednisolone, as part of the immunosuppressive regimen, because of their high GC activity. Prednisone is rapidly converted to its major active metabolite by the enzyme ll-/3-hydroxydehydrogenase in the liver, primarily to prednisolone. When administered orally, both of them provide comparable bioavailability of active prednisolone and immunosuppressive efficacy (11).

Mechanism of Action

The immunosuppression effect by GCs is mainly decreases in the function and numbers of lymphocytes including both B cells and T cells through multiple mechanisms (Figure 3).

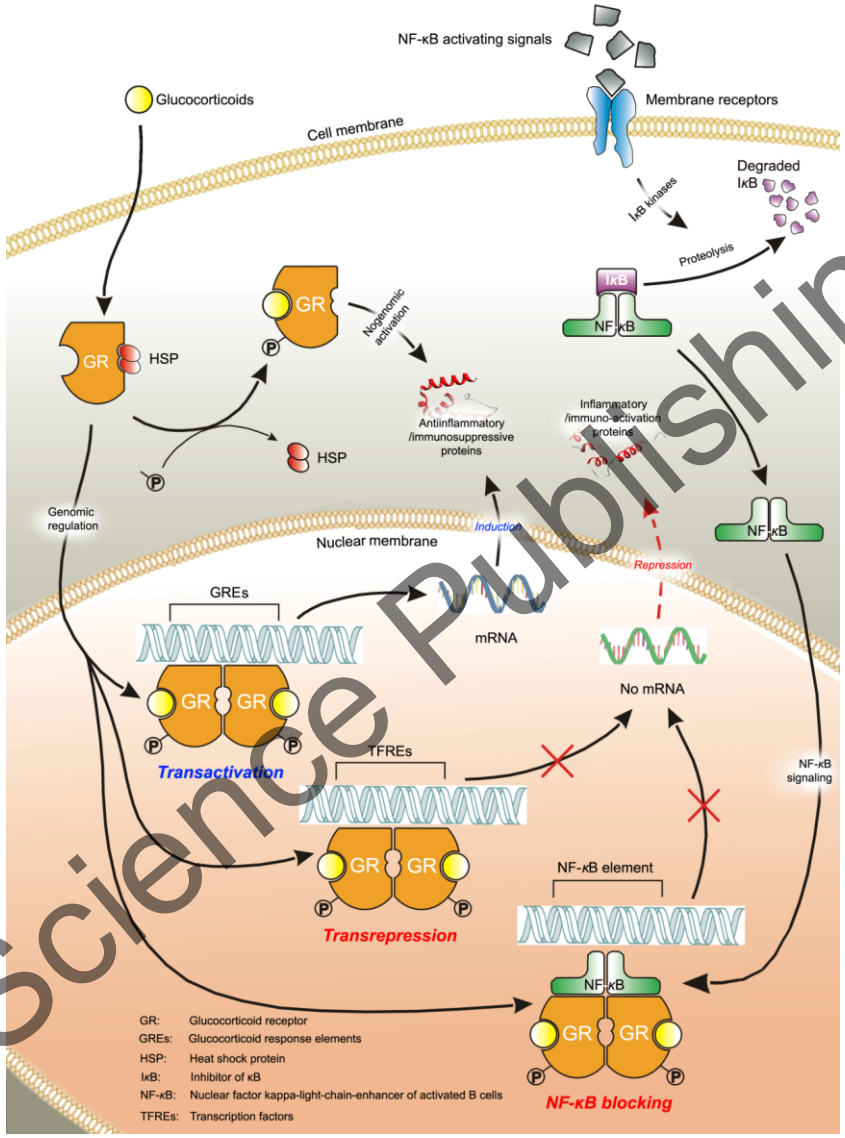

Figure 3. Mechanisms of glucocorticoid-induced immunosuppression. Binding of glucocorticoid to glucocorticoid receptor leads to activation of glucocorticoid receptor complex by dissociation of the heat shock proteins from the complex. The activated steroid-receptor complex monomers perform non-genomic action by interacting with other signaling pathway. The complex translocates itself into the nucleus where it interacts with glucocorticoid response elements as homodimers, performing genomic action and leading to either transactivation or transrepression. Figure adapted from Rhen T, Cidlowski JA. Antiinflammatory action of glucocorticoids-new mechanisms for old drugs. N Engl J Med 2005; 353:1711–1723 [23].

Regulation of Gene Transcription (Genomic Signaling)

GCs appear to exert their most critical immunosuppressive effects by blocking the expression of several cytokine/cytokine-receptors derived from T-cell and antigen-presenting cells (APCs) [12]. Unbound GCs passively diffuse through the cell membrane into the cell, where they bind with high affinity to intracellular GC receptor (GR), which is closely associated with the 90-kDa heat shock protein (HSP90). As a consequence of binding, HSP90 becomes dissociated from the steroid-receptor complex [13], and the complex translocates itself into the nucleus where it interacts, as a homodimer, with specific DNA sequences called GC response elements (GREs). GRE sequences have been localized in the critical promoter region of several target genes.

Binding of the GR complex to those target genes results in transcriptional activation (transactivation) or transcriptional repression (transrepression). The transactivation process up-regulates several anti-inflammatory proteins, e.g., lipocortin I, p11/calpactin binding protein, secretory leukoprotease inhibitor 1 (SLPI), mitogen-activated protein kinase phosphatase (MAPK phosphatase) and gluconeogenesis [14].

Binding of the complex to the DNA sequences where another transcription factor would bind competitively prevents the genes transcription activated by that factor. Those genes are mainly the ones that code for the cytokines IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, and interferon gamma (IFN-γ); the most important of which is IL-2, a principal T cell growth factor, and IL-2 signaling pathway (Signal 3) activator [15, 16]. This mechanism explains inhibition of the cytokine transcription by GCs but it is generally not accepted. Evidence indicates that during transcription, the ligand-activated GR is not interacting directly with DNA, but rather with other transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein-1 (AP-1). This may be mediated in part by GC-induced expression of MAPK phosphatase 1 [17–23]. AP-1 induces cytokine genes and growth factors such as the IL-2 cytokine, whereas NF-kB is a critical transcription factor involved in the synthesis of many cytokines and cell adhesion molecules [14, 23, 24].

Post-Transcriptional Effects (Nongenomic Regulation)

In addition to the effects on gene transcription, activated GR can directly interact with cytokine molecules or with mRNA at post-transcriptional levels. It has been shown to inhibit secretion of cytokines and messenger RNA (mRNA) encoding IL-1, IL-2, IL-6, IL-8, tumor necrosis factor (TNF), and granulocyte-macrophage-colony stimulating factor (GM-CSF) [23, 25].

Humoral Immunity Suppression

GCs not only inhibit cell-mediated immunity at transcriptional/post-transcriptional levels, but also slightly suppress the humoral immunity by reducing B cell clonal expansion via decreasing IL-2 and IL-2 receptors expression. Steroid-induced inhibition of antibody synthesis is possibly as a consequence of direct B cell depletion; it may be attributable to antibody catabolism, T cell helper/suppressor balancing, and period of immunosuppressive therapy [26–31].

Induction of Lymphocyte Apoptosis

It is generally accepted that the T cell suppression by GCs is via inhibition of T-cell proliferation, but GC-induced T lymphocyte apoptosis is also an increasing interest. The effects on T cell apoptosis depend upon the stage of T cell differentiation and T cell subtype. The effect is more prominent in immature T cells still inside the thymus such as ($CD4^+/CD8^+$) thymocytes, but peripheral T cells are also affected. Resting mature T cells are generally GC resistant. The susceptibility to GC-induced apoptosis depends on the presence of expression of B-cell lymphoma 2 (Bcl-2), a protein which is absent in immature T cells [32, 33].

Adverse Effects

The effects of GCs result from both genomic and nongenomic mechanisms [34–36]. The desired immunosuppressive/anti-inflammatory effects of GCs are mainly mediated via genomic gene transrepression. In contrast, the mechanisms for GCs-mediated side effects are complex; they appear to result predominantly from genetic transactivation process [35]. The adverse effects of GCs are not specific to lymphocytes. They may affect any organ system in the body; they appear as a reflection of their profound immunosuppressive, anti-inflammatory and hormonal action on numerous target tissues. The occurrence of many serious adverse events is associated with the patients receiving these drugs in high doses or over a long period of time. Appropriate use of GCs in organ transplantation can minimize the ubiquitous complications. GC toxicity is generally related to the average dose and cumulative duration of steroid administration, but there is no commonly accepted "threshold" dose or duration [37]. Short-term or acute adverse effects, which usually occur with initiation of therapy, include central nervous system (CNS) effects (mood changes) ranging from mild euphoria and insomnia to severe mental disorders, hyperglycemia and impaired glucose tolerance, sodium retention-related weight gain and fluid accumulation, and gastrointestinal effects. Long-term adverse effects are most likely to occur in patients who take GCs daily for months or longer and such risk is both dose- and time-dependent [38, 39]. Long-term effects include: osteoporosis and fractures, hypothalamic-pituitary-adrenal-axis (HPA-axis) suppression, iatrogenic Cushingoid appearance and weight gain, cardiovascular disease and dyslipidemia, infectious complications, myopathy, cataracts and glaucoma, growth failure/delayed puberty, as well as dermatologic disorders.

Hyperglycemia and Diabetes Mellitus

The cumulative incidence of new-onset diabetes mellitus (NODM) at 12 and 36 months post-transplantation has been reported to be approximately 16% and 24%, respectively [40]. Risk factors for NODM are varied, including use of CNIs (CsA and TAC) and GCs. Exogenous GC induces hyperglycemia by stimulating glucose secretion as well as reducing glucose clearance via inhibition of glucose transport into adipose and muscle cells. GCs can also impair the expression of glucose transporter-2 (GLUT-2), a protein-mediated glucose transporter that ferries glucose across cell membranes [41]. High-dose GCs can lead to glucose toxicity further by increasing insulin resistance in patients with pre-existing and new-onset diabetes. In general, GC-induced hyperglycemia improves with dose reductions and usually reverses when steroid therapy is discontinued.

Cushingoid Appearance and Weight Gain

Prolonged GC-based immunosuppressive regimen is the most common cause of Cushingoid features or Cushing's syndrome which may have a characteristic appearance of moon face, truncal obesity, skin changes, resulting from weight gain and redistribution of adipose tissue. The Cushingoid appearance occurs within the first two months of therapy. Patients may develop hypertension and edema. The occurrence of Cushingoid abnormalities appears to be dependent on both the dose and duration of treatment. In children, Cushing's syndrome induced by GC therapy can suppress growth (42); also they are at higher risk of experiencing adrenal suppression (AS). Therefore, HPA-axis function should be evaluated prior to discontinuing steroid therapy in children with Cushingoid features [43, 44].

Dyslipidemia and Hypertension

Approximately 30–75% of renal transplant recipients have developed an abnormal lipid profile, both in adult and in children [45, 46]. The prevalence appears lower than that observed in heart and liver recipients following transplantation [47–52]. In addition to sirolimus and CsA, GCs are major factors contributing to hyperlipidemia. Persistent dyslipidemia can eventually lead to accelerated atherosclerosis and ischemic heart disease [53]. As a consequence, interest in regular monitoring and attempt to prevent and to treat the hyperlipidemia as well as other traditional risk factors for cardiovascular diseases in the post-transplant period has increased dramatically. GCs have a number of dose-dependent effects on renal function and systemic hemodynamics: Higher-dose GCs commonly promote fluid retention, particularly in patients with underlying heart or kidney diseases. There is no clear explanation that how GC therapy can raise the blood pressure in both normal and hypertensive subjects [54, 55]. However, significant hypertension occurred in the patients receiving low doses of GCs which may be the results of older age and initial blood pressure [56]. Successful blood pressure control (130/80 mm Hg or lower) after renal transplantation can reduce the risk of progressive graft loss.

Cardiovascular Disease (CVD)

Cardiovascular risk factors, often existing before transplantation, include hypertension, hyperlipidemia, hyperglycemia and diabetes mellitus, obesity, ischemic heart disease, vascular disease, long-term dialysis, physical inactivity, and older smoking males [57–59]. Post-transplantation GC-induced hypertension, hyperglycemia, and obesity are known to be associated with an increased risk of serious adverse cardiovascular events, particularly ischemic heart disease and heart failure. CVD risk was significantly greater with high GC doses (prednisone ≥7.5 mg daily or its equivalent) [60] and with those current/recent use (within 1 month) or with long-term use of GCs. It was demonstrated that there is a positive correlation between GC doses and incidence of new-onset atrial fibrillation and atrial flutter [61, 62].

A significantly increased risk of heart failure and ischemic heart disease in GC users has been also noticed. CVD remains the leading cause of death in kidney transplant recipients. Because CVD has occurred primarily in patients with underlying diseases, the control of those diseases is crucial for CVD risk reduction. However, it was difficult to determine whether this adverse effect was more likely attributable to GCs or to the underlying disorders necessitating the therapy.

Osteoporosis, Osteonecrosis and Fracture

Osteoporosis is one of the more serious GC-induced complications of prolonged GC therapy. Loss of bone matrix and reductions in bone mineral density (BMD) are followed by a decrease in bone formation by suppressing osteoblast activity and function, and promoting the apoptosis of osteoblasts and osteocytes [63–65]. The incidence of developing osteonecrosis subsequent to GCs therapy ranges varying from 21% to 37% and appears to increase with higher doses and prolonged treatment [66]. It has been found that the risk is dose-dependent in the studies of prednisone therapies, which was less than 3% with 15 to 20 mg/day and exceeded 90% in the highest-dose (40 mg/day) of therapy [67]. Increased bone fragility due to GC-induced osteoporosis or osteonecrosis can lead to spontaneous vertebral compression and bone fractures. In children, of the various fracture types, the risk of humerus fracture was doubled; their vertebral fractures were often asymptomatic [68–71].

HPA-Axis Suppression (Adrenal Suppression, AS)

AS is the phenomenon of inadequate (commonly decreased) cortisol production that results from exposure of the HPA-axis to exogenous GCs (72). Use of longer-acting GC formulations tends to be associated with a higher risk of AS [73]. Abrupt withdrawal of corticosteroids is a risk factor of AS development [74].

This may be prevented by a more gradual tapering of GCs but there is no reliable tapering guideline. AS is the most common cause of the adrenal insufficiency in children. Adrenal crisis caused by AS is associated with higher mortality; clinically this may present with

profound weakness, low blood pressure, dehydration, rapid heart rate, seizures or even death among others [43, 44, 75–77].

Central Nervous System Effects

GCs induce a wide range of central nervous system effects, from euphoria to depression, and cognitive symptoms, probably as a result of increased brain excitability. The psychiatric and cognitive disturbances include emotional lability, agitation, irritability, hypomania, mania, anxiety, depression, insomnia/or lethargy, and even psychosis. Rare cases of pseudotumor cerebri have been reported with GC use [78, 79]. Central nervous system disorders can emerge few days after initiating GC therapy, and appear to be dependent on dose and duration of therapy [80, 81]. The patients with a past psychiatric history are more susceptible of developing a given neuropsychiatric disorder following GC therapy [79]. A short-term GC therapy is commonly associated with euphoria; the long-term GC courses induce depressive outcomes [81–83]. In most patients, these psychiatric symptoms are mild and reversible as they usually recover with dose reductions or upon interruption of therapy. GC-induced psychosis only occurs exclusively at high doses (prednisone above 20 mg/day or equivalent) given for a prolonged period [37, 84–85].

Cataracts and Glaucoma

One of the most common side effects of GC therapy is cataract formation, which commonly occurs after prolonged GC use. The GC-induced cataracts can usually be distinguished from senile cataracts as they develop in the posterior subcapsular area (in the rear region of the lens capsule), are often bilateral and develop slowly, occur more commonly in a younger age group, and usually require earlier surgical intervention/removal [86]. The risk of cataract formation is dose- and time-dependent, with doses ≥10 mg/day of oral prednisone (or equivalent) which lasted more than one year [86−88].

Glaucoma may be induced or aggravated by GC ophthalmic preparations after corneal transplantation [89]. To a lesser extent, systemic GC use can also increase intraocular pressure leading to optic nerve damage which is often permanent.

Gastrointestinal Impacts

GCs have been associated with an increased risk for a number of adverse gastrointestinal events, mostly peptic ulcer formation. However, combined use of GCs with non-steroidal anti-inflammatory drugs (NSAIDs), which results in a synergistic increase in the incidence of gastrointestinal events, increases significantly the risks of peptic ulcer disease rather than use of GCs or NSAIDs alone [90, 91].

Cutaneous Adverse Events

Skin thinning/fragility and purpura are common cutaneous adverse events attributable to steroids-included immunosuppressive therapy post-transplant. GCs reduce collagen synthesis, keratinocyte growth factor expression, and inflammatory cells infiltration, thus impairing the natural wound-healing process [92]. Data demonstrated that skin changes were seen in 46 percent of patients treated for three months with prednisone at doses greater than 20 mg daily [93].

As a consequence of immunodeficiency, the risk of non-melanoma skin cancers (squamous cell and basal cell types) was increased in oral GC users compared with the controls without skin cancer [94].

Myopathy

Decrease in protein synthesis and increase in protein degradation both contribute to the muscle atrophy which is an infrequent complication of GC therapy. Steroids appear to play a crucial role in muscle atrophy observed in various pathological conditions [95]. Skeletal muscle atrophy is characterized by a decrease in the size of the muscle fibers. GCs have been shown to cause atrophy of fast-twitch or type II muscle fibers with less or no impact observed in type I fibers [96, 97].

Myopathy generally develops over several weeks to months of GC use. Patients typically present with proximal muscle weakness and atrophy in both the upper and lower extremities [98, 99]. The resulting weakness of peripheral and respiratory muscles may have major clinical implications such as loss of quality of life, fatigue, impaired wound healing, compromised lung function, and poor immune response. Symptoms generally improve within 3 to 4 weeks of dose reductions, and usually resolve after discontinuation of GC therapy [100].

Growth Suppression

GC therapy has been associated with growth suppression in children. They inhibit growth through several different mechanisms which may involve reduced endogenous growth hormone production, inhibition of bone and collagen formation, and nitrogen retention.

The growth suppression can be an independent adverse effect of GC therapy or can develop with other symptoms of GC excess, known as Cushing's syndrome; it can also be a sign of AS. The effects are related to the type, dose, and duration of the exposure. Growth impairment is most pronounced with daily therapy and with long-acting agents, as compared with an alternate-day regimen and with short-acting agents (e.g., dexamethasone > prednisone > hydrocortisone) respectively [101]. Affected children may experience some catch-up growth after GCs are discontinued. Prolonged systemic GC (prednisone) treatment may have persistent growth effects [102], especially in girls.

Immunodeficiency

Immunodeficiency is the common adverse effect of concomitant immunosuppressive therapies post-transplant. Systemic uses of GCs act in multiple ways to inhibit the immune system and so their use is associated with a dose-dependent increased susceptibility to infection, especially with common bacterial, viral, and fungal pathogens. In addition to GC dose, factors influencing infection risk include patient age, the underlying disorder, the presence of concomitant immunosuppressive therapies [103, 104], and the location of the patient (i.e., whether the patient is hospitalized).

Malignancy is a widely recognized complication of transplantation, and the effects of various immunosuppressive drugs on malignancy risk remain controversial. Immunosuppressive therapy appears to increase the risk of both non-melanoma skin cancers and non-Hodgkin lymphoma among patients with renal transplantation [105–110]. It has been reported that the overall risks for squamous cell carcinomas and basal cell carcinomas, as well as non-Hodgkin lymphoma were increased in a large cohort of GC users, which suggests that use of GCs may be a shared risk factor for certain skin cancers and lymphomas [111].

Immunosuppressive Strategies in Solid Organ Transplantation

GC-Based Immunosuppression Regimens

Immunosuppressive treatment of the transplantation patient begins with an induction phase which is subsequently followed by a maintenance therapy. Induction and maintenance strategies use different agents at specific doses to achieve target therapeutic levels for long-term graft survival. Biologic induction with monoclonal or polyclonal antibody is used recently for initial immunosuppression in most of solid organ transplants except in liver transplants procedures [112]. High-risk recipients including GC-resistant patients typically receive antibody induction therapy beginning perioperatively and immediately after transplantation to avoid early acute rejection.

Maintenance immunosuppression is the key to prevention of acute and chronic rejections throughout the life of the graft. The optimal maintenance immunosuppressive therapy in organ transplantation is not established. The major immunosuppressive agents used for many years consisted of 3 types of drugs in combination: GC (methylprednisolone /prednisone), a purine antagonist (Aza or mycophenolate mofetil, MMF), and a calcineurin inhibitors (CsA or TAC). Dose ranges of GCs vary with transplant centers and individual patients. A common and steroid-included regimen in kidney transplantation is to give large intraoperative dose of methylprednisolone subsequently to taper to fixed physiological dose of 5 mg daily of oral prednisone by postoperative day 3–5.

GC Withdrawal and Avoidance Strategies

Over the course of the last century, GCs have been the mainstay of immunosuppression. However long-term use of steroids has been associated with well-described adverse events, including immunodeficiency, cardiovascular disease, "steroid diabetes", cataracts, osteoporosis, adrenal insufficiency, central nervous system, hypercortisolemia, and skin/muscle side effects. The immunosuppressive benefits derived from the GC regimen are offset. These numerous complications contribute to increased long-term morbidity and mortality post-transplant.

To reduce toxicity and decrease overall immunosuppression, minimizing GC use (tapering, ultimate withdrawal or complete avoidance) has been attempted for many years [4–6]. Although GC regimens are currently still used in most of transplant centers, steroid withdrawal/avoidance strategies are increasingly accepted. However, the benefits of steroid withdrawal/avoidance must be weighed against the risk of precipitating rejection.

The immunosuppressive effects of steroid minimizing strategies are dependent in part upon the ability of the remaining non-steroid immunosuppressive agents to suppress the anti-allograft immune response. The continuing introduction of newer and more potent induction and maintenance immunosuppressive agents may eventually make it possible to abandon GCs in maintenance treatment.

In kidney transplantation, the most common type of solid organ transplantation, GC administration can be further minimized via four strategies: very lower doses maintenance therapy, earlier or late-stage withdrawal, very early withdrawal, and complete avoidance.

Lower Doses Maintenance

Initialed with an antibody induction therapy and in the absence of acute rejection, GCs generally are tapered to a dose of 5 mg daily by one month post-transplant then continue maintenance indefinitely. The benefit of low-dose steroid maintenance may include decreasing acute rejection associated with improvement of immunosuppression and avoiding chronic kidney allograft nephropathy directly or via modulation of calcineurin nephrotoxicity, compared with early steroid withdrawal regimen [113].

Early (Weeks to 6 Months) and Late (after One Year) GC Withdrawal

The attempts made to gradually withdraw steroids months post-transplantation (≤6 months) in selected recipients showed an increased incidence of AREs and possible decreased long-term allograft survival [114, 115]. It is not recommended and may only be attempted in those who have received lymphocyte-depleting therapy. The results of studies evaluating late steroid withdrawal are conflicting and remain a controversial issue. The higher failure rate of early/late steroid withdrawal strategies in most studies underlines the importance for caution [116–124].

GC Avoidance (Rapid Steroid Discontinuation or Complete Steroid Avoidance)

For minimizing any early steroid-related side effects and preventing steroid dependence caused by late steroid withdrawal, the strategy of rapid steroid discontinuation (withdrawal of steroids within the first weeks post-transplantation) or complete steroid avoidance (without any perioperative i.v. methylprednisolone) has been attempted by the introduction of mycophenolic acid (MPA) therapy, together with the widespread adoption of antibody induction [125–128]. The results of short- and intermediate-term studies have shown no difference between rapid steroid discontinuation and maintenance prednisone groups in recipient and organ survival, in severity of ARE, or in renal function [129]. The studies showed that steroid withdrawal increases MMF and TAC exposure, possibly resulting in lower acute rejection occurrence [130, 131]. However further long-term follow-up studies are required to characterize the safety and the benefits of rapid steroid discontinuation. There is preliminary evidence from nonrandomized trials which has suggested that complete steroid avoidance may be feasible for appropriately selected patients [132, 133].

An increasing number of studies conclude that GC avoidance regimens provide similar renal allograft outcomes, compared with continuation of GC therapy. However, the cohort of individuals included in part of those studies were selected relatively low-risk patients, with a majority of living-donor kidney recipients, non-African Americans, and nonsensitized recipients, and compared with historical controls receiving higher steroid doses for sustained periods of time [134].

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Chapter 7

Small Molecules in Solid Organ Transplantation with Cytostatic Antiproliferative Effects

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Abstract

Solid organ transplantation has become a daily clinical routine. Survival rates have improved tremendously due to the availability of potent immunosuppressive drugs. There are numerous drugs in the market, which are administered for organ transplant recepients on a regular basis. Conventional drugs, like Azathioprine have been essential for decades. It was first used as an anti-cancer medication and later on, its use was extended to organ transplantation. Azathioprine replaced 6-Mercaptopurine due to the toxic effects exerted by the latter.

Later on, Azathioprine was nearly completely replaced in clinical practice by Mycophenolate mophetil as a drug of choice in solid organ transplantation. Mycophenolate mofetil gained popularity in the late nineties as a new drug in the field of immunosuppressive therapy. It is widely used due to its low toxicity in comparison to Azathioprine. Apart from transplantation, it is also applied to treat certain autoimmune diseases.

Mizoribine is another promising immunosuppressive drug, which is mostly used in Japan in solid organ transplantation. As a nucleoside imidazole and a natural product, Mizoribine, has proven to be less toxic compared to Azathioprine. It has been approved for renal transplantation, as well as, for treating lupus nephritis and rheumatoid diseases.

Apart from the above-mentioned purine biosynthesis targeting drugs there are pyrimidine biosynthesis inhibitors such as Leflunomide and Malononitrilamides. Leflunomide affects pyrimidine biosynthesis by inhibiting a key enzyme, the dihydroorotate dehydrogenase. This in turn inhibits rapidly-dividing cells such as lymphocytes. The role of Malononitrilamides in transplantation settings is not clear yet, however, they have been used in renal transplant cases.

In this chapter, we discuss some of the major purine and pyrimidine synthesis inhibitors such as, Azathioprine, Mycophenolate mofetil, Mizoribine, Leflunomide and Malononitrilamides, respectively. Apart from these, we also shortly discuss other small molecules with antiproliferative and cytostatic properties, such as, Farnesyl-S-transferase inhibitors, 2-Chlorodeoxyadenosine (Cladiribine), PG 409-88, VX-497 and VX-148 *etc*.

Keywords: purine analogues, pyrimidine analogues, immunosuppressive drugs, mycophenolate mofetil, mizoribine, malononitrilamides

Abbreviations

6-MP: 6-Mercaptopurine
ATP: Adenosine triphosphate
AUCs: Area under the curves
AZA: Azathioprine
BS: Brequinar sodium
CMV: Cytomegalovirus
CsA: Cyclosporine A
DHODH: Dihdyroorotate dehydrogenase
DNA: Deoxyribonucleic acid
EC-MPS: Enteric-coated mycophenolate sodium
FTIs: Farnesyl-S-transferase inhibitors
FDA: Food and drug administration
GI: Gastrointestinal
GMP: Guanosine monophosphate
GTP: Guanosine triphosphate
HCV: Hepatitic C virus
HGPRT: Hypoxanthine-guanine phosphoribosyltransferase
HIV: Human immunodeficiency virus
IMPDH: Inosine-5′-monophosphate dehydrogenase
iNOS: Inducible nitric oxide synthase
LEF: Leflunomide
MMF: Mycophenolate Mofetil
MNA: Malononitrilamide
MPA: Myophenolic acid
MPAG: Mycophenolic acid glucuronide
MZB: Mizoribine
MZB-5-P: Mizoribine-5-Phosphate
NO: Nitric oxide
PRPP: 5-phosphoribosyl-1-pyrophosphate

RA: Rheumatoid arthritis
RNA: Ribonucleic acid
ROS: Reactive oxygen species
TPMT: Thiopurine S-methyltransferase
TWHF: Tripterygium Wilfordii Hook F
VCAM-1: Vascular cell adhesion molecule-1
VLA-4: Very late antigene-4

Introduction

During the last fifty years, a very fascinating development could be observed in transplantation medicine, concerning the understanding of the immune responses involved in rejection and tolerance, as well as, regarding the introduction of new immunosuppressive agents. Despite all these impressive strides, there are still several problems to be solved and questions to be answered: there is no absolute method, molecular target or medication available, which could specifically prevent rejection without impairing the physiologically essential immune responses.

One of the first observations about the characteristics of an alloimmune response was the presence of a prominent lymphocyte proliferation. Thus the initial attempt to treat this immune reaction was aimed at inhibiting lymphocyte proliferation [total body/lymphoid irradiation, 6-Mercaptopurine (6-MP)] [1]. Accordingly, targeting lymphocytes with the usage of antimetabolites or cytostatic agents is not a new approach in immunosuppressive therapy. Most of the agents detailed in this chapter have a long history; nevertheless, few of them are still parts of the routine immunosuppressive regimes used in clinical transplantation. However, those developed in the first line - especially Azathioprine - have several unwanted side effects. The research activity for new compounds with higher selectivity, and newer formulations with acceptable side effect profiles but with a potent anti-proliferative effect, is still highly important.

In this chapter, we attempted to give an overview of the most frequently used drugs targeting the purine biosynthesis: azathioprine (AZA), mycophenolate mofetil (MMF), mizoribine (MZB) or pyrimidine biosynthesis: leflunomide (LEF). Furthermore, to provide a more complete overview of this field we also summarized the data accumulated about alternative or second-line agents and newer compounds in different phases of preclinical or clinical development.

Agents Targeting Purine Biosynthesis

The details of purine biosynthesis are beyond the scope of this chapter as they represent basic knowledge in biochemistry. Briefly, via the de novo pathway 5-phosphoribosyl-1-pyrophosphate (PRPP) is synthesized from ribose-5-phosphate, adenosine triphosphate and as the last step of the pathway guanosine monophosphate (GMP) is derived through the inosine monophosphate dehydrohenase (IMPDH) enzyme (Figure 1). The end product of the so-

called salvage pathway is likewise GMP, but synthesized from PRPP and guanine by the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (Figure 1).

The immunosuppressive drugs discussed in this section AZA, MMF, MZB are targeting the purine biosynthesis at one or more essential steps of this pathway.

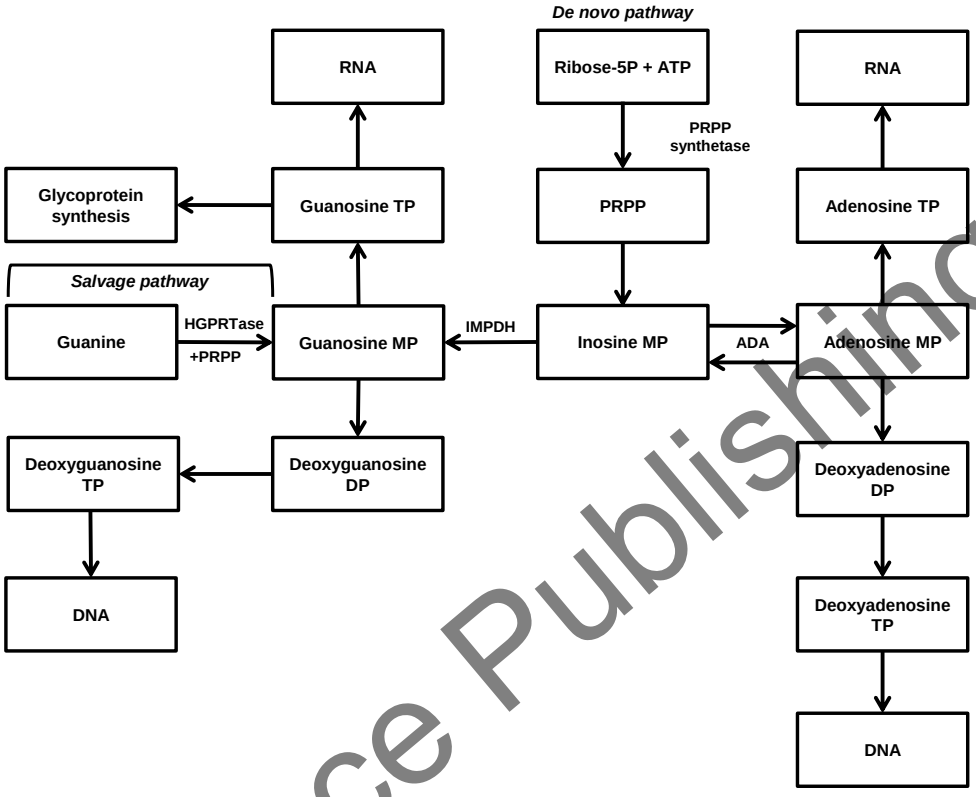

Figure 1. *De novo* and salvage purine biosynthesis pathways. GMP plays a central role in purine biosynthesis. The two rate-limiting enzymes in lymphocytes (PRPP synthetase, IMPDH) are activated by guanosine ribonucleotides and deoxyguanosine triphosphate, but inhibited by AMP, ADP and by dATP, respectively. The abbreviations used are: DNA: deoxyribonucleic acid; RNA: ribonucleic acid; MP: monophosphate; DP: diphosphate; TP: triphosphate; PRPP: 5-phosphoribosyl-1-pyrophosphate; AMP, ADP, ATP: adenosine mono-, di, tri-phosphate; dATP: deoxyadenosine triphosphate; IMPDH: inosine monophosphate dehydrogenase; ADA: adenosine deaminase; HGPRTase: hypoxanthine-guanine phosphoribosyltransferase. (Adapted from: Allison AC, Eugui EM. Immunpharmacology. 2000; 47(2−3): 85−118) .

Azathioprine

AZA, a purine analogue, was widely used for treating malignant diseases in the late fifties. The compound was first discovered by Hitchings and Elion in 1942, which among others highly contributed to the award of Nobel Prize in Medicine in 1988 for their imprescriptible work in this field [2].

In the 1950s−60s, 6-Mercaptopurine was first tested in different experimental settings [3]. Subsequently, it was also used in treating acute lymphocytic leukaemia [3−5]. Later on,

Azathioprine, the prodrug of 6-MP, was proven to be less toxic and to have more favourable pharmacokinetics compared to 6-MP.

The immunosuppressive potential of AZA was revealed by Schwartz, Stack and Damashek [6]. Thereafter, AZA made its way into solid organ transplantation as an immunosuppressive drug. Based on the impressive preclinical results, it was was firstly applied in kidney transplant recipients at Peter Bent Brigham Hospital in Boston by Joseph E. Murray (Nobel prize in Medicine, 1990) and John P. Merrill [7].

AZA has been used as an immunosuppressant for more than fifty years. The compound is widely manufactured under the trade name of Imuran® (Glaxo Smithkline). The other trade names include Azasan®, Azamun®, and Imural®.

Apart from interference with purine synthesis, AZA is known to exert cytotoxic effect. The drug is also indicated as an immunosuppressive agent in autoimmune diseases (rheumatoid arthritis-RA, systemic lupus erythematosus etc.), and inflammatory bowel diseases [8, 9].

Mechanism of Action

Azathioprine consists of two moieties: 6-MP and an imidazole derivative. It is metabolised to 6-MP and aminoimidazoles (Figure 2). Azathioprine interacts with nucleophiles, such as glutathione to form 6-Mercaptopurine. This compound is further transformed into metabolites that inhibit de novo purine synthesis. It has been shown that AZA is metabolised, both *in vitro* and *in vivo*, to 6-MP and 6-thioguanine [10]. These compounds are further converted in the body to its respective monoribonucleotides, which in turn hinder the conversion of inosine monophosphate to adenine and guanine. Furthermore, they are known to play an important role in the feedback inhibition of *de novo* purine synthesis.

As mentioned earlier, initial transformation of AZA to its biologically active products is a nonenzymatic reaction propitiated by the nucleophilic attack on the C (5i) atom of the imidazole ring [11]. This reaction, both *in vivo* and *in vitro*, depends on the available sulphydryl compounds like glutathione, cysteine, other thiols and some proteins (Figure 2). On the other hand, 6-thio-ionosine monophosphate is converted to 6-thio-guanosine monophosphate and finally to 6-thio-guanosine triphosphate. This is later incorporated into DNA, RNA or both, thereby arresting cell proliferations.

Due to these changes, various lymphocyte functions are known to be affected. After consumption, sulphydryl groups present in compounds, such as glutathione and cysteine convert most of Azathioprine to 6-MP. 6-MP in the presence of xanthine oxidase gets converted to 6-thiourate. Later on, thiopurine S-methyltransferase (TPMT) converts 6-thiourate to 6-methyl monophosphate (Figure 2). HGPRT acts upon 6-methyl monophosphate and converts it to 6-thioguanine. Research has shown that TPMT pathway is vital for Azathioprine-mediated side effects such as myelosuppression. Simultaneously, 6-thioguanine, produced via HGPRT pathway, is known to exhibit immunosuppressive properties of 6-MP [12]. It has been shown that, AZA exerts its immunosuppressive action via synergistic cooperation of weak cytostatic effect of 6-MP and chemo-sensitizing effect exerted by highly reactive imidazole groups [11]. Once AZA is incorporated into the replicating DNA, it blocks

the de novo pathway of purine synthesis [10]. Since lymphocytes lack salvage pathway, the above-mentioned action contributes to the relative specificity to lymphocytes.

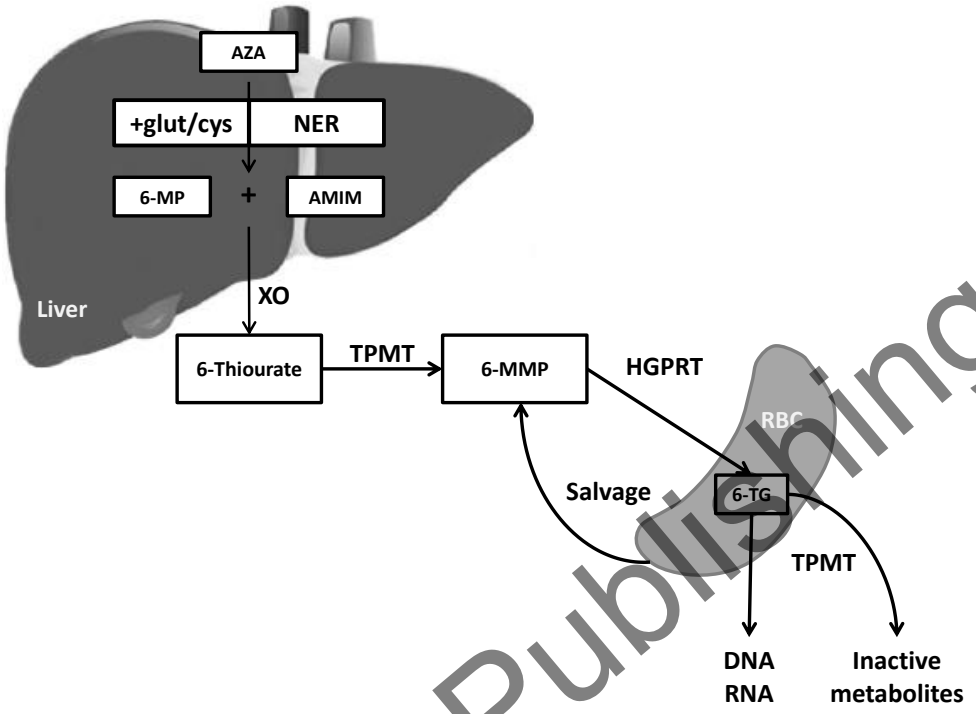

Figure 2. Metabolism of AZA and 6-MP. AZA is a pro-drug of 6-MP, which is converted within the liver via a non-enzymatic reaction to 6-MP. During the metabolism, different substances, responsible for the clinical immunosuppressive activities, as well as, toxic effects are created by enzymatic reactions. The abbreviations used are: AZA: azathioprine; glut: glutathione; cys: cysteine; NER: non-enzymatic reaction; 6-MP: 6-Mercaptopurine; AMIM: aminoimidazoles; XO: xanthin-oxidase; TPMT: thiopurine S-methyltransferase; 6-MMP: 6-methyl-monophosphate; HGPRT: hypoxanthine-guanine phosphoribosyltransferase; RBC: red blood cell; 6-TG: 6-thioguanine; RNA: ribonucleic acid; DNA: deoxyribonucleic acid.

It has been shown that AZA acts via inflammatory pathways [3]. Upon administering AZA, both T and B-lymphocytes in peripheral blood were significantly decreased [13]. Patients also showed normal primary and secondary humoral immune response *in vivo* [14].

Tiede et al., reported that *in vitro* stimulation of primary human T-lymphocytes in the presence of AZA or 6-MP leads to increased levels of apoptosis [12].

Another important feature exhibited by AZA and 6-MP is antigen-specific tolerance. However, the tolerance induction effects of AZA have failed to show promising results in human solid organ transplants. This resulted in opting for newer immunosuppressive drugs including calcineurin inhibitors, Cyclosporine A (CsA) and FK506 and antiproliferative agents such as MMF and rapamycin [10].

Pharmacokinetics and Pharmacodynamics

Formulations and Dosage

AZA is a prodrug of 6-MP. The initial dose of AZA is 3−5 mg/kg/day in renal transplantation and approximately 1−2 mg/kg/day in case of rheumatoid arthritis [15, 16].

It should be noted however, that the TPMT enzyme polymorphism considerably interferes with the metabolism of AZA, thus it has a significant impact on the therapeutical effect and on toxicity. These genetic differences between individuals must be routinely monitored as a part of the therapy planning [17].

Imuran®, Transimune®, and Azoprine® are available in formulations containing 25 or 50 mg AZA.

Pharmacokinetics

Azathioprine is known to be well absorbed orally (bioavailability 80%) and reaches maximum blood levels 1−2 hours after administration. Half-life of AZA is approximately 10 minutes, whereas, 6-MP has a half-life of almost 1 hour. Both, AZA and 6-MP are moderately bound to plasma proteins (up to 30%) and hence, are partially dialyzable. They are both metabolized in the liver and erythrocytes and are rapidly removed from the blood (Figure 2). It is important to monitor complete blood count and liver function [15].

Drug Interactions

Allopurinol interferes with the inactivation of AZA, thus patients receiving AZA and allopurinol concomitantly should have a dose reduction of AZA, to approximately 1/3 to 1/4 the usual dose [18]. Aminosalicylates can inhibit the TPMT enzyme *in vitro*. In case of co-administration of these agents with AZA, the patients must be followed carefully [19]. The use of ribavirin for hepatitis C virus (HCV) in patients receiving AZA has been reported to induce severe pancytopenia and may increase the risk of AZA-related myelotoxicity [20]. Drugs which may affect myelopoesis should be adminsitered carefully. Concomittant use of angiotensine converting enzyme inhibitors might induce leukopenia and anaemia [21].

Side Effects and Toxicity

The major side effect is bone marrow suppression, presented as leucopenia, anaemia, and thrombocytopenia. Some adverse effects are noticed upon prolonged usage of AZA, which includes hepatotoxicity, gastrointestinal (GI) toxicity, alopecia, pancreatitis, and increased risk of neoplasia [17, 22]. Most of these toxic effects are found to be associated with increased oxidative stress.

Clinical and Experimental Development and Indications

Azathioprine in Renal Transplantation

During the pioneering era of transplantation, AZA was used as an exclusive immunosuppressive medication in renal transplantation, which was then combined with steroid preparates [7, 23].

Starzl et al. used the combination of AZA and steroids in a series of renal transplant recipients and reported, at that time, very convincing results [23]. These early findings supported the fast spreading of this combinantion therapy in kidney transplantation [23, 24]. Following the introduction of Cyclosporine A in clinical solid organ transplantation, the triple therapy, based on CsA, AZA, and steroids, became the most frequently used regime for a long period, albeit with the recent introduction of newer agents (FK506, MMF), treatment regimes with AZA are significantly losing their popularity [25].

Azathioprine in Lung Transplantation

AZA and MMF were used in a multicentre study with 81 lung transplantation cases. The results showed no significant difference between these drugs (26, 27). Another larger, randomized multicentre study with 315 patients compared CsA and streoid with either AZA or MMF. Analysis of results after 3 years showed no significant differences between the two drugs in terms of incidence of acute rejection, infection or bronchiolitis obliterans syndrome [26].

Azathioprine in Cardiac Transplantation

The first successful cardiac transplantation cases were also performed under conventional immunosuppresion. The continuously improved early outcome with allogenic heart transplant induced a remarkable media attention, which resulted in a wider public acceptance of organ transplantation and donation.

AZA has shown inferior results compared to MMF in cardiac transplantation cases. Survival and graft rejection rate in randomized trials were significantly higher in AZA treated cases in comparison to MMF or CsA [28].

Experimental Studies with Azathioprine

A recent study showed toxic effects of AZA in rats. The authors induced macrocytic anaemia, leucopenia and thrombocytopenia by intraperitoneal injection of AZA. They also reported gradual hepatotoxicity and myelotoxicity in those animals [29]. Studies have shown that AZA-induced liver toxicity is due to depletion of gluthation which leads to mitochondrial damage and ATP depletion, resulting in necrotic cell death [30, 31]. Yet another study

showed involvement of reactive oxygen species (ROS) in AZA-induced liver toxicity [32]. They demonstrated the role of oxidative damage, ROS and inflammation-related factors in hepatotoxicity due to Azathioprine. The study also suggested that oxidative stress and ROS might be involved in the initiation phase of AZA-induced hepatotoxicity [32].

Indications and Further Applications

AZA is indicated as an adjunct for the prevention of rejection in renal transplantation. It is also indicated alone or more frequently in combination for the management of active rheumatoid arthritis to reduce its signs and symptoms and has been used with benefits in patients suffering from other disesases (systemic lupus erythematosus, inflammatory bowel disease, dermatomyositis/polymyositis, autoimmune chronic active hepatitis, pemphigus vulgaris, polyarteritis nodosa, autoimmune haemolytic anaemia, chronic refractory idiopathic thrombocytopenic purpura) [22].

Summary

AZA was the drug of choice for immunosuppression for decades. It has proven to be highly effective in preventing acute graft rejection. However, its side effects, including thrombocytopenia, bone marrow suppression and hepatotoxicity make it a second choice. Azathioprine is still employed as an immunosuppressant but it is mainly used in treating different autoimmune and inflammatory bowel diseases. Newer drugs like MMF, CsA, tacrolimus etc. gained ground during the last decades.

Mycophenolate Mofetil

MMF is the product of Hoffmann-La Roche Ltd., commercially available under the name CellCept®. The product was approved by the FDA (Food and Drug Administration) in 1995 in the USA and one year later, it was introduced in Europe. MMF is the morpholinoethyl ester and pro-drug for the myofenolic acid (MPA), the potent inhibitor of the Inosine-5′-monophosphate dehydrogenase (IMPDH) enzyme. In 2004, the newly-designed enteric-coated salt of MPA: Enteric-coated mycophenolate sodium (EC-MPS) produced by Novartis (Myfortic®) was also approved by the FDA with the aim to reduce unpleasant gastrointestinal side effects [33].

The history of this effective cytostatic and antiproliferative agent dates back to 1893, when the Italian scientist Bartolomeo Gosio isolated mycophenolic acid from a strain of *Penicillium* [34]. Gosio reported that the crystalline compound is able to inhibit the growth of Bacillus anthracis. In this pioneer work, in fact, he identified and isolated the first agent with antibiotic effect in medical history; nevertheless these observations did not draw significant attention within the scientific community [35]. During the 1970s−80s, after almost 100 years of vicissitudinous history, MPA got into the spotlight of scientific interest again, thanks to the increasing demand for new cytostatic agents for cancer treatment (and also for organ

transplantation immunosuppression). This phase of drug development is strongly associated with the Syntex Company and the names of Allison, Eugui, and Morris. Meanwhile, the indefatigable work of the German origin transplantation surgeon and immunologist, Hans Werner Sollinger, in the field of transplantation surgery and immunology made an essential contribution to the preclinical and translational testing, which paved the way for MMF to be introduced for clinical transplantation.

Mechanisms of Action

From 1982, Allison and Eugui worked at a feverish pace to find new agents, which could selectively inhibit lymphocyte proliferation without severe adverse effects on hemopoesis, liver, and kidney function. Their extensive experience in the investigation of purine metabolism led them to select IMPDH, the key enzyme of *de novo* purine biosynthesis as potential molecular target [36].

Eugui showed that therapeutically attainable MPA concentrations could effectively inhibit the proliferation of human peripheral mononuclear cells as well as of B- and T-cells induced by various mitogens [37]. In these concentrations (Inhibitory Concentration 50 was less than 100 nM), MPA had no *in vitro* effect on fibroblast and endothelial cell proliferation. It has been demonstrated that due to MPA-induced guanosine triphosphate (GTP) depletion, the cells are blocked in the S phase of the cell cycle [38]. It should be noted, that clinically effective MPA concentrations can inhibit the proliferation of smooth muscle cells and mesangial cells, henceforth, positively influencing the graft proliferative aretriopathy and glomerulopathy changes as well [37, 39]. These findings also provided a basis for the development of drug-eluting stents coated with MMF for the treatment of coronary diseases [40].

In the above-detailed system, several factors are contributing to the success of MPA as a selective cytostatic agent: (i) Prominent differences can be found between cell and tissue types regarding purine synthesis [41]. While in neurons, synthesis of purine nucleotides is predominantly catalyzed by HGPRT through the salvage pathway; in leukocytes it is mostly dependent on the *de novo* cascade. Other cell types (fibroblasts, endothelial, smooth muscle cells, intestinal epithelial cells) are able to suffice the purine nucleotide need using both pathways [36]. (ii) Besides the metabolic differences, the existence of different IMPDH isoforms and the sensitivity of these isoforms for MPA inhibition provide additional cornerstones for MPA selectivity. As it was reported by several authors, the Type I enzyme is mostly expressed in resting human leukocytes and in other cells, while the Type II isoform is predominantly present in activated T- and B-cells [42, 43]. MPA inhibits, five times more potently, the isoform Type II compared to Type I.

Besides the main antiproliferative effect of MPA, several alternative mechanisms play important role when this agent is used in clinical transplantation settings (Figure 3). Allison et al. reported that MPA could also inhibit antibody formation of human B-leukocytes in vitro [44]. An interesting observation is, that MPA not only inhibits leukocytes, but also has a strong apoptosis-inducing effect for T-lymphocytes and T-lymphocytic cell lines (38). This effect might be selective for lymphocytes [45].

During the last two decades, data showed the role of nitric oxide (NO) and inducible nitric oxide synthase (iNOS) in allograft rejection. Numerous teams showed the intense iNOS-origin NO production in association with rejection response [46−48]. Senda et al., in 1995 reported the selective iNOS-inhibiting effects of MPA on rodent endothelial cells [49]. The authors postulated that this effect is due to the depletion of the enzyme co-factor, tetrahydrobiopterin [49, 50]. Moreover, considering the role of cytokines (TNF-alpha, IFN-gamma; Tumor necrosis factor-alpha, Interferon-gamma) in iNOS activation, the inhibitory effect of long term MPA administration on these cytokines demonstrated by Nagy et al., might contribute to this phenomenon [51, 52].

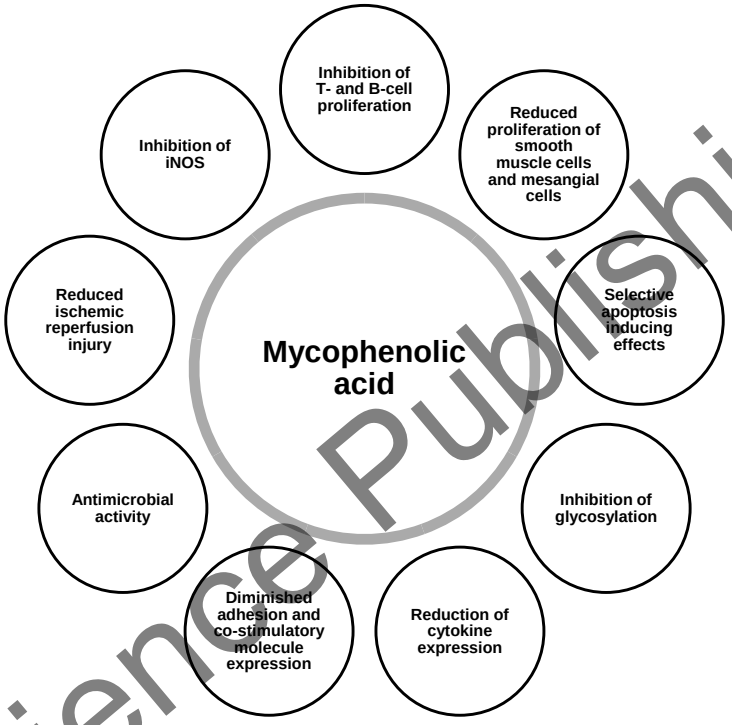

Figure 3. Effects of mycophenolic acid. Multiple mechanisms are contributing to the global mechanisms of action and to the clinical effects of mycophenolic acid. The abbreviations used are: iNOS: inducible nitric oxide synthase.

Besides these essential mechanisms, MPA also has the ability to antagonize the expression of adhesion molecules; due to this effect, MPA can alleviate lymphocyte and monocyte recruitment to the site of inflammation during the rejection processes. This effect was already proved for very late antigene-4 (VLA-4) on lymphocytes, as well as, for vascular cell adhesion molecule-1 (VCAM-1), E- and P-selectins on endothelial cells [36, 53]. Beyond the influence of MPA on leukocyte-endothelium interactions and thereby on graft ischemic-reperfusion injury and on leukocyte recruitment, this compound might also have a direct effect on the antigen-presenting cell - T cell receptor interaction, thus affecting co-stimulation and allo-activation. This hypothesis was supported by the findings of team Morris. Their results demonstrate that MPA possesses an inhibitory effect on the leukocyte CD154 and CD28 co-stimulatory factors [54]. The main mechanism behind the inhibition of adhesion

molecules and co-stimulatory factors is the negative effect of MPA on protein glycosylation [55]. As mentioned earlier, MPA was the first antimicrobial agent isolated and purified in medical history. MPA more effectively inhibits the IMPDH enzyme of the eukaryotes, but also has a weak effect on prokaryotes [56]. Over and above, it is known to have also certain anti protozoa and antimycotic potential as well as synergistic activity when combined with antiviral agents [36].

Pharmacokinetics and Pharmacodynamics

Formulations and Dosage

MPA is used as its 2, 4-morpholinoethyl ester pro-drug, MMF, because of the low bioavailability of MPA. CellCept® is available in capsules (250 mg) and tablets (500 mg) as well as in powder form for intravenous administration. This is supplied in glass vials containing 542 mg mycophenolat mofetil hydrochloride. Myfortic® is for oral use provided as delayed-release capsule in 180 or 360 mg. Two 500 mg MMF tablets deliver an equimolar dose of MPA as two 360 mg capsules of Myfortic® (739 *vs.* 720 mg MPA).

Detailed guidelines for dosage provided by manufacturers are divided for transplant groups [57, 58].

Pharmacokinetics

After oral intake, MMF and EC-MPS are hydrolysed resulting in rapid disappearance from the plasma. Oral bioavailability is 94% in healthy volunteers, which might be reduced in transplant recipients [59, 60]. In blood, the main portion of the MPA (99.99%) is in plasma fraction and is extensively bound to serum albumin (97−99%) [61]. Considering that only the free MPA can inhibit IMPDH (*in vitro*) and is available for metabolism, in diseases accompanied by hypoalbuminaemia the free MPA concentration and MPA clearance might be altered. The main mechanism for MPA metabolism is glucuronidation in the liver, kidney and gastrointestinal tract. The metabolite MPA glucuronide (MPAG) has no inhibitory effect on IMPDH. Further minor metabolites are 7-0-glucoside and Acyl MPAG. Moreover, MPAG has an enterohepatic recirculation. The main metabolite is excreted into the bile, deconjugated and the resorption of MPA occurs in the colon resulting in a second peak of MPA. Excretion is predominantly via urine, 87% as MPAG [61].

Drug Interactions

The administration of other immunosupressants might have an effect on the plasma concentration of MPA and MPAG. Significant alterations in MPA pharmacokinetics were reported with the co-administration of Tacrolimus or Sirolimus [61, 62]. Cyclosporine A also has a known effect on MMF pharmacokinetics via the inhibition of multidrug-resistance-associated protein 2 (MRP-2) transporter in the biliary tract and thus, via the inhibition of

enterohepatic circulation. MMF is not recommended to be administered with Cholestyramine or other agents interfering with its enterohepatic circulation, due to the significant drop of MPA concentrations. Cattaneo et al., reported the altered bioavailability of MMF when administered together with glucocorticoids [63]. Inhibitors of tubular secretion - like Probenecid should be avoided because of the considerable increment in MPA and MPAG-area under the curves (AUCs). Further information about drug interactions can be found in the manufacturer's guidelines and in comprehensive reviews [61, 64].

Side Effects and Toxicity

In contrast to some other immunosuppressants, MMF has a rather favourable side effects profile without diabetogenic potential, nephrotoxicity, and cardiovascular events. The major groups of side effects described are either gastrointestinal or haematological.

The unwanted GI side effects such as diarrhoea, abdominal pain and gastritis are occurring in high numbers of patients [65, 66] and leading to dose reduction or interruption of the therapy, resulting in an inferior clinical outcome [67]. More severe GI side effects (pancreatitis, GI bleeding, gastrointestinal perforation) were also observed [66]. Most frequent haematological side effects are leucopenia and anemia [66].

Increased risk of opportunistic infections during MMF treatment is important when compared to Azathioprine [68]. Notable increment in cytomegalovirus (CMV) [69] and BK virus [70] infections were reported in the recipients of kidney allografts treated with MMF.

Clinical and Experimental Development and Indications

Mycophenolate Mofetil in Renal Transplantation

Three pivotal, Phase III, double-blinded, randomized, multicentric studies were implemented with MMF in renal transplant recipients. The study performed in the USA at 14 sites and the trial organized in Europe, Canada, and Australia at a total of 21 centers used Azathioprine as control [71, 72]. The European study was placebo controlled [66]. In all three works, the drugs were used in combination with CsA and steroid.

For the primary endpoint (incidence of treatment failure in the first 6 months following transplantation; see the studies for details), a significant improvement was observed with both MMF regimens (2 g/day, 3 g/day) when compared to the control group.

Efficacy and side effect profile of EC-MPS and MMF were compared by two randomized studies [73, 74]. Besides the similar therapeutic efficacy, the results were not impressive concerning the side effects profile. Enteric coating was not able to convincingly reduce major side effects.

MMF in Cardiac Transplantation

One important prospective, randomized, double-blinded study with the inclusion of 650 patients was performed in 28 centres worldwide and published in the Transplantation journal in 1998 [75]. This work reported a positive effect of MMF in regard to the first year mortality and acute rejection in the first 6 months, when compared to AZA.

In a comprehensive analysis based on the data of 5599 cardiac transplant patients, Hosenpud et al. reported the superiority of MMF concerning the 3 years survival rate when compared to Azathioprine [76].

MMF in Liver Transplantation

A prospective, double-blinded, randomized study performed in 23 centers on three continents, with the enrolment of total 565 liver transplant patients showed a lower rate of acute rejection at 6 months and a similar numbers of death or retransplantation at first year with MMF combination therapy when compared to AZA [77]. There were further concerns regarding the effects of MMF on the recurrence of hepatitis C after liver transplantation in HCV positive patients [78].

Experimental Studies with MMF

The first results concerning the experimental testing of MMF as transplantation immunosuppressive agent were published by the team of Morris in the model of rat allogenic heterothopic heart transplantation [79, 80]. In this model, they demonstrated an impressive improvement in graft survival. The convincing preliminary results were also proved by numerous studies in different experimental transplantation settings [81−83]. Further studies confirmed that MMF is not only able to prolong graft survival, due to its B- and T-cell antiproliferative effects, but also can potently reduce graft vasculopathy, thus graft dysfunction [84, 85].

Further Applications

Besides the applications and possibilities reported in transplantation settings (pancreas, lung, etc.), MMF was also used for treating several other diseases: primary glomerulonephritis, different types of pemphigus, multiple sclerosis, autoimmune haemolytic anemia, systemic lupus erythematosus, rheumatoid arthritis, Crohn disease *etc* [86−89].

Indications

Currently, MMF is indicated for rejection prophylaxis in patients undergoing allogenic renal, cardiac, and hepatic transplants. MMF should be used in combination with cyclosporine and corticosteroids.

Summary

The introduction of MMF into the clinical practice has strongly contributed to the success of the clinical transplantation programmes and improved the outcome in patients and graft survival as well as quality of life for recipients.

Nevertheless, there are still further possibilities to achieve improvements in MMF immunosuppressive therapy. To reduce the unpleasant complications and side effects, two approaches have been already introduced: (i) The development of the enteric-coated MPA; (ii) Controlled-dose regimes and therapeutic drug monitoring, instead of fixed-dose administration. There are still several open questions in this field, which need further investigations to improve outcome and contemporaneously reduce adverse effects.

A recent study showed the potential benefit of the use of a novel nanogel-based drug delivery vehicle containing MPA for systemic lupus erythematosus in mice [90]. This is only one example demonstrating that after decades of experience with the use of MMF, there are still new possibilities on the horizon.

Further interesting research direction might be the in-depth investigation of the alternative mechanisms behind the effects of MPA. One fascinating perspective would be to exploit the potential effects of MPA on HCV and HIV.

AZA versus MMF

Athough the differences in clinical efficacy between AZA and MMF were tangentially discussed in the respective sections, it is necessary to provide a separate, comprehensive overview for the reader on this issue, based on transplant groups.

In the field of renal transplantation, the pivotal multicenter studies of the USA and the Tri-continental MMF study groups showed the superiority of MMF for the acute rejection when compared to AZA [71, 72]. The observations on the efficacy and safety of MMF in this transplant group were confirmed by others [91, 92]. Ojo et al., analyzed the data of 66, 774 renal transplant recipients from the US renal transplant scientific registry and reported a higher censored 4 years graft survival with the use of MMF compared to AZA [93]. The authors also observed that MMF decreased the relative risk of development of chronic allograft failure and concluded that this improvement is partly caused by the decrease in the incidence of acute rejection observed with MMF treatment, but it is also caused by an independent effect. Similar reports can be found concerning cardiac and pancreas transplantation. Sollinger et al. showed an unequivocal improvement in pancreas graft and patient survival with the use of MMF compared to AZA in 500 simulataneous pancreas-kidney recipients [94]. The superiority of MMF in cardiac transplantation was published by Hosenpud et al., as it was already detailed above (see MMF in cardiac transplantation) [76].

The benefit of MMF compared to AZA is more controversial and less clear in the field of liver and lung transplantation. The meta-analysis and systematic review of Germani et al., demonstrated a little, if any, clinical benefit of MMF versus AZA in liver transplantation [95]. Various studies suggested that AZA may even have an advantage over MMF regarding the HCV reccurrence after liver transplant [95]. Previous reports showed that MMF might be superior versus AZA during the early phase (6−10 months) concerning the acute rejection

episodes after lung transplantation [96, 97], although the results are controversial [27, 98]. According to McNeil et al., no differences can be seen in the incidence of acute rejection and in bronchiolitis obliterans syndrome, or in the 3 years suvival of lung transplant recipients treated with MMF or AZA [98].

Mizoribine

MZB is a nucleoside analogue, available commercially under the name Bredinin® in Japan. *Eupenicillium brefeldianum* was isolated from the soil of Hachijo Island, Tokyo, Japan, in 1971 [99]. The antimicrobial agent produced by this ascomycetes microbe was reported to inhibit the growth of *Candida albicans* and vaccinia virus [100]. MZB was investigated in Japan in several studies during the 80s and was approved by the Ministry of Health and Welfare, Japan in 1984, for prevention of rejection in renal transplantation [99]. Based on its efficacy for the inhibition of lymphocyte proliferation and the relative friendly toxicity profile and fewer side-effects compared to AZA, Bredinin® became popular in Japan. MZB is a pro-drug activated via the phosphorylation by adenosine kinase to Mizoribine-5-phosphate. MZB-5-P is an inhibitor of the purine biosynthesis [101].

Encouraged by the aforementioned findings, several studies were initiated with MZB as a part of long-term immunosuppressive therapy regimes. Thanks to these efforts, MZB was approved in Japan for the treatment of lupus nephritis, rheumatoid arthritis and primary nephritic syndrome as well [102].

Mechanisms of Action

Koyama reported that MZB is transformed into its active form (MZB-5-P) by adenosine kinase [101]. The pivotal works of Sakaguchi [103, 104] and Mizuno [105] demonstrated that low concentrations of MZB can strongly inhibit the DNA and RNA, but not the protein synthesis in mouse L5178Y cell line. The addition of GMP, but not IMP (inosine monophosphate) to the culture, was able to suspend this inhibitory effect. Based on these early findings, the authors concluded, that MZB inhibits the synthesis of GMP from IMP, without incorporation into DNA or RNA. Later studies revealed more details of the mechanisms [99]. Kusumi et al., reported that the main target of MZB-5-P is the IMPDH enzyme, nevertheless, it inhibits GMP synthetase as well, thus acting at two steps of the *de novo* purine biosynthesis [106]. Due to these above-detailed molecular mechanisms, MZB is able to suppress the blastogenic response of lymphocytes to different mitogens (107, 108). MZB administration results in a selective lymphocyte proliferation block, acting at the G_1−S phase transition of the cell cycle [109, 110]. Novel findings concerning the mechanisms behind the effects of MZB were reported as well. Takahashi et al., revealed that MZB affects the conformation of 14-3-3 protein *in vitro* [111]. These conformation changes enhanced the interaction between the glucocorticoid receptor and 14-3-3 protein. The 14-3-3 protein interacts, among others, with the glucocorticoid receptor and increases its transcriptional activity. According to the observations of Picard-Jean et al., MZB also inhibits the human RNA capping enzyme, which is critical for the splicing of the cap-proximal intron, the

transport of mRNAs from the nucleus to the cytoplasm, and for both stability and translation of mRNAs [112]. Mizoribine has several structural similarities with the antiviral agent, Ribavirin, thus it is not surprising that it was reported to exhibit *in vitro* activity against several viruses (CMV, parainfluenza virus, influenza virus, HCV, respiratory syncytial virus *etc*.) [113−116]. The significance of these novel effects remains to be elucidated, although the contribution of these to the global mechanisms of action of this substance is assumed.

Pharmacokinetics and Pharmacodynamics

Formulations and Dosage

In the early studies the regular MZB dosage was 3 mg/kg/day, which resulted in 0.5 μg/mL peak blood levels [117, 118]. It has been reported that peak blood MZB levels (3.0–6.0 μg/dL) are necessary for the inhibition of the human lymphocyte proliferation [119]. According to the novel reports, a higher starting dose (6−12 mg/kg/day) is recommended for kidney transplant recipients during the early post-transplantation phase [99]. A still favorable side-effects profile was reported with the use of this elevated MZB dosage protocol.

Mizoribine is available as Bredinin® Tablets 25 mg and 50 mg (Asahi Kasei Pharma Corporation).

Pharmacokinetics

Murase et al., found in early pharmacokinetic studies that, following oral administration in rats, MZB rapidly absorbs, blood concentration peaks at 1.5 hours and the elimination is almost complete within 24 hours [120]. Eighty-five percent of the drug was excreted, without metabolisation, in the urine, 9.7% in the feces and approximately 1% to the bile. The first report examining MZB pharmacokinetics in renal transplant patients is attributed to Inou et al., [117]. The investigators found a peak MZB concentration 2−3 hours after p.o. administration. The substance was predominantly eliminated by 12 hours und was not measurable at 24 hours. Other authors reported a strong correlation between MZB concentrations and kidney function [121, 122]. When 100 mg MZB was administered orally to kidney transplant recipients with good kidney function, T1/2 was found to be 2.2 hours [99]. Shinoda et al. reported peak serum levels at 4 hours after MZB administration in adult living donor liver transplantation recipients [123].

Drug Interactions

Because of the relative limited experience with MZB treatment, there is only a handful of data about the interactions of MZB with other drugs. Several reports showed synergic effects of the co-administration of MZB and CsA or tacrolimus [124−126]. Morimoto et al., observed MZB induced rhabdomyolysis when it was administered simultaneously with Bezafibrate®, a fibric acid derivate [127].

Side Effects and Toxicity

Mizoribine has been characterized as a well-tolerated and safe agent with a favorable side-effects profile. Leucopenia, abnormal hepatic function, rash, increased uric acid levels, nausea and vomiting were reported during the use of MZB, albeit the drug does not appear to be significantly hepato-, nephrotoxic or myelosuppressive in clinical situation [100].

Clinical and Experimental Development and Indications

Mizoribine in Clinical Studies

Following the introduction of MZB in Japan, it was tested in numerous Japanese centers during the 80s−90s. In the early studies, MZB was applied as a conversion therapy in cases of AZA-induced toxicity with promising results [117, 128]. Later, different studies demonstrated the favorable effect of MZB as a part of a double therapy (MZB-steroid) [129] or a triple-drug regime (MZB-AZA-steroid) [102] compared with historical controls treated with AZA-steroid alone. During late 80s and the 90s, Tanabe et al., enrolled 116 kidney transplant recipients and performed a randomized, prospective study to assess the effects of MZB (130). The participants received either MZB or AZA for 9 years after transplantation. The 9 year patient and graft survival rates for the MZB and AZA groups were 88%, 83% and 58%, 52%, respectively. These differences were not significant, although AZA had to be converted to MZB in 16 patients (due to adverse effects). In contrast to this, no major adverse effects were observed in the MZB group. In a small volume study, Funahashi tested the efficacy of MZB in renal transplant recipients with BK nephropathy. In five out of seven patients, urine BK virus DNA turned negative; furthermore for the remaining two patients, there was also a significant reduction observed in BK virus DNA in the urine [131].

Mizoribine in Experimental Studies

Mizoribine was not only tested in the experimental and clinical setups of renal transplantation, but, multiple reports can be found with the use of MZB in different models of heart [132, 133], pancreas [134] and liver transplantation [135]. These new, unrevealed fields might help to extend the indications of MZB in the future.

Further Applications and Indications

MZB (Bredinin®) is currently approved only in Japan, for the prevention of acute rejection in renal transplantation. Furthermore, it is indicated for the treatment of lupus nephritis, chronic rheumatoid arthritis, and primary nephritic syndrome as well [99].

Summary

Mizoribine was mostly used in Japan, accordingly, there is only limited data with the use of Bredinin® in clinical situations. Nevertheless, this nucleoside analogue seems to be a promising and safe immunosuppressive agent. Considering its *in vitro* antiviral effects and the positive results with the use of Bredinin® in renal transplant patients suffering from BK nephropathy, MZB might be a potential solution for this important problem in renal transplantation. Moreover, MZB was also successfully used for the treatment of other renal diseases. New randomized control trials would be necessary to further evaluate the effect of MZB and to optimize therapy.

Agents Targeting Pyrimidine Biosynthesis

During the 80s−90s, the intensive research for new immunosuppressive agents was not limited to the investigation and targeting of the purine synthesis but components of *de novo* pyrimidine synthesis were also in the spotlight of scientific interest. Hence, dihdyroorotate dehydrogenase (DHODH), the enzyme catalyzing the fourth step in *de novo* pyrimidine synthesis, became the other pivotal target of drug development (Figure 4).

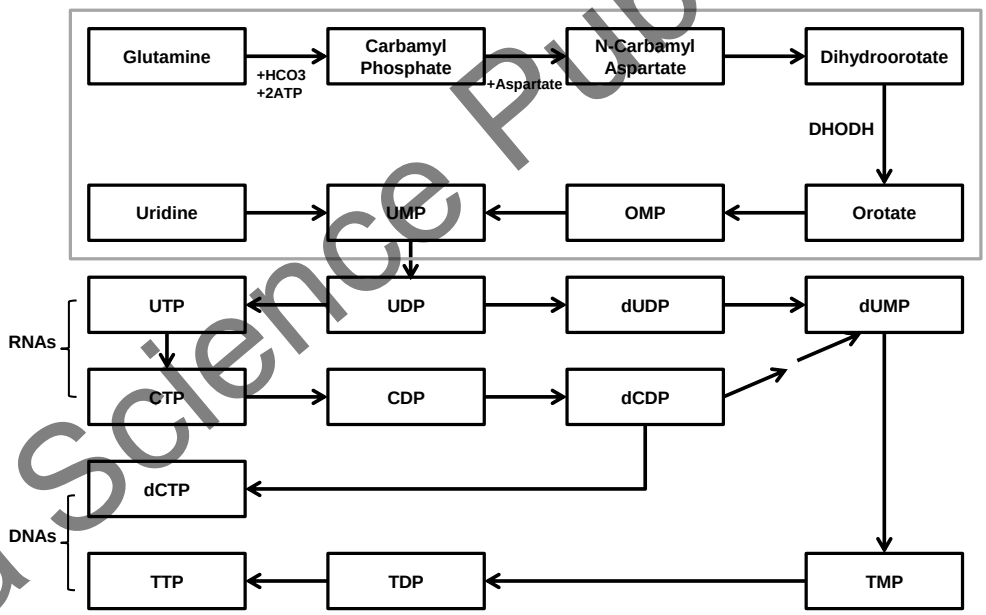

Figure 4. Pyrimidine nucleotide synthesis. Through several enzymatic steps, UMP is produced. The 4th crucial reaction is catalyzed by the DHODH enzyme. The abbreviations used are: HCO3: bicarbonate; ATP: adenosine tripshosphate; DHODH: dehydroorotate dehydrogenase; OMP: orotidine monophosphate; UMP, UDP, UTP: uridine mono-, di-, triphosphate; dUDP, dUMP: deoxyuridine di-, monophosphate; RNA: ribonucleic acid; DNA: deoxyribonucleic acid; CDP, CTP: cytidine di-, triphosphate; dCDP, dCTP: deoxycytidine di-, triphosphate; TMP, TDP, TTP: thymidine mono-, di-, triphosphate. (Adapted from: Cooper AR, Perry S, Breitman TR. Cancer Res. 1966; 26: 2267-2275).

Leflunomide and Malononitrilamides

Leflunomide (LEF), the product of Sanofi Aventis Company (Arava®) is a potent inhibitor of the DHODH enzyme. The immunomodulatory effects of this drug led to its successful application in rheumatic diseases, hence to its FDA approval in 1997. The potent immunosuppressive and immunomodulatory effects of this isoxazol derivate were already demonstrated in preclinical transplantation settings, albeit the average 15 days long plasma half-life, the lack of data about interactions with other drugs, as well as the hardly predictable target blood concentrations impeded the application in clinical scenarios [136].

LEF is a pro-drug which is metabolized *in vivo* to its active form: A77 1726 (teriflunomide) [136]. This malononitrilamide (MNA) active metabolite compound provided the basis for further drug development. Emboldened by the strong effects of LEF in preclinical investigations, the attention turned to other synthetic derivates. Two of these synthetic MNAs: FK778 and FK779 were intensively investigated [137]. Considering the shorter serum half-life and more favourable pharmacokinetics of the derivates, they were already more suitable for the use in solid organ transplantation. Despite the hopes that FK778 might be a promising new agent in humans, on 30th June 2006 Astellas Pharma Inc. announced to discontinue the development of FK778 as immunosuppressive agent for transplantation, referring to the not convincing Phase II trial results.

Mechanisms of Action

As mentioned above, the main mechanism behind the immunosuppressive and antiproliferative effects of MNAs is the inhibition of the mitochondrial key enzyme (DHODH) of *de novo* pyrimidine synthesis (Figure 4) [138−140]. The result is decreased levels of uridine monophosphate, thus a cell cycle arrest in the G1 phase and the inhibition of cellular as well as humoral immune responses [141]. As it was previously discussed in detail in relation to purine synthesis, the way of pyrimidine synthesis is likewise different in resting and activated lymphocytes [142]. The fact that activated T- and B-cells are dependent mostly on the *de novo* pathways provides the basis for the relatively good selectivity of MNAs. Elder et al., reported that the effects of LEF is not only dependent on the pyrimidine synthesis inhibition but, at significantly higher concentrations, also can be attributed to the decreased activity of several tyrosine kinases, playing key role in numerous signal transduction pathways [143]. It has been demonstrated as well that the active metabolite of LEF can induce apoptosis in human T lymphocytes after mitogen activation, via disturbing the cell cycle [142]. Pyrimidine nucleotides also have an important role in protein glycosylation, thus the observations that MNAs can interfere with the mononuclear cell adhesion (presumably due to the inhibition of the adhesion molecule glycosylation) are not surprising [144].

It is very promising that MNAs possess potent antiviral effect against CMV (also in ganciclovir-resistant cases), herpes simplex viruses and BK virus [145−148]. This feature makes the use of these agents even more attractive in transplantation settings, particularly in the cases of kidney transplantation where BK virus nephritis represents a major concern. In this situation, MNAs are not only able to help the polyoma virus elimination but they also have the ability to reduce concomitant interstitial inflammation [136].

Pharmacokinetics and Pharmacodynamics

Formulations and Dosage

Arava® tablets are available for oral use in 10, 20 and 100 mg form [149].

There is no general agreement concerning the dosage of MNAs in solid organ transplantation. In a retrospective review, Williams et al. analyzed the results of LEF administration in kidney and liver transplant recipients [150]. They concluded that a total loading dose of 1200-1400 mg, taken over 7 days is necessary to achieve a target serum level of 100 μg/mL. Following the loading dose, they administered 40−60 mg/day LEF for maintenance. In a prospective crossover study, published in the same year in the American Journal of Transplantation by Hardinger et al., the authors were able to demonstrate the positive effects of Leflunomide in 22 kidney transplant recipients converted from MMF or AZA to 20 mg/day Leflunomide for the treatment of chronic renal allograft dysfunction [151]. In the studies reporting impressive results with the treatment of BK nephropathy, the effective target serum levels were 40−100 μg/mL provided by a loading dose and then 20−60 mg/day Leflunomide for maintenance [136, 152−154]. So far, two randomized, double-blinded clinical studies were published in kidney transplant recipients investigating the safety and efficacy of the FK778 MNA. Both studies used different doses of FK778 in combination with Tacrolimus and steroids compared to either placebo [155] or standard MMF treatment [156]. The authors concluded that low dose FK778 treatment (loading doses of 600 mg given 2 times on days 1st and 2nd followed by doses of 100 mg/day) is comparable in efficacy to standard regimes and can be safely applied in renal transplant recipients.

Clinical Pharmacokinetics

After oral administration of LEF, this 270 Dalton molecule is completely metabolized, via the opening of the isoxazol ring to its active metabolite: A77 1726, resulting in very low or undetectable serum levels of the mother substance. Bioavailability of LEF tablets is 80%. Following oral administration, the peak active metabolite levels are reached after 6−12 hours. A loading dose is used in practice to facilitate the rapid attainment of steady-state levels. A77 1726 is more than 99.3% albumin-bound, although the free active metabolite fraction can be two times higher in renal failure [142, 149].

Location of LEF metabolism is most probably the gastrointestinal tract and the liver, albeit no concrete evidence is available about the specific site of metabolism. The active metabolite is eliminated in its original form in the feces (48%) and as glucuronide in the urine (42%) [22].

Drug Interactions

No pharmacokinetic interactions were observed when Arava® and methotrexate were administered, although, the administration of the two drugs together increases the risk of

hepatotoxicity and pancytopenia. Rifampin can increase the active metabolite levels of LEF [136].

Side Effects and Toxicity

The most characteristic side effect observed during the use of MNAs in transplantation settings was a dose-dependent anaemia, especially, in kidney transplant recipients [22]. The most frequent side-effects reported in the pivotal multicenter studies with Leflunomide in RA, were respiratory tract infections, diarrhoea, headache, nausea, dyspepsia, rash and reversible alopecia [157, 158]. More severe adverse effects such as hepatotoxicity, severe bone marrow suppression, Steven-Johnson-syndrome, were documented rarely.

Clinical and Experimental Development and Indications

MNAs in Clinical Studies

There is only scarce experience with the use of MNAs in clinical scenarios of organ transplantation. Two studies were published in 2002 reporting the successful use of LEF as rescue-therapy. Williams et al., performed a retrospective review of the data of 53 transplant recipients (45 kidney and 8 liver) receiving Arava® [150]. In these 53 patients, Leflunomide was introduced based on different complications, such as chronic renal allograft nephropathy, chronic hepatic allograft rejection, or calcineurin inhibitor intolerance. In 12 of 18 renal patients, the dose of CsA or tacrolimus was reduced by a mean of 38.5% and stopped in one patient. The prednisone dose was also reduced by a mean of 25% in the same patients. CsA or FK506 was stopped completely in four liver recipients and reduced by 65% in another patient. This study showed that LEF is a potent and safe immunosuppressive agent in kidney and liver transplant recipients and can be administered for more than 300 days.

The prospective, crossover study by Hardinger et al., reported 22 kidney transplant recipients who were converted from conventional drugs (MMF, AZA) to LEF with the aim to slow progression of chronic transplant dysfunction [151]. The mean serum creatinine was stabilised and the six-month post-conversion patient and graft survival were 100% and 91%, respectively. In two cases, LEF therapy was aborted due to side effects.

Considering that the clinical development of FK778 was discontinued in 2006, only a few reports are available investigating the safety and efficacy of FK778 as immunosuppressive drug in organ transplantation. The randomized, double-blinded, 4-group, multicenter, phase IIb study published by Wlodarczyk et al., was conducted over 12 months in 37 European transplant centers [156].

Three hundred and sixty-four kidney transplant recipients were randomized either into one of the three groups of FK778 treatment regimes (high-level, mid-level, and low-level FK778 groups), or to the control group (MMF). Both drugs were given in combination with tacrolimus and corticosteroids. A low dose of FK778 was comparable in terms of efficacy to

the standard immunosuppressive regime. Increased exposure to FK778 did not result in increased efficacy and it was poorly tolerated.

It should be noted however, that one of the most interesting conditions where LEF and MNAs were tried, is transplant recipients with BK nephropathy. MNAs have a strong effect *in vitro* against CMV and BK viruses, although the *in vivo* results are controversial and further studies are necessary to find the right place for LEF treatment in this population of patients [136, 159, 160].

MNAs in Experimental Studies

LEF as well as FK778 were tested in numerous models of experimental transplantation. Several impressive preclinical results can be found concerning the potent immunosuppressive effects of MNAs investigated in different renal, intestinal, lung, cardiac, skin, islet cell transplantation models in small [161−168] as well as in big animals [169−173].

In these studies, MNAs were highly effective in attenuation of acute [162, 163] and chronic [161] rejection. Furthermore, a synergic effect was reported when MNAs were administered in combination with calcineurin inhibitors [162, 167].

Further Applications

Besides the attempts for the use in clinical transplantation, LEF was also tested pre-clinically as well as in human studies in several other autoimmune diseases [22, 174−176] and with different malignant tumours in vitro and in vivo [177−180].

Indications

Currently, Arava® tablets are indicated in adults for the treatment of rheumatoid arthritis, to reduce symptoms, structural damage and improve function [149].

Summary

There is no general agreement currently concerning the use MNAs in the settings of organ transplantation. LEF is a safe and effective drug, albeit due to its pharmacokinetical properties, it is very problematic to control in transplant recipients. Unfortunately, the development of other MNAs (such as FK778) with optimal PKs was run down a blind alley during the Phase-II clinical trials.

The improvements in drug development [181] or alternative fields of application [182] may help us in the future to vanquish these obstacles and to take advantage of the multiple favourable effects of this interesting group of drugs.

Other Small Molecules with Cytostatic, Antiproliferative Effects

There are several new immunosupressive agents in different phases of preclinical or clinical evaluations. In this section, we would like to describe the compounds, which might have the potential to gain importance in future clinical transplantation settings or have historical significance.

Some of these drugs are in the early phase of modern drug development (e.g., VX-497); others have failed during the last decades due to inappropriate pharmacological features (e.g., Brequinar sodium). These agents are waiting for the novelties in drug design and development, in order to overcome these difficulties, via finding effective derivates or formulations without unpleasant effects.

Farnesyl-S-transferase inhibitors (FTIs) are inhibiting Ras GTPases, thus they were developed as anticancer medications [183]. However, recently, investigations on these inhibitors revealed that FTIs are effective for the treatment of other diseases such as progeria, P. falciparum-resistant malaria, trypnosomiasis, *etc* [184]. Ras GTPases also play key roles in T cell activation and function, therefore the idea was proposed that FTIs might have immunomodulatory properties and may be used in transplantation settings.

A 228839 and ABT-100 are two FTIs already tested *in vitro* or in preclinical experimental studies. A 228839 potently inhibited the lectin and antigen-presenting cell-induced lymphocyte proliferation *in vitro* [185]. Si et al., reported that ABT-100 can inhibit peripherial blood mononuclear cell proliferation and it was able to prolong allograft survival time in a rat model of heterotopic cardiac transplantation [186]. However, no clinical studies are available so far.

2-Chlorodeoxyadenosine (Cladiribine) was proved to have immunosuppressive activities. Górski et al., reported that Cladiribine produces a significant inhibition of lymphocyte proliferation in human mixed lymphocyte reactions at a concentration of 10 nM [187]. The potential of this adenosine-resistant deoxyadenosine analogue as immunosuppressive agent was demonstrated in different allogenic transplantation models (skin, small bowel, heart, liver) [188−190].

VX-497 and VX-148 are the first IMPDH inhibitors generated in a structure-based drug design program, addressing the tolerability problems of currently available immunosuppressive drugs [191, 192]. VX-497 is currently in Phase II trials in psoriasis and hepatitis C [193] and it has been shown to prolong skin graft survival and improve graft versus host disease in a murine model [191].

The substance brequinar sodium (BS) was produced by the DuPont Merck Pharmaceuticals (Wilmington, USA). The initial aim was the development of new drugs for the treatment of malignant diseases. This compound can exert very potent antiproliferative effects, via the inhibition of DHODH, thus of *de novo* pyrimidine synthesis (similarly to the above described Leflunomide or MNAs) [194]. Based on the very impressive preclinical results in cardiac, renal and hepatic transplantation and the good bioavailability, water-solubility, BS was selected for clinical development [195, 196]. During the clinical studies, an extremely narrow therapeutic window was observed, with severe side effects (including gastrointestinal symptoms and bone-marrow suppression). Considering these findings, the clinical development of BS was stopped. Designing new derivates with favorable

pharmacological and toxicity profile may provide a future for this very attractive immunosuppressive agent.

Tripterygium Wilfordii Hook F (TWHF) is an extract of the Chinese medical herb, traditionally used in China for the treatment of autoimmune diseases [197]. PG490 was identified as the active component of the extract, predominantly responsible for the imunosuppressive effects of TWHF [198]. PG490 has been shown to be a potent inhibitor of lymphocyte proliferation in vitro [199]. PG490-88 is a semi-synthetic, water-soluble ester pro-drug, reported to be effective against renal allograft rejection in rats and cynomolgus monkeys [200, 201].

Lymphoid cell-specific kinase is activated after T cell stimulation and it is required for T-cell proliferation and interleukin IL-2 production [202]. A 770041 is a potent inhibitor of this tyrosine kinase. It has been recently demonstrated that this compound can prevent rejection of cardiac grafts transplanted heterotopically in rats across a major histocompatibility barrier, using Brown Norway donors and Lewis recipients [203].

A novel immunosuppressive agent, FR252921, was isolated from *Pseudomonas fluorescens*. This compound has been demonstrated to inhibit lymphocyte proliferation *in vitro* and has a synergic activity *in vivo*, when co-administered with FK506 in a skin allograft transplantation model. The target of this drug is the activating protein-1 (AP-1), thus it acts predominantly against antigen-presenting cells rather than against T cells [204].

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Chapter 8

Calcineurin Inhibitors

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Abstract

The calcineurin inhibitors (CNIs), including cyclosporin A (CsA) and tacrolimus (FK506), are the cornerstone immunosuppressive agents administered to solid organ transplant recipients to prevent and treat allograft rejection. CNIs have significantly reduced the incidence of acute rejection and improved 1-year allograft survival. CNIs have a narrow therapeutic window. The balance between efficacy and toxicity is aimed to maintain long-term functional graft. Long-term CNI-based immunosuppression causes nephrotoxicity and other adverse events. It should be balanced between the potential risk of side effects of CNI use and the risk of rejection. Long-term graft survival has not improved. The effect of chronic CNI toxicity in the failure to improve outcomes is suggested although it is controversial. Reducing or removing CNIs from immunosuppressive regimens is designed to improve outcomes. This chapter discusses pharmacokinetics, pharmacodynamics and pharmacogenetics of CNIs, current and future CNI-based immunosuppressive strategies, major toxicities of CNIs and CNIs sparing strategies.

Keywords: Calcineurin inhibitor, cyclosporine, tacrolimus, pharmacokinetics, pharmacodynamics, pharmacogenetics, transplantation

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Abbreviations

CNIs	Calcineurin inhibitors
CsA	Cyclosporin A
FK506	Tacrolimus
TDM	Therapeutic drug monitoring
JNK	c-Jun N-terminal kinase
TGF-β1	Transforming growth factor β 1
FKBP	FK-binding proteins
NFAT	Nuclear factor of activated T cells
AP-1	Activator protein-1
NF-κB	Nuclear factor κ light-chain enhancer of activated B cells
MAPK	Mitogen activated protein kinase
ERK	Extracellular signal regulated kinase
AUC	Area under the curve
CYP	Cytochrome P450
AR	Acute rejection
C_{max}	Peak concentration
MMF	Mycophenolate mofetil
MPAG	Mycophenolate glucuronide
MPA	Mycophenolic acid
IL	Interleukin
GFR	Glomerular filtration rate
NKT	Natural killer T
FK778	Malononitrilamide 715
CMV	Cytomegalovirus
EBV	Epstein-Barr virus
ABCB1	ATP-binding cassette, subfamily B, member1
SNPs	Single nucleotide polymorphisms
KDIGO	Kidney disease improving global outcomes
NODAT	New-onset diabetes mellitus after transplantation
MPS	Mycophenolate sodium
BP-CAN	Biopsy-proven chronic allograft nephropathy
SRL	Sirolimus
CAESAR	Cyclosporine avoidance eliminates serious adverse renal- toxicity trial
EVR	Everolimus
DSA	Donor-specific HLA antibodies
BPAR	Biopsy proved acute rejection
PyVAN	Polyomavirus-associated nephropathy
NAT	Nucleic acid testing
HBV	Hepatitis B virus
HCV	Hepatitis C virus
NAs	Nucleos(t)ide analogs
DAAs	Direct acting antivirals
UTIs	Urinary Tract Infections

Introduction

The discovery of Calcineurin inhibitors (CNIs), including CsA and FK506, has been major advances in immunosuppression. In the early 1980s, the introduction of CsA marked a new era in clinical transplantation and increased one-year graft survival rates to well over 80% from 50% [1]. CsA reduced the incidence and severity of acute rejection during the critical first three months post-transplant when the graft is at greater risk of acute immunological injury to increase the safety and success of transplantation [2]. The application of CsA has gradually decreased since new agents have been adopted in clinical practice. It is not the single major agent and is a selected component of combination therapy.

FK506 has gradually become more widely used CNI over the last twenty years owing to a lower risk of acute rejection and allograft loss after it was approved by FDA in 1994 [3, 4]. The one-year graft survival rates were about 90% and the acute rejection rates were less than 20%. The therapeutic indices of both drugs are narrow and large intrapatient and interpatient pharmacokinetic variability exists [5, 6]. The adequate CNI dose is necessary for preventing rejection. CNI overdose can induce toxicities which decrease tolerability and affect long-term allograft and patient survival [7]. Therapeutic drug monitoring (TDM) is mandatory for both drugs. The therapeutic target range is recommended for both drugs. But similar immunosuppressant concentrations do not have the same effect due to individual difference. Safe drug concentration does not promise absence of drug toxicity and complete immunosuppressant effect.

Long-term CNI-based immunosuppression induces nephrotoxicity and other adverse events and long-term improvement in allograft-survival has not been achieved [8]. Chronic CNI toxicity is one of the contributing factors in failure to improve outcomes. The research on withdrawing or avoiding CNIs from immunosuppressive regimens has been performed.

This chapter will summarize the molecular actions and pharmacology of CNIs; it will review the current and future CNI-based immunosuppressive strategies, major toxicities of CNIs and CNIs sparing strategies.

Structure and Molecular Effects of CNIs

CsA and FK506 act in a similar manner, although they have different chemical structures. CsA is a cyclic endecapeptide [9] and FK506 is a macrocyclic lactone [10]. The pathways of CsA and FK506 immunosuppression include inhibiting calcineurin, the c-Jun N-terminal kinase (JNK) and p38 pathways, and increasing expression of transforming growth factor β 1 (TGF-β1) [11].

Effect on Calcineurin and NFAT

Both CsA and FK506 bind with high affinity to proteins known as immunophilins after they enter T cell. CsA binds mainly to cyclophilin A and FK506 binds to immunophilin FK binding protein 12 (FKBP-12). The affinity of immunophilin for calcineurin is enhanced after binding of CsA or FK506 and causes inhibition of the protein's activity [12]. Calcineurin is a

calmodulin dependent phosphatase and is stimulated during T cell activation by a chain of events involving calcium and calmodulin [13, 14]. After calcineurin is activated, it dephosphorylates members of the nuclear factor of activated T cells (NFAT) family to activate these proteins [15]. NFAT proteins translocate to the nucleus after they are activated [16]. They associate with other transcription factors, such as members of the activator protein-1 (AP-1) family, and bind to DNA to promote the transcription of interleukin (IL)-2 [17]. They bind to a large variety of other_cytokine genes on promoter sites, including those coding for IL-4, IL-10, and IL-17 [18]. Calcineurin inhibition prevents dephosphorylate, NFAT activation and affects the transcription of cytokines important in the immune response. The transcription of IL-2 is downregulated which plays a major role in the immune response, including the maintenance of regulatory T cells and differentiation and survival of $CD4^+$ and $CD8^+$ T cells [19].

Nuclear factor κ light-chain enhancer of activated B cells (NF-κB) is also involved in the induction of IL-2 transcription [18]. NF-κB is a group of dimeric transcription factors with both positive and negative effects on gene transcription [20]. NF-κB has a large effect on the development, homeostasis, survival, and function of T cells [21]. It has a lot of target genes within T cells and is also involved in the regulation of cytokines such as tumor necrosis factor-β [22] and interferon-γ [23]. Calcineurin is also involved in the activation of NF-κB. It indirectly degrades a protein known as IκB which is bound to inactive NF-κB. It prevents NF-κB from associating with its nuclear target genes as an inhibitory protein. Calcineurin inhibition by both drugs affects the ability of NF-κB to exert its action on the genes of immune system [21, 24].

Effect on the JNK and p38 Pathways

CsA and FK506 are involved in the inhibition of the mitogen activated protein kinase (MAPK) pathway. The MAPK pathway is a signaling cascade involved in a wide variety of processes, particularly in cells within the immune system [25]. It consists of three protein kinases: MAPK, MAPKK, and MAPKK-K. MAPKK-K phosphorylates and activates MAPKK, which in turn phosphorylates and activates MAPK [26]. Three distinct MAPK subgroups include extracellular signal regulated kinase (ERK), JNK, and p38 [25]. CsA and FK506 inhibited the JNK (MAPK8) and p38 (MAPK14) pathways. CsA and FK506 reduced the JNK and p38 protein levels in Jurkat T lymphocytes [27]. JNK and p38 are activated through the MAPK signaling cascade by T cell and CD28 costimulatory receptors [28] and translocate to the nucleus where they can fulfill their various roles after activation [29]. It includes the regulation of AP-1 members [30]. AP-1 factors are involved in bolstering the transcription of IL-2 [17] and other cytokines [31]. IL-2 gene expression was prevented by the blockade of the p38 and JNK pathways [28]. CsA and FK506 prohibited JNK and p38 by inhibiting the activation of a MAPKK-K known as MAP3K1 [27].

Effect on TGF-β1

TGF-β1, a member of the TGF-β family, is a cytokine critical for the regulation of cells in the immune system. TGF-β inhibits IL-2-dependent T cell proliferation [32] and exerts a variety of other immunosuppressive effects within T cells [33]. TGF-β1 protein and mRNA expression was increased in renal transplantation patients with CsA treatment [34]. TGF-β1 protein and mRNA expression was also significantly increased by FK506 in T cells [35]. TGF-β1 protein or mRNA levels cannot be affected by CsA and FK506 when IL-2 production is successfully inhibited [36, 37]. The fibrogenic properties of TGF-β1 can cause the development of nephrotoxicity [38]. TGF-β1 mRNA expression was increased in kidney biopsies of kidney transplantation patients with either CsA or FK506 nephrotoxicity. This suggests that increased levels of TGF-β1 may lead to the CNIs nephrotoxicity [39].

Pharmacokinetics/Pharmacodynamics of CNIs

Absorption

CsA and FK506 are usually administered orally [7, 9]. Sandimmune was the first-generation of CsA and was developed as a crude oil-in-water emulsion. Its median bioavailability was 30% [40]. Neoral was the second-generation of CsA and was established as a stable microemulsion formulation with both liquid and gel caplet forms. It showed superior bioavailability and consistency and increased linear dose-exposure relationships over a wide therapeutic range. It quickly emerged as the preferred formulation owing to better therapeutic effect [41]. Modified polysaccharide vehicles or nanoparticles were used for the third generation formulation which showed effect in animal models [42]. The third generation formulation has not yet been adopted in clinical practice. An intravenous formulation is available for CsA and is kept in steady state with 100mg/ml cremophore. It is used either by intermittent or continuous infusion for 4−24h. Microemulsion formulation Neoral is assimilated rapidly with approximately dose-linear due to the homogeneous dispersion of uniform particles at the absorptive surface [43]. It has a short absorption lag and reaches the peak concentration (C_{max}) at about 1.5 h. C_{max} is increased by more than 60% and overall bioavailability by 30−50% compared with the first-generation formulations. About 50% CsA binds to erythrocytes, 10% to leukocytes, and 30−40% to the plasma proteins; only 1−6% normally exists in the free state. CsA stores easily in heart, lung, liver, pancreas, kidney, spleen, lymph nodes, blood and body fat. The CsA concentration in mononuclear leukocytes is about 1000 times higher than in erythrocytes. CsA does not easily traverse the blood-brain barrier or placenta.

Commercial oral formulations of FK506 include an immediate-release hard gelatin capsule (Prograf®) for twice-daily administration and an extended-release capsule (Advagraf®) for once-daily dosing. The absolute bioavailability of FK506 is about 20% after oral administration. Food coadministration is one of the major factors affecting the oral absorption of FK506 [44]. High-fat meal reduces about 25−37% of the overall area under the curve (AUC) for both the immediate-release and extended-release oral formulations. Low-fat diet also has a significant effect in reducing both the rate and extent of absorption and the

impact is slightly less than high-fat diet. The timing of food has an impact on the oral absorption of immediate-release formulation. Oral intake of FK506 capsules 1h before breakfast has a small effect. However, the administration either immediately following the consumption of food or 1.5h after beginning consumption of food has a similar significant effect in reducing the AUC [45]. Blood levels of FK506 can be increased by grapefruit juice and pomelo [46, 47]. The absorption difference of FK506 between the African population and Caucasian may be related to differences in the CYP3A5 genetic polymorphisms [48, 49]. Other factors affecting FK506 absorption include formulation and circadian rhythms [50].

Metabolism

CsA and FK506 are metabolized by gastrointestinal CYP3A isozymes, predominantly CYP3A4 and CYP3A5 [51, 52] after they enter enterocytes. CsA is primarily metabolized by CYP3A4 [53] and FK506 is primarily metabolized by CYP3A5 [54, 55]. Parent drug that escapes intestinal metabolism enters the hepatic portal system and the liver where CYP3A4 and CYP3A5 metabolize CsA and FK506 [56, 57]. Both CsA and FK506 bind broadly to erythrocytes after they enter systemic circulation [58]. The only unbound drugs can exert its main_immunosuppressive effects after they enter lymphocytes. 25 metabolites of CsA are formed. AM1, AM9 and AM4N are the major metabolites found in blood [59]. CsA is transformed into AM1, AM9, and AM4N by CYP3A4 and only AM9 by CYP3A5 [53]. Immunosuppressive activity of all these metabolites is reduced compared with CsA [60].

FK506 may form 15 metabolites [61] and the most prevalent metabolite is 13-O-demethyl-tacrolimus, which is about 10% as active as FK506 [62]. 31-O-demethyl-tacrolimus, a minor metabolite has comparable immunosuppressive activity with FK506 [63].

CsA and FK506 are largely metabolized and less than 0.5 to 1% of the parent drug appears unchanged in urine and feces [64, 65]. The majority of CsA metabolites is discharged in the bile and only about 3% of the drug was excreted in urine [64]. 95% of FK506 metabolites are eliminated by the biliary route and 2% are excreted from urinary tract [65].

The efflux transporter P-glycoprotein on the apical surface of cells has an important role in the pharmacokinetics of CsA and FK506 [66]. It transports both CsA and FK506 [67]. High concentrations of P-glycoprotein exist in the villus tip of enterocytes of the small intestine [68, 69] and decrease intracellular concentrations of both drugs by evacuating them from enterocytes into the intestinal lumen [70]. Intestinal P-glycoprotein variation explained about 17% of the variability in the oral clearance of CsA. Higher P-glycoprotein level suggested higher CsA clearance. Variation of liver CYP3A4 activity and enterocytes expression of P-glycoprotein can interpret 75% of interpatient variability in CsA clearance [70]. A strong inverse correlation was seen between the concentration/dose ratio of FK506 and the intestinal mRNA level of ATP-binding cassette, subfamily B, member1 (ABCB1) for the first 4−7 days after liver transplant [71,72]. P-glycoprotein also transports drugs across membranes within hepatocytes [73], kidney cells [74] and lymphocytes [69]. P-glycoprotein content may affect the real concentration of CsA and FK506 available for immunosuppression within these cells.

Pharmacodynamic Monitoring

TDM

TDM of CsA and FK506 is performed by adjusting drug dosage according to concentrations within the blood. It is widely accepted that TDM is beneficial owing to narrow therapeutic indices of these agents and their large interindividual pharmacokinetic variability [75]. Full dose interval area under the concentration-time curve from 0 to 12 h (AUC0-12) is generally considered the best marker of overall drug exposure. However, it is impossible to collect multiple samples over a 12 h period [75, 76]. Trough blood concentration (C0) or 2 h postdose blood concentration (C2) is used in most transplant centers to guide CsA dosing [77] and C0 is used to guide FK506 dosing [75]. The strength of correlation between FK506 AUC and C0 is still a matter of debate, with some studies finding better relationships between C3 [78], C4 [79], and C5 [80] and AUC. For CsA, C0 monitoring was initially used, though C2 was later found to correlate better with CsA AUC0-4, the time period in which CsA shows the greatest pharmacokinetics variability [77]. C2 was also exhibited to have solid associations with clinical results compared with C0 [77]. Guideline targets for CsA C2 levels have been proposed by the Consensus on Neoral C2: Expert Review in Transplantation (CONCERT) committee [79]; target concentrations for kidney and liver transplant patients can be seen in Table 8.1. Targets for FK506 C0 in kidney, heart, and liver transplant patients have also been proposed by a recent expert consensus document [75]. A systematic review of CsA C2 monitoring studies found that there was a lack of good quality studies comparing C0 and C2 monitoring, with the majority being observational or nonrandomized with highly heterogeneous results. Prospective studies endorse the short-term clinical benefits of C2 monitoring and propose that better evidence from randomized and high quality trials is essential to evaluate the benefits of using C2 as a timepoint [81]. TDM is broadly accepted as crucial for patient treatment. However, more studies are required to explain which clinically practical time points provide the most precise evaluation of drug exposure. Albring et al. report that analysis of intracellular interleukin (IL)-2 production and determination of the IL-2 release are accurate parameters for monitoring the pharmacodynamics of CNIs [82]. The best FK506 trough blood target after 3 months post-tranplant to prevent rejection of transplanted allografts is not clear. FK506 concentration is deceased in most clinics 2–3 months post-transplant in rejection free kidney transplant patients. Israni et al. [83] determined the association of time-varying FK506 trough levels with acute rejection (AR) in the first 6 months post-transplant. The results from 1930 renal transplantation patients suggested lower FK506 trough levels were significantly associated with increased risk of AR in the first 6 months post-transplant with additional risk of AR between months 3–6 post-transplant. The time and practice of FK506 dose decrease should be personalized according to the individual risk factors.

Table 8.1. Summary of suggested therapeutic target ranges for CsA and FK506

Organ	CsA (Neoral)	FK506		
	Months post-transplant	*C*2 target (µg/ml)	Months post-transplant	*C*0 target (ng/ml)
Kidney	2	1.5	0–3	10–15
	3	1.3	3–12	5–15
	4–6	1.1	> 12	5–10
	7–12	0.9	–	–
	12 +	0.8	–	–
Liver	0–6	1.0	0–1	10–20
	6–12	0.8	1–3	5–15
	> 12	0.6	> 3	5–10

Adopted from Ref.11 Barbarino JM, Staatz CE, Venkataramanan R, Klein TE, Altman RB. PharmGKB summary: cyclosporine and tacrolimus pathways. Pharmacogenet Genomics 2013; 23:563–585.

Drug-Drug Interactions

Many drugs can modulate cytochrome P450 enzyme activity, altering the metabolism and clearance of CsA [84]. Phenytoin, rifampin, and nafcilin cause a fall in CsA levels within 72 h of administration and may lead to graft rejection if the interaction is not recognized and corrected. CsA absorption can be affected by cholestyramine or the somatostatin analog octreotide through direct or indirect way. The cytochrome P450 enzyme system can be suppressed by erythromycin, ketoconazole, diltiazem, colchicine, and certain fluoroquinolones and antiretroviral agents. CsA concentration can be increased by these agents within days to induce acute nephrotoxicity. So the CsA dose needs to be decreased by 50% or more. CsA has less potent interactions with the macrolide derivatives josamycin, ponsinomycin, roxithromycin, with fluconazole and with verapamil. Nifedipine and nitrendipine do not affect CsA pharmacokinetics. Oral contraceptives are weak inhibitors of cytochrome P450 enzyme, and may increase CsA concentration in renal and liver transplant patients. CsA and corticosteroid metabolism is prohibited by the same hepatic microsomal enzymes. CsA trough levels may be transiently increased by high-dose intravenous methylprednisolone [85].

CsA may also change the concentration and potency of other therapeutic agents with important consequences. This effect is typified by the interaction with mycophenolate mofetil (MMF). CsA inhibits the biliary secretion and/or hepatic extraction of mycophenolate glucuronide (MPAG), leading to a reduced rate of enterohepatic recirculation of mycophenolic acid (MPA). Several concurrent mechanisms, such as CsA-induced changes in renal tubular MPAG excretion and enhanced elimination of free MPA through competitive albumin binding with MPAG, can also contribute to the altered MPAG pharmacokinetics observed in the presence and absence of CsA [86].

The immunosuppression or toxicity of CsA may be influenced by pharmacodynamic drug interactions [87, 88]. The production of vasodilatory prostaglandins is inhibited by nonsteroidal *anti-inflammatory* drugs to decrease intra-renal blood flow and induce acute renal dysfunction in patients receiving CsA. Serum creatinine can be increased by septra,

cimetidine, and ranitidine due to inhibition of tubular secretion. CsA may decrease the elimination of prednisone and digoxin, and potentiate hyperkalemia. Changes in CsA bioavailability during coadministration of herbs or herbal extracts have been analyzed. CsA blood concentrations can be reduced by ginger, liquorice, scutellaria radix, and quercetin as shown *in vivo* animal studies. CsA concentrations can be increased in patients concomitantly taking grapefruit juice, chamomile, or berberine, and in animal studies with cannabidiol and resveratrol. The findings from animal studies are an indicator of a potential interaction. It would be reasonable to avoid concomitant use of these herbal extracts until human clinical studies have excluded any possible interaction and potential for decreased therapeutic effect or toxicity [89]. Physicians are suggested to interview their patients carefully about their use of herbs and herbal supplements before CsA administration. Moreover, patients already receiving CsA treatment should be warned about the possible interactions between herbal preparations and CsA and the potential outcomes.

Agents that induce CYP3A metabolism result in decreased oral bioavailability and/or increased hepatic clearance of FK506. Consequently, increased FK506 doses are required to achieve targeted trough whole blood concentrations. The prototype for CYP3A inducers, rifampin, has been formally studied with FK506 [90]. Rifampin significantly increased FK506 hepatic clearance and significantly decreased bioavailability. The co-administration of Rifampin caused an abrupt decrease in FK506 trough concentrations and increased dosage requirements for renal transplant recipient [91]. Co-administration of ketoconazole, the prototype potent CYP3A inhibitor, significantly increased FK506 bioavailability but did not consistently affect the hepatic clearance. The magnitude of the drug-drug interaction with ketoconazole is greater in patients lacking the *1 CYP3A5 allele [92]. The clinical impact of this interaction on kidney transplant patients has been assessed. With long-term therapy, the dose of FK506 needs to obtain target trough whole blood concentration was significantly reduced in ketoconazole-treated patients [93].

Pharmacodynamic interactions with FK506 include agents that enhance toxicity and those that increase efficacy through additive or synergistic immunosuppression. The major foucus for drug interactions, enhancing the toxicity of FK506 has been affecting renal function. The combination of FK506 with nonsteroidal anti-inflammatory drugs (both nonselective, diclofenac, and Cox-2 selective, rofecxib) impaired renal function [94]. Ibuprofen, nonselective inhibitor of cyclooxygenase, was reported to cause acute renal failure in transplant recipients [95]. Other nephrotoxic agents, including aminoglycoside antibiotics, CsA, and amphotericin B, also may potentiate the renal toxicity of FK506. FK506 and CsA combination therapy was reported to increase renal toxicity in initial clinical trials for liver transplantation patients [96]. Other pharmacodynamic interactions with FK506 include the potential increase in the neurotoxicity of ganciclovir, enhanced glucose intolerance in combination with corticosteroids, and enhanced gastrointestinal toxicity with MMF.

The combination of FK506 with other immunosuppressants has been studied in animal models of organ transplantation and clinical transplant studies. Concomitant therapy of low-dose (drug-optimal) FK506 and sirolimus caused a synergistic effect in the extension of kidney allograft survival in Vervet monkeys without additive drug-associated toxicities [97]. The mechanism of FK506 and sirolimus induced long-term allograft survival in primates relates to up-regulated FasL expression, natural killer T (NKT) cells and dendritic cells, with downregulation of MLR sensitivity. It is also associated with late-dominant expression of Th2 cytokines [98]. A significant elongation of renal allograft survival was created when

Malononitrilamide 715 (FK778) administration was delayed by 7 days combined with FK506 in Vervet monkeys [99]. A selective inhibitor of NOS, FR260330 has a protective role in chronic aortic allograft rejection in the rat. Combination therapy of low-dose FR260330 with FK506 causes significant protection of immune injury and may serve to improve long-term graft survival and function [100]. Combination therapy of Baohuoside-1with FK506 creates synergistic effect in prevention acute cardiac allograft rejection in the rat [101]. Baohuoside-1(B1) immunosuppression of mitogen-activated T cell proliferation take places in G_1−S transition. It may be connected with the expression of cyclin A, D and p33cdk2 proteins. B1 prevents rat heart allograft rejection in vivo. The mechanism of B1 is different from FK506 and sirolimus [102]. Low-dose (2 mg/kg) or high-dose (5 mg/kg) ASKP1240, in combination with MMF (15 mg/kg) or FK506 (1 mg/kg), exhibited a significantly longer allograft survival time compared with monotherapy groups. No obvious side effects including drug-related thromboembolic complications were found [103].

Combination treatment with MMF causes additive immunosuppressive potency and a decreased incidence of acute allograft rejection compared to placebo or azathioprine. FK506 plus MMF has become the most commonly used immunosupprssion after renal transplantation. FK506 has been studied with sirolimus, corticosteroids, azathioprine, and monoclonal or polyclonal antibody preparations. These pharmacodynamic interactions create the foundation of combination immunosuppressant regimens that allow decreased dose of each agent in order to improve efficacy and reduce toxicity. The efficacy of the combination immunosuppressive treatment may also be a critical risk factor for opportunistic infections as well as malignancies.

Physicians should know the following drug interaction when immunosuppressive regimens need change. Corticosteroids discontinuation may cause elevated FK506 blood levels [104]. Conversion from CsA to FK506 in patients with MMF may cause elevated mycophenolic acid (MPA) levels owing to a lack of interaction with FK506. One agent should be terminated before starting the other owing to elevated toxicity with the combination when conversion is between CsA and FK506.

Pharmacogenetics of CNIs

The majority of pharmacogenetic studies on CsA and FK506 has focused on the effects of variants in the CYP3A4, CYP3A5, and ATP-binding cassette, subfamily B, member1 (ABCB1) genes because of the central role the enzymes and transporters they code for play in CsA and FK506 disposition. The effect of variants within CYP3A5, CYP3A4, and ABCB1 on CsA pharmacokinetics is dubious [105,106]. Highly varied results have been shown in searching an association between the CYP3A5*3 and CsA pharmacokinetics. No associations between the CYP3A5*3 and CsA pharmacokinetics have been shown in 171 renal transplant patients and 151 heart and renal patients [107]. However, CYP3A5*3 homozygotes have higher dose-adjusted trough concentrations than heterozygotes in 110 renal recipients [108]. CYP3A5*3 homozygotes retained higher dose-adjusted trough concentrations on days 1–10 of treatment and higher dose requisites on days 16–30 in 91 bone marrow transplantation patients compared to CYP3A5*1 homozygotes [109]. A meta-analysis of 1036 individuals showed no influence of the SNPs (Single nucleotide polymorphisms) rs1045642 on any CsA

pharmacokinetic parameters except increased AUC_{12} for T allele carriers [110]. The rs1128503 T allele was associated with elevated dose-adjusted maximum blood concentrations [111]. However, most of the other studies on rs1128503 and rs2032582 have shown no relationships [90]. No significant associations between ABCB1 haplotypes and CsA pharmacokinetics are reported [112]. The above results indicate that SNPs within ABCB1 may clarify only a small part of the variation in CsA pharmacokinetics.

Diverse results have shown about the association between CYP3A5*3, CYP3A4*1B, and ABCB1 alleles and CsA pharmacodynamics. A significant association between the CYP3A5*3/*3 genotype and reduced patient survival was shown compared to *1 allele carriers [113]. Negative results have been shown in other studies [112,114].

CYP3A5 gene Variations have suggested some of the solid associations with FK506 pharmacokinetics. The rs776746 SNP in intron 3 of the gene has been indicated especially to be the most powerful predictor of FK506 dose requisites [115], and to interpret up to 45% of the fluctuation in dose [116] and 30% of the variability in the oral clearance of FK506 [127]. CYP3A5*3 is connected with a range of responses to FK506, including elevated dose-adjusted trough concentrations (C0/D), reduced dose requirements, and declined oral clearance [118,119]. CYP3A5 nonexpressers are patients homozygous for the *3 allele [120]. This decrease in enzymatic activity causes the lower dose requisites for CYP3A5 nonexpresser renal transplant patients who need to reduce mean daily doses by ~ 0.05 mg/kg [121]. The association between CYP3A5 genotype and FK506 metabolism has been duplicated many times in different studies and a recent systematic review of literature indicated a very high level of evidence to support the gene–drug interaction [121].

rs2740574 (CYP3A4*1B) and rs35599367 (CYP3A4*22) both have associations with FK506 dose requirements. rs2740574 is a promoter variant which increases gene transcription [122]. Carriers of the *1B allele have been shown to have 35% lower FK506 dose-adjusted trough concentrations compared with those homozygous for the normal *1 allele [108]. However, rs2740574 was displayed to be in linkage disequilibrium (LD) with rs776746 within the CYP3A5 gene, so its effect on FK506 dosage requirements (as well as any pharmacodynamic parameters) is likely mediated by this SNP [123]. The combinations of the CYP3A4*1B and CYP3A5*3 alleles have shown significant associations with FK506 pharmacokinetics [112]. *22 allele carriers need a mean daily dose of FK506 33% lower than wild-type homozygotes to reach the same predose FK506 blood concentration [124]. rs35599367 is not in LD with rs776746, and it partially provides to the variation in FK506 dose requirement independently of the CYP3A5*3 allele [125].

Associations between FK506 pharmacokinetics and variations in ABCB1 have been variable. rs1045642 had the potential to affect drug bioavailability because it was observed to decrease intestinal P-glycoprotein expression and function [126]. A recent systematic review of available literature indicated no consistent evidence for an association between FK506 dose adjusted trough concentrations and rs1045642 (3435C > T), a well-studied SNP within the gene [127].

Genotyping before CNIs treatment has potential for preventing side effects such as nephrotoxicity, rejection, or neurotoxicity. However, currently no genes (or variations within these genes) display consistent associations with pharmacodynamic parameters. Indeed, only CYP3A5*3 presents reliably positive associations with pharmacokinetic parameters for FK506. As both FK506 and CsA are subjected to careful dose-monitoring, genotyping CYP3A5 to accurately predict dosage may not be necessary [11]. Thervet et al. [128] found

that, though patients given genotype-adapted FK506 dosing had trough blood concentrations in the target range more often than those on the standard regimen, this did not result in any positive clinical endpoints such as decreased incidence of rejection or nephrotoxicity.

Lunde et al. affirmed a crucial impact of the CYP3A5*3 allele on FK506 exposure. Patients with POR*28 and PPARA variant alleles illustrated 15% lower and 19 % higher FK506 C0/D respectively. CsA C2/D was 53 % higher among CYP3A4*22 carriers. The results endorse the utilization of pre-transplant CYP3A5 genotyping to ameliorate initial dose of FK506, and propose that FK506 dose may be further individualized by additional POR and PPARA genotyping. Furthermore, the initial CsA dose may be corrected by pre-transplant CYP3A4*22 assessment [129].

Large-scale studies should focus on whether CYP3A5 nonexpressers determination before FK506 treatment convalesces clinical outcome in order to make genetic testing relevant for these drugs. The pharmacogenetics of FK506 and CsA is complex, and a lot of factors likely endow to its variability. However, developing our comprehension in this area will have an important impact on the health and well-being of patients treated with these drugs [11].

A SNP in cytochrome P450 (CYP) 3A5 (6986A > G) has been consistently related to FK506 dose requisite. Patients expressing CYP3A5 (those carrying the A nucleotide, defined as the *1 allele) have a doseing requisite that is around 50% higher than non-expressers (those homozygous for the G nucleotide, defined as the *3 allele). A randomized controlled study in kidney transplant patients has displayed that a CYP3A5 genotype-based approach to FK506 dose causes more patients reaching the target concentration early after transplantation. However, the clinical outcomes (rejection incidence, toxicity) were not improved. CYP3A4*22 and POR*28 SNPs are the most encouraging genetic variants which may also contribute to the variability in FK506 pharmacokinetics. Patients with the CYP3A4*22 T-variant allele have a lower FK506 dose requirement than those with the CYP3A4*22 CC genotype and this effect seems to be independent of CYP3A5 genotype status. Individuals carrying the POR*28 T-variant allele have a increased FK506 dose requirement than POR*28 CC homozygotes but this association was only shown in CYP3A5-expressing individuals. For FK506 pharmacodynamics, no strong genotype-phenotype relationships have been confirmed. Certain SNPs associate with rejection risk but these observations need replication. The genetic basis of FK506-induced toxicity is unclarified. No genetic markers reliably foresee new-onset diabetes mellitus after transplantation, hypertension or neurotoxicity. The CYP3A5*1 SNP is currently the most inspiring biomarker for tailoring FK506 treatment. However, before CYP3A5 genotyping is incorporated into the routine clinical care of transplant recipients, prospective clinical trials are needed to determine whether such a strategy ameliorates patient outcomes. The role of pharmacogenetics in FK506 pharmacodynamics should be explored further by the study of intra-lymphocyte and tissue FK506 concentrations [130].

Limited evidence suggests that variation in genes involved in pharmacokinetics (ABCB1 and CYP3A5) and pharmacodynamics (TGF-b, CYP2C8, ACE, CCR5) of FK506 may impact a transplant recipients' risk to develop FK506-induced nephrotoxicity across different transplant organ groups [131].

Current CNIs Therapeutic Strategies

Advances in immunosuppressive strategies have prompted meaningful developments in organ transplantation. CsA changed organ transplant by reducing acute rejection rates and increasing short-term graft survival in the 1980s. FK506 and mycophenolic acid further improved the transplant outcomes in the 1990s. Immunosuppressive agents can be divided into three categories: "induction agents", "maintenance therapy" and "treatment for rejection". Induction agents include polyclonal antibodies (antithymocyte globulins) and interleukin (IL)-2 receptor antagonists (basiliximab). New induction agents include alemtuzumab, efalizumab and alefacept. The four drug classes that form maintenance regimens contain calcineurin inhibitors (CsA and FK506), mTOR inhibitors (sirolimus and everolimus), antiproliferative agents (azathioprine and mycophenolic acid), and corticosteroids [132].

Calcineurin inhibitors have been extensively used as immunosuppressive regimens for prevention of acute rejection and improvement of graft survival. The effect of FK506 and CsA on solid organ transplantion was compared in several landmark trials. No difference in patient or graft survival post transplantation between FK506 to CsA using the combination of calcineurin inhibitors, azathioprine and corticosteroids was reported in two multicenter studies [133,134]. The effect of FK506 and CsA was compared in transplant patients with one of three immunosuppressive regimens: [1] FK506 with azathioprine and corticosteroids; [2] FK506 with MMF and corticosteroids; and [3] microemulsion CsA and MMF with corticosteroids [135]. The incidence of corticosteroid resistant rejection was lower in the FK506 group, although acute rejection rates were similar in each group (≤ 20%). FK506 improved 3-year patients and graft survival in recipients with delayed graft function [135]. A meta-analysis reported that for every 100 patients treated with FK506 rather than CsA for the first year, 12 would be prevented from having acute rejection, 2 would be prevented from having graft failure, but 5 would develop new onset diabetes after transplantation [136]. The low dose CsA regimen was not as effective as the low dose FK506 regimen demonstrated by the Elite Symphony trial [135]. FK506 should be the first-line calcineurin inhibitor for renal transplant recipients (Level of recommendation 2A) bases on the results of the above trials as the KDIGO (Kidney Disease: Improving Global Outcomes) Clinical Practice Guidelines suggested [138].

A prolonged release FK506 formulation and voclosporin, a CsA analog, are the potential improvements to the calcineurin inhibitor class. Advagraf®, an extended release FK506 formulation, is being refined to improve adherence of the medication regimen in post-transplant patients and has been approved for use in various European countries and Canada. The efficacy and safety of prolonged release-FK506 was compared to FK506 and CsA in a large, randomized, open label, phase III study with 668 de novo kidney transplant recipients. Excellent patient and graft survival were achieved (> 93%) in all groups and efficacy failure (death, graft failure, or acute rejection) was 14.0%, 15.1% and 17.0% in extended release-FK506, FK506 and CsA groups respectively [139]. However, 10.3% of extended release FK506 patients had a BPAR compared to 7.5% in FK506 group and 13.7% in CsA group. Similar patient survival (97.5% *vs.* 96.9%) and graft survival rates (92.8% *vs.* 91.5%) were shown among extended release FK506 and twice daily FK506 patients in a Phase III trial [140]. The FK506 levels were slightly lower with an extended release FK506 group compared to twice daily FK506 patients [141,142]. The efficacy was similar in both groups. Both

formulations had very similar renal function at 1-month, 6-month and 12-month. The result suggested non-inferior nephrotoxicity profile. The incidence of BPAR was elevated slightly in the extended release FK506 groups [137,138] and therefore patients should be monitored closely when therapy is changed. Extended release FK506 is proposed to promote patient compliance due to a noninferior effect profile and the convenient daily dose. The effect of FK506 extended-release (Astagraf XL) was compared FK506 (Prograf) or CsA in a phase III, open-label, comparative, noninferiority study with 638 subjects receiving de novo kidney transplants [143]. Basiliximab induction, MMF and corticosteroids were given to all patients. Patient survival by four-year Kaplan-Meier estimates in the Astagraf XL, Prograf, and CsA groups were 93.2, 91.2, and 91.7%, respectively, while graft survival was 84.7, 82.7, and 83.9%, respectively. Patients receiving Astagraf XL and Prograf had higher incidence of new-onset diabetes after transplantation (NODAT) and superior renal function compared to patients receiving CsA, although the effect and safety profiles were comparable.

Voclosporin, a cyclosporine analogue, is being tested in solid organ transplant [144]. Voclosporin significantly increased renal allograft survival in a nonhuman primate model [145]. Low (0.4 mg/kg), medium (0.6 mg/kg) and high (0.8 mg/kg) dose voclosporin were compared to FK506 (0.05 mg/kg), in combination with a standard immunosuppressive regimen in low risk renal transplant patients with immediate allograft function. Rejection rates were non-inferior to FK506 (11%, 9%, 2%, and 6% respectively) and renal function was clinically similar (69−72 mL/min) at 6-month after transplantation [144]. The rate of NODAT was significantly decreased in the low dose voclosporin group (1.6% *vs.* 16.4% FK506), but not in the medium (5.7%) and high dose (17.7%) group [144]. Low to medium dose voclosporin may supply enough immunosuppression with a decreased frequency of NODAT. Pharmacokinetic studies suggested that voclosporin should be given on an empty stomach and that dose adjustment may be necessary in severe renal failure (< 30 ml/min) and mild to moderate hepatic impairment patients (146−148). Best trough concentrations should be proposed between 35−60 ng/ml [149].

CNIs remain a foundational therapy for organ transplantation. The use of CsA is progressively declined from approximately 79% of the kidney patients in 1996 to only 15% in 2005. FK506 is increased from 13% to 76% in the same period due to the greater simplicity of use, its superior performance in preventing graft rejection and improving graft survival [150−155].

Toxicity of CNIs

Calcineurin inhibitors have numerous toxicities which are often dose dependent. Hirsutism, gingival hypertrophy, hypertension and hyperlipidemia are more presented with CsA treatment than with FK506. FK506 treatment had more neurotoxicity, alopecia, and potentially post-transplant diabetes than with CsA [156]. The major complications of CsA treatment in organ transplantation include skin and appearance, neurotoxicity, cardiovascular diseases, nephrotoxicity, metabolic diseases, new-onset diabetes, infection and malignancy. The adverse effects of FK506 include posttransplantation metabolic, new-onset diabetes, nephrotoxicity, neurotoxicity, and gastrointestinal effects, opportunistic infections and malignancy.

Nephrotoxicity

Calcineurin inhibitors cause acute and chronic nephrotoxicity. The acute nephrotoxicity includes arteriolopathy, tubular vacuolization and thrombotic microangiopathy. Chronic nephrotoxicity includes interstitial fibrosis and tubular atrophy, medial arteriolar hyalinosis, glomerular capsular fibrosis, global glomerulosclerosis, focal segmental glomerulosclerosis, juxtaglomerular apparatus hyperplasia, and tubular microcalcifications, many of which can be caused by other factors and tend to be nonspecific findings on post-transplant biopsy [154]. CNI-caused nephrotoxicity is the principal reason of renal dysfunction after liver transplantation [155, 157, 158] and has been associated with significant morbidity and mortality [155, 157]. Incidence rates were as high as 18.1% at 5 years posttransplant [157] and the 5-year cumulative incidence of chronic renal failure was 22% [159].

Acute CNI nephrotoxicity is well reported and high blood concentration of CNIs is connected to the reduction in renal function and histological changes to the kidney. These changes can be corrected with CNI removal [160, 161]. Vasoconstriction of the afferent arterioles and direct effects of CNIs on the tubular epithelium are suggested to determine the mechanisms of acute CNI nephrotoxicity [160]. The CNI toxicity diagnosis is according to the combined manifestation of several of the criteria and the withdrawal of other possible causes because all histological changes may have other causes [162]. The extent of CNI toxicity in biopsy specimens fluctuates noticeably between studies. Nankivell et al. found that evidence of CNI toxicity existed in almost all biopsy specimens at 10 years posttransplant [163]. However, it was impossible to assess the effects of other potential factors on renal function because this study did not have a control group. Renal function can be affected by rejection, infections, other nephrotoxic drugs, comorbidities and the ageing process. The death-censored kidney graft survival rates were 95% at 10 years and suggested that CNI toxicity was not connected to late graft failure. The other factors may have a role in the development of histological lesions due to the relatively limited number of late biopsies, evidence for the occurrence of ongoing subclinical rejection and the early appearance of fibrosis with little subsequent progression [164]. Chronic CNI toxicity has been widely considered as a major factor in late graft failure. However, recent studies analyzing the reasons of late graft failure in detail have raised doubts about this view and have emphasized the importance of antibody-mediated rejection and other factors. CNI nephrotoxicity diagnosis is a challenge to physicians with the possibility for over-diagnosis and an unsuitable decrease in immunosuppressive therapy. It is necessary to correctly ascertain the cause of the kidney disease for effective long-term management of the patient when graft function is deteriorating. A complete clinical examination is needed for the diagnosis and a definite cause can be recognized in the majority of cases [165].

Endothelin-1 (ET-1) is shown to be connected to increased vascular resistance, CNI-caused nephrotoxicity and chronic rejection. Ambrisentan, a selective ETA receptor blockade, ameliorates the outcome of rats with liver transplantation partially through reducing nephrotoxicity [166]. A role for Nox2 in CNI-induced renal fibrosis is specifically shown in animal study. Specific Nox2 inhibition strategies may ameliorate chronic CNI nephrotoxicity in solid organ transplantation if it is confirmed in humans [167].

The mechanisms causing CNI nephrotoxicity [153] include a decrease in vasodilators such as prostaglandin E2 and nitric oxide as well as an increase in vasoconstrictors, such as

thromboxane, endothelin and the renin-angiotensin system. Direct toxicity to the tubular epithelium has been demonstrated both clinically and experimentally.

NODAT

New-onset diabetes mellitus after transplantation (NODAT) is a frequent complication after kidney transplantation, with an incidence of 15% to 30% [168, 169]. The incidence of diabetes mellitus is very high in the first year after transplantation. NODAT and type 2 diabetes mellitus (T2DM) share common risk factors of decreased insulin sensitivity and decreased insulin secretion. T2DM risk factors are older age, ethnicity (African American, Hispanic, and Native American), family history of T2DM and obesity. The risk factors unique to the posttransplantation environment comprise immunosuppression, cytomegalovirus infection, hepatitis C seropositivity and weight gain after transplantation. Immunosuppression drugs commonly used, including CNIs (CsA and FK506), corticosteroids, and mammalian target of rapamycin inhibitors (sirolimus and everolimus) have been implicated to be diabetogenic. [170, 171]. The diabetogenic effects of CNIs are partially attributed to pancreatic β-cell apoptosis, impaired insulin secretion [172,173] and CNI-induced insulin resistance [174−176] and insulin secretion decrease [177]. The correlation of calcineurin inhibition and diminished β-cell survival and its independent effect on insulin resistance needs further investigation [176]. An in-progress, open-label, multi-center study (SAILOR) is under way to evaluate whether a steroid-free immunosuppressive protocol, based on ATG induction and a low FK506 dose, reduces the incidence of NODAT [178].

Malignancy

FK506 and CsA can activate proto-oncogenes and pathways of cancer, such as TGF-b in a dose-dependent fashion to promote tumour proliferation and metastasis by *in vitro* studies and animal models [179−181]. The effect of two CsA doses on *de novo* malignancy was compared in randomized controlled trial with 231 kidney transplant recipients. The normal-dose group (trough levels 150–250 ng/ml) had significantly increased rates of *de novo* tumours at 6 years compared to low-dose group (trough levels 75−125 ng/ml) [182]. The risk of malignancy related to CNI may come from the dosing rather than the type of CNI used. CsA was reported to increase risk of malignancy in liver transplant patients compared with FK506 [183]. In 385 liver transplantation patients between 1986 and 2007, 50(13.0%) recipients developed *de novo* malignancy. The cumulative incidence of de novo cancer at 1, 5, 10, and 15 years after liver transplantation was 2.9% ± 0.9%, 10.5% ± 1.8%, 19.4% ± 3.0%, and 33.6% ± 6.8%, respectively. Liver transplantation patients ≤ 50 years had a higher cancer rate when treated with CsA compared to treatment with FK506 [183]. These patients displayed a meaningfully lower acute rejection rate which might reflect a more robust immunosuppressive status caused by the CsA-C [2] blood concentration at 2 hours post dose.

A multivariate analysis of posttransplant malignancies in 33,249 deceased donor primary solitary renal recipients summarized by 264 kidney transplant programs to the Organ Procurement and Transplantation Network database from July 1, 1996 to December 31, 2001

was carried out. Maintenance immunosuppression with the TOR inhibitor drugs is connected to a meaningfully decreased risk of developing any posttransplant de novo malignancy and non-skin solid malignancy compared to CNIs [184].

Cancer incidence was substantially increased after kidney transplantation. Most of these cancers are those with known or suspected viral causes. In contrast, cancer incidence was only slightly increased before kidney transplantation [185].

Infection

Infections come second as the reason of death in patients with allograft function and infections are a common reason of morbidity and mortality after transplantation. The rate of first infections in the initial 3 years after kidney transplantation is 45.0 per 100 patient-years of follow-up, as estimated using Medicare claims data collected by the U.S. Renal Data System [186].

Cytomegalovirus Infection

Cytomegalovirus (CMV) infection is the most common opportunistic infection in kidney transplant recipients and occurs 8% of patients [187]. In the cohort of 1528 lung transplant recipients from 12 transplant centers, delayed-onset CMV disease occurred in 13.7% and early-onset CMV disease occurred in 3.3% [188]. CMV infection (36−100%) and disease (11−72%) can be expected to happen within the first 3−4 months after liver transplantation without any prevention strategy [189]. CMV risk factors include donor seropositivity (especially if the recipient is seronegative), use of induction immunosuppression, simultaneous kidney-pancreas transplantation, older donors, presence of allograft rejection, and coexisting infection from other viruses [190]. It is important to distinguish CMV infection from CMV disease for treatment options. CMV infection is ascertained by evidence of CMV replication, regardless of symptoms. CMV disease needs both proof of infection and symptoms, including viral syndrome with fever or malaise, leukopenia, thrombocytopenia, or proof of tissue invasion (e.g., pneumonitis, hepatitis, retinitis, gastrointestinal disease) [191]. CMV infection within 100 days of transplant is an independent risk factor for overall recipient mortality and early CMVdisease is associated with increased cardiovascular mortality beyond 100 days [190]. CMV disease is also connected to post-transplant lymphoproliferative disorder (PTLD), transplant renal artery stenosis, post-transplant diabetes mellitus irrespective of immunosuppressive drugs, and repeated thrombotic microangiopathy after kidney transplant [191–194]. Prophylaxis with antiviral medications is suggested to be used routinely in cytomegalovirus-positive recipients and in cytomegalovirus-negative recipients of organs positive for the virus because it decreases the risk of cytomegalovirus disease and associated mortality in recipients of solid-organ transplants [192].

EBV Infection and PTLD

Epstein-Barr virus (EBV) is associated with about 62–79% of PTLD and is an important reason of post-transplant morbidity and mortality. EBV-naive recipients who receive EBV-seropositive organs are at greatest risk for PTLD [138]. The risk factors of EBV-associated PTLD include primary EBV infection, younger recipient, CMV disease, OKT3 or polyclonal antilymphocyte antibody administration, and the type of organ transplanted [138]. Primary EBV_infection in EBV-seronegative recipients usually occurs in the first 3–6 months [138]. PTLD usually occurs after primary infection. PTLD diagnosis requires histologic confirmation, preferably via excision biopsy with immunologic cell-typing, cytogenetics, immunoglobulin gene arrangements, and EBV-specific staining [195]. PTLD staging is based on location, metastasis, and histologic type (monoclonal, polyclonal, T cell, or B cell) [196]. Immunosuppression decrease is routinely prescribed for the treatment of PTLD which can cause remission in 23–86% of patients [197]. The therapeutic interventions include surgery, monoclonal antibody therapy, chemotherapy, and radiation. Rituximab (monoclonal antibody to CD20) is generally provided to treat EBV-positive PTLD in patients requiring therapy besides immunosuppression reduction. No evidence supports the effect of antiviral therapy [193]. When rituximab fails, chemotherapy can be administered [198]. Regular therapy is not available for preventing PTLD. Effective CMV prevention may impede EBV disease by restraining immunomodulation by CMV infection [199]. EBV viral load monitoring recognizes recipients with fast growing viral loads who are risk of acquiring PTLD and permits for the timely decrease of immunosuppression before the development of PTLD. The EBV viral load is examined by nucleic acid testing (NAT) in the first post-transplant week, then monthly for the first 3–6 months, then every 3 months until the end of the first post-transplant year by KDIGO guidelines. Patients with primary EBV, CMV, and hepatitis C virus (HCV) infection are at elevated risk for PTLD and should be observed closely [138].

BK Polyoma Virus Infection

BK polyomavirus (BKV) is the major cause of polyomavirus-associated nephropathy (PyVAN) to cause 1–15% of kidney transplant patients at risk of premature allograft failure, but is less common in other solid organ transplants. BKV replication screening in urine and blood of kidney transplant patients has been adopted as the key recommendation to guide the decrease of immunosuppression in patients with BKV viremia owing to no effective antiviral therapies. Late diagnosis is faced with an irrevocable functional decrease, miserable treatment response, and graft loss. Retransplantation after PyVAN is overall successful, but close observation is needed for recurrent BKV viremia [200]. BKV viremia usually leads BKV nephropathy by an average of 8 weeks. About 50% of patients who grow BKV viremia will develop it by 3 months after transplantation. BKV nephropathy happens almost in the first 2 years after transplant, with only 5% of cases coming between 2 and 5 years after transplantation [201].

BKV with quantitative plasma nucleic acid testing (NAT) should be analyzed for all kidney transplant recipients at least monthly for the first 3–6 months after transplant, every 3

months until the end of the second year if an unexplained expansion in serum creatinine happens; and after treatment for acute rejection [138].

PyVAN may happen in 1−10% of renal transplant patients with loss of the transplanted organ in 30% up to 80% of the cases. Pathogenesis of PVAN is still unknown. Immunosuppression decline is the first line of intervention, however, clinical controlled trials are needed to distinguish the best therapeutic strategies [202].

Hepatitis B and C

In general, the induction of immunosuppressive treatment bears the risk of HBV reactivation. The major innovation for transplantation patients with hepatitis B virus (HBV) and hepatitis C virus (HCV) is the utilization of nucleos(t)ide analogs (NAs) and direct acting antivirals (DAAs). NAs form the mainstay in the treatment of patients with HBV in the non-transplant setting as well as before and after liver and kidney transplantation [203].

Urinary Tract Infections

Urinary tract infections (UTIs) are the most common bacterial infections requiring hospitalization in kidney transplant recipients. Women have the greatest risk for UTIs. The risk factors include deceased-donor transplant, kidney-pancreas transplantation with bladder drainage, prolonged catheterization, uretero-vesical stents, and increased immunosuppressed state [204]. Cumulative UTI incidence was 17% during the first 6 months after transplantation, 60% for women and 47% for men at 3 years revealed by U.S. Renal Data System database of 28,942 primary renal transplant recipients [205]. Escherichia coli is the most common uropathogen in kidney transplant recipients. Patients may present with classic UTI symptoms, gastrointestinal symptoms, or asymptomatic bacteriuria [206].

Mycobacterium Tuberculosis Infection

Mycobacterium tuberculosis infection incidence among kidney transplant recipients is 0.5–1.0%, 0.7–5%, and 5–15%, respectively in North America, Europe, and India/Pakistan [207]. Active tuberculous disease after transplantation happened in 20–25% patients with positive tuberculin skin test reactions before transplantation [208]. Clinical presentation of TB in immunocompromised patients differs from that in the normal host, with approximately one third of all cases of active infection involving disseminated disease or extra-pulmonary sites, compared with approximately 15% of cases in normal hosts [207]. Active TB diagnosis in transplant patients needs a high index of suspicion and, in some cases, biopsy for microbiologic diagnosis. Neither tuberculin skin tests nor IFN-g release assays are suitable for the diagnosis of active infection. Screening and identification of individuals with prior latent infection with TB are important to decrease posttransplant infections. Immunosuppression increases the risk for reactivation because of impairment of microbial specific cytotoxic T cell response which is a key host defense against mycobacterial infection.

Consequently, all transplant candidates and live donors should be assessed before transplantation to minimize the likelihood of post-transplant disease [209]. Screening should include a careful history regarding previous exposures, review of previous and current results of tuberculin skin testing or IFN-g release assay, and chest radiography. IFN-g release assays can be utilized to differentiate latent TB infection from reactivity due to bacille Calmette-Guérin vaccination. Historical or radiographic evidence may be enough to assist the diagnosis of latent infection, especially because transplant candidates may have diminished responses with either tuberculin skin test or IFN-g release assay. Nine months isoniazid should be given to previously untreated patients with confirmation of latent TB infection [208]. Organ transplantation may be carried out in patients with treatment for latent TB.

Cardiovascular Disease

The annual risk of a cardiovascular event for kidney transplant patients is up to 50-fold higher than for the general population [209, 210] and cardiovascular disease accounts for over a third of all deaths following kidney transplantation [211, 212]. CNIs interact negatively with the endothelium [213, 214] which is the critical defence line for protection against the initiation and progression of atherosclerotic and arteriosclerotic changes. The major cause of death in transplant recipients is cardiovascular disease because these patients have a complicated set of conditions including hypertension, diabetes mellitus, and post-transplant hyperlipidaemia [215]. Cholesterol and triglyceride levels are still high in kidney transplant recipients after intervention [212]. Hypertension was reported in 20–70% of patients using CNIs [216, 217] and it was more in patients with CsA than FK506 [218]. Hypertension after transplantation increased the risk of cardiovascular complications [219] and was associated with earlier graft failure in kidney transplant recipients [220]. CNIs affected several systems involved in blood pressure regulation, including the renin-angiotensin system, endothelin-1, nitric oxide and the sympathetic nervous system [221]. The mechanism of CNI-induced hypertension involved vasoconstriction stimulation by increased endothelin-1, angiotensin II and the activity of the sympathetic nervous system [222], impaired vasodilation through inhibition of nitric oxide [223] increased salt-sensitive [224] and the renal sodium chloride cotransporter activation [225].

Neurotoxicity

CNIs are associated with a wide range of neurotoxic adverse events ranging from peripheral manifestations such as neuropathy, to centrally mediated complications such as altered cognition, visual disturbances and seizures [226]. Headache, tremor and fatigue are common manifestation of treatment with CNI which represent a potentially systemic neurotoxicity [227]. The mechanisms of neurotoxicity are still unclear, although CNI-related neurotoxicity is well recognized in the clinical practice [228]. Magnetic resonance imaging (MRI) may supply important diagnostic information in transplant recipients [228]. 10−28% of patients with CsA treatment face some form of neurotoxic adverse event. Both sensorial motoric functions may be adversely influenced, and thus patients show a wide range of

neurological and psychiatrical disorders. Mild symptoms are common and include tremor, neuralgia, and peripheral neuropathy. Severe symptoms, including psychoses, hallucinations, blindness, seizures, cerebellar ataxia, motoric weakness and leukoencephalopathy, influence up to 5% of patients. FK506 is associated with similar neurotoxic adverse events. Neurotoxicity may result in serious complications for some patients, particularly recipients of orthotopic liver transplants. Factors that may advocate the development of serious complications include advanced liver failure, hypertension, hypocholesterolemia, elevated CsA or FK506 blood levels, hypomagnesemia, and methylprednisolone. Occipital white matter seems to be uniquely susceptible to the neurotoxic effects of CsA; injury to both the major and minor vasculature may induce hypoperfusion or ischemia and local secondary toxicity in the white matter. Calcineurin inhibition by CsA and FK506 modifies sympathetic outflow which may have a role in the mediation of neurotoxic and hypertensive adverse events. The symptoms of CsA- and FK506-associated neurotoxicity may be corrected in most patients by substantially decreasing the dose of immunosuppressant or discontinuing these drugs. After dose decrease or discontinuation permanent or even fatal neurological damage cannot be corrected in some patients. CsA-sparing and FK506-sparing drug regimens using the immunosuppressant MMF without neurotoxic effects may decrease the incidence and severity of neurotoxic adverse events and keep enough level of immunoisuppression [226].

Calcineurin Inhibitor Sparing Strategies

A majority of the success of organ transplantation is connected to the ability of CNIs to decrease acute rejection rates. However, long term graft survival rates have not ameliorated over time, and although controversial, evidence does indicate a role of chronic CNI toxicity in this failure to rectify outcomes. Decreasing or eliminating CNIs from immunosuppressive regimens could correct outcomes. Several strategies remain to spare calcineurin inhibitors, including application of agents such as MMF, mycophenolate sodium (MPS), sirolimus, everolimus or belatacept to favor late calcineurin inhibitor withdrawal, beyond 6 mo post-transplant; or utilizing these agents to plan early withdrawal within 6 mo; or to avoid the CNIs all together employing CNI-free regimens [229]. Three basic strategies are accessible for calcineurin- sparing, “Avoidance”, and “Early” and “Late” reduction or withdrawal. Late, characterized as calcineurin inhibitor decrease withdrawal or exclusion over 6 mo (> 6 months) after kidney transplant, is a strategy that has been frequently utilized when patients are faced with declining renal function, possibly related to established toxicity. Early, characterized as calcineurin inhibitor withdrawal or decrease within the first 6 mo (≤ 6 months) after the kidney transplant, is generally performed to prevent anticipated calcineurin inhibitor toxicity or in response to early evidence of dropped renal function. Calcineurin inhibitor withdrawal or calcineurin inhibitor-free regimens are characteristically a proactive strategy in response to the considerations about the potential toxicity of the calcineurin inhibitors and their failure to advocate long-term graft survival, despite dramatic decrease in the risk of acute cellular rejection.

Endeavours to decrease the toxicities of CNIs, in particular nephrotoxicity, include various strategies intend to eliminate (withdrawal), minimize and avoid these agents, such as: [1] CNIs withdrawal happens either in CNI exclusion, with the elimination of the drug after a

predetermined time, thereby decreasing CNI side effects, or in CNI substitution, with the application of alternative agents, keeping the total amount of immunosuppression comparable; [2] CNI minimization is a decrease in the dose of CNIs followed by therapeutic drug monitoring to target CNI levels lower than in the standard treatment; and [3] CNI avoidance is the intentional non-application of the drug from the beginning of transplantation [230].

In general, the CNI-sparing strategies may include mycophenolic acid (MPA), proliferation signal inhibitors [sirolimus (SRL) or everolimus] or the newer agents such as Belatacept.

CNI Withdrawal with mTORi

CNI discontinuation after kidney transplantation is often carried out early after transplantation or more often late due to grafts with impairing function. CNIs early withdrawal after transplantation with mTOR inhibitors in patients with good renal function seems to be reliable and potent. GFR is elevated in CNI discontinuation recipients with sirolimus [231] or everolimus [232] both at one and three years; however, the withdrawal rate in patients on mTOR inhibitors is high and is induced by drug related adverse events. Acute rejections happen soon after CNI discontinuation. The incidence of acute rejection was not different in the sirolimus group at one year and was higher in the everolimus group at three years. The data advocate longer studies with more patients and highlight that only some patients have a better advantageous effect of switching to mTOR inhibitors from CNIs.

Late CNI Withdrawal with mTORi Immunosuppression

Excellent 2 years patient and graft survival were reported by SRL conversion among patients with baseline GFR more than 40 mL/min compared with CNI continuation. There was no difference in BPAR. Increased urinary protein excretion and a lower incidence of malignancy was observed. Better renal function was shown among patients who stayed on SRL through 12 to 24 months [233]. The data show that renal biopsy prior to conversion is helpful to choose patients without mild to severe chronic renal allograft damage in whom conversion from CNIs to mTOR inhibitors can be completed safely and effectively.

The renal effect of late conversion is confined, except in patients with good renal function and without proteinuria. Early conversion to mTOR inhibitors in the first 6 months, in combination with MMF, could be a suitable strategy for maintenance therapy in renal transplant recipients with a low immunological risk after careful screening at the time of conversion. Good renal function (glomerular filtration rate >40 ml/minute), weak proteinuria (<1 g/day), an absence of previous acute rejection and subclinical rejection, and appearance of donor-specific anti-human leukocyte antigen antibodies seem to be the most important standard in recognizing patients for whom conversion to an mTOR inhibitor may ameliorate renal function at 5 years. Whether the benefits examined in these trials could affect long-term graft and patient survival remain to be ascertained [234].

CNI Withdrawal with MMF

Elective CNI elimination: MMF and steroids were used to replace CsA in recipients with stable renal function [235,236]. The CsA-withdrawal group had better renal function and lipid profile at one and five years, and the incidence of BPAR was higher in the CsA-withdrawal group. Increased graft loss in patients experiencing acute rejection was shown although improvement in GFR was kept at five-year follow up [237]. The incidence of BPAR at 12 month was significantly increased in the CsA-withdrawal group compared with the other two groups in the Cyclosporine Avoidance Eliminates Serious Adverse Renal-toxicity (CAESAR) trial which assessed the outcomes of reduced-dose CsA (50% lower) either with or without early withdrawal at 6-month, in primary kidney allograft recipients receiving daclizumab induction, MMF and steroids [237]. These studies indicate that complete elective elimination of CNI is a "doubleedged sword": the improvement in GFR was balanced by an increase in acute rejection rates that in turn cause reduced graft survival over the long term. The strategies in the mTORi studies, including refining the criteria for patient selection to identify those patients at low immunological risk, the timing of CNI elimination and immunosuppression monitoring, may decrease acute rejection episodes to expand graft survival.

CNI Elimination for Transplant Dysfunction

CNIs elimination with MMF in patients with renal dysfunction confirmed by renal biopsy significantly increased GFR without acute rejection [238−239].

A randomized multicenter trial included 212 kidney patients transplanted between 1997 and 1999. All patients were initially treated with MMF, CsA, and prednisone (pred). At 6 months after transplantation, 63 patients were randomized for MMF/pred, 76 for MMF/CsA, and 73 for MMF/CsA/ pred. Within 18 months after randomization 23 patients experienced a rejection episode: MMF/pred (27.0%), MMF/CsA (6.8%) and MMF/CsA/pred (1.4%) (p<0.001). Fifteen years after conversion to a CNI free regimen, benefit regarding graft and patient survival or regarding prevalence of or death by comorbidities was not shown. However, rejection shortly after CNI withdrawal was connected to decreased graft survival [240].

MMF or MPS replacing CNI seems to improve serum creatinine and creatinine clearance/GFR in a majority of patients with biopsy-proven chronic allograft nephropathy (BP-CAN), without an increased risk of proteinuria [241]. This strategy improves blood pressure, lipid profile and serum glucose [242]. Patients should be carefully selected because benefits of mycophenolic acid derivatives may be compensated by in increased risk of acute rejection and infection. These adverse events may be reduced by concentration-controlling the administration [243]. Recent reviews and meta analyses support a potential role of late CNI elimination with mycophenolic acid derivatives [241, 244].

The early discontinuation of CNIs after transplantation with mTOR inhibitors in patients with good renal function seems to be reliable and potent. Overall, acute rejections happen soon after CNI discontinuation, and physicians should know this timetable. GFR is better in CNI withdrawal recipients both at one and three years; however, the discontinuation rate in

patients on mTOR inhibitors is increased and is induced by drug related adverse events. Overall, these data suggest longer studies that enroll a higher number of patients and highlight that only some patients have a better beneficial effect from switching to mTOR inhibitors from CNIs. However, identifying which patients will have a better effect before withdrawal stays a major problem.

mTOR inhibitors (mTORi) have been used as nephroprotective strategies in CNI-free or CNI-reduced regimens. Renal function was improved when patients were converted to mTORi with [245] or without [246–247] continuation of CNI at low dose. mTOR inhibitors are often used for transplant patients with cancer because they reduce the recurrence of skin cancer [248] and of hepatocellular carcinoma [249]. They may also delay appearance or decrease the frequency of de novo malignancy compared with other immunosuppressive drugs such as CsA [250].

Everolimus (EVR), an antiproliferative agent, is utilized as an alternative to CNI or in combination with attenuated CNI in solid organ transplantation. EVR prohibits the activity of the mTOR and induces the inhibition of downstream signaling and progression from the G1 to the S phase of the cell cycle [251]. Perbos et al. [252] reported a retrospective, monocentric case–control study including heart, lung, kidney, and liver transplant recipients. Conversion to EVR was carried out about six yr after the transplant, and low-dose CNI was provided in 60% of patients. Donor-specific HLA antibodies (DSA) were detected at transplantation, pre-switch, and at three, six, and 12 months post-switch. The author showed no statistical difference for rejection, evolution of preformed anti-HLA antibodies or de novo DSA, after conversion to EVR or not. Incidence of anti-class II DSA is disposed to expand at month 12 whatever the immunosuppressive regimen. The study suggests that late conversion to EVR seems to be safe and to not change the natural evolution of anti-HLA antibodies in organ transplantation. As 60% of patients received EVR and low doses of CNI, it appears that such combinations could be used with a good outcome.

Sotrastaurin, a low molecular weight immunosuppressant, selectively impedes protein kinase C isoforms and prohibits early T-cell activation by a calcineurin-independent pathway. Sotrastaurin was assessed in de novo renal recipients in a phase II study and the study was blocked because the acute rejection rate increased to unacceptable levels after FK506 was withdrawn at 3 months [253].

CNI Minimization

CNI minimization is a different approach to decrease CNI toxicity and nephrotoxicity which consists of a decline in CNI dose followed by therapeutic drug monitoring to target CNI levels lower than in the control group.

Salvadori et al. summarized all of these minimization studies and documented no differences in renal function, rejection rates or survival among recipients receiving a lower dose of CsA in combination with everolimus. A lower dose of FK506 with everolimus was also tried, but no difference in renal function or rejection rates was discovered between these drug associations [244]. CNI minimization protocols display an advantageous impact of kidney function and agreeable acute rejection rates mostly in patients who have been

recipients of a graft for >3−5 years. However, the graft survival has not improved significantly [254].

Alemtuzumab is a CD52-specific monoclonal antibody that induces profound and sustained lymphocyte depletion. It is increasingly used as induction therapy in organ transplantation to decrease CNI in maintenance therapy. Morgan et al. [255] systematically reviewed and performed a meta-analysis of 10 randomized controlled trials (RCTs) with 1223 patients comparing alemtuzumab with other induction therapies such as rabbit antithymocyte globulin (rATG) and the interleukin-2 receptor antibodies (IL-2Ras). Alemtuzumab induction decreases the danger of BPAR compared to IL-2RAs but not rATG. The analysis suggests that it is a more acceptable induction agent according to safety outcomes and/or costs because the incidence of other effect outcomes (graft loss, delayed graft function, and patient death) was identical.

CNI Avoidance

CNI avoidance is the complete elimination of CNIs from the immunosuppressive regimen beginning after transplantation.

Salvadori et al. summarized the results of 2688 patients using CNI avoidance with MMF and/or mTOR inhibitors. It concluded that the overall combination of mTORi and mycophenolate was connected to elevated graft failure compared to CNI-based regimens, although several studies showed good effect results [244].

Belatacept, a fusion receptor protein biological agent, is given intravenously for chronic immunosuppression and promoted as a substitute for CNIs. Belatacept, a second-generation CTLA4Ig, connects with high affinity to CD86/CD80 and prohibits the delivery of costimulatory signals through the CD28 receptor, causing T-cell anergy [256, 257].

The effect of belatacept for kidney transplantation was recently reviewed [258, 259]. Belatacept improved GFR at the end of 3−5 years and is very attractive over CNIs in the maintenance immunosuppression in renal transplantation regarding the further favorable cardiac and metabolic end points. The longer term follow-up was suggested to confirm whether belatacept improves long-term graft and patient survival [257]. There was no difference between belatacept and CNI in preventing acute rejection, graft loss and death. The advantages of belatacept treatment are less chronic kidney scarring, better kidney transplant function, better blood pressure/lipid profile and a lower incidence of diabetes versus treatment with a CNI. The relative advantages and disadvatages of using belatacept stay obscure because side effects (particularly PTLD) remain poorly reported. Long-term study comparing belatacept versus FK506 is necessary to aid clinicians determine which patients might profit most from using belatacept [259]. However, the results of the multicenter belatacept liver transplant trial disappoint with respect to safety and efficacy and new approaches will be required before this agent plays a role in liver transplant immunosuppression [260].

Grannas et al. [261] followed-up 20 patients in a randomized multicenter Belatacept phase 2 study for 10-year. The patients were included three different groups: 1) primary calcineurin inhibitor-based (CNI-based) immunosuppression (n=5); 2) an early switch from a belatacept-based to a CNI-based regimen within the first 14 months (n=8); and 3) completely CNI-free belatacept immunosuppression (n=7). Five patients are still on belatacept. Kidney

function deteriorated in the CNI group and the belatacept to CNI switch group during long-term follow-up. Stable kidney function was exhibited in patients with belatacept throughout follow-up. Acute rejections happened mainly within the first 12 months after transplantation and were accountable for four of seven switches from belatacept to CNI-based immunosuppression in the first 14 months. Belatacept is efficacious and innocuous in renal transplant patients and was not connected to graft loss owing to chronic allograft nephropathy. Belatacept was well endured in all patients and produced less nephrotoxic side effects and was well received by most patients.

CNI nephrotoxicity is not the only significant factor deciding the lack of progress in long-term graft survival. Only early CNI withdrawal with conversion to mTOR inhibitors showed good results regarding effect. The belatacept study is the only trial with positive results respecting the progressive GFR improvement; however, a longer follow-up period is needed to evaluate the long-term results.

Conclusion

CNIs are a cornerstone for the maintenance immunosuppressive treatment in solid organ transplantation by significantly increasing short term graft survival and decreasing rejection rate. However the long-term graft or patient outcomes was not significantly improved. The major causes of late graft loss include chronic allograft nephropathy, death with a functioning graft and chronic immune injury mediated by anti-donor antibodies which CNIs are relative ineffective. Early conversion of CNIs to mTORi has confirmed to be effective only in some patients. The belatacept trial appears effective and it causes a stable improvement of GFR in CNI-free patients without emerging posttransplant DSAs. The long-term effect of belatacept needs to be evaluated. Immune-monitoring of every patient should be performed to balance the risk of rejection and risk of chronic allograft nephropathy.

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Chapter 9

Inhibitors of Mammalian Target of Rapamycin

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Abstract

The mammalian target of rapamycin (mTOR) is an evolutionary conserved serine-threonine kinase that regulates various environmental stimuli in most cells primarily to control cell growth. It is known that mTOR functions as two complexes, mTOR complex 1 and 2 (mTORC1/2). mTOR inhibitors first form a complex with the intracellular receptor FK506 binding protein 12 (FKBP12) and then bind a domain separated from the catalytic site of mTOR, thus blocking mTOR function. The role of mTOR inhibitors is undergoing intense investigation in organ transplantation and cancer fields. In clinic trials with kidney, heart, lung, small bowel, pancreas, islet and liver transplantation, mTOR inhibitors combined with calcineurin inhibitors (CNIs) (CsA, tacrolimus) showed equivalent or superior rejection rates compared to those in combinations of CNIs with mycophenolate mofetil (MMF). Despite this status, clinical application of mTOR inhibitors in transplantation remains limited because of side-effect profile, especially elevation in serum lipids and nephrotoxicity when combined with CNIs. However, the ability of mTOR inhibitors to reduce fibrosis and neovascularization suggests other areas of potential use. In this chapter, we describe the immunosuppressive effect of mTOR inhibitors and its relevance with special emphasis to organ transplantation.

Keywords: Mammalian target of rapamycin inhibitors, sirolimus, everolimus, rejection, organ transplantation

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Abbreviations

AUC:	Area under curve
AZA:	Azathioprine
CMV:	Cytomegalovirus
CNIs:	Calcineurin inhibitors
CsA:	Cyclosporine A
DEPTOR:	DEP domain-containing m-TOR-interacting protein
eIF4EBP1:	Eukaryotic initiation factor 4E-binding protein 1
EVL:	Everolimus
FDA:	Foods and drugs administration
FKBP:	FK506 binding protein
FRB:	FKBP12–rapamycin-binding domain
HPLD:	Hyperlipidemia
IRS1:	Insulin receptor substrate 1
MHC:	Major histocompatibility complex
mLST8:	Mammalian lethal with SEC13 protein 8
mTOR:	Mammalian target of rapamycin
mTORC1:	Mammalian target of rapamycin complex 1
mTORC2:	Mammalian target of rapamycin complex 2
mTORi:	Mammalian target of rapamycin inhibitor
mSIN1:	Mammalian stress-activated protein kinase interacting protein 1
MMF:	Mycophenolate mofetil
MPA:	Mycophenolic acid
NK:	Natural killer cell
PRAS:	Proline-rich akt1 substrate
PDK1:	Phosphoinositide-dependent protein kinase-1
PI3K:	Phosphatidylinositol-3 kinase (PI3K)
PKCα:	Protein kinase C α
RAPA:	Rapamycin
Raptor:	Regulatory-associated protein of mTOR
RICTOR:	Rapamycin-insensitive companion of mTOR
S6K1:	S6 kinase
SRL:	Sirolimus
TGFβ:	Transforming growth factor-β.
Th:	T-helper
Treg:	T-regulatory
TLR:	Toll-like receptor
TSC 1:	Tuberous sclerosis complex 1
TSC 2:	Tuberous sclerosis complex 2
TTP:	Thrombotic thrombocytopenic purpura
HUS:	Hemolytic uremic syndrome
VEGF:	Vascular endothelial growth factor
VSMC:	Vascular smooth muscle cells

Introduction

Mammalian target of rapamycin inhibitors (mTORi) are potent immunosuppressive agents with convincing antiproliferative and anti-inflammatory effects [1, 2]. These inhibitors block the activity of an important cellular kinase called mammalian target of rapamycin (mTOR) which is necessary for physiological cellular activities, and belongs to the phosphatidylinositol 3-kinase (PI3K) cell survival pathway [3]. The mTOR is a central regulator of cell growth, proliferation, metabolism and angiogenesis. Its frequent dysregulation was reported to be linked with the development process of various human diseases, and has thus attracted great interest in developing drugs that target mTOR [3, 4].

mTOR Signaling Pathway

The mTOR is a 289-kDa serine/threonine-specific kinase which belongs to phosphatidylinositol-3 kinase (PI3K) related kinases (PIKKs) family. Its pathway is activated by a variety of different classes of stimuli, and it is known to regulate a wide array of cellular processes and also participates in numerous pathological conditions. It operates by forming two functionally distinct multiprotein complexes: mTOR Complex 1 (mTORC1) and mTOR Complex 2 (mTORC2) [5−7] (Figure 9.1).

mTORC1 known as mammalian target of rapamycin complex 1 is a protein complex that regulates cell growth and size by controlling mRNA translation, ribosome biogenesis, autophagy and lipid metabolism. It is composed of mTOR itself, regulatory-associated protein of mTOR (Raptor), mammalian lethal with SEC13 protein 8 (mLST8), the non-core components Proline-rich akt1 substrate 40 (PRAS 40) and DEP domain-containing m-TOR-interacting protein (DEPTOR). The role of mTORC1 is to activate translation of proteins by signals from growth factors (insulin or IGF-1), co-stimulatory signals, Toll-like receptor (TLR) ligands, cellular energy levels, hypoxia, cellular stress, various cytokines and DNA damage [6, 7−11]. These signals mediate their effects through the effector tuberous sclerosis complex 1 (TSC1)-tuberous sclerosis 2 complex (TSC2) (TSC1-TSC2 complex), leading to the activation of m-TORC1. Activated mTORC1 phosphorylates 70S ribosomal protein S6 kinase (S6K1) and the eukaryotic initiation factor 4E binding protein 1 (eIF4E-BP1) to promote mRNA translation by stimulating S6K1 and inhibiting eIF4E-BP1 [9] (Figure 9.1).

mTORC2 is a protein complex that regulates cellular metabolism as well as the cytoskeleton. It is composed of the interaction of mTOR and of rapamycin-insensitive companion of mTOR (RICTOR), mLST8, and mammalian stress-activated protein kinase interacting protein 1 (mSIN1) (Figure 9.1). Previous studies demonstrated its important function as regulator of the cytoskeleton through its stimulation of F-actin stress fibers, paxillin, RhoA, Rac1, Cdc42, and protein kinase C α (PKCα) [12−15]. mTORC2 leads to full activation of PKB/AKT by phosphorylating the serine/threonine protein kinase Akt/PKB at the serine 473 residue which activates Akt phosphorylation at a threonine T308 residue by PDK1 and leads to full Akt activation [6, 10 −13] (Figure 9.1).

mTOR plays a central role in the differentiation of T-cell subsets, and also controls aspects of B-cell and antigen-presenting cells (APC) development (Figure 9.1). In

transplantation, mTOR also serves as the downstream effector of epithelium-mensenchymal transitions which are normally recognized to be initiated and maintained by transforming growth factor-β (TGFβ).Thus, mTOR plays multifunctional roles in innate and adaptive immunity [16, 17].

Mechanism of mTOR inhibitors

Immunosuppression Mechanism of mTORi

mTOR inhibitors (mTORi) binds to the immunophilin FK506-binding protein 1A, 12 kDA (FKBP12) to form a drug receptor complex that particularly and effectively blocks mTORC1 activity. The FKBP12-rapamycin complex binds the amino-terminal to the kinase region of mTOR (in the FKBP12-rapamycin-binding domain, FRB) and disrupts the *in vitro* and *in vivo* activity of mTORC1, potentially by disrupting the interaction between Raptor and mTOR. mTORC2 was reported to be resistant to direct inhibition by mTOR inhibitors. The mechanism reponsible for prevention of the interaction between the FKBP12-rapamycin complex and the FRB on mTORC2 is unknown.

mTOR inhibitors are able to potently inhibits T-cell proliferation. This immunosuppressive activity is mediated through the G_1/S transition of the cell cycle, and blocks both IL-2R signalling and growth-factor-mediated signal transduction [18, 19]. Two major biochemical events are involved in this immunosuppressive activity of rapamycin: First, the inhibition of phosphorylation/activation of $p70^{s6k}$ by mTOR inhibitors reduces the translation of certain mRNAs encoding for ribosomal proteins and elongation factors, thereby decreasing protein synthesis. Second, inhibition of the enzymatic activity of the cyclin-dependent kinase cdk2-cyclin E complex, which functions as a crucial regulator of G_1/S transition, results from prevention of the decline of the p27 cdk inhibitor that normally follows IL-2 stimulation [20–25] (Figure 9.1).

mTOR inhibitors interrupts CD28 costimulation signalling via blockade of the downregulation of inhibitor kappa B alpha (1κBα), thereby blocking generation of c-Rel, a transcriptional regulator of lymphokine and lymphokine receptor genes [26]. On a cellular level, mTOR inhibitors have beneficial effects on the induction of Tregs that is totally different from action of CNIs. On one side, they inhibit differentiation of pro-inflammatory Th17 cells and promote TGFβ-induced development of $CD4^+CD25^+$FoxP3T cells (Tregs) on the other side [27, 28]. In experimental animals, mTORis were shown to promote the emergence of noncytotoxic IgG_{2B} anti-donor antibodies which provides an additional layer of protection against rejection phenomena [29].

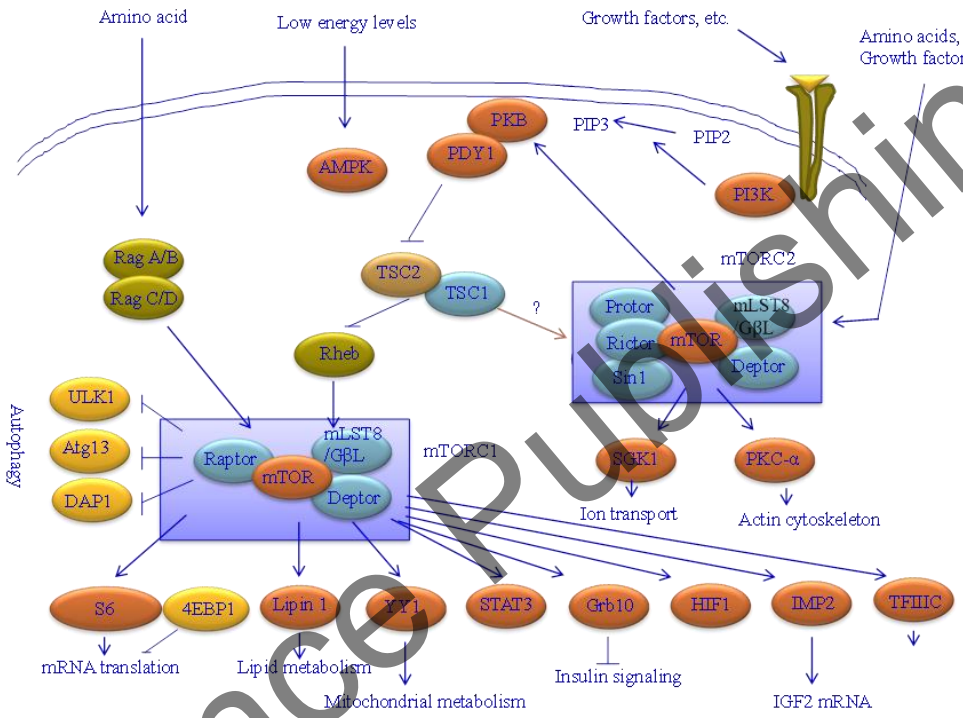


Figure 9.1. mTOR signalling pathways.

Upstream targets of mTOR includes the tuberous sclerosis complex 1 (TSC 1) and tuberous sclerosis complex 2 (TSC2), the AKT-PI3K axis and the extracellular-regulated kinase ERK1/ERK2. The TSC1/2 complex is an upstream key node that regulates the ras homolog enriched in brain (RHEB) kinase that further impacts the mTORC1 activity. mTORC1 integrates inputs from intracellular or extracellular cues (growth factors, stress, energy, oxygen and amino acid sensing). mTORC1 controls protein synthesis by S6K1, which activates mRNA biogenesis and 4E-BP1 that regulates cap-dependent translation. mTORC1 also, negatively regulates autophagy and controls the activity of transcription factor implicated in lipid and cholesterol biosynthesis, mitochondrial metabolism and biogenesis of lysosome. mTORC2 responds mainly to growth factors and activates three main downstream effectors: AKT kinase, glucocorticoid induced protein kinase 1 (SGK-1) and PKCa. All are crucial for the control of ion transport, cell growth and cell shape by modulating in a cell-specific manner the actin skeleton.

Impact on Innate Immunity

mTORi enhance pro-inflammatory cytokines such as IL-12 and IL-1β and inhibit the anti-inflammatory cytokines such as IL-10, and also increase major histocompatibility complex (MHC) antigen presentation via autophagy in monocytes/macrophages and dendritic cells [16]. In addition, mTORi controls IFN-type1 production and the expression of chemokine receptors and co-stimulatory molecules. mTORi blocks progression from G_1 to S phase in natural killer (NK) cells without affecting IFN-γ production in primary NK cell lines [23, 25].

Impact on Adaptive Immunity

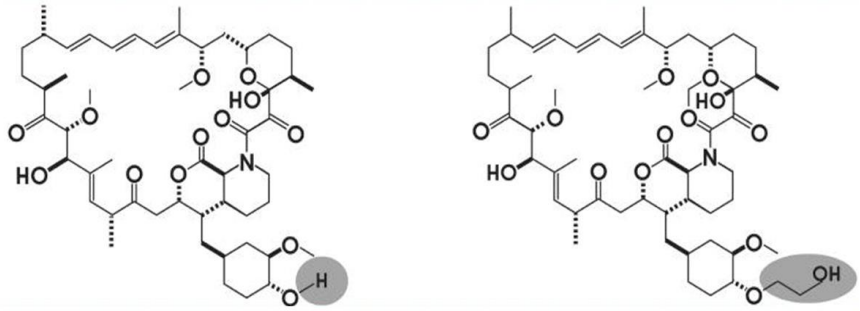
mTORi potently decrease the proliferation of $CD4^+$ T lymphocytes. mTOR was shown to direct the differentiation of Th1 cells by inducing the production of IFN-γ after IL-12 receptor activation on T cells [30]. Therefore, mTORi might be able to enrich $CD4^+CD25^+$ regulatory T (Treg) cells *in vitro [31]*.

mTOR Inhibitors and Derivatives

Sirolimus (Rapamycin, Rapamune®, SRL). SRL (by Pfizer (formerly by Wyeth-Ayerst), also known as rapamycin or AY-022989 was first discovered in 1975 in a soil sample from Easter Island of South Pacific, called Rapa Nui, from where its name is derived. It is a lipophilic macrolide, a natural fermentation product of *Streptomyces hygroscopicus* with $C_{51}H_{79}NO_{13}$ as chemical formula, and a molecular weight of 914.2 (Figure 9.2). It showed antifungal properties and was found to have potent immunosuppressive properties. Sirolimus was approved by the US Food and Drug Administration (FDA) in September 1999 as an immunosuppressant for prevention of renal allograft rejection [18, 19]. Previous *in vitro* studies found that SRL was able to inhibit cellular proliferation and cell cycle progression, and suppressed multiple tumor-promoting intracellular signalling pathways [18, 19].

Everolimus (Zortress, EVL). EVL (INN, earlier name RAD001)) is the 40-O-(2-hydroxyethyl) derivative of sirolimus and works similarly to sirolimus as an inhibitor of mTOR to prevent rejection of organ transplants and for the treatment in oncology. It is marketed by Novartis under the tradenames Zortress (USA) and Certican (Europe and other countries) in transplantation, and the trade name Afinitor in oncology (Figure 9.2). Everolimus has greater polarity, is more hydrophilic and was developed to improve the pharmacokinetic and pharmacodynamic properties of sirolimus, especially oral bioavailability [32, 33].

SRL, EVL, temsirolimus, and deforolimus have been approved and used in clinic as potent mTOR inhibitors. Despite pharmacological improvement in their safety profile, these mTORi revealed numerous side effects that are in some cases serious and/or debilitating, and often unpredictable.



Sirolimus Everolimus

Figure 9.2. Chemicals Structures of sirolimus and everolimus.
Sirolimus (Wyeth-Ayerst/Pfizer): Ester derivative, water soluble; oral/i.v. Kidney cancer, Mantle cell lymphoma. **Everolimus** (Novartis)**:** Hydroxyethyl ether derivative; taken orally, Inhibit graft rejection, Kidney cancer, pancreatic neuroendocrine tumors.

Temsirolimus (CCL-779) (Wyeth, Philadelphia, PA). It is a dihydroxymethyl propionic acid ester of rapamycin. It was formulated for intravenous administration. It has greater water solubility (± 120μ/mL), requiring co-formulation with ethanol. Temsirolimus was identified in the 1990s and subsequently developed as an agent for the treatment in oncology.

SRL is predominantly used as an immunosuppressant in prevention of rejection following solid organ transplantation [1], while everolimus, temsirolimus, deforolimus are used mainly as antineoplastic agents in various cancers [4]. SRL and EVL have been developed due to increased solubility and bioavailability for prevention and treatment of graft rejection, and both are the focal point of this chapter.

Pharmacological Properties of mTOR Inhibitors

Chemically, SRL and EVL differ by the presence of a 2-hydroxyethyl group at position 40 [18, 19, 32] (Figure 9.2). Both SRL and EVL have shown important pharmacokinetic and pharmacodynamic differences. EVL was shown to have a higher oral bioavailability (20% *vs.* 14%), and the lower plasma protein binding (74% *vs.* 92%), and have a shorter time to reach steady-state (4 days *vs.* 5–7 days) (34, 35). Due to an increased oral bioavailability, EVL is twice as potent *in vivo* as SRL based on an oral dose comparison (Tables 9.2) [36–41].

Pharmacological studies demonstrated that both SRL and EVL are substrates for hepatic and intestinal cytochrome P450 (CYP) 3A4 enzymes, as well as for P glycoprotein [40, 41]. The difference in potency between SRL and EVL may be explained by the different target trough blood levels, which are 3–8 ng/mL for EVL and 4–12 ng/mL for SRL [37, 38].

EVL is absorbed rapidly, with peak blood concentrations (Cmax) of 1–2 hours approximately after an oral dose. Its steady-state is generally achieved by day 4 [37, 38]. Its absorption kinetics was proved to be dose-proportional in renal transplant recipients after oral doses of 1.5 mg/day or 3 mg/day in combination with CsA microemulsion and corticosteroids in a 6-month study [37].

In a single dose and multiple doses of up to 5 mg/day of EVL, a dose-proportional kinetics for area under the concentration time curve (AUC) was reported [42, 43]. Systemic

exposure AUC and Cmax was proved to be dose-proportional in cardiac transplant recipients [37].

Previous studies have shown that mTOR inhibitors, SRL and EVL, have a large intra- and inter-patient variability in drug exposure, and narrow therapeutic windows (trough levels [C0] of 3−8 ng/mL and 5−15 ng/mL, respectively) [42, 43]. Consequently, routine therapeutic drug monitoring of SRL and EVL is recommended to optimize efficacy and minimize toxicity in individual renal transplant patients [38]. EVL tissue distribution in humans has not been determined but the highest tissue concentrations in monkeys have been measured in the gall bladder, pancreas, transplanted lung, cerebellum, kidneys, and spleen [44].

Oncology research studies showed that SRL and EVL have an important antineoplastic and antiproliferative effect on tumor cells and endothelial cells via mTOR inhibition. Indeed, the both agents also impair tumor cells by increasing E cadherin, decreasing the cyclin pathway and the cell cycle and by decreasing IL-10 and the Jak/STAT signalling.

Drug interactions between mTOR Inhibitors and Other Agents

Studying immunosuppressant drug interactions is necessary for prevention of undesirable immunosuppression side effects. mTOR inhibitors (SRL and EVL) interact concomitantly with substances that inhibit or induce CYP 3A4 and P-glycoprotein. These interactions may lead to a modification of the levels of immunosuppressive drugs in the blood [45−47].

Table 9.1. Pharmacokinetic characteristic of Everolimus and Sirolimus

	Sirolimus	Everolimus
Oral bioavailability	14%	20%
Time to reach peak plasma concentration	1-2 hours	1-2 hours
Elimination half-life	62 hours	28 hours
Dosing interval	Once a day	Twice a day
Time to steady state	5-7 days	4 days
Plasma protein binding	92%	74%

Source: adapted from Transplant Proc 2010; 42:3050−3052

mTOR Inhibitors – CNIs

Previous *in vitro* experiments reported an antagonistic effect between mTOR inhibitors (SRL and EVL) and calcineurin inhibitors (CsA, tacrolimus). *In vivo* studies did not reveal a competitive inhibition between mTOR inhibitors and tacrolimus agents which share the same binding protein (FKBP-12). Simultaneous and separate administration of sirolimus and tacrolimus revealed no significant interactions in pharmacokinetic parameters, including AUC and Cmax [39]. The combination treatment of SRL and tacrolimus in clinical trials has shown a nephropathy-like delayed allograft function and extensive tubular damage in few patients [48−51].

Concomitant administration of SRL and CsA showed a significantly higher peak/trough levels and AUC when compared with their administration alone, with interactions and

additive side effects such as hypertriglyceridemia and possibly thrombotic thrombocytopenic purpura-hemolytic uremic syndrome (TTP-HUS). EVL and CsA displayed synergistic immunosuppressive activity when used in combination *in vitro* and in preclinical models, predicting that they could be used together at lower doses in humans [52−60].

mTOR Inhibitors − MMF and Steroids

The pharmacokinetic profiles revealed a significantly higher mean AUC for mycophenolate in MMF + SRL-treated patients when compared with MMF + CsA-treated patients [61]. Similarly, mean mycophenolic acid (MPA) trough concentrations were found to be significantly higher in MMF + SRL-treated patients, thus, suggesting mycophenolate dose reduction when using combination of MMF-SRL to avoid potential toxicity [61, 62].

mTOR Inhibitors and Pharmacogenomic

Recent studies data indicate that genetic mutations may influence the sensitivity of mTOR inhibitors [63, 64]. Huang et al., have reported that mTOR inhibitors insensitivity could result from mutations of FKBP12 or mTOR as well as mutations or defects of mTOR-regulated proteins such as S6K1-, 4E-BP1, PP2A-related phosphatases and p27 can [64, 65].

Previous drugs studies showed that SRL and EVL were metabolized primarily by enzymes of the cytochrome P450 family, CYP3A4/CYP3A5 and transported by the MDR-1 P-gp system [66, 67]. Results from these studies reported a lower sirolimus concentration/dose ratio in the CYP3A5*1 (CYP3A5 expresser) carriers than in the CYP3A5*3/*3 carriers (non-expressers), and concluded that CYP3A5 non-expressers required a lower SRL daily dose to achieve adequate blood concentration; patients with CYP3A5*1/*1 were more presumably to have a higher liver metabolism and require a higher daily dose to achieve adequate blood sirolimus levels [68, 69]. Also, patients carrying the CYP3A4*1B (−392A > G) allele and associated with a higher enzymatic activity required higher SRL doses to achieve adequate blood concentrations [69]. All these results were observed only for SRL in some studies and specifically in patients not treated with CNIs [70–73].

Clinical Effects of mTOR Inhibitors in Organ Transplantation

Immunosuppressive Efficacy of mTOR Inhibitors

The mTOR inhibitor drugs, SRL and EVL have undergone extensive clinical trials for a variety of solid organ grafts and have been licensed for use in human organ transplantation. mTOR inhibitors show synergy with the calcineurin inhibitors in antirejection effects but also augment the nephrotoxicity of both cyclosporine and tacrolimus [74−76]. They allow marked reduction in CNIs drug doses, which also reduces the nephrotoxicity of the combinations [77,

78]. In kidney, heart, lung, small bowel, pancreas, islet and liver clinical trials transplantation, mTORi combined with CNIs (CsA or tacrolimus) showed equivalent or superior rejection rates compared to those in combination of CNIs with MMF [79−84]. The side-effect profile observed in clinic, particularly an elevation in serum lipids and nephrotoxicity when combined with CNIs, creates a limitation for the use of mTOR inhibitors [85].

In heart transplantation, immunosuppressive potency of mTORi coupled with its capacity in suppression of growth factor-driven smooth muscle cell proliferation significantly reduces *de novo* cardiovascular artheriovascular disease [86, 87]. Previous studies also reported significant reduction in cytomegalovirus (CMV) infection in mTORi-treated patients which might contribute to the the minimization of intimal vascular changes [87, 88].

Antineoplastic Efficacy

Genetic deregulations of components in PI3K, Akt, and mTOR pathway promote tumorigenesis [89, 90]. The PI3K/Akt/mTOR pathway was proven to impact cellular hypoxia and energy depletion. The inhibition of mTOR in cell signaling through the PI3K/Akt/mTOR pathway was shown to block cell proliferation and angiogenesis. This is due to the prevention of vascular remodeling and interference with hypoxia inducible factors. These factors cause overexpression of hypoxia inducible factor-1 target gene products such as vascular endothelial growth factor (VEGF). VEGF and other factors are thought to be the key drivers of tumor angiogenesis, enabling the growth and progression of cancers. EVL inhibits the expression of hypoxia-inducible factor and reduces the expression of VEGF during management of malignancy [89, 90].

In post kidney transplants, both mTOR inhibitors, SRL and EVL have been associated with a significant decrease of malignancies possibly through above mentioned mechanisms. Therefore, specific clinical guidelines recommend mTORi for renal transplant recipients who had a pretransplant malignancy or who have developed *de novo* cancer after transplant [91, 92].

mTORi have been used for treating transplanted patients who develop tumour such as primary hepatocellular carcinoma or those who develop malignant tumours post-transplant, such as Kaposi's sarcoma [93, 94]. In addition, another analogue of sirolimus, temsirolimus has been developed as an anti-neoplastic agent and is licensed for use in advanced renal carcinoma [95]. Randomized, prospective and controlled trials have shown a lower incidence of new malignancies in patients receiving mTOR inhibitors compared with those receiving other immunosuppressive agents [96, 97].

Refering to short follow-up duration of the mTORi trials, everolimus, specically does not permit any conclusions regarding the magnitude of an epidemiologically significant antineoplastic effect of mTORi in kidney transplantation and the efficacy of mTORi in tumors. However, outside the field of transplantation, mTORi use has been documented in renal cell carcinoma, in neuroendocrine tumors, and in hepatocellular cancer [87, 97−99].

Antineoplastic effects of mTORi in liver have been evaluated in few clinical, randomized, controlled trials, in particular those related to hepatocellular carcinoma and liver transplantation [100−104].

Vasculopathy Effect

Allograft vasculopathy remains the most important cause of late graft deterioration and death. Vascular remodeling develops over a period of months or years and results in decreased caliber of arterial lumens, ischemia, and graft failure. Vasculopathy is associated with immunologic mechanisms, such as the activation of alloreactive T cells and antibodies, and nonimmunologic factors, including hyperlipidemia (HPLD), obesity, ischemia or reperfusion injury, older age of the donor, and CMV infection [105−107]. Antiproliferative effect of mTOR inhibitors that inhibit proliferation of smooth muscle cells and endothelial cells represents an additional benefit for preventing vascular remodelling implicated in the development of allograft vasculopathy, particularly in cardiac graft recipients.

mTOR inhibitors have been proved to reduce neointima formation and in-segment restenosis after the implantation of drug-eluting stents [107−113] and to decelerate the process of transplant vasculopathy [113−115] and consenquently exert cardioprotective effects. Animal data suggested that mTOR inhibitors may restrict the pathogenesis of atherosclerosis which is consistent with preliminary clinical data showing that switching from calcineurin inhibitors to everolimus can stabilize markers of arterial stiffness. Use of mTOR inhibitors also has shown the potential to reduce the burden of cardiovascular disease following kidney transplantation [87].

Despite the protective effect of EVL on atherosclerosis, it has been found to cause blood lipid increase. This antiproliferative effect of EVL could be associated to an antiproliferative p27 Kip-dependent mechanism. It involves abrogation of the upregulation of MCP-1 mRNA expression, which promotes monocyte chemoattraction, stabilization of vessel wall architecture via increased transforming growth factor-β (TGF-β); suppresses low-density lipoprotein, very low-density lipoprotein receptors and CD36 gene expression; and increases cholesterol efflux from human mesangial cells [116, 117].

Adverse Events and Risks

The immunosuppressive and antineoplasic properties of mTOR inhibitors can interfere with the progression of many disorders including metabolic disorders (e.g., diabetes, hyperlipidemia) [119], renal disorders (e.g., proteinuria, delayed graft function) [74, 120, 121] (Table 9.2), dermatologic and mucosal disorders (e.g., stomatitis, rash) [122], hematologic disorders (e.g., anemia, microcytosis, thrombocytopenia and leucopenia) [123], hemodynamic disorders (e.g., hypertension) [124], and hormonal conditions (e.g., impaired gonadal function, ovarian toxicity) [125, 126] , as well as impaired wound healing disorders (e.g., lymphocele, hernia) [127]. Although mTOR inhibitors adversely affect glomerular compartment of kidney and induce massive proteinuria, studies have reported less nephrotoxic effects and uncommon diabetes with mTORi than with CNIs. A fatal pneumonitis that was resolved after mTORi treatment withdrawal, was also reported in renal-transplant recipients treated with SRL [128].

In heart transplantation, an antiproliferative effect of mTOR inhibitors on fibroblasts was reported to be associated with wound healing problems and lymphocele formation [126]. SRL was particularly reported to be associated with delayed recovery from ischemia-reperfusion

injury. This effect could be associated with initial oral load of SRL necessary to reach steady state [74].

mTOR Inhibitors and Proteinuria

mTOR inhibitors used in kidney transplantation have been complicated by the development or exacerbation of proteinuria [129−131]. The pathogenesis of proteinuria is certainly multi-factorial and involves tubular and glomerular contributions. Recent data derived from biopsy sub-studies of clinical trials, which compared CsA with SRL, demonstrated a close association of SRL use with tubular damage and tubular proteinuria [132]. In the glomerular compartment, other studies have demonstrated reduced nephrin expression [133] and reduced VEGF, particularly in patients with significant proteinuria [134, 135]. VEGF is a mitogen, critical for endothelial function in vascular, lymphatic and glomerular vessels [136, 137] and belongs to the family of platelet-derived growth factors. It is mainly produced by macrophages, endothelial cells and activated T-cells [138]. Physiological level of VEGF is pivotal for maintaining vascular homeostasis and survival of glomerular cells under both physiological and pathological conditions; VEGF also maintains vascular permeability which is a requirement for normal glomerular filtration and homeostasis [139, 140].

VEGF signalling is mediated by two high-affinity receptor-binding sites: tyrosine kinase receptor VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR). Activation of VEGFR-1 mediates migration of monocytes and endothelial cells during angiogenesis under pathological conditions and has a regulatory role in the development of inflammatory diseases. VEGF signalling through VEGFR-2 induces the main mitogenic, angiogenic and vascular permeability effects of VEGF activity on endothelial cells [141]. mTOR inhibitors, particulary SRL has been shown to inhibit production of VEGF *in vivo* and *in vitro* through the inhibition of mTOR pathway. mTORi also was proven to interrupt down-stream tyrosine kinase signalling in response to VEGF-receptor (VEGFR) activation [142]. Proteinuria is described as a hallmark of progressive deterioration of renal function and has been reported in clinical studies as a consequence of therapies inhibiting VEGF [143, 144] (Table 9.2)

Clinical Strategies and Issues in Organ Transplantation

mTOR inhibitors (SRL, EVL) have been utilized mostly in kidney transplantation and their *de novo* use has been limited to some transplantation centers because of the early post–transplantation complications of SRL, such as delayed allograft function, poor wound healing, adverse short-term outcomes, and an increased incidence of lymphoceles [145].

Short-Term and Long-Term Use of mTOR Inhibitors

The benefit of a long-term CNI-free mTOR inhibitor-based regimen after renal transplant is the promise to reduce development of chronic damage to the graft mediated by CNI, and to lower incidence of post-transplant malignancy [146]. CNI-free mTOR inhibitor-based regimens are less efficacious for rejection prophylaxis and their use in the *de novo* transplant is not recommended. When conversion from a CNI to mTOR inhibitors is performed late, the patient has GFR < 40 ml/minute or pre-existing proteinuria, as in the CONVERT study, then benefit is blunted. Studies that have demonstrated the greatest benefit in terms of renal allograft function are those where conversion is attempted between 1 and 6 months after transplantation and benefits in that medium term are better allograft function, a lower incidence of cancer, and possibly a lower rate of viral infection [147].

EVL was approved as therapeutic option for liver transplant recipients, particularly in post-transplant nephrotoxicity and other adverse events associated with long-term administration of CNIs [148]. Clinical trials have shown that EVL provides improved protection against renal dysfunction while maintaining immunosuppressive efficacy, particularly when introduced early after liver transplantation [148, 149]. Compared with standard-exposure of FK506, EVL combined with reduced-dose of FK506 provides a significant improvement in renal function, sustained over 2 years [149].

Very Low CNIs Exposure in Association with EVL

Therapeutic regimen using EVL and CNIs involves therapeutic dose monitoring of both agents because of a potential nephrotoxicity as a consequence of interference by EVL with CNIs. Possibility of CNI (CsA) dose reduction was proven evident since the early studies when EVL and CsA were administered as fixed doses, and several trials documented the efficacy and safety of EVL when given with a low CsA dose [53]. Association of very high EVL exposure with very low CsA exposure has been shown to be a promising therapeutic regimen in heart and lung transplantation in recent studies [150].

In cardiac transplant recipients, combination of EVL with a reduction in CNIs dose is increasingly used [151–153]. There is evidence that this immunosuppressive regimen is able to maintain the low cellular rejection rates seen with the standard therapy [85] and may protect kidney function [57,154].

CNI withdrawal – mTOR inhibitors

Recent and important study of CNIs withdrawal with EVL has shown similarity of efficacy and safety and significantly improved renal function in EVL and enteric-coated mycophenolic sodium group [155]; this reflected a therapeutic approach of maintaining stable renal function without compromising efficacy and safety of EVL and CNIs. Proteinuria has been described in patients switched from CNIs to EVL, and also in renal transplant patients treated *de novo* with EVL.

mTOR inhibitor (EVL) has been also introduced in maintenance of heart transplant patients to support minimization or withdrawal of CNIs therapy. mTOR inhibitor (EVL)

should be initiated as soon as possible after heart transplantation due to its potential to inhibit the progression of cardiac allograft vasculopathy and to reduce cytomegalovirus infection. Immediate and adequate reduction of CNIs exposure is mandatory from the start of mTOR inhibitors (EVL) therapy.

A study showed that in chronic renal allograft nephropathy, conversion to mTORi (EVL) with complete withdrawal of CNIs is safe and simple when recipient's cGFR is more than 30 mL/min. EVL along with MMF or AZA and prednisolone as a maintenance immunosuppressive therapy was found to be effective and safe in patients with CNI withdrawal either due to chronic allograft nephropathy or CNIs toxicity [148].

The efficacy of mTOR inhibitor (SRL) was proved in refractory renal allograft rejection in limited preliminary studies [156]. Conversion of CsA to SRL was shown to be associated with stable kidney allograft function, and CsA discontinuation should be considered early in such indication [147].

Corticosteroid Withdrawal

Withdrawal of corticosteroids from the immunosuppressive regimens in kidney transplantation may eliminate a number of the well-known side effects of these agents [157]. However, the benefits of withdrawing steroids must be weighed against the risks of precipitating acute and chronic allograft rejection. In attempting to minimize corticosteroid-induced morbidity, SRL has been administered to renal transplant recipients in whom corticosteroids were eventually withdrawn [158]. SRL and low doses of FK506 have allowed successful islet cell transplantation without the use of corticosteroid therapy [159]. Excellent short-term outcomes with an acute rejection rate of approximately 7% in the first 3 months after transplantation were reported in the combination of SRL, FK506 and and prednisone in African Americans [160]

mTOR Inhibitos and Antiviral Activity

CMV infection and dissemination in the host requires viability of the epithelial cells of the host that are primarily infected. CMV then replicates and spreads to monocytes in the peripheral blood. Infected monocytes promote CMV migration into host organ tissues. mTOR inhibitor (EVL) have an intrinsic property which is due to a proapoptotic action against CMV. This activity of mTOR inhibitors against CMV is noteworthy, because CMV disease is associated with allograft rejection, decreased graft and patient survival, and predisposition to malignancies [161, 162].

A lower incidence and severity of CMV infection in transplant recipients treated with mTOR inhibitors has been observed in several clinical trials and meta-analyses [163−165]. Most studies have demonstrated a very low incidence of CMV, human herpesvirus 8, BK virus infections associated to SRL use [166, 167].

Conclusion

The importance of mTOR pathway in numerous biological processes significantly impacts the benefits *vs*. adverses effects linked to the use of mTOR inhibitors in organ transplantation. mTOR inhibitors have pleiotropic actions which could influence the morbidity and mortality of patients, through antiviral, anticancer and antifibrotic properties, besides its immunosuppression-associated large range of side effects. The antiproliferative effect of mTOR inhibitors has a relevant antiviral activity which could impact the development of anti-virus compounds. This antiproliferative effect on vascular smooth cells and endothelial cells could also help to avoid vascular remodelling after transplantation, but on fibroblasts, this effect might be an inconvenience by inducing lymphoceles, delayed wound healing, and proteinuria. More research studies are still needed to define the actual long-term eventual benefit of mTORi regimens, particularly in comparison/combination with the upcoming new immunosuppressant agents. Targeting either upstream or downstream components of mTOR signalling, rather the mTOR itself, may offer an alternative for added specificity and therapeutic opportunities. Current mTOR inhibitors that act through an unusual allosteric mechanism that involves interaction with FKBP12 and a new series of synthetic small molecules that inhibit both mTORC1/C2 are available (Torin1, mTORC1/2 catalytic inhibitor, or dual PI3k/mTOR inhibitors). They all have shown promising effects on tumor growth and could be tested in the field of organ transplantation.

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Chapter 10

Immunomodulation Therapy by the Control of Immune Cell Trafficking

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Abstract

Allograft rejection is mainly arbitrated by T and B cell-mediated immune responses. The control of T cell subsets and lymphocyte homing affects the clinical outcome of organ transplantation and autoimmune disorders. Specifically, a potent biologically active sphingolipid metabolite, sphingosine-1-phosphate (S1P), plays critical roles in the regulation of immune processes. The S1P analogs FTY720 and KRP-203 are remarkably effective in animal models of organ transplantation and autoimmunity. Use of FTY720 has achieved promising results in clinical phase studies of renal transplantation. Unlike conventional immunosuppressants such as calcineurin inhibitors [e.g., cyclosporin A (CsA) and FK506] and mycophenolate mofetil (MMF), these S1P analogs neither suppress the whole immune status nor impair lymphocyte activation and proliferation in the host, but can alter lymphocyte homing, leading to a reduction of peripheral lymphocytes. This article focuses on the action mode of S1P receptor agonists and their potential use in combination with other classes of immunosuppressants.

Keywords: sphingosine-1-phosphate (S1P) analogs, FTY720, KRP-203, Cell Trafficking, Rejection

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Abbreviations

ALT	Alanine aminotransferase
CCL	Chemokine receptor ligand
CCR	Chemokine receptor
CCR	Chemokine receptor
CD	Cluster of differentiation
CD62L	CD62 ligand
Con A	Concanavalin A
CsA	Cyclosporine A
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
EMA	European Medicines Agency
ERK	Extracellular signal-regulated Kinase
FoxP3	Forkhead box protein P3
GPCR	G protein-coupled receptor
HEVs	High endothelial venules
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
MHC	Major histocompatibility complex
S1P	Sphingosine-1-phosphate
MMF	Mycophenolate mofetil
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NFAT	Nuclear factor of activated T-cells
TCR	T cell receptor
Th	T helper
Treg	Regulatory T cell

Introduction

T cell-mediated immune responses have been known to play a critical role in allograft rejection [1, 2]. Recent interest has focused on T cell subsets and lymphocyte homing as a means to account for clinical outcomes. It is known that chemokines, chemokine receptors (CCRs) and other adhesion molecules are necessary for lymphocyte trafficking to lymph nodes or to areas of inflammation [3, 4]. In addition to this, a potent, biologically active sphingolipid metabolite, sphingosine-1-phosphate (S1P), also plays critical roles in the regulation of those inflammatory cell processes [4−6]. The inflammatory cell trafficking and related processes have been established in rodents and increasingly followed in humans. The rationales are now being explored and then adapted in allograft recipients and patients with autoimmune disease. In this chapter, we describe recent advances in our understanding of immune trafficking concerning organ transplantation and discuss newly identified S1P-mediated immune cell controls. Finally, we elaborate on a therapeutic scheme involving new drugs that target S_1P functions for organ transplantation.

Basic Understanding of Major Immune Responses in Organ Transplantation

In adult humans, more than 60% of peripheral blood T cells express antigen-positive effector and memory phenotypes [8, 7]. In particular, immunological memory is a hallmark of the adaptive immune system and results from the clonal expansion and differentiation of antigen-specific lymphocytes [8, 7]. Differences in T cell differentiation and subtype are involved in the pathophysiology of allograft rejection. Specifically, $CD4^+$ helper T cells play a central role in execution of the alloimmune response. Classically, once naïve $CD4^+$ T cells received antigenic stimulation, they were believed to undergo dichotomous differentiation into T helper-1 (Th1) or T helper-2 (Th2) cell lineages with their own set of cytokines and functional properties [9]. In the last decade, a suppressive subset of $CD4^+$ T cells has been identified; the regulatory T cells (Tregs) are characterized by high expression of the interleukin (IL)-2 receptor α chain (CD25) and intracellular expression of the transcription factor forkhead box protein P3 (Foxp3) [10−13]. These cells have been physiologically implicated in the prevention of various autoimmune diseases [10], host tolerance to chronic infections [14], and escape of immune surveillance by malignant cells [15]. There is also increasing evidence suggesting that high Treg frequencies following organ transplantation are associated with reduced severity of allogenic immune responses [16, 17]. Moreover, a distinct lineage of $CD4^+$ T helper cells, referred to as T helper-17 (Th17) cells, has been described as playing an important role in tissue inflammation [18, 19]. With the discovery of induced regulatory T cells (iTregs), descriptions of the novel Th17 cells and the plasticity among these subsets have favored defining $CD4^+$ T cell subsets as fixed T cell lineages [20−22]. Since then, a few more subsets of $CD4^+$ T cells have been identified, namely IL-9-secreting Th9 cells [23−26] and IL-22-producing Th22 cells [27, 28]. It has been established that Th9, Th17 and Th22 cells are separate lineages from Th1 and Th2 cells, and play a pivotal role in the pathogenesis of autoimmune diseases and inflammation [9−28]. Many studies have shed light on the emerging role of Th9, Th17 and Th22 cells in allograft rejection. A detailed description of the precise role of Th1, Th2, Th9, Th17 and Th22 cells in transplant rejection are beyond the scope of this review and the reader is referred to Ref 29 for details.

Cell Trafficking of Effector and Memory T Cells for the Allografts

The migration of T cells from the vascular compartment to the lymph node followed by lymph node egress and migration to the peripheral tissues requires a multi-step adhesion cascade involving CCRs, selectins and integrins [30−32]. Naïve and central memory T cells express high levels of CD62 ligand (CD62L, L-selectin) and CCR7, which facilitate their migration to lymph nodes. CD62L and CCR7 can interact with peripheral node addressing (PNAD) and chemokine receptor ligand (CCL)21, respectively, which are constitutively expressed on high endothelial venules (HEVs) and allow entry of the lymphocyte into the lymph node [30, 33, 34].

Accumulated evidence suggests that memory T cells may play a critical role in inhibiting allograft acceptance [35]. Briefly, memory T cells are heterogeneous, but two well-described subsets exist within most antigen-specific memory populations. Central memory T cells ($CCR7^{+}$ $CD62L^{high}$) migrate primarily to secondary lymphoid tissues (e.g., lymph node and spleen) and are responsible for generating a burst of new effectors following recall. Effector-memory T cells ($CCR7^{-}$ $CD62L^{low}$) migrate to non-lymphoid tissues and provide immediate effector function at peripheral sites [36−38]. It is unclear whether these two populations derive from one another or have distinct origins, and there is evidence to support both paradigms [39−41]. These memory cells have recently been shown to have the ability to migrate into allograft tissues before the 4-6 days required for donor-specific priming in the spleen [38]. Furthermore, tolerance achieved in cardiac allografts using a costimulatory pathway-blocking agent (anti-CD154) is rejected when alloreactive effector memory T cells are generated in recipients through sensitization with donor-type skin grafts [42, 43]. Therefore, these experimentations strongly suggest that donor-reactive effector memory T cells can be generated by homeostatic mechanisms through immune cell trafficking.

Control Agents of Cell Trafficking and Adhesion

Given the role of alloreactive memory T cells in initiating effector function in the periphery, the interruption of trafficking could have selective effects in reducing effector T cell function. To date, various immune suppressive agents have been employed in clinical transplantation (e.g., T cell depletion using antibodies, costimulatory pathway blocks using CTLA-4-Ig and anti-CD154 antibody, and signaling blocks through the T cell receptor (TCR) and cytokine receptors) [35]. Among these, the most commonly used immunosuppressants, the calcineurin inhibitors, CsA and tacrolimus/FK506, target TCR-mediated signaling. These agents prevent translocation of the nuclear factor of activated T-cells (NFAT) that is required for gene transcription of IL-2, which is important for optimal expansion and survival of T cells. The calcineurin inhibitors have been shown to be unique among the clinically available immunosuppressants in preventing memory and effector T cell proliferation and cytokine production [44]. To date, calcineurin inhibitors have been important contributors in the prevention of allograft rejection in humans.

With regard to the interruption of immune trafficking, one such candidate is FTY720 (Fingolimod). This agent binds sphingosine-1-phosphate (S1P) receptor as an agonist and prevents the S1P receptor from performing its function of allowing lymphocyte migration from the thymus and peripheral lymphoid tissues, effectively sequestering T cells in the lymph nodes and inhibiting their trafficking to peripheral graft sites [37, 45, 46] (Figure 10.1). Currently, FTY720 has been approved by the U.S. Food and Drug Administration and European Medicines Agency (EMA) as the first orally active immunomodulatory drug for relapsing-remitting multiple sclerosis (MS) [47].

Characterization of FTY720 As a S1P Agonist

FTY720 was first described in 1995, and was designed as a chemical derivative based on the fungal metabolite myriocin (known as ISP-1) [48, 49]. An early animal study demonstrated that FTY720 induced long-term graft acceptance in rat and dog allotransplantation [50]. Further, first animal studies showed that FTY720 synergized with authentic calcineurin inhibitors for allograft survival prolongation [51−54]. In animal models and in humans, FTY720 decreased the number of peripheral blood lymphocytes, affecting $CD4^+$ T cells, $CD8^+$ T cells, and B cells [52, 55, 56]. Since then, it was thought that this agent might promote the homing of lymphocytes into lymph nodes [52, 53, 57]. Initial *in vitro* studies suggested that FTY720 could promote T cell apoptosis [58]. However, these apoptotic events occurred at higher drug concentrations more than 100-fold in excess of the low nanomolar (nM) exposures required for *in vivo* activity [59]. These hypotheses had to be revised when it was discovered that the mode of action of FTY720 is linked to G protein-coupled receptors (GPCRs) for S1P [60, 61] and altered lymphocyte trafficking [45, 62]. The close structural homology of FTY720 with sphingosine (Figure 10.2), a metabolite of the cell-membrane constituent sphingomyelin, prompted investigations to determine whether FTY720 affects intracellular sphingolipid metabolism. This led to the discovery that FTY720 is a substrate of sphingosine kinases and that the generated FTY720 phosphate could act as a new class of GPCRs referred to as S1P receptors (Figure 10.1). Five subtypes have been identified ($S1P_{1-5}$ receptors), which were formerly referred to as Edg1, Edg5, Edg3, Edg6 and Edg8 [63, 64].

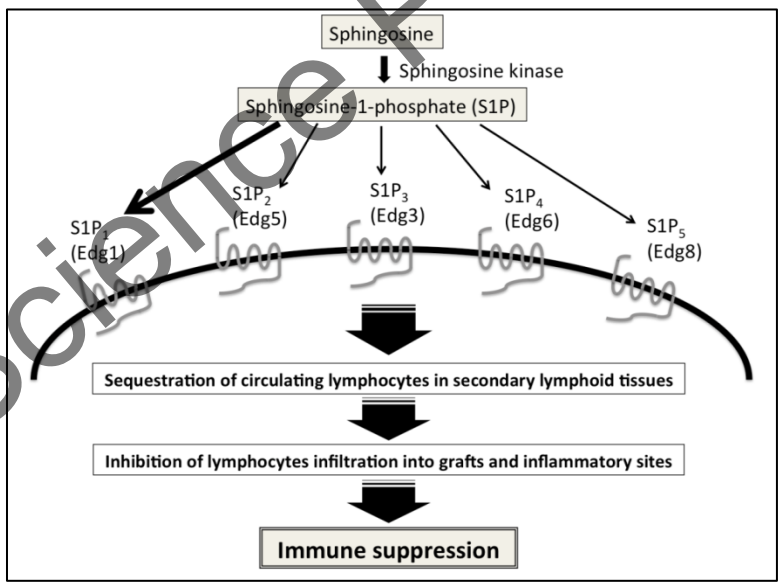

Figure 10.1. Outline of $S1P_1$ receptor ($S1P_1$)-mediated immune suppression. The production of S1P can lead to intracellular or extracellular actions. S1P was produced by the action of sphingosine kinase. Extracellular actions are mediated by $S1P_{1-5}$ receptors, which are classified as G protein-coupled receptors (GPCRs). Biological actions of S1P are affected by tissue distribution and function of the receptor subtypes. The $S1P_1$ receptor is markedly expressed by immune cells and plays a crucial role in sequestration of circulating lymphocytes in secondary lymph nodes.

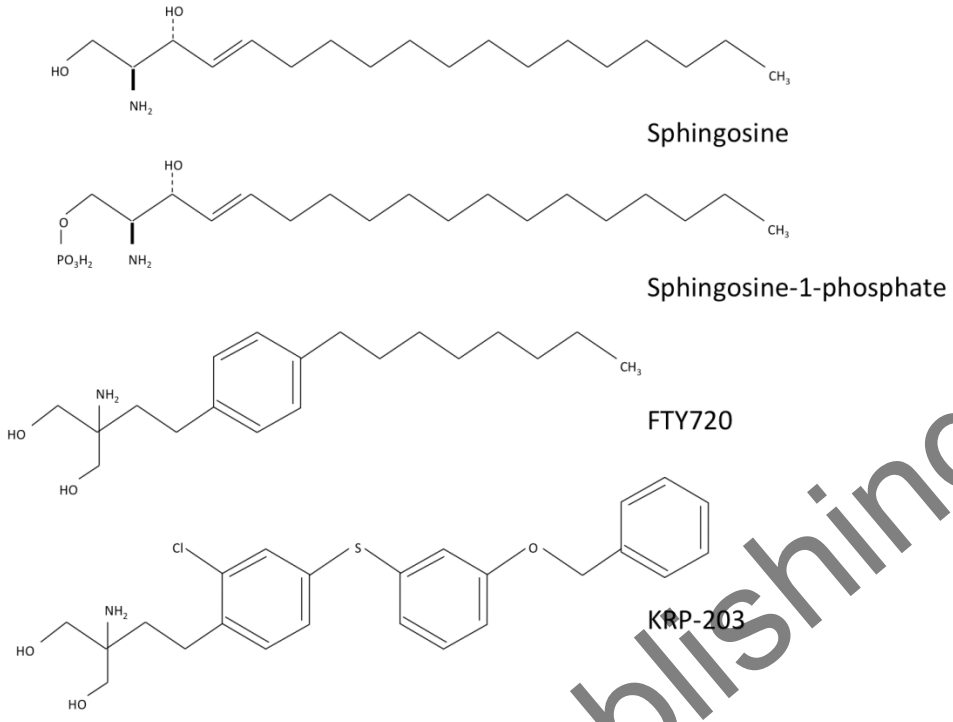

Figure 10.2. Representative structures of S1P agonists. FTY720 and KRP-203 are structural analogs of sphingosine and sphingosine 1-phosphate (S1P).

Many studies have examined the immunomodulatory actions in S1P and S1P receptors as described above. S1P concentrations are high in plasma but low in tissues [65, 66], and excessive production of the pleiotropic mediator at inflammatory sites may occur under various pathological conditions. Such biological actions are affected by tissue distribution and function of the receptor subtypes. Marked expression of the $S1P_1$ receptor is affected by immune cells, neural cells and endothelial cells [63, 67−69]. Investigation of $S1P_1$ receptor-deficient mice suggests that the $S1P_1$ receptor plays a critical role in angiogenesis and neurogenesis, as well as in the regulation of immune cell trafficking and endothelial barrier function [70]. The $S1P_2$ receptor also shows widespread expression [63], and its loss leads to a large increase in the excitability of neocortical pyramidal neurons, demonstrating a role in the development and/or mediation of neuronal excitability [71, 72]. Furthermore, the $S1P_2$ receptor is essential for proper functioning of the auditory and vestibular systems [73], and $S1P_2$ receptor deficiency results in deafness. The $S1P_3$ receptor is expressed in the heart, lung, spleen, kidney, intestine, diaphragm and at certain cartilaginous regions [63], but genetic deletion of the $S1P_3$ receptor does not result in an obvious phenotype [74]. This receptor may play a role in fine-tuning some cardiovascular functions, including the regulation of heart rate, although no consensus has been reached concerning the role of the $S1P_1$ receptor relative to the $S1P_3$ receptor in heart rate regulation in humans [75, 76]. The $S1P_4$ receptor has a more restricted expression pattern and has been detected predominantly within immune compartments and on leukocytes [77]. Given that $S1P_4$-deficient mice have yet to be described [64], its functional role remains unclear. The $S1P_5$ receptor is identical to rat nerve growth factor-regulated GPCR neuregulin 1 [78], and is predominantly expressed in

oligodendrocytes in the white matter tracts of the central nervous system [78, 79]. Deletion of this receptor alters natural killer cell trafficking [80], and studies using another $S1P_5$ receptor-deficient mice reported that this receptor influences immunological natural killer cell egress through a T-bet/Tbx21 transcription factor mechanism involving various immunological compartments [81]. However, no defects in myelination are observed in $S1P_5$ receptor-deficient mice and its precise function in oligodendrocytes remains to be elucidated.

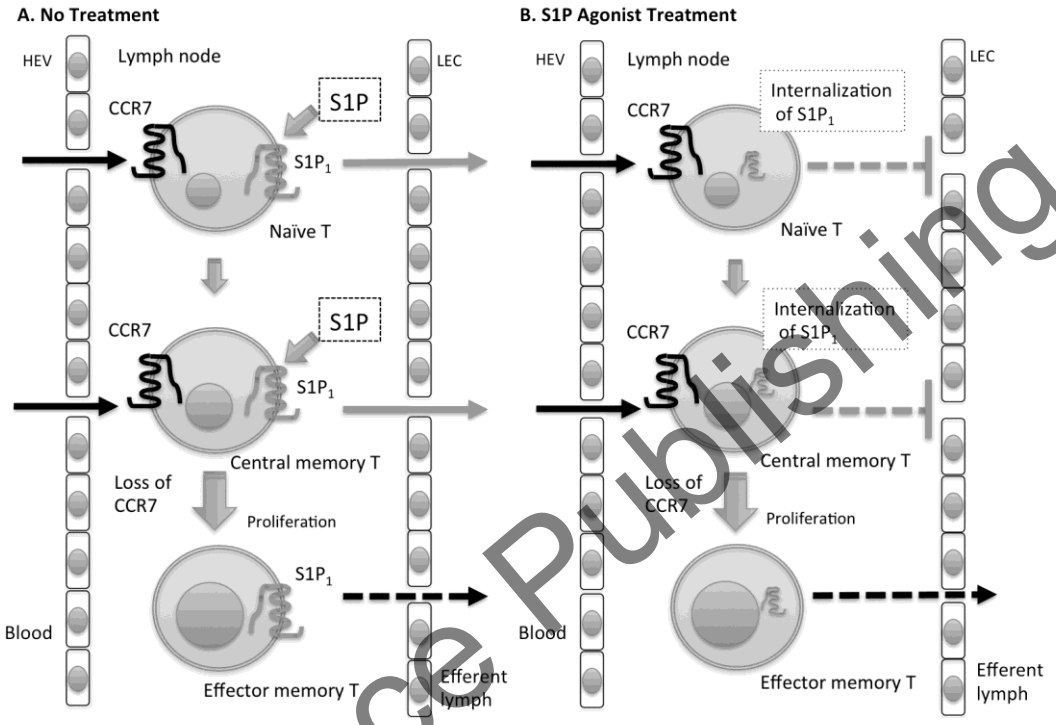

Figure 10.3. Possible mechanism of T cell retention by S1P analogs in lymph nodes. To generate protective immune responses, naïve T cells require antigenic stimulation in lymph nodes. This strong signals produces CC-chemokine receptor 7 $(CCR7)^-$ effector memory T cells, which move into infected tissues and exert effector function [7]. In contrast, weak antigenic stimuli predominantly generate $CCR7^+$ central memory T cells, which home to T cell areas of secondary lymphoid organs (central memory T cells have little effector function but await antigenic restimulation to proliferate and differentiate into effector memory cells). The naïve and central memory T cells recirculate between blood and secondary lymphoid tissues and home to lymph nodes through high endothelial venules (HEVs) in a CCR7-dependent fashion (black arrows). To egress from lymph nodes, activation of the sphingosine 1-phosphate receptor 1 ($S1P_1$) by lymphatic endothelial cell (LEC)-derived S1P is required, and this overrides CCR7-mediated retention in lymph nodes [84] (light grey arrows). Sufficiently differentiated T_{EM} cells have irreversibly lost CCR7 [7] and related retention signals and, as a consequence, are less dependent on $S1P_1$ for egress [84] (dotted arrows). S1P agonists (e.g., FTY720), following phosphorylation, bind to $S1P_1$ receptors [60, 61] on T cells and aberrantly internalize the receptor [84] (see part B of the figure). This event reduces the responsiveness of T cells to the S1P egress signal and enhances CCR7-mediated retention in lymph nodes [84].

Biological Mode of Action of FTY720

Studies using transfected cell lines showed that FTY720 caused S1P receptors to internalize from cell membranes [82]. This raised the possibility that the drug might act as a functional antagonist to inhibit S1P-mediated migration. In other words, although FTY720-phosphate acts initially as an agonist at S1P receptors, its effects are inhibitory for the longer term on S1P receptor function. The reduction in blood lymphocyte counts associated with FTY720 treatment could be mimicked by conditional depletion of $S1P_1$ receptors from haematopoietic cells or T cells [45, 83]. Treatment of wild-type mice with FTY720 also induced internalization of cell-membrane-expressed $S1P_1$ receptors in lymph nodal T cells [84]. Thus, the decrease in $S1P_1$ receptor activity was correlated with the accumulation of lymphocytes at the lymphatic endothelial barriers of lymph nodes [61, 85]. These findings suggest that FTY20 may block the egress of lymphocytes from these organs into cortical sinuses and efferent lymphatics (Figure 10. 3). In fact, this hypothesis was successfully verified using conditional $S1P_1$-deficient mice. Interestingly, imaging studies also showed that $S1P_1$ receptors play a critical role in the initial attachment and rolling of T cells on lymphatic endothelium [86]. Specifically, the $S1P_1$ signal was required to overcome retention signals involving the lymph node-homing receptor CC-chemokine receptor 7 (CCR7) [84]; $S1P_1$ receptor-deficient T cells and CCR7-overexpressing T cells were retained in lymph nodes for longer periods, whereas CCR7-deficient T cells left lymph nodes more rapidly than wild-type cells [84]. These findings suggested antagonistic roles for the $S1P_1$ receptor and CCR7. Collectively, $S1P_1$ signaling in T cells overrides CCR7-mediated retention to promote their egress from lymph nodes, and functional antagonism of $S1P_1$ by FTY720 supports lymphocyte retention in lymph nodes.

Phenotypic and functional analysis of blood lymphocytes from FTY720-treated mice [87], from patients following renal transplant [62, 88, 89], and from patients with MS [56, 90] provided evidence that this agent preferentially reduced $CCR7^+$ naive T cells and memory T cells in blood, but spared $CCR7^-$ effector memory T cells, which is consistent with $S1P_1$- and CCR7-dependent regulation of egress from lymph nodes. Therefore, it is likely that FTY720 may retain all memory T cell subsets in lymph nodes.

Pharmacological Characteristics of FTY720

The clinical pharmacology of FTY720 has been investigated in more than 1,000 subjects in 30 studies [90]. FTY720 has a slow absorption period and attains maximal concentration at 12–24 hours post administration [91, 92], when comparable concentrations of FTY720 and FTY720-phosphate are detectable in the blood [91]. The half-life of FTY720 is ~9–10 days [93]. With daily dosing of FTY720, pharmacokinetic steady state is achieved after 1–2 months. FTY720 is cleared by a metabolic pathway that predominantly utilizes cytochrome P450 4F2 [94]. Pharmacodynamic studies show that the drug has a rapid onset. Within several hours of the first dose of FTY720, a dose-dependent decrease in the peripheral lymphocyte number emerges. With continued daily dosing, both a stable blood concentration and a stable reduction in the number of circulating blood lymphocytes are observed [94], with an average reduction of 77% and 73% with doses of 1.25 mg and 0.5 mg, respectively; cell

counts remained stable for the total administration period [95, 96]. An increase in the peripheral blood lymphocyte count was evident within days of stopping the administration, and returned to a normal range within 6 weeks [97].

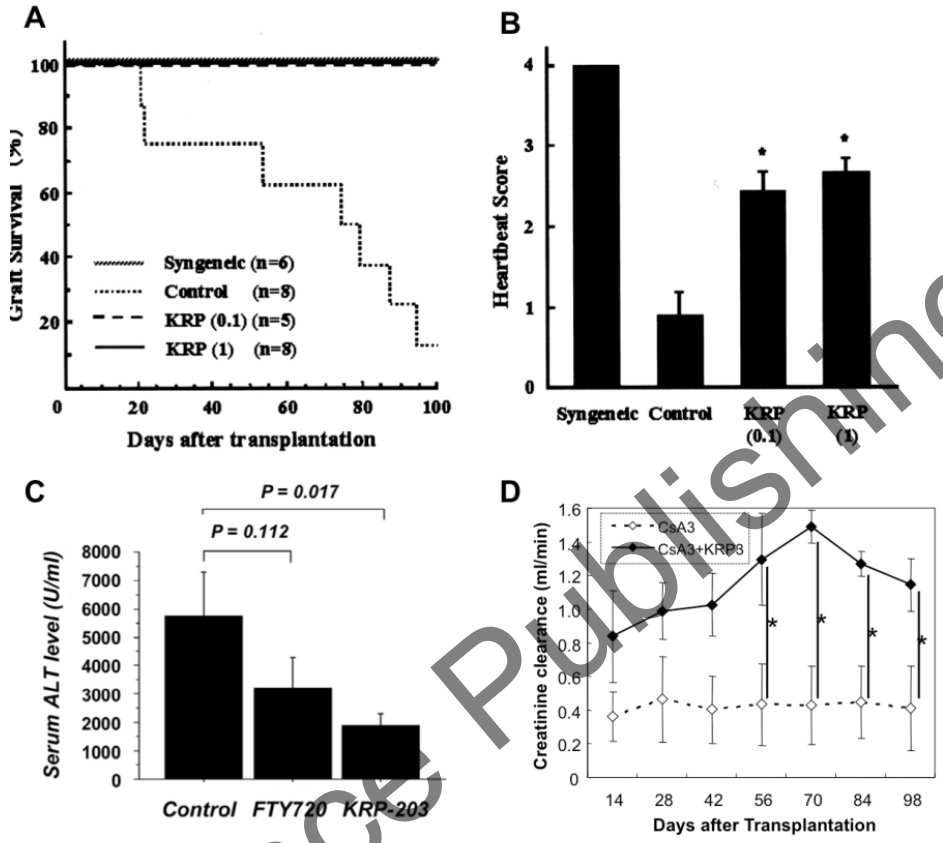

Figure 10.4. Potential effects of KRP-203 on rat allograft rejection and mouse T cell-mediated hepatitis. (A and B) KRP-203 attenuates chronic rejection in MHC-compatible rat heart allografts [103]. (A) Rat heterotopic abdominal heart transplantation (*Lewis* to F344) was performed using 8 to 10-week-old male rats, and allograft function was followed daily by palpation of the graft heartbeat. All allografts survived without rejection following KRP-203 treatment. Control, saline administration; KRP (0.1), 0.1 mg/kg KRP-203; KRP (1); 1 mg/kg KRP-203. (B) Heartbeat scores for the viability of heart allografts. The heartbeat score was significantly higher in KRP-203-treated rats than in control rats (data shown are the mean ± SEM; *p<0.05 vs. control group). (C) As with the case of FTY720, KRP203 reduced the number of liver lymphocytes in concanavalin A (Con A)-induced hepatitis conditions [106]. FTY720 (1 mg/kg) or KRP-203 (1 mg/kg) was orally administered to Balb/c mice 24 hours prior to intravenous injection of Con A (40 mg/kg), and serum alanine aminotransferase (ALT) levels were examined 24 hours following Con A injection. While FTY720-treated mice showed moderately less serum ALT levels compared with control mice, KRP203 could significantly block the increase in ALT levels. (D) Combinational use of KRP-203 with subtherapeutic dose of CsA improves graft function and recipient status in orthotopic rat renal transplantation (Brown Norway to Lewis rats) [104]. Renal transplantation was performed in a MHC-mismatched combination and recipients underwent contra-lateral nephrectomy at day 7. Creatinine clearance was shown in recipient rats receiving long-term treatment with 3 mg/day CsA (CsA3) alone or 3 mg/day CsA plus 3 mg/kg KRP-203 (CsA3 + KRP3) for 100 days (*, p<0.01).

While initial clinical pharmacological studies were conducted largely using renal transplant patients, the first clinical benefits using FTY720 was obtained using relapsing MS patients [98]. Phase III trials demonstrated that oral administration of FTY720 had greater efficacy compared with intramuscular interferon (IFN)-β and placebo with regard to reducing the rates of MS relapse and MRI evaluation for inflammation activity [95, 96]. The progression of clinical disability was statistically significantly reduced by FTY720 over 2 years compared with placebo.

Most of the adverse effects observed with FTY720 in the clinical trials appeared to be associated with its mechanism of action as a modulator of $S1P_1$ receptors. The transient and/or asymptomatic reduction of heart rate and slowing of atrioventricular conduction was observed on starting FTY720 treatment (mainly after the first dose). These cardiac events may have resulted from short-term, $S1P_1$–dependent activation of the G protein-gated potassium channel IK_{ACh} in atrial myocytes, before internalization and/or desensitization of $S1P_1$ receptors [99]. This mechanism is similar to the heart rate reduction generated by acetylcholine acting at muscarinic receptors [90]. Animal data suggested that the transient bradycardia produced by FTY720 [100] and natural S1P [90, 101] may involve predominantly $S1P_3$ receptors [102]. However, there appeared to be species differences and this adverse effect may be related to higher expression levels of the $S1P_1$ receptor in human ventricular, septal and atrial cardiomyocytes in comparison with the $S1P_3$ receptor [90].

Development of S1P-Related Immunomodulators

FTY720-mediated immune regulation is mainly dependent upon its effects on $S1P_1$ (Figures 10.1, 3). Thus, a further distinct advantage can be obtained with the development of next-generation S1P receptor modulators by selectively targeting $S1P_1$. The $S1P_1$ receptor-selective agonist, KRP-203, has structural similarity to FTY720 (Figure 10.2). KRP-203 is also rapidly phosphorylated *in vivo* and KRP-203-phosphate acts as the S1P agonist [103, 104]. In animal models of organ transplantation investigated by ourselves and others, KRP-203 was found to prolong skin, heart, kidney, and pancreatic islet allograft survival and attenuated chronic rejection [103−105] (Figure 10. 4A, 4B). More recent preclinical studies using animal models for inflammatory and autoimmune diseases have revealed that KRP-203 treatment ameliorated the injury in concanavalin A-induced hepatitis [106] (Figure 10. 4C), experimental autoimmune myocarditis [107], chronic colitis [108], and lupus pathogenesis [109]. KRP-203 sequestered circulating lymphocytes into lymphoid tissues and the agent could inhibit Th1 proinflammatory cytokine release. Notably, in contrast to FTY720, KRP-203 has a lower potential to induce heart rate reduction in guinea pigs [103]. Since KRP-203 selectively targets $S1P_1$ but not $S1P_3$ (EC_{50}: >1000 nM), the use of KRP-203 can potentially avoid the adverse effects associated with the use of FTY720 [104, 108].

Another $S1P_1$-selective agonist SEW2871 is structurally unrelated to S1P but is capable of activating multiple signals that are triggered by S1P. Both SEW2871 and S1P activate ERK and Akt signaling pathways and induce $S1P_1$ internalization and recycling [76, 108]. SEW2871 also promotes lymphocyte trafficking *in vitro* and induces lymphopenia in mice via a $S1P_1$-dependent mechanism [76]. Animal models demonstrate that SEW2871 ameliorates

renal ischemia/reperfusion injury by inhibiting lymphocyte egress and reducing pro-inflammatory molecules [111].

Several antagonists that interfere with S1P receptor activation by the physiological agonist S1P have also been described, including 3-amino-4-[3-hexylphenylamino]-4-oxobutylphosphonic acid (W146) [112, 113], VPC23019 [114], and JTE-013 [115]. Some of these compounds are useful for basic research but might not have potential use in humans.

Potential Therapeutic Options: S1P Agonists for Organ Transplantation

To date, calcineurin inhibitors have been widely administered for clinical organ transplantation [1, 35]. However, long-term use of calcineurin inhibitors remains problematic since it induces various adverse effects in clinical settings such as nephrotoxicity, hypertension, and hyperlipidemia. Furthermore, calcineurin inhibitors themselves accelerate transplant arteriosclerosis (i.e., chronic rejection) [116−118]. Clinical studies, in which serial biopsies from renal transplant patients were conducted, demonstrated that CsA was unsuitable as a universal and long-term immunosuppressant for renal transplantation [119]. Therefore, a new effective strategy is urgently required to reduce clinical morbidity in renal and other organ transplant patients. Indeed, FTY720 was introduced as an immunomodulator with a distinct mode of action resembling authentic calcineurin inhibitors and clinical trials of FTY720 for renal transplant patients also demonstrated its effectiveness [88, 93]. However, treatment with S1P agonist alone remained partially effective, and the agent alone is insufficient to inhibit acute immunological rejection. As the former animal studies demonstrated, FTY720 and its relevant developing agents (such as KRP-203) may need to be employed together with calcineurin inhibitors. If the therapeutic dose of calcineurin inhibitors could be minimized, this would provide a great benefit in reducing the toxicity associated with long-term use of calcineurin inhibitors [103, 104]. In fact, animal studies have demonstrated this possibility and KRP-203 in combination with a limited dose of CsA [3 mg/kg/day] showed substantial graft survival in a rat renal transplantation model [104] (Figure 10. 4D). Additionally, besides CsA, use of the anti-metabolic agent mycophenolate mofetil (MMF) with a S_1P agonist has been demonstrated to be exclusively effective in cases of allotransplantation [120, 121]. These findings suggest that the clinical use of S1P agonists should be considered in appropriate combination with other immunosuppressants for successful allotransplantation.

Conclusion

In an effort to explore the new mode of action for FTY720, the precise mechanisms of S1P-mediated immune cell trafficking have been elucidated and the concept of ‘immunomodulator’ is an important consideration in the continued challenge of developing real pharmaceutical products. Although the use of FTY720 and its derivatives would improve the current therapy of patients with autoimmune diseases, the use of S1P agonists is still an attractive option in therapeutic regimens for clinical organ transplantation. Further clinical

observation and long-term follow-up studies of S1P agonists are required; a more informed assessment of the benefits and risks of relevant treatment strategies would facilitate the development of new, safe and effective strategies for clinical organ transplantation.

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Chapter 11

Protein Kinase C Inhibitors

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Abstract

Protein kinase C (PKC) is a family of multifunctional isoenzymes involved in a variety of physiological and pathological processes. A number of PKC-specific inhibitors have been evaluated as therapeutics in immune regulation (or to be specific, graft protection). Despite the apparent promise of PKC modulators in control of transplant rejection, results in preclinical research and clinical trials have been mixed and largely negative. This chapter gives an overview of the biology in the members of the PKC family and analyzes the well-documented PKC regulators that have been applied in preclinical research or clinical trials in transplantation.

Keywords: protein kinase C, transplantation, PKC inhibitor

Abbreviations

AUC	Concentration-time curve
BPAR	Biopsy-proven acute rejection
CCR7	C-C chemokine receptor type 7
CNI	Calcineurin inhibitor
CsA	Cyclosporine A
DAG	Diacylglycerol
DCs	Dendritic cells

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ER	Endoplasmic reticulum
FoxP3	Forkhead box protein 3
GFR	Glomerular filtration rate
GvHD	Graft-versus-host disease
GvHR	Graft versus host reaction
iNOS	inducible nitric oxide synthase
IRI	Ischemia-reperfusion injury
IP3	Inositol trisphosphate
LPS	Lipopolysaccharide
MMF	Mycophenolate mofetil
MPA	Mycophenolic acid
MST	Median survival time
MLR	Mixed lymphocyte reaction
NO	Nitric oxide
OLT	Orthotopic liver transplantation
PIP2	Phosphatidylinositol-bisphosphate
PKC	Protein kinase C
PKCI	Protein kinase C inhibitor
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PRK	PKC-related kinases
PS	Phosphatidylserine
RhoA	Ras homolog gene family member A
Tregs	Regulatory T cells

Introduction

Protein kinase C (PKC) is a family of protein kinases that has been an enticing target for drug discovery since it was first identified over three decades ago. The family is comprised of a group of structurally and functionally related serine/threonine kinases represented by at least 13 different isoforms in humans. Members of this family of isozymes are activated in a variety of physiological and pathological conditions. There is well-grounded evidence for a critical role of PKC in cancer, cardiac ischemia, autoimmune diseases and many other important human diseases [1–3].

PKC members (PKCs) can be categorized into four groups on the basis of their protein architectures and catalytic activities. Of the four groups, the most studied and best understood is the conventional PKCs (cPKCs), which comprise α, βI, βII and γ isotypes; the second group is novel PKCs (nPKCs) consisting of the ε, η, δ and θ isotypes; the atypical PKCs (aPKCs), ζ and ι, comprise the third; the fourth is the kinases related to PKC, including two subgroups, the PKCμ and the PKC-related kinases (PRKs) that consist of at least three members (PRK 1-3). Similar to many other protein kinases, PKC has a regulatory region and a catalytic region. In the inactive state, the regulatory region is bound to the catalytic region. The catalytic region resides in the C-terminal half of PKC. It contains two conserved binding sites, one for ATP/$Mg^{++}$and the other for phospho-acceptor sequence in the substrate proteins.

The N-terminal half of the enzyme is the regulatory region. A typical regulatory region is comprised of two conserved domains, C1 and C2. The C1 domain, present in all of the isoforms of PKC has a binding site for diacylglycerol (DAG) as well as non-hydrolysable, non-physiological analogues called phorbol esters. This domain is functional and capable of binding DAG in both conventional and novel isoforms; however, the C1 domain in atypical PKCs is incapable of binding to DAG or phorbol esters. The C2 domain acts as a Ca^{++} sensor and is present in both conventional and novel isoforms, but is functional as a Ca^{++} sensor only in the conventional. Although originally considered a new member of the PKC family, PKCμ differs from PKC isozymes in its regulation and substrate selectivity. PKCμ contains an amino-terminal putative transmembrane (TM) domain and a pleckstrin homology (PH) domain, but no pseudosubstrate domain. The distinct feature of PRKs is that their kinase activity is directly regulated by small GTP-binding proteins. PRKs possess a binding site for the Rho small GTP-binding proteins and belong to the family of Rho-activated kinases that includes rhotekin and rhophilin. Dissociation of this inhibitory binding results in activation of the enzyme. Both the catalytic and the regulatory domains can be targeted for generating drugs to affect PKC activity [2–4]. (Figure 11.1)

In resting cells or in the absence of lipid hydrolysis, PKCs are localized primarily to the cytosol, and they usually remain catalytically inactive due to the pseudosubstrate domain binding to the catalytic region. PKC isozymes can be activated by a variety of molecules, such as hormones, growth factors, and neurotransmitters. Typically, these stimulators activate members of the phospholipase C (PLC), resulting in hydrolysis of phosphatidylinositol-bisphosphate (PIP2) to form the second messengers inositol trisphosphate (IP3) and DAG. DAG binds to the C1 domain and increases the affinity of PKC for membrane phospholipids, as a consequence, not only is the residence time of PKC on the membrane increased, but also the conformational changes associated with phospholipid binding displaces the pseudosubstrate moiety from the catalytic domain, enabling PKC to phosphorylate protein substrates. The IP3 acts on the IP3-sensitive Ca^{++} channel at endoplasmic reticulum (ER) and triggers the release of calcium, causing a rise in cytosolic Ca^{++} concentration. The rise in DAG and Ca^{++} leads to activation of PKC and its translocation from the cytosol to the plasma membranes as well as to other subcellular locations, where each isozyme interacts with its anchoring protein, phosphorylating a number of substrates that are nearby, and then leading to diverse cellular responses. (Figure 11.2)

However, the activation of PKC can also occur in the absence of the above second messenger. High levels of cytosolic calcium can directly activate PLC, thus leading to PKC activation in the absence of receptor activation. In addition, PKC can be activated by a number of post-translational modifications including tyrosine phosphorylation, oxidation of a cysteine-rich domain within the C1 domain, nitrosylation, acetylation, binding of other metabolites (e.g., retinol) and proteolytic cleavage of the enzyme at the hinge region between the catalytic and the regulator halves of the enzyme. When Ca^{++} and DAG are present in sufficient concentrations, they bind to the C2 and C1 domain, respectively, and recruit PKC to the membrane. This interaction with the membrane results in release of the pseudosubstrate from the catalytic site and activation of the enzyme. Among the differences in the four groups, the cPKC isotypes are activated by phosphatidylserine (PS) in a Ca^{++}-dependent manner and bind to DAG that increases the specificity of the enzyme for PS and shifts the affinity for Ca^{++} into the physiological range; the nPKCs are Ca^{++}-insensitive, but are still activated by DAG or phorbol esters in the presence of PS; the aPKCs, like the nPKCs, are

Ca^{++}-insensitive and do not respond to PMA (phorbol myristate acetate)/DAG; PKCμ can be activated in cells through PKC-mediated phosphorylation and can therefore function downstream of PKCs; similar to the aPKCs, PRKs are insensitive to Ca^{++}, DAG and phorbol esters, however, PRK1 has been shown to bind to the activated Ras homolog gene family member A (RhoA), which leads to a 4-fold activation of the kinase *in vitro*. In addition, it has recently been shown that PRK2 is able to bind RhoA, suggesting that this is a general property of this group (2, 4). PKCs demonstrate relatively broad in vitro substrate specificity, yet have distinct *in vivo* functions. The diverse and distinct roles of individual PKCs are, at least in part, attributed to differences in their structural features and the mechanisms that modulate their activation. These various regulatory mechanisms work together to define tissue- or cell-selective function of individual PKCs.

Some PKC members play important roles in immune system, and distinct functions of individual PKCs in the immune system have been highlighted from *in vivo* studies of PKC isoenzyme-selective knockout and transgenic mice [5]. The genetic analyses, along with biochemical studies utilizing PKC isoenzyme-specific cDNA, antisense oligonucleotides, RNA interference, and pharmacological inhibitors, revealed that PKC-regulated signaling pathways play a significant role in immune cell development, differentiation, activation and many other aspects of immune responses.

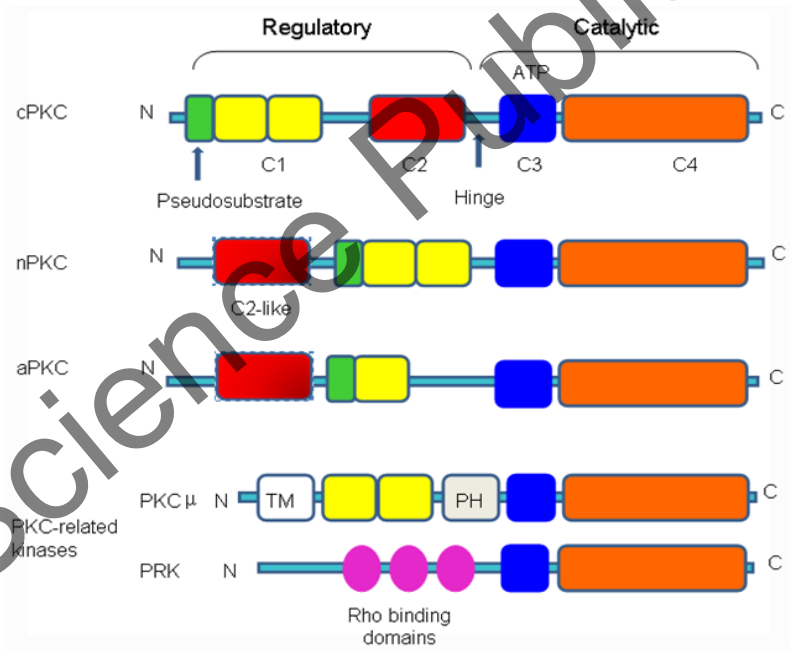

Figure 11.1. Schematic representation of the protein architecture of the four groups of the PKC family members. The arrows indicated are the pseudosubstrate domain (green) and the hinge region. C1 domain comprises one or two Cys-rich motifs (yellow orange). C2 domain (red) is of the regulatory half, and the ATP-binding lobe (C3, blue) and substrate-binding lobe (C4, Orange) of the catalytic region. The nPKCs and aPKCs lacks amino acids involved in binding calcium but have key conserved residues of C2 homology (hence referred as "C2-like"). PKCμ contains transmembrane (TM) and Pleckstrin homology (PH) domains instead of pseudosubstrate and C2 domains. Atypical PKCs have only one Cys-rich motif, and phorbol ester binding has not been detected. PRKs have domains that can bind to Rho GTPase.

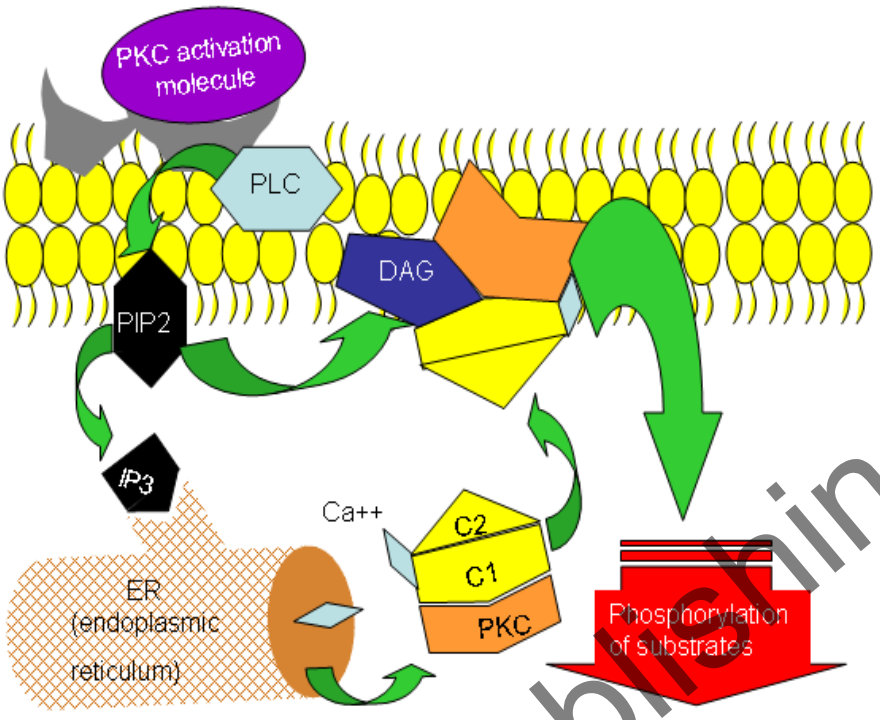

Figure 11.2. The processes leading to activation of the PKC. PKC activation molecule can bind to the receptor on cell surface and activate PLC, resulting in hydrolysis of PIP2 to DAG and IP3. The IP3 acts on the IP3-sensitive Ca^{++} channel at ER and triggers the release of Ca^{++}, raising the cytosolic Ca^{++} concentration. The translocation and binding of PKC to DAG make for the PKC-DAG complex in the cell membrane, phosphorylating a number of substrates that are nearby, thus leading to diverse cellular Responses.

Accumulated data have shown that PKCθ may play an important role in development, activation, tolerance and survival of immune cells. Originally, PKCθ was suspected to play an important role in TCR-induced activation. Over-expression and inhibition of PKCθ revealed that PKCθ can mediate activation in response to TCR/CD28 co-stimulation [6, 7]. PKCθ was further demonstrated to play its role in T cell activation through co-localization with the TCR [8]. The role of PKCθ for TCR activation and signaling was also verified *in vivo* using PKCθ -knockout mice [9]. Meanwhile, the unimpaired thymocyte differentiation and activation in those mice suggested that specific functions of PKCθ might be determined by developmental stages and could be compensated by other PKC members in thymocytes. In addition, studies from several independent labs have linked PKCθ to AP-1 (activator protein 1) and NF-κB activation in T-cells, and indentified the involvement of a number of molecules, such as the family of caspase recruitment domain-containing proteins (10–13). Moreover, PKCθ can impact T-cell activation through regulation of the interaction of the actin cytoskeleton with signaling molecules downstream of TCR [14].

The early *in vivo* evidence for a role of the cPKC member in B-cell functions came from the work of PKCβ-knockout mice that showed reduced splenic B-cells, a lower number of B-1 lymphocytes and low levels of serum IgM and IgG3 [15, 16]. Although the contradictory results remain to be reconciled, evidences suggest that BCR engagement results in the

activation of not only the pro-mitogenic PKCβ, but also of the other PKC members. The role of another PKC, the PKCδ, in the control of B-cell tolerance has been uncovered by characterization of PKCδ-knockout mice, showing that PKCδ is involved in negative regulation of proliferation [17, 18]. Besides, B-cells from PKCζ-deficient mice showed increased spontaneous apoptosis, and impaired proliferation and survival in response to IgM cross-linking, whereas both peripheral T-cells and thymocytes seemed to develop and proliferate normally. In addition, PKCζ-null mice showed compromised T-cell-dependent immune response, in spite of the fact that they exhibited no major defects in the sub-populations of B-cells [19, 20].

Macrophage-specific inhibition has also been linked to PKCs. Stimulation of the murine macrophage RAW 264.7 cell line with phorbol esters fails to promote nitric oxide synthesis as occurs in rat hepatocytes or peritoneal macrophages. PKC-epsilon can regulate a pathway that promotes inducible nitric oxide (NO) synthase (iNOS) expression in macrophages in response to phorbol ester activation [21]. In addition, PKCα, βI, βII, γ, δ and ε isoform inhibitors completely inhibited lipopolysaccharide (LPS)-stimulated NO production in murine RAW 264.7 macrophages [22]. PKCε-deficient mice were unable to clear bacterial infections and demonstrated a significantly decreased period of survival [23]. Given the emerging and cell-specific functions of the PKCs in the regulation of the cellular immune responses, selective inhibition of individual PKC isoenzymes might be effective in treating a wide range of human diseases associated with aberrant immune functions.

Although it is attractive, the inhibition of PKC isozymes is very intractable. Many PKC isozymes are present within the same cell and are activated by the same stimuli, and additional distant subfamily of PKC isozymes exists, though they do not respond to the same second messenger. Besides, PKC isozymes play unique and sometimes opposing roles in both normal signaling and disease states. Even more, depending on the context of the stimulation, the same isozyme can have opposing roles in the same cell, further complicating the scenarios of PKC activation, and thus of the inhibition.

To develop PKC-specific inhibitors has long been a goal of researchers, where significant progress has been made. Several patents related to inhibitors of PKC that represent a new and promising strategy for the prevention and treatment of illnesses have been reviewed [24–26]. Some of the PKC isoenzymes are unique in terms of tissue distribution and an elevation in any isoform level results in different disease conditions. Although different PKC isoenzymes may have high sequence identity, they could be involved in different diseases. PKCs have been shown to be highly potential therapeutic targets for treating diabetic complications and oncological, inflammatory, immunological and dermatological disorders. However, the clinical trial candidates of PKCs inhibitors mainly target the catalytic domain, which is highly conserved throughout the PKC family, making it difficult to target a particular isoform selectively. Relatively less PKC isoenzyme-specific inhibitors targeting both ATP and regulatory domain have been explored.

Table 11.1. PKC inhibitors with potential for immunosuppression or graft protection

Product Name	Targets	Description	Application	Approval status	Information Resource
Sotrastaurin	PKCθ, β1, α, η, δ, ε	a potent and pan-PKC inhibitor, mostly for PKCθ; inactive to PKCζ.	melanoma; lymphoma; immunosuppres-sion; graft protection	Clinical trials phase I-II	[27]
Go 6983	PKCα, β, γ, δ, ζ	a pan-PKC inhibitor; less potent to PKCζ and inactive to PKCμ	decrease ischemia/reperfusion damage; graft protection		[28,29]
Ro 31-8220 Mesylate	PKCα, βI, βII, γ, ε	a pan-PKC inhibitor, also inhibits RSK2, MSK1, GSK3β and S6K1	immunosuppressive potential in transplantation		[30,31]
Ro 32-0432	PKCα, βI, βII, γ and ε	inhibits IL-2 secretion, IL-2 receptor expression and proliferation of peripheral human T-cells	chronic inflammation; adjuvant-induced arthritis host vs. graft reaction (HvGR)		[32,33]
SPC-100270	PKC regulatory domain		reducing reperfusion injury		[34]
Chelerythrine	PKC catalytic domain	cell-permeable inhibitor of PKC	anti-platelet, tumor, and inflammatory; cold ischemia protection		[35,36]
Bisindolylm-aleimide I	PKC α, β, δ, ε and γ.	ATP binding site of PKC	immunosuppression; prevention of graft-versus-host disease (GvHD)		[37,38]

While the inhibition of protein to protein interaction between PKC and cognate protein is complicated, the efforts towards this aim continue. So far, three classes of agents have been developed: ATP-competitive small molecule inhibitors; Activators and inhibitors that mimic DAG-binding; Inhibitors of protein/protein interactions between PKC and cognate proteins. Among them, the best-characterized PKC inhibitors (PKCIs) are the ATP-competitive small molecules. The typical one in this class is Staurosporine, which has pan-PKC activity and binds several other serine/threonine kinases. The Enzastaurin gave an expectation of more selective inhibitor for PKCβ over other isozymes. However, subsequent studies demonstrated that it inhibits other PKC isozymes as well. Even so, some newer generations of inhibitors, e.g., Ruboxistaurin, show some selectivity over conventional PKC isozymes.

Despite the apparent promise of PKC modulators in human disease, results in clinical trials have been mixed and largely negative. Clinical trials have been conducted for the treatment of various pathogenic processes such as cardiovascular diseases, malignancies, neuropsychiatric disorders, transplant rejection, and diabetic complications. Thus far, the clinical trial results of PKC modulators have been disappointing, largely due to inadequate therapeutic effect and/or unanticipated adverse reactions. Among the many agents tested to date, only Ruboxistaurin achieved an adequate therapeutic signal to warrant an FDA review for potential market approval for use in non-proliferative diabetic retinopathy. This chapter will present general information of those well-documented PKCIs that have shown immunosuppressive or graft-protective potential (Table 11.1), and focus on those that have been well-studied in preclinical research or clinical trials in transplantation.

Immunosuppressive or Graft-Protective PKCIs

Concerning organ transplantation, there are two pathologies that are closely related to PKCs. The first relates to the ischemia-reperfusion injury (IRI) to the transplanted organ, which, depending on its severity, can lead to both acute and chronic rejection (39, 40); the second is the immune response to the transplanted organ that may lead to organ rejection [5]. Research data have shown the role of PKCδ/ε in IRI. The vasculopathy due to increased inflammatory stimulation is inhibited if IRI is reduced by treating with an activator of PKCε and inhibitor of PKCδ at the minutes before organ harvest and right after organ transplantation [41]. PKCθ, a critical isozyme for T cell activation, has also been implicated in the immunorejection of the transplanted organ and inhibition of this isozyme can reduce T cell activation, subsequent migration and further immune response [42, 43].

Ro 32-0432

Early in 1994, a potent, cell-permeable and selective inhibitor of PKC, Ro 32-0432, was tested by Birchall et al. They reported that Ro 32-0432 inhibited interleukin-2 (IL-2) secretion, IL-2 receptor expression, and proliferation of the peripheral human T-cells that were stimulated with phorbol ester together with phytohemagglutin or anti-CD3, but did not inhibit IL-2-induced proliferation in cells already stimulated to express IL-2 receptors. Oral administration of Ro 32-0432 inhibited subsequent phorbol ester-induced edema in rats.

Induction of more physiologically T-cell driven responses such as host vs. graft responses and the secondary paw swelling in adjuvant-induced arthritis were also inhibited by Ro 32-0432. Their data demonstrate a systemic efficacy of the compound to inhibit PKC-driven responses and a crucial role for PKC in T-cell activation, suggesting that inhibition of PKC could be a promising approach to prevent T-cell activation and compounds of this class may have important therapeutic applicability to transplant rejection [33]. Later on, Nilsson et al. studied the function of Ro-32-0432 on human internal mammary arteries in organ culture, using in vitro pharmacology, real-time PCR and Western blot techniques. The involvement of PKC in the endothelin receptor regulation was examined by cell culture in the presence of antagonist. Their results suggested that Ro-32-0432 may play a role in regulation endothelin type B, and inhibiting the intracellular signal transduction pathways may provide a future therapeutic target for hindering the development of vascular endothelin receptor type B changes in cardiovascular disease [44]. However, so far no further data directly related this PKCI to the intervention of graft rejection.

SPC-100270

During the same period, another PKCI, SPC-100270, was reported by Thurman et al., in that *in vivo* rat liver transplantation was performed using nonarterial and rearterial techniques. Livers from syngenic rats were harvested surgically, prepared with vascular cuffs and a splint. Just prior to completion of vascular reconstruction, the organ was intravascularly rinsed with solution containing SPC-100270 (up to 500 microM). Low doses of SPC-100270 were ineffective at reducing both parenchymal and nonparenchymal cell death, yet significant ($p < 0.05$) reductions were observed with 500 microM. Further, nonparechnymal cell viability was improved nearly four-fold by the drug. SPC-100270 (500 microM) tended to increase survival following 48 h cold storage, but the improvement was not statistically significant. SPC-100270 also did not diminish carbon-centered free radical formation in transplanted livers from alcohol-treated rats. Although the authors believed that the pretreatment of donor livers with an inhibitor of PKC is effective *in vivo* at reducing reperfusion injury, particularly to nonparenchymal cells, following orthotopic liver transplantation in the rat [34], though more convincing evidence is still missing. To the best of our knowledge, in addition to the efficacy of this inhibitor in protecting transplant, the chemical, physical and toxicological properties have not been fully investigated.

Ro 31-8220

Later on, a new member of selective PKCIs, Ro 31-8220, was introduced by Geiselhart-et al. in 1996. They assessed the immunosuppressive properties of this potent PKCI in human. Peripheral blood mononuclear cells were isolated from the blood of normal donors and utilized in a series of standard immunological assays. Three discrete activations, mitogen-induced interleukin (IL)-2 production, IL-2-dependent T lymphoblast proliferation, and IL-2Ralpha (CD25) expression, respectively, were inhibited by Ro 31-8220. In addition, noninhibitory doses of cyclosporine A (CsA) (8 nM) or FK506 (0.2 nM) suppressed mitogen-induced IL-2 production by 60−80% when combined with a noninhibitory dose (25 nM) of

Ro 31-8220. The ability of Ro 31-8220 to inhibit both early and late activation events and to synergize with CsA/FK506 suggests that this family of compounds has great potential as immunosuppressive agents in organ transplantation [31]. However, so far no remarkable progress has been made in this regard. More works still need to be done to verify the applicability of this promising agent in the control of transplant rejection.

Chelerythrine

It was known that a transient period of warm ischemia prior to a longer ischemic episode (ischemic preconditioning) protects the hepatic graft from cold ischemia. To understand the role of PKC in ischemic preconditioning responses, Ricciardi et al. took livers from Yorkshire pigs and set them into two groups, one was subjected to a cold ischemia for 2 hours as a control, the other one was given ischemic pretreatment for 15 minutes followed by 15-minute of *in situ* perfusion with or without a PKCI, chelerythrine. Following cold ischemia, all grafts were reperfused on a perfusion circuit and four variables, i.e., hepatic graft function, graft circulatory impairment, hepatocellular damage, and endothelial cell damage, were evaluated. PKC levels in the cytoplasm of all grafts were also measured by Western blot. Their data showed that ischemic preconditioning could improve graft function, reduce graft circulatory impairment, and reduce endothelial cell damage as compared to the controls. Meanwhile, the Ischemic preconditioned grafts demonstrated decreased levels of PKC. When preconditioned grafts were pretreated with chelerythrine, graft function, graft circulatory impairment, and endothelial cell damage were no different than cold ischemia controls. There was no change in PKC levels in cold ischemia controls or chelerythrine-pretreated grafts prior to cold ischemia. These results suggest that modulation of PKC might be essential for protective mechanism of ischemic preconditioning and the use of PKCI may not benefit the graft in such scenarios [36].

Go 6983

Documented as a fast-acting, lipid-soluble and a broad-spectrum PKCI, Go 6983 was investigated in a reperfusion injury model 20 years ago. When administered at the beginning of reperfusion, it restored cardiac function within 5 min and attenuated the deleterious effects associated with acute ischemia/reperfusion. The cardioprotection was associated with decreased leukocyte superoxide release and increased endothelial-derived NO from vascular tissue. *In vitro* studies of human tissue showed that Go 6983 significantly inhibited antigen-induced superoxide release from leukocytes of patients previously sensitized to tree pollen. In human vascular tissue, Go 6983 inhibited intracellular Ca^{++} accumulation, suggesting a mechanism for its vasodilator properties. These studies suggest that Go 6983 would be an effective compound to use in a clinical ischemia/reperfusion setting of organ transplantation where inhibiting superoxide release and vasoconstriction in post-ischemic tissues would benefit restoration of organ function during reperfusion. However, given the broad-spectrum action of Go 6983, careful titration of the dose regimen would be recommended to ensure a successful outcome in the setting of organ transplantation and/or cerebral ischemia [29].

Recently, Matsumoto et al. demonstrated that, in addition to working on T cells, B cells and macrophages, Go 6983 may also modulate the function of dendritic cells (DCs). From libraries of bioactive lipids, nuclear receptor ligands, and kinase inhibitors, they screened conventional PKCIs Go 6983, bisindolylmaleimide I, and Ro 32-0432 with strong tolerogenic potential. Human DCs were treated by subjecting PKCI to a maturation process after differentiation of immature DCs. The PKCI-treated DCs had a semi-mature phenotype and were able to efficiently induce IL-10-producing T cells and functional $Foxp3^+$ regulatory T cells from naïve $CD4^+$T cells. Besides, they also showed C-C chemokine receptor type 7 (CCR7) expression and sufficient capacity for migration toward CCR7 ligands. Additionally, PKCI-treated DCs were highly stable when exposed to inflammatory stimuli such as proinflammatory cytokines or LPS. Moreover, both PKCI-treated mouse and human DCs showed properties to prevent GvHD [38].

Sotrastaurin (AEB071)

Comparing to all of the promising PKCIs mentioned above, so far, sotrastaurin is the most well-studied one in transplantation settings.

Sotrastaurin is a pan-PKCI with activity against both conventional and novel isozymes. In both rodent and human primary T cells, sotrastaurin treatment can effectively abrogate T-cell activation molecules, such as CD25 expression and IL-2 secretion. Upon T-cell stimulation, sotrastaurin prominently inhibit PKC catalytic activity and selectively affect both the canonical NF-κB and nuclear factor of activated T cells transactivation pathways. Accordingly, the CD3/CD28 costimulation-induced T-cell responses are potently inhibited by sotrastaurin. In addition, sotrastaurin can markedly inhibit lymphocyte function-associated antigen-1-mediated T-cell adhesion at nanomolar concentrations. The mode of action of sotrastaurin seems different from that of calcineurin inhibitors (CNIs) such as CsA, as it has shown complementary effects on T-cell signaling pathways [45]. The potential of sotrastaurin for preventing allograft rejection and reducing the inflammatory response has been documented since 2009 and is currently under extensive investigation in preclinical and clinical trials, representing the emergence of novel immunosuppressive agents that can deliver immunosuppression without long-term toxicity [46–50].

As of now, the most explored function of sotrastaurin is in kidney transplant. In the early preclinical studies, sotrastaurin not only showed remarkable efficacy in prolonging allograft survival in kidney and heart transplant models, but also demonstrated to be safe in up to 750 mg single-dose treatment [51]. However, disappointing data soon followed. In two clinical studies of allograft renal transplantation, immunosuppressive regimens including sotrastaurin and mycophenolic acid (MPA) were compared to standard of care regimens. In each case, the groups receiving sotrastaurin had dramatic increases in acute rejection [52, 53]. In the 12-month Phase II study, *de novo* renal-transplant patients were randomized to sotrastaurin (200 mg b.i.d.) plus standard- or reduced-exposure of tacrolimus, and standard-exposure of tacrolimus plus MPA as control. In each groups, patients were converted from tacrolimus to MPA after Month 3, achieving CNI-free immunosuppression. The composite efficacy was evaluated based on the treated biopsy-proven acute rejection (BPAR), glomerular filtration rate (GFR), graft loss, death or loss to follow-up. The initial sotrastaurin plus tacrolimus regimen was efficacious and well-tolerated but the postconversion aotrastaurin plus MPA

regimen showed inadequate efficacy. The study was discontinued due to adverse events (e.g., Leukopenia and neutropenia) that occurred more frequently in the aotrastaurin-treated group [52]. In the other *de novo* renal transplant study, recipients with immediate graft function were randomized 1:2 to tacrolimus or sotrastaurin (300 mg b.i.d.). All patients received basiliximab, MPA and steroids. The primary endpoint was the composite of treated BPAR, graft loss, death or lost to follow-up at month 3. The main safety assessment was estimated GFR. Composite efficacy failure at month 3 was higher for the sotrastaurin *vs*. control regimen (25.7% *vs*. 4.5%), and median (± standard deviation) estimated GFR was higher for sotrastaurin vs control at all time points from day 7. The most common adverse events were gastrointestinal disorders. This study demonstrated a lower degree of efficacy but better renal function with the calcineurin-inhibitor-free MPA plus regimen compared to the tacrolimus-based control [53].

Although some data from early trials in transplant recipients were less encouraging, the investigation proceeded. On account of that the immunosuppressive regimens after kidney transplantation consist of a combination of several agents, several trials were done to further reveal the pharmacokinetic properties and to determine the efficacy and the optimal immunosuppressive regimen to benefit from sotrastaurin's distinct mechanism of action [54, 55]. In renal transplant clinical trials reported by Kovarik et al., the pharmacokinetics, pharmacodynamics and clinical efficacy of sotrastaurin was evaluated using a validated liquid chromatography method and other up-to-date techniques. Administered at doses of 200 to 300 mg twice daily, steady-state predose blood concentrations averaged approximately 600 and 900 ng/mL at these dose levels, respectively, and the average elimination half-life was 6 hours. The immunosuppressive efficacy of sotrastaurin was also verified *in vitro*. Blood samples from renal transplant patients receiving sotrastaurin were stimulated *ex vivo* by protein kinase C-dependent pathways. Inhibition of cytokine production, expression of CD69, and thymidine uptake served as biomarkers that demonstrated the ability of sotrastaurin to inhibit T-cell activation and proliferation at the doses used in these studies [56]. In another multicenter phase II trial of renal transplantation, 216 *de novo* renal transplant recipients were randomized to MPA with standard-exposure tacrolimus, 200 mg sotrastaurin twice daily with standard-exposure tacrolimus or reduced-exposure tacrolimus (three groups). After month 3, tacrolimus was replaced with MPA in last two groups. The longitudinal pharmacokinetics of sotrastaurin and tacrolimus were prospectively evaluated through month 6. The result showed that predose drug concentration (C0) was 0.6 ± 0.4 μg/mL and did not differ when combined with standard-exposure *vs*. reduced-exposure tacrolimus, nor when tacrolimus was replaced by MPA. Sotrastaurin peak concentration was 1.6 ± 0.6 μg/mL, and the intersubject variability in area under the drug concentration-time curve (AUC) over a dosing interval was 27%, which was not significantly influenced by age (18–67 years), weight (47–121 kg), sex, or creatinine clearance (36–173 mL/min). C0 was positively correlated with AUC ($r=0.62$, $p<0.0001$). Sotrastaurin increased tacrolimus concentrations by a pharmacokinetic interaction inasmuch as the tacrolimus dose needed to achieve a given C0 was up to 47% lower when combined with sotrastaurin *vs*. with MPA. These data indicated that pharmacokinetics of sotrastaurin were similar when combined with reduced-exposure or standard-exposure tacrolimus or with MPA. Tacrolimus exposure was significantly increased by sotrastaurin in the initial weeks post-transplant by a pharmacokinetic interaction [57].

In addition to the clinical trials, the efficacy and mechanisms of sotrastaurin was also assessed in rodent and non-human primate recipients of kidney allografts. To explore if the

inhibition of signal transduction downstream from PKC may reduce renal IRI and confer renal graft protection, Fuller et al. evaluated the effect of sotrastaurin in comparison with MPA on rat renal transplants with prolonged cold preservation. Donor kidneys from male Lewis rats were cold stored in UW (University of Wisconsin) solution for 24 hours before syngeneic grafting. Recipients received sotrastaurin (30 mg/kg twice daily), MPA (20 mg/kg/day), or vehicle through gavage starting 1 hr after surgery. Renal function was evaluated by serum creatinine and histology on day 2 (acute injury) and day 7 (repair phase) after transplantation. The sotrastaurin treatment enhanced immediate transplant function, attenuated epithelial injury, and accelerated renal function recovery compared with the MPA. Based on the real-time PCR of proinflammatory genes and histopathalogical examinations, sotrastaurin treatment significantly and persistently reduced apoptosis of tubular epithelial cells. Western blotting and immunohistochemistry revealed that sotrastaurin could decrease the phosphorylation of extracellular signal-regulated protein kinase and p66Shc adaptor protein, both involved in cellular stress and apoptosis. The result demonstrated the significant potential of sotrastaurin for ameliorating ischemia-reperfusion organ damage and promoting cytoprotection in organ transplantation [58]. In a non-human primate renal transplant model, *cynomolgus* monkey recipients of life-supporting kidney allografts were treated orally with sotrastaurin alone or in combination with CsA. Sotrastaurin monotherapy at 50 mg/kg once daily prolonged recipient survival times to the predefined endpoint of 29 days (n=2); when given at 25 mg/kg twice daily, the median survival time (MST) was 27 days (n=4). Neither once-daily monotherapy of sotrastaurin 20 mg/kg nor CsA 20 mg/kg was effective (MST 6 days and 7 days, respectively). In combination, however, sotrastaurin 20 mg/kg and CsA 20 mg/kg prolonged MST to more than 100 days (n=5). By combining lower once-daily doses of sotrastaurin (7 or 2 mg/kg) with CsA (20 mg/kg), MST was more than 100 (n=3) and 22 days (n=2), respectively. Sotrastaurin blood levels in transplant recipients during combination therapy were dose related (20 mg/kg, 30–182 ng/mL; 7 mg/kg, 7–41 ng/mL; and 2 mg/kg, 3–5 ng/mL). Sotrastaurin at a daily dose of up to 20 mg/kg was relatively well tolerated. These data suggested that sotrastaurin could be able to prolong survival times of kidney allograft recipients both as monotherapy and most effectively in combination with CsA, and pharmacokinetic interactions might not be responsible for the potentiation of immunosuppressive efficacy by coadministering sotrastaurin and CsA [59].

In a dose-ranging non-inferiority study in renal transplantation, the efficacy and safety of sotrastaurin with tacrolimus were assessed. A total of 298 patients were randomized 1:1:1:1 to receive sotrastaurin 100 or 200 mg b.i.d. plus standard tacrolimus (5–12 ng/mL), sotrastaurin 300 mg b.i.d. plus reduced tacrolimus (2–5 ng/mL) or enteric-coated MPA plus standard tacrolimus; all patients received basiliximab and corticosteroids. Composite efficacy failure (BPAR ≥ grade IA, graft loss, death or loss to follow up) rates at month 12 were 18.8%, 12.4%, 10.9% and 14.0% for the sotrastaurin 100, 200 and 300 mg, and MPA groups, respectively. The median estimated GFR were 55.7, 53.3, 64.9 and 59.2 mL/min, respectively. Higher sotrastaurin doses increased the mean heart rates and the discontinuations due to adverse events. The patients in sotrastaurin groups experienced fewer leucopenia than those in the MPA group (1.3–5.5% *vs*. 16.5%). sotrastaurin 200 and 300 mg had comparable efficacy to MPA in prevention of rejection with no significant difference in renal function between the groups [60]. In another two-stage Phase II renal transplant study, the efficacy and safety of sotrastaurin in a CNI-free regimen were evaluated. In this study, 131 *de novo* kidney transplant recipients were randomized 2:1 to sotrastaurin 300 mg or CsA

at the Stage 1, and 180 patients were randomized 1:1:1 to sotrastaurin 300 or 200 mg or CsA at Stage 2. All patients received basiliximab, everolimus and prednisone. Primary endpoint was composite efficacy failure rate of treated BPAR, graft loss, death or lost to follow-up. Main safety assessment was GFR estimated using the four-variable Modification of Diet in Renal Disease (MDRD) formula at month 12. Composite efficacy failure rates at 12 months were higher in sotrastaurin-treated groups (16.5% and 10.9% for sotrastaurin 300 mg and CsA at Stage 1; 27.2%, 34.5% and 19.4% for sotrastaurin 200 mg, 300 mg and CsA at Stage 2, respectively). The estimated GFR was significantly better in sotrastaurin groups *vs*. the CsA at most of the time points, although it was not the case at month 12. In agreement with the previous study, gastrointestinal and cardiac adverse events were more frequent with sotrastaurin treatment, and higher treatment discontinuation, deaths and graft losses occurred with sotrastaurin 300 mg. These data demonstrated that sotrastaurin combined with everolimus could lead to higher efficacy failure rates and some improvement in renal allograft function compared to a CsA-based therapy [61].

It is well known that humoral rejection processes may lead to allograft injury and subsequent dysfunction. A considerable fraction of renal transplanted patients is susceptible to humoral rejection. The impact of sotrastaurin, on human B-lymphocyte function was assessed by analyzing proliferation, apoptosis, CD80/CD86 expression and immunoglobulin and IL-10 production in primary stimulated B cells. B-cell co-cultures with pre-activated T cells were performed to evaluate the effect of the different immunosuppressive agents on T-cell-dependent immunoglobulin production. The result showed that sotrastaurin did not inhibit B-cell proliferation, CD80/CD86 expression, and IgG production and had only minor effects on IgM levels at the highest concentration administered, although it could cause decreased immunoglobulin levels in T-cell-dependent B-cell cultures [62]. In addition to the *in vitro* study, the efficacy of sotrastaurin on B cells was further verified in renal transplanted patients. The use of sotrastaurin in combination with everolimus or MPA was proved to be highly effective in suppressing B-cell activation, whereas sotrastaurin showed an unexpected and reverse impact on various B-cell functions when applied in combination with the mammalian target of rapamycin and the inosine monophosphate dehydrogenase inhibitor [63].

Considering that sotrastaurin inhibits NF-κB, which directly promotes the transcription of forkhead box protein 3 (FoxP3), the key molecule for the development and function of regulatory T cells (Tregs), the possible effect of sotrastaurin on Tregs was recently investigated. In a randomized trial of renal transplantation, de Weerd et al. conducted *ex vivo* mixed lymphocyte reaction (MLR) and analyzed these patient samples by flow cytometry, in comparison with the study on samples of blood bank volunteers. Their data showed that Treg numbers remained stable after transplantation and correlated with higher trough levels of sotrastaurin ($r = 0.68$, $p = 0.03$). A dose-dependent inhibitory effect of sotrastaurin on alloactivated T cell was observed (the half maximal inhibitory concentration to inhibit proliferation was 45 ng/ml). In contrast, Treg function was not affected in the presence of in vitro-added Sotrastaurin (50 ng/ml), with a suppression of 35% in comparison to the 47% in the absence of the drug ($p = 0.33$), and the signal transducer and activator of transcription 5 (STAT-5) phosphorylation in Tregs remained intact after incubation with sotrastaurin. The suppressive function of Treg of patients treated with sotrastaurin did not show significant difference in MLR at month 6 post-transplant, with inhibition of 67% in comparison to the 82% pretransplantation. These data indicate that sotrastaurin is a potent inhibitor of

alloreactivity *in vitro*, while it does not affect Treg function in patients after kidney transplantation [64].

In addition to the kidney transplantation, the role sotrastaurin may play was also investigated in other transplant models. The immunosuppressive effects of oral sotrastaurin were demonstrated in a rat local graft versus host reaction (GvHR) and rat cardiac transplantation models by Weckbecker et al. in 2010. Activation of blood T-cell from sotrastaurin-treated rats was suppressed by 95%, with a positive correlation between T-cell inhibition and Sotrastaurin blood concentration. In GvHR studies, sotrastaurin inhibited lymph node swelling dose-dependently. Cardiac allografts from Brown Norway (BN) and Dark Agouti (DA) rats were acutely rejected within 6−10 days post-transplantation in untreated Lewis rats. Sotrastaurin prolonged BN graft survival to a mean survival time of 15 and >28 days, and DA grafts to 6.5 and 17.5 days, respectively. In the DA to Lewis model, combining a nonefficacious dose of sotrastaurin (10 mg/kg b.i.d.) with a nonefficacious dose of cyclosporine, everolimus or FTY720 led to prolonged median survival times (26 days, >68 days and >68 days, respectively). Pharmacokinetic monitoring excluded drug-drug interactions. These results demonstrated that sotrastaurin could prolong rat heart allograft survival safely as monotherapy and in combination with nonefficacious doses of cyclosporine, everolimus or FTY720, suggesting that sotrastaurin may have the potential to offer as an alternative to CNI-based therapies in cardiac transplantation [65].

To compare the effects of sotrastaurin versus tacrolimus on acute rejection in rats heterotopic heart transplantation, Fang et al. investigated the BN to Lewis (LEW) cardiac graft survival. Various doses of oral sotrastaurin and tacrolimus monotherapy were given during the 30 days after transplantation. Grafts were monitored by daily palpation, and histologic examination of cardiac graft was performed at day 7 post-transplant. In untreated recipients, allograft MST was 6.83 ± 0.41 days. Sotrastaurin at 15, 30, or 60 mg/kg significantly prolonged the MST to 12.33 ± 1.21, 16.67 ± 1.21, and 19.33 ± 3.83, respectively. Histologic assessment revealed that high-dose sotrastaurin significantly decreased the rejection score, with decreased inflammatory cell infiltration in the graft. These results demonstrated that the sotrastaurin monotherapy (medium or high dose) may mitigate acute rejection and significantly prolong the MST of heterotopic cardiac graft in rat [66]. Later on, the same group of researchers further investigated the efficacies of sotrastaurin in combination with various doses of tacrolimus in the same rat heart transplantation model. Cardiac allograft survival was assessed by monitoring heartbeats in six recipients of each experimental group, and other four recipient rats were sacrificed in each group at day 7 post-transplant for histologic examination. Serum transaminases, blood urea nitrogen, and creatinine concentrations were measured. Their results showed that compared with monotherapy groups with higher dose of tacrolimus, a combination of sotrastaurin and tacrolimus could prolonged MST better. Sotrastaurin combined with tacrolimus (0.6 mg/kg/d) significantly decreased the rejection grade as indicative of decreased inflammatory cell infiltration into the graft. No experimental group was found with any abnormal histologic or serologic evidence of liver and kidney toxicity. The authors concluded that sotrastaurin combined with a smaller dosage of tacrolimus may be clinically possible to establish CNI minimization protocol in solid organ transplantation [67].

The cytoprotective functions of sotrastaurin in a rat hepatic cold ischemia model followed by orthotopic liver transplantation (OLT) were explored by Kamo et al. in 2011. Livers from Sprague-Dawley rats were stored at 4^{o}C for 30 h in UW solution, and then transplanted to

syngeneic recipients. Sotrastaurin treatment of both liver donors and recipients or recipients alone prolonged OLT survival (up to >90% in compare to the 40% in controls), decreased hepatocellular damage and improved histological features of IRI. Sotrastaurin treatment decreased activation of T cells, and diminished macrophage/neutrophil accumulation in OLTs. These beneficial effects were accompanied by diminished apoptosis, NF-κB/ERK signaling, depressed proapoptotic cleaved caspase-3, yet upregulated antiapoptotic Bcl-2/Bcl-xl and hepatic cell proliferation. This study demonstrated positive effects of sotrastaurin on liver IRI in OLT rat model that may translate as an additional benefit of sotrastaurin in clinical liver transplantation [68]. In a pharmacokinetic study, 13 *de novo* liver transplant recipients received 100 mg sotrastaurin once between days 1−3 and once between days 5−8 post-transplant. Sotrastaurin absorption based on the AUC of total drug in blood was similar to that of healthy subjects in a previous study. However, the sotrastaurin-binding protein, alpha1-acid glycoprotein, was nominally higher in patients yielding a 60% lower AUC compared to that in healthy subjects, based on free drug. There was minor excretion of sotrastaurin in drained bile (1% of dose) consistent with the fact that sotrastaurin is extensively metabolized leaving little unchanged drug to excrete. In the first week post-transplant, sotrastaurin is bioavailable after oral administration. However, patients with elevated alpha1-acid glycoprotein levels may have lower free drug concentrations. Whether a higher dose of sotrastaurin is needed to compensate for this in the short-term after surgery, awaits future study [69].

Although few data are available, the effect of sotrastaurin on islet allotransplantation is noteworthy. In a rodent allogeneic islet transplant model, efficacy of sotrastaurin was investigated by Merani et al. in 2009. The islet from LEW rats was grafted to Wistar Furth (WF) rats. Sotrastaurin was administered alone or in combination with CTLA4-Ig, mycophenolate mofetil (MMF), or CsA. In addition, the Lewis rat was used to screen for any sotrastaurin-associated toxicities in glucose homeostasis *in vivo*. Their data showed that sotrastaurin alone (30 mg/kg per os, p.o., b.i.d.) delayed rejection to a MST of 22 days (in comparison to that of 7 days in vehicle-treated control). When combined with CsA (5 mg/kg p.o., b.i.d.), sotrastaurin prolonged survival from 12 (CsA alone) to over 100 days in 80% of animals. No delay in allograft rejection was observed when sotrastaurin was combined with a sub-therapeutic dose of CTLA4-Ig or MMF. The frequency of allospecific IFN-γ-secreting splenocytes, assessed *ex vivo* by enzyme-linked immunosorbent spot (ELISPOT) assay, was lower in sotrastaurin-treated recipients compared to the controls. Sotrastaurin treatment did not alter the intraperitoneal glucose tolerance, the glucose-dependent insulin release, or the insulin content of the native pancreas. These data indicate that sotrastaurin can prolong islet graft survival alone or in combination with CsA and has no toxicity on glucose metabolism, suggesting that sotrastaurin is an appropriate immunosuppressive agent for islet transplantation [70]. Soon afterwards, the toxicity of sotrastaurin on the function of transplanted islets was further investigated by the same research group in a xenograft model. Human islets were transplanted into C57BL/6RAG$^{-/-}$ mice randomly assigned to vehicle control, sotrastaurin or sirolimus treatment groups. Non-fasting blood glucose levels, body weight and glucose tolerance was measured in recipients. In a separate experiment, human islets were cultured in the presence of sotrastaurin and assayed for glucose-dependent insulin secretion and level of beta-cell apoptosis. Eighty-six percent of the sotrastaurin-treated recipients achieved normoglycemia following transplant (compared with none in sirolimus-treated group, $p < 0.05$). Sotrastaurin-treated recipients exhibited similar glucose homeostasis

as vehicle-treated controls, which was better than in sirolimus-treated recipients. Human islets cultured with sotrastaurin showed similar rates of beta-cell apoptosis (p = 0.98 by one-way ANOVA) and glucose-stimulated insulin secretion (p = 0.15) as those cultured with vehicle. These results further confirmed the safety of sotrastaurin in islet transplantation, and suggest that sotrastaurin could be an appropriate immunosuppressive candidate for clinical trials in islet transplantation [71].

Aside from the direct effect on the transplant function and survival, sotrastaurin also affects numerous cellular processes that are potentially important for the replication of hepatitis B virus (HBV) and hepatitis C virus (HCV), major blood-borne pathogens prevalent in solid organ transplant recipients. The study reported by von Hahn et al. in 2011 assessed the direct, non-immune-mediated effects of sotrastaurin on HBV and HCV. By using state-of-the-art virological assays, they demonstrated that sotrastaurin had no pro-viral effect on either HBV or HCV. Especially, a reduction of HCV and HBV replication was observed even in the presence of high concentrations of sotrastaurin that is well above those used clinically and close to levels where cytotoxic effects become detectable; this reduction is not likely due to direct anti-viral activity of the drug. Their data warrant further evaluation of sotrastaurin in HBV- and/or HCV-infected transplant recipients [72].

Conclusion

PKC is a family of multifunctional isoenzymes involved in a variety of physiological and pathological processes. Over the past year, a number of PKCIs have received attention and have been investigated in preclinical studies or/and clinical trials. Despite the apparent promise that some of the PKCIs have shown in preserving graft function and suppressing the alloreaction, their potency as an immunosuppressive agents is somehow insufficient. The results in clinical trials have been mixed, and some studies were prematurely ended because of concern about the high acute rejection rates in the CNI-free arm or serious adverse effects. Although the most investigated PKCI sotrastaurin has shown some promise as potential alternative agent, the feasibility of *de novo* CNI-free immunosuppression after kidney transplantation with sotrastaurin, MPA and steroids compared to a standard regimen of tacrolimus, MPA and steroids still need further verification. Long-term data needs to be reported to confirm the safety and efficacy profile of this novel compound.

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Chapter 12

Agents Targeting JAK3 in Organ Transplantation

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Abstract

Interleukin-2 (IL-2) family cytokines, including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, are critical for lymphoid development and function. Notably, this family of cytokines shares the common γ chain (γc) in their cognate receptors. JAK3 exclusively associates with the γc, and therefore plays an essential role in γc–dependent signal transduction. Consistently, mutations in either the γc or JAK3 in humans cause defects in lymphoid development, leading to the severe combined immunodeficiency disease (SCID). More specifically, unlike other tyrosine kinases, JAK3 is predominantly expressed in hematopoietic cells. Based on these observations, JAK3 has been considered to be an attractive target for immunosuppression without having effects outside the immune system. CP-690,550 (tofacitinib) was originally introduced as a selective JAK3 inhibitor by Pfizer Inc, and it has demonstrated efficacy in murine and nonhuman primate (NHP) models as well as in clinical trials for renal transplantation. However, CP-690,550 also produces an overlapping activity against other JAKs, and consequently increases the incidence of over-immunosuppression consequences, which eventually led to its discontinuation of development in organ transplantation. In this chapter, we briefly discuss the general mechanisms of JAK3 inhibition, and focus on the CP-690,550, a potential and promising new immunosuppressive agent.

Keywords: JAK3 inhibitors, CP-690,550, transplantation, immunosuppressive drugs, adverse effect

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Abbreviations

ABL kinase: Abelson family of nonreceptor tyrosine kinases
ALT: Alanine aminotransferase
AP-1: Activator protein-1
AST: Aspartate aminotransferase
ATP: Adenosine triphosphate
AUC: Area under the plasma concentration time curve
BID: Twice daily
BPAR: Biopsy-proven acute rejection
C: Carboxyl
CAN: Chronic allograft nephropathy
CD: Cluster of differentiation
CIS: Cytokine-inducible SH2-containing suppressors
C_{max}: Peak (maximum) plasma concentrations
CMV: Cytomegalovirus
CNF: Ciliary neurotrophic factor
CNIs: Calcineurin inhibitors
CsA: Cyclosporine A
CYP: Cytochrome P450
EBV: Epstein-Barr virus
EGFR: Epidermal growth factor receptor
FDA: Food and Drug Administration
FERM: Band-4.1, ezrin, radixin, moesin
G-CSF: Granulocyte colony-stimulating factor
GFR: Glomerular filtration rate
GH: Growth hormone
GM: Granulocyte/macrophage
GM-CSF: Granulocyte-macrophage colony-stimulating factor
GVHD: Graft versus host disease
GVL: Graft-versus-leukemia
HDL: High-density lipoprotein
IC50: 50% inhibitory concentration
IFNs: Interferons
IL: Interleukin
IP-10: Interferon-inducible protein 10
I/R: Ischemia-reperfusion
JAKs: Janus kinases
JH domain: JAK homology domain
LCK kinase: Lymphocyte-specific protein tyrosine *kinase*
LDL: Low-density lipoprotein
LIF: leukemia inhibitory factor
MLR: Mixed lymphocyte reaction
MMF: Mycophenolate mofetil
MST: Median survival time

MTX: Methotrexate
N: Amino
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
NHP: Nonhuman primate
NK: Natural killer
OSM: Oncostatin M
PIAS: Protein inhibitors of activated STATs
PK: Pharmacokinetics
PRL: Prolactin
PTKs: Protein tyrosine kinases
PTLD: Post-transplant lymphoproliferative disorder
RA: Rheumatoid arthritis
RAPA: Rapamycin
SCID: Severe combined immunodeficiency disease
SCr: Serum creatinine
SH2: Src homology-2
SHP: SH2-containing Phosphatases
SOCS: Suppressors of cytokine signaling
STATs: Signal transducer and activator of transcription proteins
$T_{1/2}$: Elimination half-life
TAD: Transactivation domain
TNF: Tumor necrosis factor
$T_{max:}$ The time at which the C_{max} is observed
TPO: Thrombopoietin
TYK2: Tyrosine kinase 2
Vd: Volume of distribution
VEGFR: Vascular endothelial growth factor receptor
γc: Common γ chain

1. Introduction

Since the introduction of calcineurin inhibitors (CNIs), a significant reduction in acute allograft rejection has been attained. Despite advances in organ transplantation treatments over the past 20 years, long-term transplant survival has hardly been achieved yet, with current immunosuppressive therapy. The mechanisms leading to chronic allograft failure are multiple. One possible explanation is that current immunosuppressive therapy affects not only immune system but also other systems, because their molecular targets are ubiquitously distributed. Hence, these drugs have considerable side effects leading to unacceptable consequences, such as diabetes mellitus, nephrotoxicity, diarrhea, tumor, hypertension, and so forth [1]. In fact, CNIs-induced nephrotoxicity has become a major concern for solid organ transplant recipients. Thus, there is a clear and significant need for a new generation of immunosuppressive agents, which would have efficacy, but lack the toxicity associated with commonly used immunosuppressive agents.

Interleukin-2 (IL-2) family cytokines, including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21, play an important role in controlling and regulating the survival and function of T cells [2]. These cytokines initiate their signals via multimeric receptors, which contain the common γ chain (γc) [3, 4]. Although both JAK1 and JAK3 are involved in IL-2 family cytokine signaling, only JAK3 is exclusively associated with the γc and the converse is also the case [5]. Moreover, in contrast to other JAKs, JAK3 has a limited tissue distribution, being predominantly expressed in hematopoietic cells [6]. Genetic absence or mutation in either the γc or JAK3 completely abrogates IL-2 family cytokine signaling and results in severe combined immunodeficiency (SCID) in humans and mice, characterized by the absence of T cells and natural killer (NK) cells and normal numbers of poorly functional B cells [7−11]. More importantly, any type of the SCID is specifically limited in the immune system, without apparent changes in other organ systems [12], and it is easily cured by stem cell treatments [13]. Therefore, targeting JAK3 would theoretically offer immune suppression on the cells participating in transplant rejection, and meanwhile does not produce side-effects outside of these cells, thus providing the rationale for designing novel clinically efficient immunosuppression based on JAK3 inhibition.

CP-690,550, an orally active immunosuppressant, has been originally developed as a selective JAK3 inhibitor by Pfizer Inc for the treatment of rheumatoid arthritis (RA), inflammatory bowel disease, dry eyes, ankylosing spondylitis, psoriasis, psoriatic arthritis, and for the prevention of transplant rejection [14]. In November 2012, CP-690,550 was approved by Food and Drug Administration (FDA) for use in the US for the treatment of adults with moderately to severely active RA with an inadequate response to, or intolerance to methotrexate (MTX) [15]. In allo-transplantation models, CP-690,550 has demonstrated promising results in both rodents and nonhuman primates (NHPs) [16, 17]. Clinical trials also showed that it is non-inferior to cyclosporine A (CsA) in terms of improvements of graft survival in renal transplant [1]. However, at the same time, there was a trend towards increased risk of cytomegalovirus (CMV) disease, Epstein-Barr virus (EBV) and BK virus infection, anemia and leukopenia, and post-transplant lymphoproliferative disorder (PTLD) [18], which eventually led its discontinuation of development in organ transplantation [19]. CP-690,550 was originally developed as a selective JAK3 inhibitor. However, more recently, this compound has also been shown to inhibit JAK1, JAK2 and to a lesser extent Tyk2 [20−23], which in fact contributes to its broader pharmacological profile as shown in preclinical and clinical studies [21, 24, 25]. Hence, the fact that CP-690,550 interferes with multiple JAKs raised the question of whether inhibition of JAK3 alone is sufficient to disrupt cytokine signaling as required for immunosuppression. Indeed, current studies suggested that JAK1 may have a dominant role over JAK3 in γc-dependent cytokine signalling, indicating that selective JAK3 inhibition may not be enough to achieve efficient immunosuppression [26−28]. These data, although controversial, have challenged our belief that targeting JAK3 will be effective [28]. In this chapter, we discuss the mechanism of action of the JAK3 inhibitor, focusing primarily on CP-690,550, and also briefly introduce other currently available JAK3 inhibitors relating to prevention of transplant rejection.

2. IL-2 Family Cytokines

Cytokines are identified as a collection of structurally distinct ligands, which interact with different classes of receptors [29]. In general, they represent a group of small secreted proteins or glycoproteins released by different cell types, in particular cells of the immune system. A prominent feature of these molecules is their ability for mediating biological effect on most cell types with regard to cellular proliferation, differentiation and survival. Because cytokines acting as biological messengers have a specific effect on the interactions and communications between cells, they are extremely important for both the innate and the adaptive immune response. Today cytokine-mediated biological processes have been studied in a wide range of fields, such as immunology, inflammation, atherosclerosis, cancer and allograft rejection. Based on their origin and activity, cytokines have been mainly classified as interleukins, chemokines, interferons, tumor necrosis factors (TNFs), mesenchymal growth factors and adipokines [30].

Table 1. IL-2 family of cytokines and their function

Cytokine	Functions
IL-2	Stimulate the proliferation and differentiation of T, B and NK cells Stimulate the development of Tregs Stimulate the apoptosis of antigen-activated T cell
IL-4	Induce the differentiation of naïve helper T cell (T_H0) to the T_H2 subset Inhibit T_H1 differentiation Induce immunoglobulin switching to IgE in B cells
IL-7	Stimulate the development of pluripotent hematopoietic stem cells into lymphoid progenitor cells Promote the development, proliferation and survival of T, B and NK cells
IL-9	Stimulate intrathymic T cell development Regulate lung eosinophilia and serum IgE levels Promote protective immunity to intestinal nematodes Regulate goblet cell hyperplasia and mucus production
IL-15	Promote the proliferation, cytotoxicity and cytokine production of NK cells and regulate NK-macrophage interaction Promote the development, homeostasis and activation of dendritic epidermal T cells, intestinal intraepithelial lymphocytes and NK-T cells
IL-21	Enhance primary T cell response and effector T cell differentiation Enhance B cell function after interaction between T and B cells Regulate expansion of NK cells Induce proinflammatory T_H17 cells

Adapted from ref [132].

Cytokines exert their biological effects through the interaction with specific receptors expressed on the membrane of responsive target cells. Thus, the different cytokine families are grouped in accordance to their receptor types. IL-2 family of cytokines includes IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 [31]. Their receptors consist of three subunits, α, β and γ [32]. Both the β and the γ subunits function as signal transducing subunits, whereas α subunit acts

as the ligand binding subunit [33]. Uniquely, the IL-2 receptor γ chain (IL-2Rγ, CD123) is an indispensable component of the receptor complexes for all members of IL-2 family cytokines, and it is thereby referred to as the common γ chain (γ_c) [34]. Importantly, the γ_c was later uncovered to be an essential component for the functional receptor complex. Due to their receptors sharing the γ_c, IL-2 family of cytokines is also called the γ_c cytokine family [35]. Recent studies revealed that members of the IL-2 family cytokines are particularly important for lymphoid development and function during immune responses [36]. They collectively exhibit both unique and overlapping biological actions with respect to their modulation of immune responses [37, 38] (Table 1).

In humans, the gene coding for the γ_c is localized on chromosome Xq13 [4], and its mutation abrogates signaling via γ_c-dependent cytokines, which contributes to X-linked severe combined immunodeficiency (XSCID) [4, 39]. It is characterized by profoundly decreased numbers of T cells and natural killer (NK) cells, as well as normal numbers of non-functional B cells [40, 41]. Therefore, the γ_c was thought to be a critical subunit for mediating the signals of IL-2 family cytokines in humans. In addition, gene-targeting experiments in mice also demonstrate that the γ_c is required to initiate the signals for IL-2 family cytokines [4, 42, 43].

Based on this understanding of the mechanisms by which IL-2 family cytokines act and how their signalling pathways can be modulated by the γ_c, researchers believed that blocking the γ_c signal may have therapeutic implications for organ transplantation therapy.

3. JAK Family of Tyrosine Kinases (JAKs)

JAKs are non-receptor tyrosine kinases [44]. Cytokines initiate intracellular responses through the interaction with their cognate receptors. However, these receptors do not contain catalytic domains in their cytoplasmic domains. Instead, they physically associate with JAKs at their membrane-proximal regions [45] (Table 2). Following the cytokine binding to its receptor, JAKs phosphorylate multiple tyrosine residues of downstream signaling molecules, and therefore drive cytokine-mediated intracellular signaling cascades.

Table 2. JAKs and associated cytokine receptors

JAKs	Cytokine Receptors
JAK1	IFNα/β, IFNγ, IL-10, Shared γc receptor (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21)
JAK2	IFNγ, Shared βc receptor (IL-3, IL-5, and GM-CSF), Homodimer receptors (GH, PO, Prolactin and TPO)
JAK3	Shared γc receptor (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21)
TYK2	IFNα/β, IL-10

Adapted from ref [45].

3.1. Structure of JAKs

In mammals, the JAK family tyrosine kinases consist of 4 known members: JAK1, JAK2, JAK3, and TYK2 [47, 48]. They have molecular masses ranging from 120 to 140 kDa [49,

50]. At present, their three-dimensional structure is unknown. In primary structure, seven JAK homology (JH1-7) domains from the carboxyl (C) to the amino (N) terminus have been identified (Figure 1). The JH1 domain at the C-terminus is a highly conserved and typical functional catalytic kinase domain [22, 52, 52]. Adjacent to the JH1 domain, JH2 is a catalytically inactive pseudokinase or kinase-like domain, because it lacks the key amino acid residues required for enzyme function [53]. This pseudokinase domain is a unique feature of JAKs among PTKs [54]. Despite lacking intrinsic kinase activity, it may provide critical regulatory functions [55] or a docking site for STATs [56]. A number of studies have shown that artificial and disease-associated mutations within this domain abrogate normal kinase activity, and may positively or negatively regulate basal kinase activity, vividly illustrating the critical role of the JH2 domain in regulation of JAK signaling [57−61]. Chen et al., [59] have reported that the JH2 pseudokinase domain of JAK3 is essential for JAK3 function by regulating its catalytic activity and auto-phosphorylation.

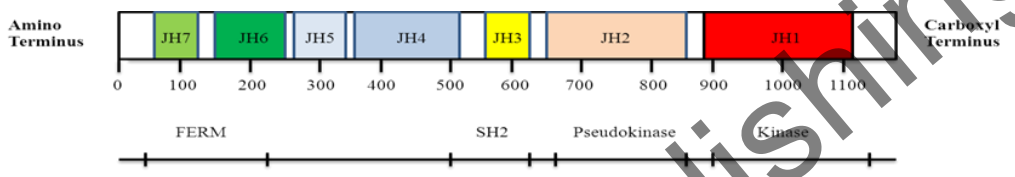


Figure 1. Primary structure of JAKs. JAKs are made up of seven distinct regions of JAK homology domains (JH- JH7). From the C-terminus, JH1 is a typical functional kinase domain. JH2 represents a pseudokinase domain, which has the conserved characteristic of the kinase domain, but lacks residues of key tyrosine kinase. The JH3 and JH4 constitute the Src homology-2 (SH2)-like domain, which shares homology with a SH2 domain. The JH5 does not belong to any part of one of the four functional domains, and it is not well documented regarding its role in JAKs. JH6 and JH7 build up the FERM (four-point-one ezrin radixin moesin) domain, which mediates binding of JAKs to cytokine receptors.

A SH2-like domain is within JH3-4 domains [62, 63]. Although this domain has many of the sequence and structure related features of a classic SH2 domain, it lacks some of the key residues that are normally conserved in the SH2 domain family [53]. Therefore, the SH2-like domain does not demonstrate the ability to bind phosphotyrosine [5]. Experimental mutation of the critical residues of SH2-like domain of JAK1 did not affect kinase activity and association with its receptor, suggesting that JAK1 SH2-like domain does not fulfill a classical SH2 function in JAK/STAT signaling [64]. Instead, this domain in JAKs may have a structural role in maintaining kinase function [65, 66]. However, the exact role of the SH2-like domain in JAKs is not yet clarified [67]. In addition, JAKs contain a Band-4.1, ezrin, radixin, moesin (FERM) homology domain within JH6-JH7 at their N-terminus [49]. The FERM domain mediates interactions of JAKs with the receptor cytoplasmic domains [68−70], and also involves regulation of kinase activity [71]. Evidence indicated that in JAK3, only FERM domain but not the other domains can effectively bind the γc subunit of cytokine receptors [72]. Mutations within the FERM domain of JAK3 result in impairment of kinase activity [73], suggesting that the FERM domain is required for both interaction and full activation of JAKs.

3.2. Function of JAKs

Following cytokine binding, the receptor become dimerized or oligomerized, consequently leading to phosphorylation and activation of associated JAKs. The activated

JAKs can phosphorylate the target tyrosine residues on the receptor sites. These phosphotyrosine residues on the receptors can then serve as docking sites for recruiting and activating other SH2 domain-containing molecules, such as STATs, thus providing a novel signaling pathway that is shared by all members of the cytokine receptor superfamily. In this regard, JAKs are key elements in cytokine-driven intracellular signalling cascades. JAKs exert their function through the selective association with different cytokine receptors in order to transmit their signals after specific cytokine binding (Table 2). JAK1, JAK2, and TYK2 are ubiquitously expressed. In contrast, the JAK3 is exclusively expressed in hematopoietic cells and appears to interact uniquely with the γc, and it is highly regulated during cell development and activation [74].

3.3. The γc-Related JAKs

Although the γc-containing receptors of cytokines, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, use both JAK1 and JAK3 to transmit their signals [75, 76], however, JAK3 is uniquely constitutively associated with the γc chain; whereas JAK1 binds IL-2Rβ chain [77, 78]. Additionally, JAK1 is also essential for gp130 family cytokines (IL-6, IL-11, oncostatin M, leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNF)) as well as granulocyte colony-stimulating factor (G-CSF) and IFNs [49]. As a result, JAK1 are known to function in a pleiotropic and redundant manner. The shared usage of JAK1 by a large number of cytokine receptor families likely accounts for its functional redundancy in mediating biological effects. The ubiquitous expression of JAK1 facilitates the pleiotropic nature of this kinase. In contrast, JAK3 is characterized as having unique properties, because it is exclusively linked to the γc. This fact makes JAK3 most relevant in transplant immunology. Furthermore, defects of the JAK3 gene have been identified as a significant cause of the autosomal recessive SCID, characterized by absent T-cells and functionally defective B-cells (T(-)B(+) SCID) [79, 80]. Most importantly, patients suffering from JAK3-SCID can be cured by the therapy of hematopoietic stem cell transplantation, confirming that JAK3 expression is limited to hematopoietic cells. These observations provided important insights into how and where JAK3 functions for lymphoid development and signaling.

4. Signal Transducer and Activator of Transcription (STAT) Proteins

In mammals, STATs, as major substrates of JAKs, play a critical role in signal transduction pathways associated with approximately 50 members of the hematopoietin family [81], including cytokines, growth factors, and hormonal factors. At present, seven members of the STAT family have been identified in mammals, namely as STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6 [45].

4.1. Structure of the STATs

The STATs share a similar structural arrangement of several conserved functional domains (Figure 2). These include an N-terminal domain, a coiled-coil domain, a central

DNA-binding domain, a linker region, a SH2 domain followed by a single conserved tyrosine residue, and a C-terminal transactivation domain (TAD) [53].

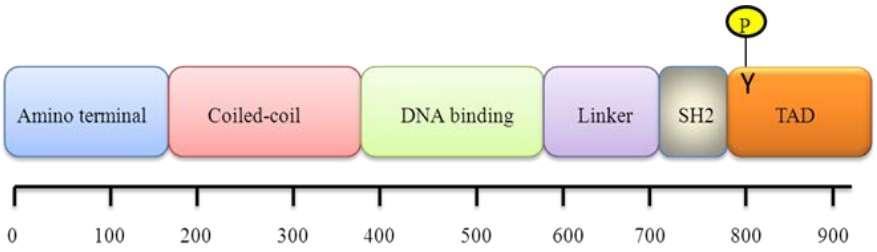


Figure 2. Basic structure of STATs. The STATs consist of several functional conserved domains: the N-terminal domain, the coiled-coil domain, the DNA-binding domain, the linker domain, the SH2 domain, and the transactivation domain (TAD). As noted, a key tyrosine residue (Y) is within TAD, and its phosphorylation is required for initiating STATs activation and dimerization [164].

The N-terminal domain plays a role in dimer-dimer interactions to form tetrameric STATs [82]. It has been reported that tetramerization of STATs enhances DNA-binding activity on weak promoters [83−86]. The coiled-coil domain is involved in interactions with other STAT cofactors, which may positively or negatively modulate their transcriptional activity [87]. The central DNA binding domain determines DNA sequence specificity of individual STATs [88, 89]. The SH2 domain is required for the recruitment of STATs to phosphorylated receptor complexes and for the interaction with JAKs, which phosphorylate the STATs. In addition, the SH2 domain is critical for reciprocal SH2-phosphotyrosine interactions between monomeric STATs to form dimers. Finally, at C-terminus a transcriptional activation domain, TAD is conserved between homologues. However, it varies in not only sequence but also length between different members of STAT family.

4.2. Function of the STATs

The STAT proteins were identified originally as a family of latent cytoplasmic transcription factors [90, 91]. Following cytokine-induced activation of JAKs, STATs are activated by JAKs-mediated phosphorylation, and subsequently dimerize and translocate to the nucleus. Once in the nucleus, the activated STAT dimers can interact with specific DNA sequence of target gene, resulting in transcriptional activation. In combination with the JAKs, STAT proteins are an important element of the JAK-STAT pathway, which is broadly involved in diverse biological processes, such as cell growth, development, differentiation, apoptosis, inflammation, and immune response, through mediating cytokine and growth factor signal transduction [92]. The fundamental roles of various STATs in signalling of a number of different cytokines have been recognized by studies of targeted deletion of STAT genes in mice (Table 3). IL-2 family of cytokines transduces their signals through JAK1 and JAK3 and several distinct downstream STATs (Table 4).

Table 3. Phenotypes of STAT knock-out mice

STATs	Cytokine affected	Knockout phenotype
STAT1	IFN α/β, γ	Impaired IFN-dependent immune responses Susceptibility to bacterial/viral infections
STAT2	IFN α/β	Impaired type I IFN-dependent immune responses
STAT3	IL-2, IL-6, IL-7, IL-9, IL-10, IL-11, IL-15, IL-21, EGF, OSM, G-CSF, TPO, LIF, GH	Embryonic mortality Impaired T-cell proliferation in response to IL-6 and IL-2 Impaired IL-10–mediated anti-inflammatory responses Defective wound healing in skin Delayed involution of mammary gland after weaning
STAT4	IL-12	Impaired Th1 cell development
STAT5a/b	IL-2, IL-3, IL-5, IL-7, IL-9, IL-15, G-CSF, GM-CSF, EPO, TPO, GH, PRL	Loss of mammary gland development and lactogenesis Loss of sexually dimorphic growth in males Defective granulocyte proliferation in response to GM-CSF Impaired cell growth in response to IL-2 Defective NK cell development Infertility in females Fetal anemia Reduced number of NK cells and impaired IL-2 induced T-cell proliferation
STAT6	IL-4, IL-13	Impaired Th2 differentiation, defective IgE class switch

Adapted from ref [92].

Table 4. γc-dependent cytokines and their related STATs

Cytokines	STATs
IL-2	STAT5a, STAT5b and STAT3
IL-4	STAT6
IL-7	STAT5a, STAT5b and STAT3
IL-9	STAT5a, STAT5b and STAT3
IL-15	STAT5a, STAT5b and STAT3
IL-21	STAT3, STAT5 and STAT1

5. JAK/STAT Signalling Pathway

The discovery of the JAK/STAT signalling pathway provided a simple mechanism for the regulation of cellular gene expression that dramatically advanced our understanding of the action of cytokines and growth factors [29]. In mammals, the JAK/STAT pathway is the principal signalling cascade for a wide array of biological events, such as cellular proliferation, differentiation, migration and apoptosis. These cellular events are required for many important biological processes, such as hematopoiesis, immunity and the inflammatory response. Here we illustrate the primary mechanisms of JAK/STAT pathway with respect to its activation and regulation.

Mechanistically, JAK/STAT signalling is relatively simple. There are only a few principal components, including seven latent transcription factors (STATs) and four non-receptor tyrosine kinases (JAKs) as mentioned above, involved in JAK/STAT signalling pathway. In the typical model of JAK/STAT signalling (Figure 3), a cytokine binding to the extracellular surface of its cognate receptors, induces the multimerization of receptor subunits. As mentioned above, the cytoplasmic domains of receptor subunits are associated

with JAKs. Due to the multimerization of receptor subunits, JAKs are brought into close proximity, allowing auto-phosphorylation and/or trans-phosphorylation by the opposing JAK kinase. Once activated, JAKs subsequently phosphorylate the cytoplasmic domains of receptor to create docking sites for recruitment of STATs. Upon binding to the receptor through their SH2 domain, STATs are in turn tyrosine-phosphorylated by JAKs. This phosphotyrosine permits the dimerization of STATs through reciprocal interactions of phosphotyrosine and the conserved SH2 domain. Dimerized STATs finally translocate into the nucleus and bind to specific regulatory sequences to regulate the transcription of target genes. Thus, the JAK/STAT cascade provides a direct mechanism of the cellular transcriptional response to an extracellular stimulation.

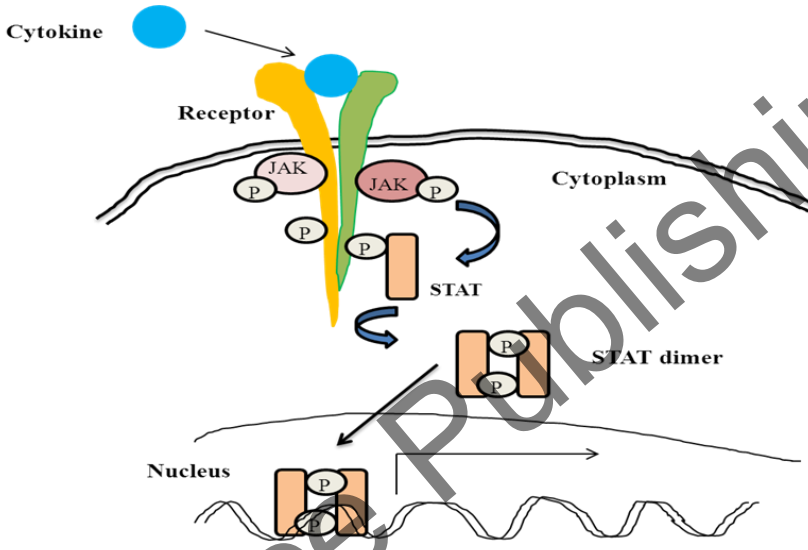


Figure 3. An overview of cytokine-induced JAKs/STATs signaling pathway. Cytokines bind to their receptors, leading to oligomerization of the receptors, which in turn induces trans- and/or auto-phosphorylation of associated JAKs. Once activated, JAKs phosphorylate the specific tyrosine residues on receptors, which then serve as docking sites for recruitment of STATs. Upon bound to receptor, STATs are also phosphorylated by JAKs. Phosphorylated STATs are released from the receptors, and subsequently form dimers, which translocate into the nucleus and activate transcriptions of target genes.

JAK/STAT pathway is negatively regulated by three major families of proteins: the suppressors of cytokine signaling (SOCS), the SH2-containing Phosphatases (SHP), and the protein inhibitors of activated STATs (PIAS) [93, 94]. SOCS proteins are induced by cytokine-stimulation, and they in turn inhibit cytokine-induced JAK/STAT signalling pathway. Thereby cytokine signalling is tightly regulated by SOCS proteins via a negative feedback loop. SOCS proteins, particularly SOCS1, SOCS3 and CIS, exert their function via several distinct mechanisms, including directly inhibiting JAKs activity, physically blocking the recruitment of STATs and other signal transducers to the receptor, and specifically targeting receptor complex and other signaling proteins for proteasome by ubiquitination [95]. Unlike SOCS proteins, both SHP and PIAS are constitutively expressed. With regard to attenuation of cytokine signal transduction, SHP directly dephosphorylate activated JAKs and receptors, and PIAS repress STATs activity by preventing them from binding to target DNA

[96]. In addition, PIAS-mediated sumoylation of STATs have been implicated in repressing STATs activity [96]. However, the mechanism remains to be elucidated.

6. Rationale for Targeting JAK3 in Preventing Organ Transplant Rejection

Solid organ transplantation has become one of the main therapeutic options for patients with end-stage disease, which creates huge needs for immunosuppressive drugs. Unfortunately, all currently used immunosuppressants cause unwanted side effects, due to the fact that they have ubiquitous molecular targets. This justifies a search for drugs with better efficacy and safety profiles. Thus, JAK3 emerged as a promising therapeutic target for preventing organ transplant rejection and treating autoimmune diseases, because of its highly selective expression and precise functions.

IL-2 family cytokines are critically important with respect to lymphoid development and function. Therefore, suppression of signaling by this family of cytokines would be predicted to have strong suppressive effects on T cells. Specifically, for initiating signalling, this family of cytokines uses the γc, a subunit of receptor complexes, which interacts uniquely with JAK3. As a result, inhibition of JAK3 will be expected to disrupt signals from the γc, thus blocking lymphocyte activities. As expected, genetic deficiency of the γc or JAK3 results in the SCID due to defects in both T cell and B cell development and activation [97].

Moreover, because JAK3 is highly expressed in hematopoietic cells, JAK3 deficiency-related SCID only affects immune system and does not cause pleiotropic defects. Therefore, patients with JAK3-SCID are easily cured by following successful stem cell transplantation. These observations suggest that selective JAK3 inhibition should be sufficient for immunosuppression without having effects outside the immune system [98]. Given very limited and precise effects of a specific JAK3 inhibitor, it likely has advantages over the current agents, which are directed against ubiquitous targets, consequently causing diverse side effects. Thus, targeting JAK3 seems to be an excellent strategy for developing immunosuppressive agents.

7. Controversy over Targeting JAK3

From the insight into the role of JAK3 in modulation of the γc-dependent cytokines that control development and function of lymphocytes, development of JAK3 inhibitors was initiated, with possible applications for the prevention of organ transplant rejection and the treatment of autoimmune diseases and inflammation [16, 17, 97]. Given the more restricted expression and precise functions of JAK3, highly specific JAK3 inhibitors were hypothesized to be more effective, but less toxic. Thus, drug discovery projects aimed at selective JAK3 inhibitors without activities on the other JAK kinases have been considered to be an important goal of a number of academic and industrial research groups [26].

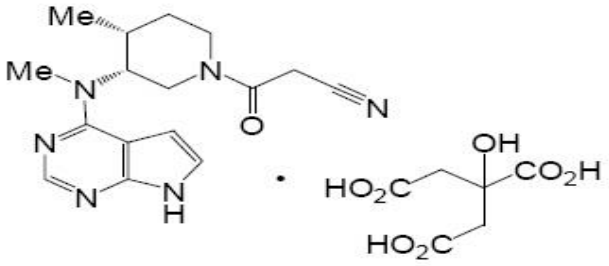
CP-690,550 was initially designed as a selective JAK3 antagonist, and it has been found to be efficacious in preventing transplant rejection in the murine heterotopic heart [16, 17] and NHP renal transplant models [6, 17, 99], as well as in a number of clinical trials

[100−102]. However, subsequent analyses of this compound by the originating labs and others revealed that, in addition to excellent selectivity of JAK3, it also displays a potent inhibition of the other JAKs, including JAK1, JAK2 and to a lesser extent TYK2 ~~Tyk2~~ [20−23]. These data raised the question of whether selective JAK3 inhibitor alone is sufficient to disrupt cytokine signalling and achieve efficient immunosuppression. Indeed, the currently available data did not provide the strong evidence that selective inhibition of JAK3 leads to the same cellular potency as dual inhibition of JAK1 and JAK3. Great success has not been achieved yet with previous attempts to design JAK3-specific inhibitors. For example, *in vitro* kinase assays demonstrated that NIBR3049 has a 50% inhibitory concentration (IC50) value of 8, 1017, 2550 and 8055 nM for JAK3, JAK1, JAK2 and TYK2 ~~Tyk2~~, respectively [23], indicating that NIBR3049 is much more selective towards JAK3 over other JAK kinases. However, despite very high selectivity for JAK3, this compound displayed more than 20-fold less potency than CP-690,550 in an IL2-induced STAT5 phosphorylation assay [23]. Based on these observations, Thoma et al., [26] hypothesized that specific inhibition of JAK3 is not sufficient to efficiently block γc-dependent cytokine signalling in which JAK1 may have a dominant role over JAK3. It is well-known that both JAK1 and JAK3 are essentially associated with the intracellular domains of the γc-containing receptors, in which JAK3 binds to the γc and JAK1 binds to other chains [76, 103−105]. Thus, JAK1 may probably cooperate with JAK3 in signalling through γc-containing receptors. However, the involvement of JAK1 in signaling via γc-containing receptors raised concerns about its exact role. Haan et al., [28] therefore explored the relative contributions of JAK1 and JAK3 in the γc-cytokine signaling, by generating analog-sensitive (AS) mutations in JAK1 and JAK3 to reconstitute IL-2 signaling. Their data exhibited that absence of JAK1 but not JAK3 results in complete abolishment of STAT5 phosphorylation. Moreover, Specific inhibition of JAK1 but not JAK3 leads to abrogation of IL-2 signal transduction. These results clearly indicated that JAK1 has a dominant role in the γc-cytokine signaling, while JAK3 activity is not critical and its activity merely enhances the effect of JAK1 [28]. In agreement with this result, earlier work performed by Liu et al., [78] with *in vitro* JAK1 and JAK3 kinase assays had already showed that JAK1 has an intrinsically higher activity over JAK3 toward STAT5. These findings suggested that inhibition of JAK1 seems to be required for complete suppression of the γc cytokine signaling, and thus questioned the potency of JAK3-specific inhibitors as the desired safe and effective immunosuppression. Given the importance of JAK1 in the γc cytokine signalling, the development of such an inhibitor that targets JAK1 may be desirable and needed if it is able to show potent activity and a good safety profile. However, since JAK1 is involved in the majority of cytokine receptor signalling, it raises concerns about target-related side effects. To date, neither specific JAK1 inhibitors nor dual JAK1/JAK3 blocking agents (devoid of JAK2 and TYK2 activity) are available [28]. If any, whether they will pass or fail in clinical trials remains to be investigated with respect to their activity and toxicity.

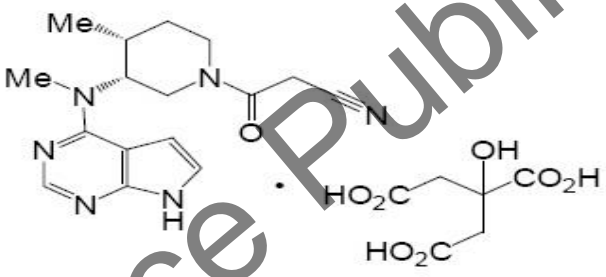
8. JAK3 Inhibitor, CP-690,550

CP-690,550 (Tofacitinib, Tasocitinib), an orally active immunosuppressant, has been originally developed by Pfizer Inc [106]. It is designated as CP-690,550-10 with a molecular

formula of C16H20N6O•C6H8O7 ~~(106)~~ and the chemical name of (3R,4R)-4-methyl-3-(methyl-7H-pyrrolo [2,3-d] pyrimidin-4-ylamino)-ß-oxo-1- piperidinepropanenitrile, 2-hydroxy-1,2,3-propanetricarboxylate (1:1) [106]. Two chiral centers of 3R,4R-isomer in CP-690,550-10 are the primary stereoisomer, and responsible for JAK3 inhibition [106]. Its chemical structure is shown below [106].



This drug has been developed in citrate salt form, which is soluble in water. Its commercial formulation (Xeljanz®; Pfizer, Inc., New York, NY, USA) is supplied for oral administration as 5 mg tofacitinib, which is equivalent to 8 mg of tofacitinib citrate [106].



8.1. Mechanism of Action

CP-690,550 is a potent JAK kinase inhibitor, which inhibits JAK3, JAK1, JAK2, and to a lesser extent TYK2. It modulates the kinase activity via competitively binding to the active site of the adenosine triphosphate (ATP) kinase domain, resulting in the prevention of phosphorylation and subsequent activation of STATs [107, 108]. *In vivo* kinase assays with CP-690,550 have demonstrated the greatest potency of inhibition of JAK3, followed by JAK1, JAK2, and TYK2 [107, 108]. The inhibition potency is 20- and 112-fold higher for JAK3 over JAK1 and JAK2, respectively [109]. CP-690,550 via inhibition of JAK3 and JAK1 blocks the γc-dependent signaling, affecting lymphocyte activation, proliferation, and function, and thus modulating multiple aspects of the immune response. In addition, inhibition of JAK1 is responsible for decreased pro-inflammatory cytokines signaling via IL-6 and Type I interferons [106]. Its JAK2-inhibiting ability will result in attenuation of erythropoietin signalling [106].

8.2. Pharmacokinetics (PK)

CP-690,550 is rapidly absorbed following oral administration, reaching the peak (maximum) plasma concentrations (C_{max}) at around 1 hour (T_{max}) [110] with an absolute oral bioavailability of 74% [106]. When administered with a high-fat meal food, the C_{max} is reduced by 32%, but the area under the plasma concentration time curve (AUC) remains unchanged; therefore this agent was given without regard to meals during clinical trials [111]. CP-690,550 is rapidly eliminated with elimination half-life ($T_{1/2}$) of about 3 hours [106]. Steady-state concentrations can be achieved in 24–48 hours with twice daily administration. The volume of distribution (Vd) is 87 L, with equal distribution between red blood cells and plasma, and CP-690,550 is approximately 40% bound to human plasma protein, mainly albumin [106]. Approximately 70% of the drug is metabolized in the liver mainly via Cytochrome P450 (CYP) 3A4, and slightly via CYP2C19 [110]. Thus, in concomitant administration of CP-690,550 with drugs that alter CYP enzyme activities, drug-drug interactions are of concern. *In vitro* data have found that CP-690,550 does not influence the CYP enzyme activity in humans [112]. However, when co-administrated with potent CYP3A4 inducers (for example rifampin), CP-690,550 AUC and its clinical efficacy can be significantly reduced ~~(106)~~ [106].On the other hand, when co-administered with potent CYP3A4 inhibitors (for example ketoconazole), or with drugs exhibiting both moderate CYP3A4 inhibition and potent CYP2C19 inhibition (for example fluconazole), the dose of CP-690,550 should be reduced by 50%, according to the manufacturer recommends [106].

Renal elimination accounts for the remaining 30% of drug clearance [106, 110]. In a study of the assessment of PK where patients with mild, moderate and severe renal impairment, and end-stage renal disease, received single CP-690,550 doses of 10 mg, the C_{max} were similar across the four treatment groups [113]. However, $T_{1/2}$ increased with severity of renal impairment [113]. Relative to patients with normal renal function, mean (90% CI) AUC (0−∞) ratios were 137% (97−195), 143% (101−202), and 223% (157−316) in patients with mild, moderate, and severe renal impairment, respectively [113].

8.3. Efficacy of CP-690,550 in Experimental Allograft Rejection

8.3.1. Rodent Organ Transplantation

Prolonged allograft survival by CP-690,550 treatment had been observed in rodent organ transplantation studies. The first indications of the efficacy of CP-690,550 to induce long-term allograft survival were reported by Changelian et al., [17], in a murine model of cardiac allograft transplant. Recipients receiving CP-690,550 for 28 days led to allograft median survival time (MST) of > 60 days, whereas control vehicle-treated mice rejected the allografts within 12 days. Meanwhile, substantially reduced chemokines and immune cell effector molecules, such as Fas ligand, granzyme B and interferon-inducible protein 10 (IP-10), were observed in the graft cells of the treated groups [17]. In another group, CP-690,550 was evaluated in the murine model of cardiac transplantation into ear pinna, in which neonatal Balb/c mouse hearts were implanted into the ear pinnae of MHC-mismatched adult C3H/HEN mice and viability was determined by the presence of heartbeat [16]. Results showed that CP-690,550 mono-therapy (5, 10, 15 and 30 mg/kg/day) dose-dependently prolonged allograft survival with MST of 10, 13, 17 and 18 days, respectively, vs. 11 days in the control vehicle-

treated group. *In vitro* CP-690,550 potently inhibited the murine mixed lymphocyte reaction (MLR) (IC50: 91 nM), and dramatically altered lymphocyte subsets in a dose- and time-dependent manner [16]. In addition, the ability of CP-690,550 to prevent allograft vasculopathy was investigated in the model of the rat aorta transplantation [114]. Aortas from AxC Irish rats were heterotopically transplanted into the infra-renal aorta of Lewis recipients and harvested at 28 or 56 days. Results showed that CP-690,550 effectively prevented allograft vasculopathy, which was associated with the significant reduction of donor-specific IgG production and the gene expression for suppressor of cytokine signaling-3 [114].

8.3.2. NHP Kidney Transplantation

Furthermore, the anti-rejection efficacy of CP-690550 was examined in the NHP model of kidney transplantation. Life-supporting kidney transplantations were performed between MLR-mismatched and ABO blood group-matched *cynomolgus* monkeys [6, 17]. Control animals rejected their graft constantly with a MST of 7 ± 1 days [6]. When animals were administered with CP-690,550 and adjusted to maintain trough levels of 200–400 ng/ml and 50–100 ng/ml, a significant prolongation of graft survival with a MST of 83 ± 6 and 62 ± 6 days, respectively was achieved [17]. Importantly, this outcome was much better than CsA monotherapy in the same animal model with a MST of 39 days (22–71 days) [115]. Thus, CP-690,550 alone seems to be more effective than CsA, with respect to successfully blocking allo-immune responses. However, dose-related anemia was observed with CP-690,550 treatment, likely as a result of cross-activity of JAK2 inhibition. In another study, animals were treated with CP-690,550 and were divided into 4 different groups according to drug exposure [6]. Results showed that CP-690,550 treatment significantly prolonged allograft survival (mean survival time of 53.2 ± 6.8 days), compared with vehicle control (mean survival time of 7.0 ± 0.6 days). As expected, survival time varied with the level of drug exposure, ranged from a mean of 83.2 ± 5.9 days for the highest level to 18.8 ± 6.3 days for the lowest level of drug exposure [6]. *In vitro* a significant reduction in numbers of NK cells and $CD4^+$ and $CD8^+$ T cells were observed in treated animals. Moreover, Borie et al., [99] performed an additional follow-up study to compare MMF in combination with CP-690,550 to MMF alone in the same animal model. Their data showed that addition of CP-690,550 to MMF significantly improved the allograft mean survival time of treated animals to 59.5 ± 9.8 days vs. 23 ± 1 days of animals receiving MMF alone. As expected, in the combination therapy group, animals that were exposed to higher levels of CP-690,550 had a significantly better survival (75.2 ± 8.7 days) than animals that received less CP-690,550 (33.3 ± 12.6 days). In combination therapy animals, a decrease in NK cell counts was observed, but $CD4^+$, $CD8^+$ and B cell numbers were unaffected, which is in contrast to studies in NHP treated with CP-690,550 monotherapy, in which $CD4^+$, $CD8^+$ and NK cell counts were all reduced [6, 116]. In combination therapy animals, anemia and gastrointestinal intolerance were seen [99], which was consistent with previous findings.

8.4. Clinical Trial with CP-690,550 in Transplantation

8.4.1. Phase 1 Clinical Trial

A phase 1 study for a treatment course of 28 days of CP-690,550 in stable renal allograft recipients had been conducted to evaluate the safety, tolerability, effects on lymphocyte

subsets, and PK of CP-690,550 when co-administered with MMF [100]. A total of 28 patients completed the trial, receiving CP-690,550 5 mg twice daily (BID), 15 mg BID, 30 mg BID and placebo, respectively. Patients with treatment of CP-690,550 5 mg and 15 mg BID were co-administered with MMF and CNI with or without corticosteroids; whereas patients in CP-690,550 30 mg BID group were required to have been on a CNI-free regimen for at least 6 months prior to study entry, and in current study only concomitant use of MMF with or without corticosteroids. The most common side events were infections. However, most of them were mild or moderate in severity and easily cured with routine medical care. In addition, gastrointestinal symptoms, such as abdominal pain, diarrhea, dyspepsia, and vomiting were often observed. As noted, the majority of severe infections occurred in the higher CP-690,550 dose groups (15 mg BID and 30 mg BID), confirming its immunosuppressive potency. A mean decrease in hemoglobin of approximately 11%, which was corroborated by a concurrent decrease in the reticulocyte count, was observed in patients who received CP-690,550 15 mg and 30 mg BID treatment. This suppressive effect on erythropoiesis is consistent with previous findings in NHP treated with CP-690,550 monotherapy [6], representing an overlapping effect resulting from the inhibition of JAK2 by CP-690,550. However, this effect was reversible, after discontinuation of the drug [100]. Similar to that observed in NHP co-administered with CP-690,550 and MMF [99], a mean decrease in absolute NK cell counts (50%), but no significant changes in the number of neutrophils, total lymphocytes, platelets, or $CD4^+$ or $CD8^+$ T cells were seen in the CP-690,550 dosed at 15 mg BID and 30 mg BID. The inhibitory effect on IL-15 activity through JAK3 inhibition of CP-690,550 may constitute a possible explanation for the observed decrease in NK cells, since these cells are dependent on IL-15 for homeostasis [100]. In addition, a mean increase in absolute $CD19^+$ B lymphocytes (130%) was observed in patients with CP-690,550 30 mg BID treatment. The underlying mechanism is unknown. Other clinical laboratory tests, such as alanine aminotransferase (ALT) and serum creatinine (SCr) were unchanged with CP-690,550 treatment of kidney allograft recipients in this study. A limitation of this study is its lack of evaluation of serum lipids. Similar to that observed in non-transplant subjects of CP-690,550 co-administered with MTX [117], there was no clinically significant effect on the PK profile of CP-690,550, observed with 30 mg BID with co-administration of MMF or steroids [100].

8.4.2. Phase 2a Clinical Trial

In a phase 2a clinical trials (A3921009), CP-690,550 had been studied on *de novo* kidney allograft recipients to compare a CP-690,550 regimen at 15 mg BID (CP15) and 30 mg BID (CP30) with tacrolimus [101]. The study was conducted in 16 transplant centers in the United States, based on a 6-month pilot study with a 12-month follow-up. All patients received an IL-2 receptor antagonist (daclizumab or basiliximab) induction, as well as concomitant MMF and corticosteroids. The primary efficacy endpoint was 6-month incidence of first biopsy-proven acute rejection (BPAR), being 1 of 20, 4 of 20 and 1 of 21 for CP15, CP30 and tacrolimus groups, respectively [101]. The 6-month estimated glomerular filtration rate (GFR) was similar across the three groups. However, the 12-month extension study of estimated GFR was 83.6, 77.6 and 73.3 ml/min for CP15, CP30 and tacrolimus group, respectively [101]. It should be noted that 20% (4 of 20) patients in the CP30 group developed BK virus nephropathy (BKN), suggesting that co-administration of CP-690,550 30 mg BID with MMF is associated with over-immunosuppression. As a result, MMF was discontinued over 2–4

weeks in this group. The 6-month rates of CMV disease was observed only in the two CP-690,550 groups, being 10% (2 of 20) and 20% (4 of 20) for CP15 and CP30 groups, respectively (101), suggesting that CP-690,550 treatment may increase susceptibility to CMV infection. In addition, serum lipid levels were modestly elevated in the CP-690,550 treatment groups, compared with the tacrolimus group. The mechanism for this is unclear. Other abnormalities noted with CP-690,550 treatment were trends towards higher anemia and neutropenia during the first 6 month of the treatment, likely due to cross-reacts of CP-690,550 on inhibition of JAK2, which mediates effects of various hematological growth factors. Similar to that observed in NHP studies, NK cells were reduced by ≤ 77% in CP-690,550 treated patients. Overall, this study demonstrated that CP-690,550 15 mg BID co-administered with MMF resulted in similar outcomes to the tacrolimus control group, but was associated with a relatively higher rate of viral infections [101].

8.4.3. Phase 2b Clinical Trial

Given the promising results obtained from the phase 2a clinical trial with CP-690,550 in preventing acute rejection in kidney allografts [101], a phase 2b trial, (ClinicalTrials.gov identifier NCT00483756) in 57 centers in 15 countries had been performed to evaluate the efficacy and safety of two different dose regimens of CP-690,550, compared to a control of CsA based regimen [102]. Patients were randomized to a more intensive (group 1) or less intensive (group 2) regimen of CP-690,550, or a control group (group 3) of CsA. In group 1, the CP-690,550 was started at 15 mg BID in months 1–6, and then switched to 10 mg BID in months 7–12. In group 2, the CP-690,550 was started at 15 mg BID in months 1–3, and then changed to 10 mg BID in months 4–12. In the control of group 3, patients received a standard CsA regimen, maintaining the 12-h trough blood levels of 125–400 ng/mL in months 1–3 and 100–300 ng/mL in months 4–12. The primary efficacy endpoints were the BPAR with a SCr increase of ≥ 0.3 mg/dL, and ≥ 20% from the pre-rejection baseline at month 6. The co-primary efficacy endpoint was measured GFR at month 12. In terms of efficacy, three groups showed the similar incidences of clinical BPAR at month 6, being 11%, 7% and 9% for group 1, 2 and 3, respectively. At month 12, two CP-690,550 groups demonstrated a significant improvement of measured GFRs, compared with CsA group (64.6 [group 1] and 64.7 [group 2] vs. 53.9 [group 3] mL/min, $p < 0.01$). The observed improvement in measured GFR was consistent with a lower incidence of chronic allograft nephropathy (CAN) in two CP-690,550 groups (25% [group 1] and 24% [group 2] respectively), compared to control of CsA group (48%). However, CP-690,550 treatment tended to increase serious infections in group 1 (45%) and group 2 (37%) vs. group 3 (25%). In addition, anemia, neutropenia and PTLD occurred more frequently in CP-690,550 groups compared with CsA control.

Taken together, these data suggested that CP-690,550 combined with MMF is non-inferior to CsA in preventing acute allograft rejection, and appears to improve renal function and prevent the development of CAN. However, it is clear that this result was achieved at the expense of over-immunosuppression and in particular JAK2 inhibition-related hematological toxicity. The increased rates of infection and PTLD associated with the CP-690,550 regimens in this study still remain the major concerns of the overall safety profile. This fact finally led the discontinuation of its development in transplant therapy. Nevertheless, CP-690,550 has been approved in November of 2012 by the United States FDA for the treatment of moderately to severely active RA in patients who have had an inadequate response to MTX,

although the European Medicines Agency has twice refused the marketing authorization for this drug [111].

8.5. Adverse Events

Due to its effect on inhibition of all JAKs, specifically, JAK1/3 or JAK2, CP-690,550 can cause mild to moderate and sometimes serious side effects, mainly including infections, malignancies, tears (perforations) in the stomach or intestines and certain changes in laboratory test results [106].

CP-690,550 can potently lower the ability of the immune system to fight infections. As a result, the most frequently reported adverse events for CP-690,550 treated patients were infections, probably due to bacterial, mycobacterial, invasive fungal, viral, or other opportunistic pathogens. Serious infections included nasopharyngitis, gastroenteritis, pharyngitis, pneumonia, and pneumococcal sepsis, cellulitis, herpes zoster, and urinary tract infection [118−120]. However, the most commonly reported infections were upper respiratory tract infection (4%), nasopharyngitis (3%) and urinary tract infection (2%) [118−122]. In addition, reported opportunistic infections include tuberculosis and other mycobacterial infections, cryptococcus, esophageal candidiasis, pneumocystosis, multidermatomal herpes zoster, cytomegalovirus, and BK virus [120]. Notably, the most common infections that resulted in discontinuation of therapy were herpes zoster and pneumonia [106]. Although serious infections were frequently reported in patients receiving CP-690,550, it should be mentioned that the most cases were mild or moderate in severity and infrequently resulted in permanent discontinuation of therapy [118, 119, 121−123].

CP-690,550 administration was frequently associated with changes in certain laboratory tests, specifically lymphocytes, neutrophils, lipid parameters, liver enzymes and SCr [118,124−126]. In clinical trials of active RA patients with CP-690,550 treatment, dose-dependent decreases in neutrophil counts were observed following 2 weeks of treatment [127]. The study suggested that this decrease in neutrophil counts may result from the anti-inflammatory action of CP-690,550, but not suppression of granulopoiesis through JAK2 inhibition [108]. In addition, CP-690,550 treatment-related neutropenia was also seen in renal allograft patients [101, 102], but not in psoriasis patients [128] or normal volunteers [129]. Changes in lymphocyte counts following CP-690,550 therapy were also seen. At first one month of CP-690,550 exposure, patients showed an increased incidence of lymphocytosis, followed by a gradual decrease in lymphocyte counts below the baseline of approximately 10% during 12 months of therapy [106]. Severe reduction in lymphocytes was more likely to contribute to an increased incidence of serious infections [106]. On the contrary, dose-related elevations of lipid parameters, including total cholesterol, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were observed following initiation of CP-690,550 therapy and remained stable throughout the study periods [111]. Elevations in SCr concentration also occurred in a dose-related manner, being 0.02 and 0.04 mg/dl greater than placebo in patients treated with the 5 mg and 10 mg CP-690,550, respectively, at third month [106]. An increased incidence of elevation in liver enzyme, including aspartate aminotransferase (AST) and ALT was also reported in patients with CP-690,550 treatment, compared to the placebo [122].

There have been cases of malignancy and lymphoproliferative disorder observed in clinical studies of CP-690,550. Patients with known malignancy or developing a malignancy during treatment should thereby be cautioned before using this drug [120].

An increased rate (16% to 17%) of gastrointestinal disorders, including diarrhea, dyspepsia, and nausea was also reported with CP-690,550 therapy, compared with placebo of 14% [120]. In addition, cases of gastrointestinal perforation associated with CP-690,550 therapy have been reported [120]. Additional common adverse events reported from several phase 3 and 2 studies included headache, vomiting, dizziness, and disorientation [111].

9. Other Agents Targeting JAK3

JAK3 inhibitors have been extensively investigated by medicinal chemists and immunologists [130]. In addition to the JAK3 inhibitors mentioned herein, there have been a variety of such agents reported in literatures. Because many cytokine receptors are associated with more than one member of the JAK family, it is not easy to sharply discriminate actions between JAK1, JAK2, or JAK3 [105]. On the other hand, the comparability with regard to JAK3 specificity is difficult based on the published data, due to major differences in assays, and thereby should be interpreted with caution [131]. Although they have been described as JAK3 inhibitors in detail, however, how exactly specific they are for JAK3 remains to be firmly established. In fact, most of these compounds do not have high selectivity for JAK3 and have certain side effects. We only briefly discuss several agents that have been reported in organ transplantation studies.

9.1. AG-490

AG-490 is a member of the tyrphostin family of tyrosine kinase inhibitors, first synthesized in the early 1990s [132]. It was originally described as a JAK2 inhibitor, as it was shown to inhibit constitutive JAK2 activity in B-lineage acute lymphoid leukemia cell lines [133]. The role of AG-490 has been widely documented in the inhibition of JAK2. For example, studies revealed that after AG-490 treatment, the cytokine-induced activation of JAK2 is significantly inhibited in eosinophils stimulated with granulocyte-macrophage CSF and in vascular smooth muscle cells and cardiac myocytes activated by angiotensin II [134, 135]. In addition, AG-490 can effectively block IL-6-induced JAK2, ERK2 and STAT3 activation in IL-6-dependent multiple myeloma cell lines [136]. Subsequently, findings showed that beyond the described inhibition of JAK2 (IC50: 0.1 μM), AG-490 also blocks JAK3 activity (IC50: 4.3 μM) [137]. It has been observed to block cytokine-mediated JAK3/STAT5a/b signal transduction and cellular proliferation in antigen-activated human T cells [138]. Through inhibition of JAK3 kinase activity, AG-490 leads to suppression of IL-2-induced T cell proliferation with an IC50 value of 25 μM [138, 139]. A preclinical animal study, in which Lewis ($RT1^l$) rat hearts were heterotopically transplanted into ACI ($RT1^a$) recipients, has shown that AG-490 alone significantly prolonged the heart allograft survival, and was synergistic with signal 1 inhibitor, CsA, but additive with signal 3 inhibitor, rapamycin (RAPA) [130]. In agreement with previous findings, AG-490 also showed *in vitro*

to block IL-2-induced T-cell proliferation (IC50: around 20 μM), JAK3 auto-phosphorylation, and activation of ~~Stat~~STAT5a/5b in the rat T-cell line of Nb2-11c [130]. However, its potency and selectivity for either JAK2 or JAK3 has not been firmly established [67, 140]. The negative side effects of AG-490 have prevented its use as the immunosuppressive therapy for routine clinical applications [141].

9.2. WHI-P131

WHI-P131 (JANEX-1) is a dimethoxyquinazoline compound, and it was initially published to inhibit JAK3 with an IC50 ~~IC_{50}~~ value of 78 μM [142]. The different IC50 value of 19.2 μM for WHI-P131 against JAK3 was also reported by another group [143]. WHI-P131 does not affect the enzymatic activity of JAK1, JAK2, SYK, BTK, LYN, and IRK, even at concentrations as high as 350 μM [144]. Moreover, WHI-P131 exhibits potent anti-inflammatory activity in several preclinical animal models, including mouse models of peritonitis, colitis, cellulitis, and systemic inflammatory response syndrome [144–146], and anti-cancer activity against human cancer cells with constitutive JAK3-STAT3/STAT5 activation [147]. A recent study demonstrated that WHI-P131 attenuated myocardial ischemia-reperfusion (I/R) injury in the mouse myocardial I/R model, via directly inhibiting the JAK3/STAT signaling pathway, decreasing neutrophil or macrophage infiltration into the infarcted myocardium, and consequently reducing infarct size [148]. JAK3-deficient mice had been found to be unable to reject islet allografts [149]. Notably, WHI-P131 as a JAK3 inhibitor could effectively prevent the rejection of islet allografts in mice with a normal JAK3 expression status [149], confirming its ability to inhibit JAK3. Additionally, experimental evidence indicated that WHI-P131 attenuates the severity of acute graft-versus-host disease (GVHD), but did not impair the graft-versus-leukemia (GVL) function after bone marrow transplantation in mice [149, 150]. However, WHI-P131 was later claimed to be neither potent nor selective for JAK3 [143]. On the contrary, it was a potent inhibitor of the EGFR kinase (IC50 of 9 nM), and also against other kinase of ABL, LCK, SRC, and VEGFR [143].

9.3. R-348

R-348 is a JAK3 inhibitor, developed by the Rigel pharmaceutical Inc ~~company~~ in cooperation with Stanford University [131]. R-348 is a pro-drug of R-333. After oral intake, R-348 rapidly converts into an active compound of R-333 in the gut by an esterase [131]. R-333 is an ATP-competitive inhibitor of JAK3 with a Ki of 16 nM [151]. R-333 inhibits IL-2 signaling in T cells and IL-4 signaling in B cells with IC50 of 0.18 and 0.07 μM, respectively [131]. R-348 exerts its inhibitory effect on JAK3 through its active metabolite R-333, but demonstrates enhanced bioavailability and absorption *in vivo*. After oral administration in rat, the peak plasma concentrations of R-348 is reached within 0.5 to 1 hours (T_{max}), and rapidly declined thereafter; meanwhile, the plasma levels of active metabolite R-333 are attained up to 15-fold higher throughout 24 hours, compared to those of R-348 [152].

R-348 has been extensively studied in autoimmune diseases, including psoriasis, RA, dry eye disease (keratoconjunctivitis sicca) and asthma. Preclinical results have shown that R-348 could effectively attenuate psoriasiform skin lesions, associated with the decreased $CD4^+$ T

cell infiltration and the reduction of IL-2-induced phosphorylation of STAT5 [151], in CD18 mutant PL/J mice, a well-established model of human psoriasis [153]. R-343 had been evaluated as a potential therapeutic for patients with allergic asthma in a phase II trial. However, it failed to meet its primary and secondary endpoints in August 2013 [154]. In addition, R-348 had currently been tested in a phase II clinic trial in patients with dry eye disease, but again failed to meet the endpoints over 12 weeks of treatment vs. placebo [155]. Therefore, Rigel Pharmaceuticals Inc has recently decided to stop developing R-348 in this indication, but is continuing its phase II study of the dry eye disease in patients with the GVHD [155].

In preclinical transplant studies, R-348 has been shown to prevent acute allograft rejection in the rat heart heterotopic transplantation model, resulting in significant reduction of graft infiltration and cellular Th1 and Th2 immune responses [131]. Its efficacy is similar to that of therapeutically dosed tacrolimus or RAPA, and synergized with tacrolimus to prolong cardiac allograft survival [131]. Subsequently, R-348 was evaluated in a rat heterotopic trachea transplant model [131]. Result demonstrated that R-348 treatment significantly attenuated chronic airway allograft rejection, leading to airway luminal obliteration of 15.7−20.6% vs. 100% in untreated controls. However, R-348 has not been reported in transplant-related clinical trials yet.

9.4. PNU156804

PNU156804 is a synthetic prodigiosin derivative, developed by the Pharmacia & Upjohn Company in 1990s [156]. PNU156804 has previously been reported to have a significant inhibitory effect on IL-2-induced NF-κB and AP-1 activation and IL-2-dependent T cell proliferation [157]. Subsequently, PNU156804 was found to block IL-2-mediated JAK3 phosphorylation and STAT5a/b activation, leading to down-regulation of JAK3-dependent T-cell proliferation induced by IL-2, -4, -7, or -15 [158]. In kinase assay, PNU156804 showed the 5-fold greater selective inhibition for JAK3-dependent T-cell proliferation, compared with JAK2-driven T-cell growth [159]. More importantly, PNU156804 alone dose-dependently extended the survival of heart allografts in the model of the rat heart transplantation [158−160]. Moreover, PNU156804 acted synergistically with the signal 1 inhibitor CsA, but additively with the signal 3 inhibitor RAPA to block allograft rejection [158, 159]. However, due to cross-reactivity with JAK2, PNU156804 proved too toxic for preclinical therapeutic applications. Its further development has been discontinued [161].

9.5. NC1153

NC1153, a Mannich base, was developed by Texas University [162]. It was identified as a potent JAK3 inhibitor with IC50 value of 2.5 μM [163]. NC1153 shows at least 40-fold greater selective inhibition for JAK3 over JAK2 [161]. Preclinical study revealed that NC1153 effectively blocked IL-2-induced activation of JAK3 and its downstream substrates STAT5a/b [163]. On the contrary, NC1153 did not inhibit several other enzyme activities, including serine/threonine protein kinases, Src family kinases and growth factor receptor tyrosine kinases [163]. In a rat kidney transplantation model of ACI ($RT1^a$) recipients with

Lewis ($RT1^l$) kidney allografts, animals that received oral therapy of NC1153 for 14 days significantly extended survival of allografts [163]. Moreover, when recipients received a 90-day therapy, most of the observed allografts (75%) survived beyond 200 days. Interestingly, these long-term surviving recipients only accepted heart allografts of donor-type Lewis ($RT1^l$) (>100 days), but not third-party Buffalo ($RT1^b$), suggesting development of transplantation tolerance [163]. Notably, NC1153 treatment was free of nephrotoxic, myelotoxic, or lipotoxic effects. When co-administered with CsA, NC1153 demonstrated synergism to prolong allograft survival, but did not increase CsA-induced nephrotoxicity. *In vitro* results indicated that NC1153 is not competing with CsA for the CYP 3A4 enzyme, and instead, it is metabolized via distinct CYP enzymes, 2D6 and 2C19 [163]. Therefore, their combination displayed highly beneficial synergistic interactions and spared the recipients from multiple toxicities. Although, many benefits were proved in rodent preclinical studies, the value of the inhibitory efficacy and safety profile of NC1153 on transplant remains to be determined. To date, NC1153 has not been reported to be tested in either NHP studies or clinical trials.

Conclusion

Commonly used immunosuppressants still possess several significant dose- and time-dependent toxicities, limiting their wide-spread applications. There is therefore an urgent need to search for novel agents whose mechanisms of action are limited to immune cells. JAK3 represents an attractive target for immunosuppression owing to its hematopoietic cell-restricted distribution and specific role in modulation of γc-dependent cytokine signaling, which is critical for lymphoid homeostasis and function. CP-690,550, a potential JAK3 inhibitor, with less potency against JAK2 and JAK1, has shown efficacy similar to comparator immunosuppressants and it has been approved by FDA for the treatment of adults with moderately to severely active RA with inadequate response to, or intolerance to MTX. However, its ubiquitous inhibition of other JAK family members contributes to the drug-related adverse events, eventually leading to its discontinuation in transplantation. To date, we have seen no evidence of any JAK3 inhibition-related immunosuppressant being approved by FDA for transplantation. Taken together, whether selective inhibition of JAK3 is sufficient to achieve efficient immunosuppression for transplant remains controversial. Accordingly, additional studies to determine the exact role of JAK3 in immunity are warranted. The challenge remains to develop new, safer and more effective immunosuppressive drugs for treating transplant rejection.

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Chapter 13

Costimulation Blockers

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Abstract

Costimulatory signals play a crucial role in optimal T cell activation. Without costimulation, T cell receptor (TCR) stimulation alone can lead to T cell anergy, death or deletion. Blocking costimulatory signalling pathway(s) with costimulation blocker(s) has become a novel therapeutic strategy to prevent and treat allograft rejection. The advantage of this approach is that costimulation blockers specifically target the T cells activated by TCR stimulation, and their influences to non-target cells such as resting T cell, B cells and non-immune cells are then limited. CD28–B7-1/B7-2 and CD40–CD40L signalling pathways are two major costimulatory receptor ligand pairs have been intensively investigated. Inhibitions of these two pathways with monoclonal antibodies have shown promise in the prevention of transplant rejection. In this chapter we will briefly review several well-investigated costimulatory molecules and discuss the agents targeting T cell costimulation.

Keywords: T cell activation, costimulation blocker, belatacept, ASKP1240

Abbreviations

ALG: Antilymphocyte globulin
APCs: Antigen-presenting cells
AZA: Azathioprine

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CsA: Cyclosporine
CTLA-4: Cytotoxic T-lymphocyte-associated protein 4
GVHD: Graft-versus-host disease
FDA: Food and Drug Administration
IKK: IκB kinase
JNK: C-Jun N-terminal kinase
MAPK: Mitogen-activated protein kinase
MHC: Major histocompatibility complex
MMF: Mycophenolate mofetil
mTOR: Mammalian target of rapamycin
PI3K: Phosphoinositide 3-kinase
TBI: Total body irradiation
TCR: T cell receptor
TIM: T cell Ig domain and mucin domain
TNFR: Tumor necrosis factor receptor
ZAP-70: Zeta-chain-associated protein kinase 70

Introduction

Organ transplantation, from the idea in ancient legend, successfully became a realistic therapeutic strategy for the patient with end-stage organ failure on December 23rd, 1954 [1]. Since then, it has saved thousands of lives in the world. Organ transplantations are mostly carried out between genetically distinct individuals. Although the outcome of transplantation could be affected by many factors including surgical techniques, organ quality, age and race of recipient, post-transplant complications etc., rejection is still the most common cause of transplant failure.

To prevent and treat allograft rejection, the strategies applied in clinic include reducing the immunogenicity of allografts and inhibiting immune responses to grafted tissues. Before transplantation, a series of tests such as ABO blood typing, tissue typing and cross-matching will be conducted to minimize alloantigenic differences between the donor and recipient. Immunosuppressive regimen, usually the combination of different types of immunosuppressants, is the key for post-transplant management.

Since Medawar and his colleagues indicated that rejection was an immunologic event in the 1940s [2, 3], the development of immunosuppressive method against allograft rejection has gone through several stages. Sublethal doses of total body irradiation (TBI), as the early attempt to avoid immune rejection of transplanted organs, was applied in a skin transplantation model in the 1950s [4]. It had been previously demonstrated that TBI was able to suppress antibody production to bacteria. The rationale for this method was that through generating generalized immunosuppression by ablation of the bone marrow, we could minimize the immune response to the allograft. Meanwhile cortisone was also tested as another therapy in the rabbit skin transplant model [5]. Unfortunately, both techniques had limited success, and the skin graft survival was prolonged for only a few days. TBI was subsequently replaced by anti-cancer drugs such as azathioaprine (AZA), methotrexate, actinomycin C and cyclophosphamide in the 1960s (6–9). The immuno-suppressive effects of

these myelotoxic agents mimicked TBI with the idea of inhibiting proliferation of donor-reactive cells. As with TBI, these agents also caused severe bone marrow depression and increased the risk of serious infections. The benefit of the myelotoxic agents was that, unlike TBI, the effects of these drugs could be reversible with drug withdrawal. The regimen of antimetabolites coupled with corticosteroids then became popular in clinical practice [8–10]. Corticosteroids were the main component of maintenance therapy, and in high dose, were the effective agents to reverse rejection crises [7, 11]. The major drawback of corticosteroids was their multiple side effects including gastrointestinal bleeding, hypertension, infection, hyperglycemia, fluid retention, osteoporosis, hirsutism, weight gain, cataracts etc. Shortly afterward, heterologous antilymphocyte globulin (ALG) as a new immunosuppressive agent was introduced into transplantation realm [12, 13]. Compared to azathioprine and prednisone treated group, patients treated with ALG in addition to azathioprine and prednisone had better graft function, less acute rejection episodes, improved graft survival, and reduction of the prednisone dose [14, 15]. However, ALG was a heterologous antibody that had potential antigenic property, and it could non-specifically bind to various hematopoietic cells leading to various side effects including fever, hypotension, thrombocytopenia, anemia, neutropenia, and anaphylaxis *etc* [14, 16–20]. Polyclonal ALG was typically made in rabbits or horses. Batch-to-batch variation in potency was another problem because the method of preparation of ALG was not standardized. These drawbacks of ALG limited its application in clinical practice.

In 1976, cyclosporine – a novel antilymphocytic agent extracted from the soil fungi *Cylindrocarpon lucidum* and *Trichoderma polysporum* [21], was found to be able to prolong skin allograft survival and delay the onset of graft-versus-host disease (GVHD) [22]. Cyclosporine A (CsA) was subsequently demonstrated to have its anti-rejection effects in various organ transplantation studies [23, 24]. CsA suppresses the expression of several genes that are critical in T cell activation, and thus inhibits T cell responses to alloantigen.

Compared with steroids or AZA, the effect of CsA on immune system is more selective. CsA exhibited potent immunosuppressive effect in low dose and less risk of bone marrow suppression. It dramatically improved the transplantation results in clinical practice [25–27]. The regimens based on CsA became the standard clinical therapeutics in the prevention of allograft rejection in solid organ transplantation in 1980s. Another much more potent immunosuppressive drug, tacrolimus – an extract of the soil fungus *Streptomyces tsukabaensis* [28], was introduced into clinical practice as rescue therapy for the liver transplant recipients who had failed with conventional immunosuppression in 1989 [29]. Similar to CsA, tacrolimus was found to inhibit early events in T cell activation by suppressing calcineurin. Therefore both of them were called calcineurin inhibitors (CNIs). Tacrolimus showed better clinical efficacy than CsA [30, 31], and has gradually replaced CsA in many transplant programs. Also in the late 1980s, the mTOR inhibitors sirolimus joined the family of anti-rejection drugs [32–34]. Sirolimus inhibits a protein called mammalian target of rapamycin (mTOR) and subsequently blocks cell cycle progression from the late G1 to the S phase [35, 36]. As a result, the growth and proliferation of T cells are suppressed. At around the same time, other immunosuppressive drugs such as the anti-CD3 monoclonal antibody Orthoclone OKT3 (muromonab-CD3) [37, 38] acting on all T cells (naïve or activated), and the antimetabolites mycophenolate mofetil (MMF) targeting all lymphocytes (selective inhibition of T and B lymphocytes purine nucleotides synthesis) [39, 40], were added to the growing list of immunosuppressant drugs.

The use of CNIs has tremendously improved the short-term outcomes of solid organ transplantation due to the incidence of acute rejection was substantially decreased. However, the effect of CNIs' on the improvement of long-term survival rates in the past decades has been disappointing [41]. Although many factors influence the long-term outcome of organ transplantation, evidences suggest that the side effects of CNIs play a role in this failure [42–46]. Long-term use of CNIs is associated with higher risk of hypertension, post-transplant diabetes, nephrotoxicity and neurotoxicity *etc*. They caused graft loss or death with graft function. To minimize the negative impact of CNIs on the long-term outcome of organ transplantation, various regimens including CNIs minimization, avoidance, and withdrawal were designed to reduce CNIs exposure. MMF or mTOR inhibitors were mostly chosen to replace the role of CNIs in maintenance therapy. However, the reported results from CNIs sparing trials were mixed and lacked concrete evidences to support the elimination of CNIs from this field [47–51]. To date, CNIs remain the cornerstone of anti-rejection protocols in clinical practice. On the other hand, all conventional immunosuppressive drugs are antigen non-specific agents. They suppress the function of all lymphocytes or all T cells instead of selectively abolishing allogeneic immune responses. They increase the risk of infection and malignancy that are also the major causes of death with graft function [52]. Hence there is a need to seek novel immunosuppressive drugs which specifically inhibit the alloimmune response initiated by donor antigens while preserving the integrity of the remainder of the host immune system.

Costimulatory molecules were chosen as novel targets to fight allograft rejection after it was known that costimulatory signals play a key role in the activation of T cells [53, 54]. The rationale of this approach is that costimulatory signals are essential for effective T cell activation triggered by the engagement of T cell receptor (TCR) and major histocompatibility complex (MHC)-bound peptides. In the absence of effective costimulation, the graft-responsive T cells will enter into a state of unresponsiveness to antigenic stimulation, and/or undergo apoptosis [55]. Unlike the conventional immunosuppressants, costimulation blockade only acts on the T cells activated by TCR stimulation while sparing the effect on the remaining T cells. Because costimulatory receptors are mostly limited to T cells and/or antigen-presenting cells (APCs), targeting these pathways offers the potential to reach the allograft acceptance through the precise and efficient regulation of host immune system, and minimize the side-effects on other organs and systems. However, there is no royal road to success; it took more than two decades to add the first costimulation blockade belatacept to the list of immunosuppressive drugs for the prevention and treatment of allograft rejection in clinic.

Costimulatory Signals Required for Optimal T Cell Activation

T cells are the crucial mediators and controllers in the alloimmune response induced by foreign histoincompatible alloantigens. During the activation process of T cells, cooperation of multiple signals is required [56]. In 1987, Helen Quill and Ron Schwartz described a phenomenon called T cell anergy [57]. They found that in the absence of functional APCs, purified MHC/peptide complexes incorporated into artificial planar lipid membranes that

could lead to high-affinity TCR/MHC interactions resulted in T cells in a state of proliferative nonresponsiveness rather than activation. It was hypothesized that, besides the signal provided by the engagement of MHC/peptide complexes and TCR, accessory signals that emanated from the interaction between functional APCs and T cells were required for full T cell activation. Results from subsequent studies provided concrete evidences to support this notion [58–60].

The two-signal model of T cell activation was then recognized [61]. During T cell activation, the interaction between TCR and MHC/peptide complexes generates the triggered signal. This signal is antigen-specific and is named Signal 1. It is transmitted into the cell through the CD3 complex and activates calcium-dependent calcineurin protein. The binding of TCR with MHC/peptide complexes is quite brief [62]. The intrinsic affinity between them is very low and some coreceptors and adhesion molecules which stabilize the engagement between TCR and MHC/peptide complexes may be involved in this process to enhance the activity. Signal 1 alone is insufficient to lead to T cell being efficiently activated. An additional signal named costimulatory signal (Signal 2 or second signal) is essential for the full activation of T cells. Costimulatory signal is non-antigen-specific and generates when costimulatory receptors on T cells contact with their corresponding ligands which are only expressed by the functional APCs. When a T cell receives both TCR signal and costimulatory signal such as cluster of differentiation 28 (CD28), various intracellular pathways including mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), phosphoinositide 3-kinase (PI3K)/Akt, and IκB kinase (IKK) are activated and in turn result in the activation of multiple transcription factors such as NFAT, AP-1, CREB, and Nuclear factor-κB (NF-κB), cell is then activated to produce cytokines (e.g., IL-2) that promote the entrance into cell cycle and proliferation [56, 63, 64]. In the situation T cell is triggered by TCR with MHC/peptide complexes interaction while no costimulatory signal, the fate of T cell will be anergy, or/and apoptosis. The exact molecular mechanisms of T cell anergy are still unclear. Related studies have indicated that both immune signal transduction pathways and the ubiquitin–proteasome system associate with clonal anergy [65–68]. It may shed light on the induction of functional tolerance to the allograft.

Costimulatory Receptors and Their Ligands

Since the first costimulatory molecule CD28 was identified, numerous related molecules have been discovered. They form a large group known as second signal family. Among them, molecules that provide enhancement signals for promoting T cell activation, survival, and/or differentiation are collectively named positive costimulatory receptors and ligands. On the other hand, molecules that generate signals to antagonize TCR signaling resulting in termination of T cell activation are referred as negative costimulatory receptors and ligands. Negative costimulatory signalling pathways play important roles in the maintenance of peripheral T cell tolerance and reducing inflammation after infection. However, activations of naïve T cells are not affected because they do not express negative costimulatory receptors. Effector T cells express negative costimulatory receptors, and at the end of an immune response, their expression will be up-regulated.

Apart from classification based on the functional properties of costimulatory receptors mentioned above, costimulatory molecules can also be grouped by their structure. According to the structure of costimulatory molecules, they are classified into four groups i.e., immunoglobulin (Ig) superfamily, tumor necrosis factor receptor (TNF-R) family, cell adhesion molecules, and T cell Ig domain and mucin domain (TIM) molecules.

The Immunoglobulin Superfamily

Costimulatory molecules in the Ig superfamily all possess a characteristic domain referred to Ig domain in the extracellular region of these molecules. Ig domain contains a sandwich-like structure known as Ig fold which is formed by two sheets of antiparallel beta strands [69]. Members in this superfamily occupy a central importance in both immune activation and active immune regulation. Some molecules of this superfamily promote (costimulatory) T cell activation and differentiation, whereas other molecules exhibit opposing effects (coinhibitory) on the activation of T cells. Generally immunoreceptor tyrosine-based activation motifs (ITAM) and immunoreceptor tyrosine-based inhibition motifs (ITIM) in their cytoplasmic region involve the transduction process of stimulatory and inhibitory signals respectively [70]. However, in some circumstances, ITAM can propagate inhibitory signals and ITIM can transmit activation signals [71–73]. In addition, some molecules in this family such as CD28 also play a role in the homeostasis and function of a population of regulatory T cells (Tregs) [74].

CD28/CTLA-4–B7 Pathway

The CD28 molecule is a 44 kDa homodimeric transmembrane glycoprotein which consists of 202 amino acids. The CD28 genes are localized on human chromosome 2q33 and on band C of mouse chromosome 1 [75, 76]. The CD28 receptor extracellular region, which possesses 134 amino acids, comprises a single disulphide-linked V-like domain. A hexapeptide motif (MYPPPY) is essential for the interaction with its ligands [77, 78]. Forty one amino acids constitute the intracellular region of the CD28 receptor. It contains four tyrosine residues that can be inducibly phosphorylated. They provide the binding sites for PI3K and growth factor receptor-bound protein 2 (Grb2), and involve the regulation of downstream signaling cascade [79–82].

In humans, CD28 is constitutively expressed on the surface of 80–95% $CD4^+$ T cells and on approximately 50% of $CD8^+$ T cells. However, all naive T cells and memory $CD4^+$ Th cells express CD28. In contrast to humans, CD28 is expressed on all $CD4^+$ and $CD8^+$ mouse T cells [83–85]. CD28 is also expressed on gamma delta T cells [86], some human plasmablasts and plasma cells [87, 88], and on human fetal peripheral blood natural killer (NK) cells [89]. The expression of CD28 in humans is down-regulated with age and in chronic disease states, but it is not the case in mice.

B7-1 (CD80) and B7-2 (CD86) are two known ligands for CD28. B7-1 is a 60 kDa type I transmembrane monomeric glycoprotein, and is constituted by 262 amino acids. B7-2, a glycoprotein with molecular weight of 70 kDa, shares structural homology to B7-1 [90–94]. The genes encoding B7-1 and B7-2 are localized to the same region in human chromosome 3q13.3-3q21 and 3q13-3q23 respectively [95, 96]. Both B7-1 and B7-2 molecule contain a single IgV-like domain and a single IgC2-like domain within their extracellular region. The

amino-acid sequence analysis indicates that, in the immunoglobulin a C-like domain of them, there is a SQDXXXELY motif which is a putative CD28-binding sequence [90–94, 97].

B7-1 is expressed on activated APCs including B cells, macrophages and dendritic cells, and is also expressed on activated T cells and $Foxp3^+$ Treg. In contrast, B7-2 is constitutively present on APCs, and its levels of expression can be upregulated when stimulated by inflammatory cytokines. Activated T cells also express B7-2 [93]. Studies indicate that B7-2 probably acts as the major initial ligand for CD28 during T cell activation, whereas B7-1 is the preferential ligand for CD28 with approximately 10-fold higher binding affinity compared with B7-2 and appears to be a more potent costimulus in terms of T cell activation [98, 99].

When the TCR is properly engaged, CD28 costimulatory signal produced by the interaction between CD28 and its B7 ligands can result in a dramatic augmentation of the expression of genes induced by TCR signaling alone and this leads to full activation of T cells.

The CD28 signaling pathway involves numerous activities. It participates in the regulation of glucose metabolism [100], promotes the production of IL-2 [101], mediates entry of T cells into the cell cycle [102], increases the generation of cytokines and chemokines [103], controls regulatory T cell homeostasis [104] and reinforces resistance to apoptosis [105]. CD28 costimulation can lower the threshold of activation by decreasing the number of TCRs required for T cell activation [106]. Together with CD28 signal can also promote cytoskeleton reorganization to the TCR contact site [107], which might partially explain how CD28 exerts its co-stimulatory effects.

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is also known as cluster of differentiation 152 (CD152). It is a T cell surface glycoprotein with high sequence and structural homology to CD28. The genes for CTLA-4 and CD28 map to the same chromosomal region and share the same overall intron/exon organization. There are four exons in the CTLA-4 gene. The exon 1 consists of the leader peptide sequence, and exon 2 to 4 encodes the ligand binding site, the transmembrane region, and the cytoplasmic tail respectively [108]. Between CTLA-4 and CD28, there is about 20% identity at the gene structure level and about 30% identity at the amino acid level [75, 76, 109]. Similar to CD28, a MYPPPY motif that is necessary for binding to its ligands can also be found within the extracellular domain of CTLA-4.

CTLA-4 is mainly expressed on T cells [110]. Expression of CTLA-4 on the surface of B cells [111] and thymocytes [112] has also been reported. Full-length CTLA-4 is only detected on the surface of activated T cells, but not on resting T cells [113]. Approximately 90% of CTLA-4 exists as intracellular vesicles due to the rapid and constitutive endocytosis of surface CTLA-4 [114]. However Tregs are an exception as CTLA-4 is constitutively expressed on their surfaces. Tregs were also found to contain higher levels of CTLA-4 than conventional T cells [115, 116]. Unlike full-length CTLA-4, a splice variant of CTLA-4 known as ligand-independent CTLA-4 (liCTLA-4) which contains exons 1, 3, and 4, is expressed on murine resting T cells. But the expression level of liCTLA-4 is downregulated upon cell activation [117, 118].

CTLA-4 also binds to B7-1 and B7-2. Although it is highly homologous to CD28, CTLA-4 appears as a negative regulator on T cell function by switching off T cell activation, proliferation and IL-2 production [119, 120]. How CTLA4 exerts its inhibiting effects on T cells still remains uncertain. It may down-regulate T cell immunity via various ways. CTLA-4 is thought to directly affect CD28–B7 engagement by ligand competition because it binds to

B7 with much higher affinity compared to CD28 [121, 122]; CTLA-4 can raise the threshold of TCR signaling for full T cell activation (109, 123); CTLA-4 coligation induces the activation of a cytosolic phosphatase and then blocks TCR-transmitted signal such as to inhibit the formation of Zeta-chain-associated protein kinase 70 (ZAP-70)-containing microclusters [124–126]. The CTLA-4–B7 interaction can induce the release of indoleamine 2, 3-dioxygenase (IDO) that will result in tryptophan catabolism [127, 128]. T cell growth is then suppressed. CTLA-4 expressed on the surface of Treg cells binding to B7-1 and B7-2 on APCs can physically remove both of them by transendocytosis [129, 130].

ICOS –B7h Pathway

The inducible T cell costimulator (ICOS, CD278) is a T cell surface receptor belonging to Ig superfamily. It is a CD28 homolog and the cDNA of ICOS shares approximate 30–40 % sequence similarity with CD28 and CTLA-4 [131, 132]. ICOS expression on $CD4^+$ and $CD8^+$ T cells is induced when they are activated. But resting $CD4^+$ and $CD8^+$ T cells do not express ICOS [132]. B7h (also known as ICOS-L, B7h-2, B7RP-1, and GL-50) is the ligand for ICOS. It is a transmembrane glycoprotein and is homologous with B7-1 and B7-2 but does not possess the unique SQDXXXELY motif [133]. B7h is expressed on B cells, monocytes and dendritic cells [134]. Some non-lymphoid tissues including lung and heart also express B7h [135]. ICOS–B7h engagement can provide additional costimulatory signals for T cell activation, differentiation and cytokine production. It exhibits synergistic effects with CD28 signaling on T cell activation. Unlike CD28 signaling which mainly promotes initial T cell activation, ICOS signaling plays a role in the activation of antigen-experienced T cells [134]. When ICOS is ligated by B7h, it can promote B cells, Treg, Th17, Th1 and follicular B helper T cells responses [136–140]. ICOS is also involved T–B cell interactions [140], immunoglobulin class switching [140] and splenic germinal centre formation [141]. In addition, ICOS signaling can induce T cell expansion in a CD28-independent manner [135].

PD-1–PD-L1/PD-L2 Pathway

Programmed death-1 (PD-1, CD279), a 50–55 kDa type I transmembrane protein, is another member of the Ig superfamily that exerts coinhibitory effects. Structurally, PD-1 shares 23% similarity with CTLA-4 but does not contain the amino acid sequence MYPPPY [142]. Unlike other members of the Ig family which are restricted to T cells, PD-1 is expressed on activated $CD4^+$ and $CD8^+$ T cells, regulatory T cells, NK cells, B cells, dendritic cells, macrophages but not resting T cells (143–145). There are two known ligands named PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD274) for PD-1 [142, 146]. PD-L1 is constitutively expressed on APCs, T and B lymphocytes, mast cells, mesenchymal stem cells and certain parenchymal tissues including heart, lung, kidney, pancreas, and placenta, endothelial cells [145–149], and the expression of PD-L1 is upregulated upon cell activation. Compared to PD-L1, the expression of PD-L2 is much more restricted. It is detected on dendritic cells, macrophages, bone marrow-derived mast cells [150]. Recent study indicated that PD-L2 was also expressed on 50–70% resting peritoneal B1 cells [151].

PD-1 signal is critical for the regulation of autoimmunity, infectious immunity, transplantation immunity, and tumor immunity. Like CTLA-4, engagement of PD-1 by its ligands generates a potent inhibitory signal that inhibits T cell activation, proliferation and cytokine production by arresting the cell cycle in the G0/G1 phase [142, 146, 152, 153].

Studies [154, 155] demonstrate that PD-1 signaling is critical for the induction of anergy and the development of induced regulatory T cells (iTregs).

The TNF/TNF-R Family

Another costimulatory molecules superfamily is the TNF/TNF-R family which consists of numerous tumor necrosis factors (TNFs) and TNF-Rs [156]. Since the first receptor ligand pair (CD40 and CD40L) of TNF/TNF-R family was identified, the inventory of this family has expanded rapidly. At present, 18 different genes that encode TNF have been identified in humans [157–159]. The TNFs are important cytokines and they are all type II transmembrane proteins. All members of the family are homologous and share about 30% similarities [160]. According to their structures, TNFs are classified into three groups: conventional ligands (L-THD), EF-disulfide ligands (S-THD) and the divergent ligands (V-THD) [161, 162]. In humans, the TNF-Rs superfamily contains 29 reported members. Among them, 22 molecules belong to type I transmembrane receptors and the remaining 7 molecules are either type III transmembrane receptors or soluble receptors [158]. TNF-Rs exhibit functional difference based on their structural diversities. TNF-Rs which contain a length of approximately 80 amino acid death domain (DD) motif can activate caspase cascades and result in apoptosis [163]. In contrast, some TNF-Rs possess a TNF receptor associated factor (TRAF)-binding sequence that recruits TRAF proteins. TRAF is involved in cell activation, differentiation, and survival. Therefore signals from these TNF-Rs participate in the regulation of T cell activation instead of the induction of T cell apoptosis [164].

Most TNF-Rs including OX40, 4-1BB, and CD30 etc. are expressed predominantly by activated T cells [165–168]. Their ligands OX40L, 4-1BBL, and CD30L are not expressed by resting or immature APCs. These proteins are detected within one to several days after activation [169] which suggests that those costimulatory molecules are mainly involved in the effector and memory phases rather than the initial phase of T cell immune response. On the other hand, the herpes virus entry mediator (HVEM) and its ligand (LIGHT) are constitutively expressed by naive T cells and APCs, respectively [170]. It suggests that they play a role in the early activation of T cells and APCs.

CD40–CD40L Pathway

CD40 was firstly identified in 1985. CD40 is a 48-kDa type I transmembrane glycoprotein cell surface receptor and a member of TNF-R superfamily [171]. The CD40 genes map to chromosome 20 in humans and chromosome 2 in mice [172]. CD40 is constitutively expressed on APCs and its expression level could be dramatically upregulated upon activation of the cells. It is also expressed on some parenchymal cells including endothelial cells, fibroblasts, and smooth muscle cells [173–177]. The CD40 ligand (CD40L, CD154), a 32 to 33-kDa type II transmembrane protein, belongs to the TNF superfamily [178]. In both humans and mice, the genes that encode CD40L are located on the X chromosome [179]. CD40L is expressed on activated T cells ($CD4^+$ T lymphocyte lineage and a subset of $CD8^+$ T cells), NK cells, B cells, mast cells, eosinophils, basophils, DCs and platelets [180–182]. It has been known that CD40L expressed on platelets is involved in the formation and stabilization of thrombi [182]. The CD40–CD40L pathway is involved in both cellular and humoral immune responses. The receptor ligand interaction generates important

signals to promote B-cell activation, proliferation, Ig production, Ig isotype switching, the generation of B-cell memory, and T cell activation [183–186]. CD40–CD40L engagement is a key step in APC activation. CD40-mediated activation of dendritic cells is one of the mechanisms by which CD40 signaling promotes immune response. Upon CD40 ligation by CD40L, dendritic cells mature and become activated. Activated dendritic cells produce high levels of pro-inflammatory cytokines and chemokines, and upregulate the expression of MHC class II and other costimulatory molecules including B7-1, B7-2 [187–193]. All these factors contribute to the augmentation of immune response.

CD27–CD70 Pathway

CD27, a member of the TNF-R family, was identified in human cells in 1987. It is a 120-kDa transmembrane homodimeric glycoprotein with 55 kDa subunits which can generate a soluble form by proteolytic cleavage after T cell activation [194, 195]. CD27 is constitutively expressed on naïve T cells, B cells, and NK cells at low level and the expression of CD27 is transiently upregulated on memory or cytotoxic T cells [195–197]. CD70 is the ligand of CD27 and belongs to TNF family. Initially it was found as a marker of Reed-Sternberg cells in Hodgkin's disease and non-Hodgkin's lymphomas. CD70 is a homotrimeric type II transmembrane glycoprotein. It is expressed on APCs, activated T cells and B cells. Epithelial cells in the thymic medulla also express CD70 [198–201]. The interaction between CD27 and CD70 promotes T cell activation, proliferation and cytokine production. The costimulatory signals provided to T cells by CD27 engagement are CD28-independent. The CD27–CD70 pathway plays an important role in T cell–B cell interactions and T cell–DC communication. It is involved in regulating B cell activation and T-cell-dependent antibody production. The CD27–CD70 pathway also participates in the NK-mediated innate immunity [196, 197, 202–204]. In addition, CD27 can trigger apoptotic cell death [205] by binding to a proapoptotic protein – CD27-binding protein (SIVA).

OX40–OX40L Pathway

OX40 (also known as TNFRSF4, ACT35, TXGP1L, or CD134), a membrane-bound member of TNF-R superfamily, was initially characterized as a T-cell activation marker. It is a 50 kDa protein that is abundantly expressed on activated T cells rather than on naive T cells. Generally OX40 is induced on T-cells 12 to 24 hours after activation instead of immediately following the TCR ligation [206]. The expression of OX40 is preferentially found on activated $CD4^+$ T cells rather than on $CD8^+$ T cells and the level of expression declines within few days [206, 207]. Th2 cell subset is the predominant group expressing OX40, but Th1 cells can also express OX40 [208, 209]. In addition, OX40 is expressed on activated $CD4^+CD25^+$ Tregs. Ligation of CD28 by its ligands can promote the expression of OX40 although CD28 is not required for OX40 expression [210, 211]. The ligand for OX40 is OX40L (CD252) that belongs to TNF superfamily. Structurally, human OX40L shares approximately 40% sequence identity with murine OX40L [212]. OX40L is expressed on activated dendritic cells, B cells, T cells, macrophages and inflamed vascular endothelial cells [156, 206, 213].

OX40–OX40L pathway exhibits an extensive impact on the T cell immunity. OX40 signaling plays a key role in the survival and homeostasis of effector T cells, as well as promoting the generation of memory T cell (especially memory $CD4^+$ T cells) [206, 210]. It upregulates the expression of antiapoptotic proteins such as Bcl-xL and Bcl-2, and inhibits

apoptosis. OX40-deficient naive $CD4^+$ T cells are capable of IL-2 production, cell division, and expansion. However, the colony expansion is at low level and the cells die by apoptosis after activation [210]. OX40 engagement delivers signal to promote IL-17 production [214]. OX40 and OX40L expression could be induced in the $CD8^+$ T cells from intraepithelial lymphocytes of the gut, and it is related to cytotoxic-effector function [215]. In addition, in activated $CD4^+CD25^+$ Tregs, OX40 stimulation can profoundly inhibit Foxp3 gene expression and therefore downregulate the expansion of Tregs [216]. OX40-OX40L interaction also enhances T cell-dependent B cell proliferation and differentiation, as well as the immunoglobulin production [217].

Agents Targeting Costimulation

Belatacept

Because CD28–B7 pathway plays a key role in the T cell immune responses, it is rational to induce allograft acceptance by intervening in CD28–B7 interaction. However, the early attempts that directly blocked CD28–B7 pathway using anti-CD28 or anti-B7-1/B7-2 antibodies did not show the desired effects [218]. CTLA-4 was then selected as a surrogate because it binds to same ligands as CD28 with higher affinity and thus blocks the binding of CD28 with B7-1/B7-2. The first CTLA-4 biological agent is CTLA-4Ig (abatacept, Orencia) which is a fusion protein by recombining the extracellular portion of human CTLA-4 with the modified fragment crystallizable (Fc) region of human IgG1 [219, 220]. In this new molecule, the portion from the CTLA-4 molecule keeps the binding specificity of CTLA-4 with B7-1/B7-2, and the Fc region of the IgG1 molecule furnishes antibody-like half-life and opsonisation properties. Experiments with CTLA-4Ig demonstrated that it could effectively suppress T cell immune response not only by preventing CD28–B7-1/B7-2 engagement, but also by inducing the expression of IDO on DCs [127]. Initial studies indicated that CTLA-4Ig was remarkably effective in the prolongation of allograft survival in the rodent models [221, 222]. When combined with other costimulation blockers such as the agent targeting CD40, CTLA-4Ig exhibited the ability to induce donor-specific tolerance in some cases [223, 224]. However, its efficacy in further studies in nonhuman primate (NHP) models of kidney transplantation and in rodent models of skin transplantation was disappointing. CTLA-4Ig was unable to prevent transplant rejection [225–227]. In 2006, abatacept was approved by the Food and Drug Administration (FDA) for clinical use in the treatment of rheumatoid arthritis but not for transplantation.

Therefore, the second-generation CTLA-4Ig was designed by mutating the B7-binding domain of abatacept to increase its binding affinity for B7-1/B7-2 [228]. A daughter molecule of abatacept known as belatacept (LEA29Y, trade name Nulojix) was then selected. The differences between belatacept molecule and its parent compound were two amino acid substitutions (L104E and A29Y) in its B7 binding domain. These changes led to belatacept possessing approximately twofold higher binding affinity to B7-1 and fourfold higher binding affinity to B7-2, and slower dissociation rates for both ligands, compared to abatacept [228]. Previous study indicated that abatacept exhibited high binding affinity for B7-1 and low binding affinity for monomeric B7-2 [121]. Thus complete and equal blockade of the

costimulation pathway that is required for durable inhibition of allograft rejection cannot be achieved [122]. It partially explained why abatacept failed to prevent allograft rejection in some transplantation models. Therefore the strategy in the development of belatacept was devoted to increase its B7 binding affinity especially for B7-2. In vitro study demonstrated that this new compound obtained an approximately 10-fold increase in the inhibition of T-cell activity. Moreover, belatacept showed significant benefit in the prolongation of renal allograft survival in NHP kidney transplant model either as a monotherapy or in combination with conventional immunosuppressive agent MMF and methylprednisolone [228].

These results encouraged further studies and the first clinical trial to test the efficacy of belatacept on kidney transplantation was then conducted. It was a large-scale, multicenter, randomized, partially blinded, parallel group, phase 2 study. Total 218 adult renal allograft non-human leucocyte antigen (non-HLA) identical recipients were included in this 12-month study to compare the safety and efficacy of belatacept as a maintenance immunosuppressant to the conventional CsA. Patients were randomly assigned to the group of intensive regimen of belatacept, less-intensive regimen of belatacept, or CsA combined with steroids. Patients of all three groups received same induction therapy with basiliximab, MMF, and corticosteroids. Belatacept was administered as an intravenous infusion 10 mg/kg body weight every two weeks at early phase, and 5 mg/kg body weight every 4 or 8 weeks at late phase. The incidence of acute rejection at six months was similar among three groups. It was 6% for less-intensive belatacept group, 7% for intensive belatacept group, and 8% for CsA group. Further, at 12 months, the glomerular filtration rate (GFR) in belatacept-treated groups was significantly higher than CsA group (62.1, 66.3, and 53.5 ml per minute per 1.73 m^2 for group of less-intensive belatacept, intensive belatacept and CsA, respectively). Additionally, belatacept-treated groups showed the trend of lower incidence of CNI-related toxicities. Although patients in the CsA group received intensive lipid-lowering and antihypertensive treatment, the lipid levels and blood-pressure values in the belatacept groups were similar or slightly lower than those of CsA group [229].

Other open-label, phase II exploratory trials were conducted to examine the possibility to entirely replace CNIs and corticosteroids by belatacept-based maintenance regimen in the kidney transplant recipients [230, 231]. Kirk et al., investigated belatacept-based regimen as the maintenance immunosuppression in twenty living donor kidney transplantations [230]. All patients received alemtuzumab induction, monthly belatacept and daily sirolimus, and were randomized 1:1 to inject unfractionated donor bone marrow. After 1 year, sirolimus was allowed to be removed from the regimen in selected patients. Belatacept in combination with sirolimus effectively prevented kidney allograft rejection irrespective of bone marrow infusion. Seven of ten patients which were selected for sirolimus weaning were maintained rejection-free with belatacept monotherapy. In another 1-year trial of Ferguson et al., [231], eighty-nine recipients of living and standard criteria deceased donors randomly received belatacept-MMF, belatacept-sirolimus, or tacrolimus-MMF. Thymoglobulin (4 doses) and a short course of corticosteroids were administered as induction to all patients. By month 6, the incidences of acute rejection in belatacept-MMF group, belatacept-sirolimus group, or tacrolimus-MMF group were 12%, 4%, and 3%, respectively. Although belatacept-MMF group had a higher rate of acute rejection, it was still much lower than reported in other CNI- or steroid-avoiding regimens. At 12 months, more than two-thirds of patients of two belatacept-treated groups continued to apply the CNI- and steroid-free regimens. They appeared to confer higher GFR than others.

The possibility of switching from CNI-based regimens to a belatacept-based regimen was also investigated. In this randomized phase II trial [232], 84 renal transplant recipients who were 6 to 36 months after transplantation with stable graft function on a CNI-based regimen were switched to belatacept-based therapy. At 12 months, the GFR in the belatacept arm was about 5 ml/min higher than that of CNI arm (89 patients). Acute rejection rate was 7% in belatacept group but there was no graft loss. No acute rejection was found in CNI group. By month 12, in the CNI group, one patient died with a functioning graft, but no patient death occurred in the belatacept group. Another phase II trial also demonstrated that switching to belatacept-based regimen from the conventional CNI-based regimens conferred the improvement of renal function in kidney transplant recipients [233].

An international phase III trial termed as the Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression Trial (BENEFIT) was designed to further compare the efficacy and safety of belatacept-based regimen with CsA -based regimen [234]. 738 adult recipients of living and standard criteria deceased donors were recruited for this trial and 686 patients who met the criteria were enrolled. The objectives of this phase III trial were to evaluate, at 12 months compared to CsA -based immunosuppression, if belatacept-based regimens (less intensive and more intensive) could maintain similar rates of acute rejection and patient/graft survival, as well as superior renal function. As expected, belatacept-based regimens exhibited superior renal function, similar patient and graft survival rate compared to CsA -based group, and a trend of less chronic allograft nephropathy. However, the incidence of acute rejection at 12 months in the belatacept groups (22% in more intensive, 17% in less intensive) was higher than CsA group (7%).

Equivalent rates of patient and graft survival was confirmed by the subsequent 3-year BENEFIT follow-up [235]. Long-term renal function advantages were also observed in belatacept-based regimens. In addition, from year 2 to year 3, no cases of acute rejection were found in the belatacept groups. However, post-transplant lymphoproliferative disorder (PTLD) was more common in the belatacept groups, although the overall incidence is rather rare. Similar outcome was observed in another phase III trial named BENEFIT-EXT where adult recipients received allografts from extended criteria donors [236, 237].

The clinical phase II and III trials demonstrate that belatacept is effective in the prevention and treatment of allograft rejection in renal transplantation. Belatacept is associated with superior graft function, but higher rates of acute rejection episodes in early stage and an increased rate of PTLD compared to CsA. It is also associated with an improved cardiovascular risk profile. Belatacept was approved for clinical use in kidney transplantation by the FDA in June 2011.

ASKP1240

ASKP1240 (4D11) is a fully human IgG4 monoclonal antibody (mAb) against CD40. This antagonist anti-CD40 mAb was generated by using trans-chromosome mice [238]. ASKP1240 interrupts the CD40–CD40L interaction by masking and thus inhibits both humoral and cellular immune responses. Previous studies indicated that Chi220, a chimeric IgG1 mAb targeting CD40, depleted circulating B cells and increased the risk for cytomegalovirus infection [239]. To avoid the cytotoxicity of IgG1 antibody, ASKP1240 was designed as an IgG4 antibody which was a non-depleting antibody not to reduce the number

of $CD40^+$ cells including B cells, DCs, macrophages, and platelets. The recombinant IgG1 anti-CD40L mAbs, such as hu5C8 and IDEC-131, were found to cause thromboembolic complications and were halted for further development. The exact mechanisms of the thromboembolic events associated with anti-CD40L mAbs are still not fully understood. One speculated mechanism is that these anti-CD40L mAbs can cross-link with platelet Fc gamma receptors (FcγRs) resulting in platelet activation/aggregation. ASKP1240 is expected to not cause these unwanted effects because IgG4 has an extremely lower binding affinity for FcγRs and extremely lower complement activation capacity compared to IgG1 [240–242]. Additionally, this molecule was designed as a fully human antibody to lower its immunogenicity and to enhance efficacy and safety [243].

In an *in vitro* study that tested the cytotoxicity of ASKP1240 to CD40 expressing cells, ASKP1240 IgG4 (100 μg/mL) did not induce lyses of CD40 positive cells via antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) activities. Contrastingly, with same concentration, rituximab and ASKP1240 IgG1 did [244]. It implicates that the change of the constant region of ASKP1240 (to be converted from IgG1 to IgG4) is associated with the reduction of ADCC and CDC activities. It was reported that CD40L was involved in the stabilization of the formed platelet thrombi [245]. The influences of ASKP1240 and mu5C8 (a mouse anti-human CD154 mAb) on the platelet thrombus formation were also examined in this study. ASKP1240 was found to bind to both unstimulated and thrombin-stimulated human platelets. In contrast, mu5C8 bound to only stimulated platelets. Under physiological high shear stress conditions, mu5C8 destabilized platelet thrombi. However, ASKP1240 did not show the influences on the formation of platelet thrombus and the stabilization of the formed platelet thrombi under either low shear stress condition or high shear stress condition [244]. ASKP1240 inhibited human, monkey and rabbit peripheral blood mononuclear cell proliferation in a concentration-dependent manner. When ASKP1240 (1 or 10 mg/kg, weekly) was intravenously injected to *cynomolgus* monkeys, it dose-dependently inhibited delayed-type hypersensitivity reaction. The CD40 receptor saturation was found to correspond with the immunosuppressive effect of ASKP1240. In the safety study, ASKP1240 was administered weekly to *cynomolgus* monkeys at doses up to 100 mg/kg for 4 weeks. No significant decreases in the number of peripheral B cells were observed during study. There were also no evidences related to thromboembolic events and the production of anti-ASKP1240 antibodies [244]. The first investigation of the effect of ASKP1240 on preventing allograft rejection in nonhuman primate was performed in *cynomolgus* monkey kidney transplantation model [246]. Although the number of animals in each group was limited, ASKP1240 had shown promise in the prevention of renal allograft rejection. ASKP1240 monotherapy was demonstrated to dramatically prolong renal allograft survival. A further study was then conducted in same experimental model to elucidate the immunosuppressive effects of ASKP1240 and to pave the way for the clinical studies [247]. Two practical and clinically relevant treatment regimens (i.e., 2-week induction therapy and 6-month maintenance therapy) were applied in this study. In order to find out an optimal therapeutic dose and blood concentration of ASKP1240, four different dosages (i.e., 1 mg/kg, 5 mg/kg, 10 mg/kg and 20 mg/kg) were examined in both regimens. The results of the mixed-lymphocyte reaction (MLR) tests indicated that T-cell-mediated alloimmune responses were significantly inhibited in all ASKP1240-treated animals. Compared to untreated control group, the renal allograft survival times in all ASKP1240-treated animals were tremendously prolonged. The better graft survival results

were observed in maintenance therapy animals that received additional weekly ASKP1240 administration after the induction treatment. Donor-specific antibody (DSA) was detected in the induction treatment groups 3–8 weeks after ASKP1240 to be ceased, but not during drug treatment. However, in the maintenance treatment groups, DSA was found in most of animals treated with lower dose ASKP1240 (1 mg/kg or 5 mg/kg) in the period of drug administration, and within 2 months after DSA formation, most grafts among them were rejected. On the other hand, no DSA was detected in the animals treated with higher dose ASKP1240 (10 mg/kg or 20 mg/kg) during the treatment course. In the induction treatment groups, after drug cessation, anti-ASKP1240 antibody was detected in some animals irrespective of the dosage of ASKP1240. Contrastingly, in the maintenance treatment groups, anti-ASKP1240 antibody was only found in animals treated with lower dose ASKP1240 instead of in the animals treated with higher dose ASKP1240 [247]. These studies, together with many prior studies, further corroborate the fundamental importance of CD40–CD40L pathway in alloimmune response. Targeting CD40 to block this pathway is a feasible strategy to prevent allograft rejection. The results provide concrete evidences to support further studies, and they also indicate a need that ASKP1240 should combine with additional immunomodulatory agents to further improve allograft survival and function [248].

The effects of ASKP1240 combined with tacrolimus or MMF on allograft survival were then investigated in the kidney transplantation model in *cynomolgus* monkeys [249]. The study results verified that ASKP1240, as a monotherapy, dose-dependently prolonged renal allograft survival. ASKP1240 (with dosage of either 2 mg/kg or 5 mg/kg), in combination with MMF (15 mg/kg) or subtherapeutic dose of tacrolimus (1 mg/kg), showed a significantly better allograft survival results than monotherapy groups (Figure 1). These results suggest that ASKP1240 is a promising agent for both CNI sparing and CNI avoiding antirejection regimens. Further study to test ASKP 1240 pharmacokinetics (PK) and pharmacodynamics (PD) was also performed in *cynomolgus* monkey model [250]. It was found that, in both normal and renal transplanted *cynomolgus* monkeys, the serum concentration of ASKP1240 was positively correlated with the CD40 receptor occupancy by ASKP1240 (Figure 2). Immediately after the first dose, the serum concentration of ASKP1240 reached the maximum level and remained at this steady state level during the induction phase. During the maintenance period, the serum concentration of ASKP1240 decreased in a dose-related manner.

Compared to the same dose-treated groups, the serum ASKP1240 concentrations in transplanted monkeys were lower than those of normal monkeys. The CD40 receptor occupancy by ASKP1240 on $CD20^+$ B cells rapidly reached an almost saturated state after the first dose and remained stable during the induction phase either at a dosage of 2 mg/kg or 5 mg/kg. The occupancy of the CD40 receptors then decreased gradually during the maintenance phase. Compared to the normal animals, transplanted monkeys presented a significantly lower CD40 receptor occupancy rate in this period. For example in ASKP1240 5 mg/kg treatment groups, on day 70, CD40 receptor occupancy rate by ASKP1240 in transplanted monkeys was 5.5% ± 14.1%, whereas it was 72.8% ± 3.4% in normal monkeys. The results imply that transplanted monkey, compared to normal monkey, may possess a different ASKP1240 metabolic profile. One possible reason to cause these differences is that CD40 overexpression may occur in the transplanted monkey. These factors have to be taken into consideration during designing optimal ASKP1240 dosing regimens. Besides renal

allograft, ASKP1240 has been also demonstrated to delay islet, and hepatic allograft rejection in nonhuman primate models [251, 252].

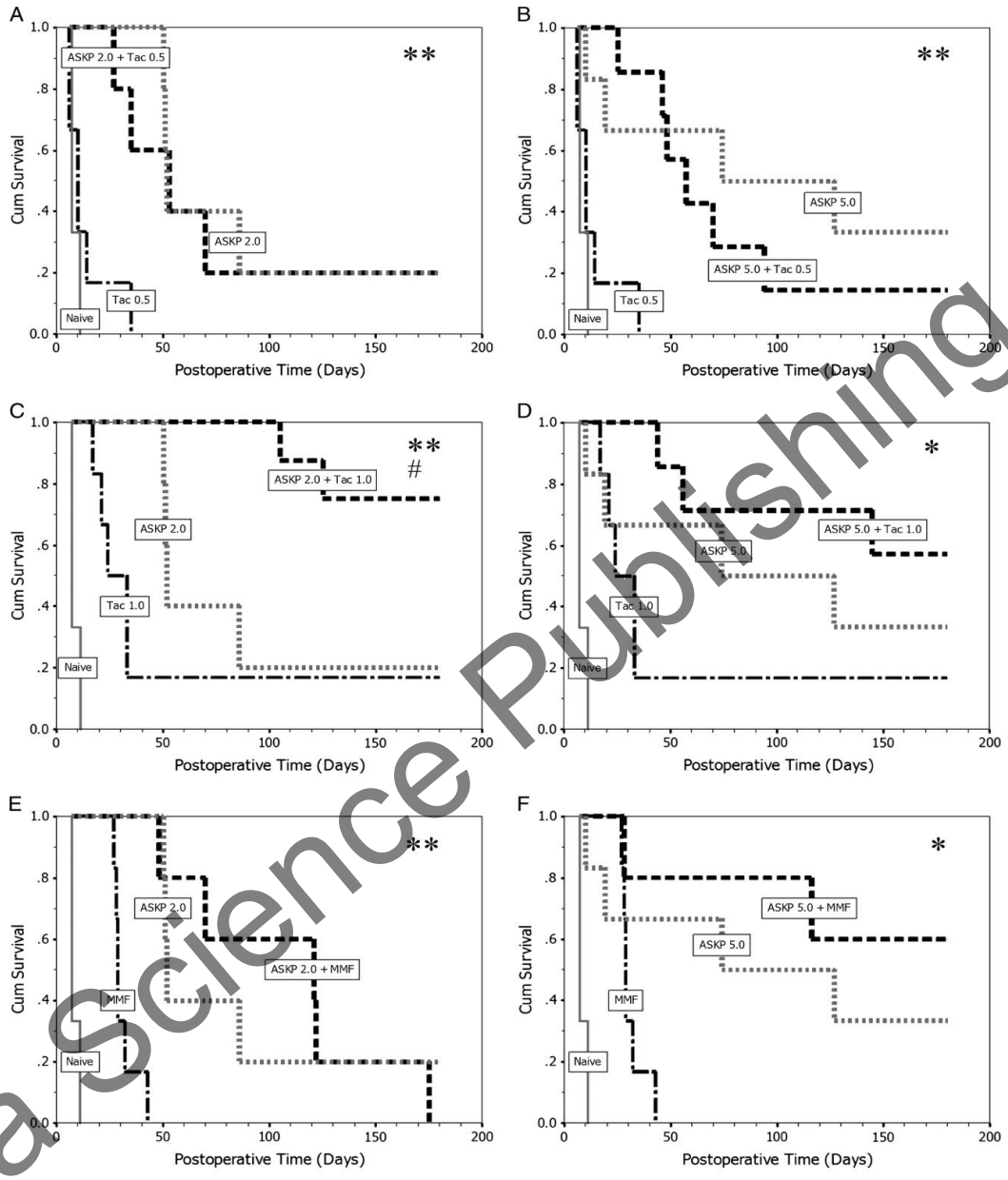

Figure 1. Renal allograft survival for each group. The curves represent renal graft survival for naive control (gray solid line), tacrolimus or MMF monotherapy (black dot dash line), ASKP1240 monotherapy (gray dotted line), and combination therapy (black dashed line) group. (Tac 0.5: tacrolimus 0.5 mg/kg, Tac 1.0: tacrolimus 1.0 mg/kg, ASKP 2.0: ASKP1240 2.0 mg/kg, ASKP 5.0: ASKP1240 5.0 mg/kg, MMF: mycophenolate mofetil 15 mg/kg; *: $p < 0.05$ ASKP1240 combination group *vs.* Tacrolimus or MMF monotherapy group, **: $p < 0.01$ ASKP1240 combination group *vs.* tacrolimus or MMF monotherapy group, #: $p < 0.05$ ASKP1240 combination group *vs.* ASKP1240 monotherapy group).

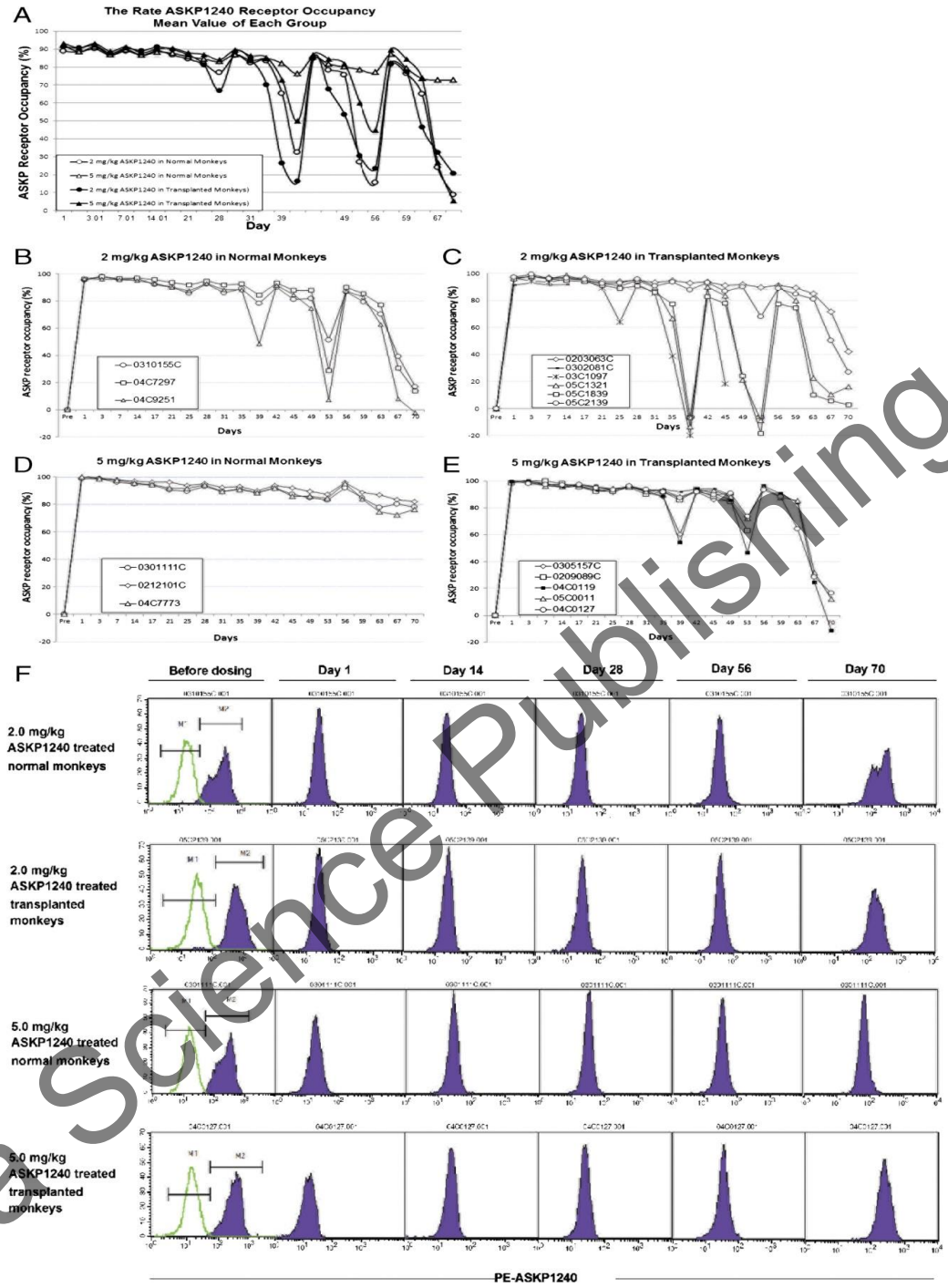

Figure 2. PD study. Mean values of CD40 receptor occupancy by ASKP1240 in the low- and high-dose ASKP1240-treated normal and transplanted monkey groups (A). CD40 receptor occupancy by ASKP1240 in the 2.0 mg/kg ASKP1240-treated normal monkey group (B), 5.0 mg/kg ASKP1240-treated normal monkey group (C), 2.0 mg/kg ASKP1240-treated transplanted monkey group (D), and 5.0 mg/kg ASKP1240-treated transplanted monkey group (E). The representative MFI data show the CD40 receptor occupancy by ASKP1240 in four groups; the green lines represent the controls (F).

ASKP1240 has shown promise in the recent phase I clinical trials [253, 254]. A randomized, double-blind, placebo-controlled, single ascending dose study was performed in 108 healthy subjects to investigate the safety, tolerability, PK and PD profile of ASKP1240. ASKP1240 was set in 12 different dose levels from 0.00003 mg/kg to 10 mg/kg, and each fasted subject was intravenously injected a single dose. ASKP1240 showed nonlinear PKs and the mean of ASKP1240 half-life ($t_{1/2}$) dose-relatedly increased. B cell CD40 receptor occupancy by ASKP1240 increased in a dose-dependent manner, and reached the maximum level when the dosage was more than 0.01 mg/kg. Among the doses above 0.01 mg/kg groups, the mean maximal CD40 receptor occupancy was similar, but the duration of receptor occupancy was dose-dependently prolonged. No drug-induced cytokine release or thromboembolic events were observed in tested subjects. It demonstrated that ASKP1240 was safe and could be well tolerated by healthy subjects [253].

To further evaluate safety, tolerability, PK and PD of ASKP1240, subsequently, a phase 1b, randomized, double-blind, parallel group, placebo-controlled clinical trial was performed in 46 *de novo* kidney transplant recipients who received their kidney from a living or deceased donor. Patients were randomly assigned to each of four ASKP1240 dose groups (*i.e.*, 50 mg, 100 mg, 200 mg, and 500 mg) or placebo group. Subjects were intravenously administered a single dose of 1 of 4 ASKP1240 dose levels or placebo. No induction therapy was allowed. All recipients received standard maintenance immunosuppressive therapy per each center's protocol. All subjects except one in 50 mg group were followed 90 days. ASKP1240 presented non-linear PKs and all four dose levels were well tolerated in kidney transplant recipients. ASKP1240 C_{max} linearly increased in a dose-related manner. Maximal receptor occupancy (MaxRO) for B cell CD40 was reached in all four dose groups, and the duration of MaxRO dose-relatedly increased. Total 7 cases acute rejection occurred in early post-transplantation period *i.e.*1 case in the placebo group and 3 cases each in the 50 mg group and the 500 mg group. The incidence of infection was not related to dose levels. No patient deaths, graft losses or malignancies occurred in the study [254]. A phase 2a, randomized, open-label, active control, multi-center clinical trial is ongoing now [255]. This study will be conducted in *de novo* kidney transplant recipients to intensively evaluate the effectiveness and safety of ASKP1240. The purpose of this study is, through comparing the efficacy of both ASKP1240 based CNI avoidance regimen (basiliximab induction + ASKP1240 + MMF + steroids) and ASKP1240 based CNI minimization-MMF avoidance regimen (basiliximab induction + ASKP1240 + tacrolimus + steroids) with the standard immunosuppressive regimen (basiliximab induction + tacrolimus + MMF + steroids), paving the road toward the clinical use of ASKP1240 against allograft rejection.

Conclusion

Theoretically, targeting costimulatory pathway with costimulation blockers is an optimal strategy for the prevention and treatment of allograft rejection. This approach acts only on the T cells activated by TCR stimulation, and thus preserves the integrity of remaining components of the immune system and avoids off-target side effects. It is also expected to exhibit the potential in the induction of immune tolerance. With the efforts over the past several decades, belatacept as the first approved costimulation blocker for transplant rejection

is now available in the market. Other promising agents are in the pipeline of preclinical and clinical studies. However, the complexities of T cell costimulation are far more than expected. Although it is clear that costimulatory signals play a key role in alloimmune response, few relative mechanistic details have been revealed. Many questions such as co-signalling how to regulate T effector/memory cells and Tregs, the expression of costimulatory molecule how to be regulated, the signaling cascades of both known and novel costimulatory and coinhibitory molecules etc. need to be answered. Belatacept ushered in the era of costimulation blockers as the agent to prevent and treat allograft rejection. It has shown the benefits in improving kidney function and reducing cardiovascular risk factors. However, it is still too early to conclude if these biologics will become the gravedigger of conventional immunosuppressants. Further studies in these areas are needed to better understand the detail roles of costimulatory and coinhibitory signals during T cell activation and differentiation. It may lead to the discovery of novel immunosuppressive agents or/and novel therapeutic approaches, and eventually to reach the goal of induction of transplantation tolerance.

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Chapter 14

Adhesion Molecules: Therapeutic Targets for Allograft Rejection and Ischemia-Reperfusion Injury

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Abstract

Migration of activated leukocytes into inflamed tissue is a major factor in contributing to graft rejection for transplanted organs and tissues. T cell infiltrates are characteristic signs of acute cellular graft rejection. Leukocytes in the unstimulated state do not readily adhere to the vascular endothelium. However, inflammatory stimuli lead to the expression of adhesion molecules on the endothelial cell surface that promotes the adhesion and extravasation of activated leukocytes from the circulation into inflamed tissue. Key molecules which play an important role in this process are P- and E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the endothelial cells, and their respective counter-structures, P-selectin glycoprotein ligand-1 (PSGL-1), leukocyte function associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4) on the leukocytes. Inhibition of these adhesion molecules prevents leukocyte adhesion and activation *in vitro*.

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In animal models, anti-adhesion molecules agents ameliorate ischemia-reperfusion injury, graft rejection and improve graft outcome. Clinical trials with humanized monoclonal antibodies against adhesion molecules have shown the usefulness and efficacy in organ transplantation setting including bone marrow, kidney and islet transplantation. Therefore, the use of anti-adhesion antibodies is one of the attractive therapeutic strategies in organ transplantation.

Keywords: adhesion molecules, organ transplantation, ischemia-reperfusion injury, selectin, integrin, immunoglobulin superfamily

Abbreviations

AS-ODN: Antisense oligonucleotides
ATG: Antithymocyte globulin
CD: Cluster of differentiation
ECM: Extracellular matrix
ICAM-1: Intercellular adhesion molecule-1
IRI: Ischemia-reperfusion injury
JAM-1: Junctional adhesion molecule-1
LAD-1: Leukocyte adhesion deficiency-1
LFA-1: Leukocyte function associated antigen-1
mAb: Monoclonal antibody
Mac-1: Macrophage antigen complex-1
PSGL-1: P-selectin glycoprotein ligand-1
VCAM-1: Vascular cell adhesion molecule-1
VLA-4: Very late antigen-4

Introduction

In organ transplantation, the control of the immune response of an organ graft recipient is of crucial importance for the post-transplant graft acceptance and function. Immunological changes occurring in the graft before and after organ retrieval, the transplantation procedure itself and the organ recipient's clinical state contribute to the immune response.

Inflammatory disorders including graft rejection are mediated by activated leukocytes, particularly T-lymphocytes, which migrate into inflamed tissue and amplify or perpetuate immune reaction. Infiltration of T-cells is characteristic of acute cellular graft rejection. It is now generally accepted that leukocyte emigration from the bloodstream to extravascular sites of inflammation or immune reactivity is a multistep process, controlled by the regulated expression of adhesion molecules and their receptors [1, 2] (Figure 1). Adhesion molecules play a central role in regulating the infiltration of leukocytes into the graft during ischemia-reperfusion injury (IRI) and rejection [3−7]. In this chapter, we summarize the role of endothelial cell adhesion molecules in immune inflammation and graft rejection, and we review the intervention by monoclonal antibodies (mAb) and blocking agents.

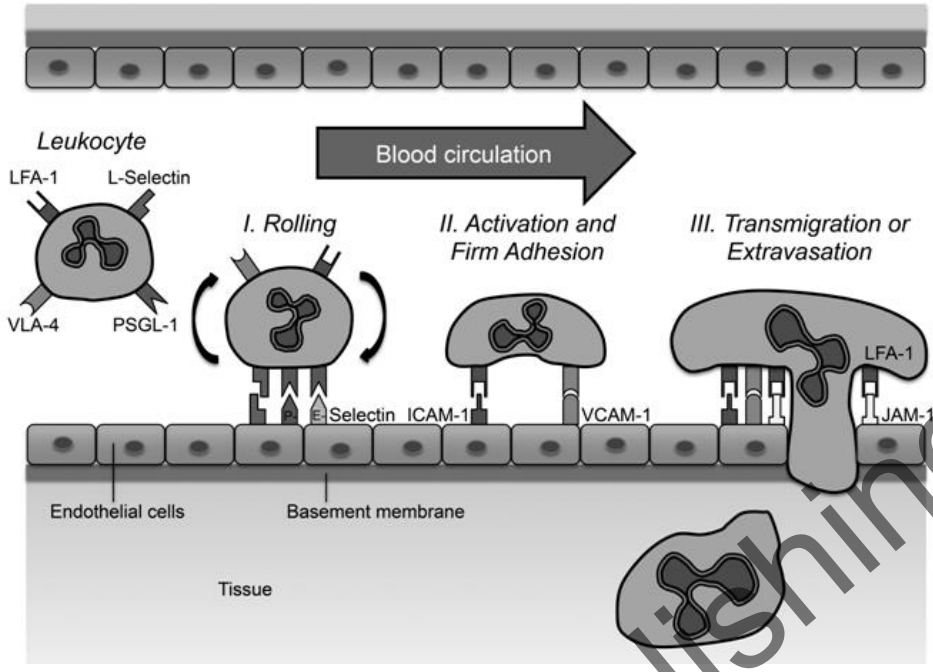

Figure 1. The multistep adhesion cascade for leukocyte adhesion to endothelium and extravasation. In response to inflammatory stimuli, adhesion molecules including selectins, ICAM-1 and VCAM-1 are upregulated on endothelial cells and leukocytes. (I) Leukocytes initially roll along the activated vascular endothelium through selectin-mediated interactions. (II) Firm adhesion of leukocytes to endothelium is induced through binding of integrins on the leukocyte surface to ICAM-1 or VCAM-1 on the endothelium surface. (III) Subsequently, leukocytes transmigrate through the microvascular endothelium into the underlying tissue via interactions with endothelial cell-cell junction molecules including JAM-1. Abbreviations: LFA-1: leukocyte function associated antigen-1; VLA-4: very late antigen-4; ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1; PSGL-1: P-selectin glycoprotein ligand-1; JAM-1: junctional adhesion molecule-1.

The Role of Adhesion Molecules in Inflammation and Graft Rejection

Adhesion molecules have been divided into three major families: the selectins, the integrins and the immunoglobulin superfamily (Table 1). Selectins (P-, E- and L-selectin, also known as CD62P, CD62E and CD62L) are small proteins that initiate leukocyte extravasation by interaction with the vascular endothelium. The initial leukocyte rolling on the inflamed endothelial surface has been shown to be mediated mainly by low-affinity interactions between the selectin family and its ligands [8−12]. P- and E-selectin are predominantly expressed on activated endothelium, induced by inflammatory mediators such as endotoxin, cytokines, histamine and complement fragments, and serve as adhesion receptors for P-selectin glycoprotein ligand-1 (PSGL-1). L-selectin is expressed mainly on polymorphonuclear leukocytes, monocytes and some T cells. Naive T cells express high amounts of L-selectin, which promotes their migration into peripheral lymph nodes where L-selectin ligands are constitutively expressed [13]. All three selectins facilitate leukocyte rolling *in vitro*, and L- and P-selectin mediate leukocyte rolling and recruitment *in vivo* [14].

Following initial rolling interactions, firm leukocyte adhesion on activated endothelium is mediated by integrin receptors and their ligands. The leukocyte function associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4) are primary antigens which facilitate firm adhesion of T lymphocytes to inflamed endothelium, and respectively bind to intercellular adhesion molecule-1 (ICAM-1, CD54) and vascular cell adhesion molecule-1 (VCAM-1, CD106) induced on activated endothelium by inflammatory mediators [15−18].

Table 1. Adhesion molecules in organ transplantation

Adhesion Molecule	CD	Expression	Ligand	Function	Inhibitor in clinical study for organ transplantation [Reference]
Selectins					
L-Selectin	CD62L	Leukocytes	Sialylated and fucosylated structures expressed on glycoproteins	Adhesion of Leukocytes to ECs; Lymphocyte homing	
P-Selectin	CD62P	ECs	PSGL-1	Adhesion of Leukocytes to ECs; Rolling phenomenon	
E-Selectin	CD62E	ECs	PSGL-1	Adhesion of Leukocytes to ECs; Rolling phenomenon	
Integrins					
LFA-1 (αLβ2)	CD11a/ CD18	Leukocytes	ICAM-1	Adhesion of Leukocytes to ECs	Odulimomab [93, 94] Efalizumab [95-97]
Mac-1 (αMβ2)	CD11b/ CD18	Monocytes, Macrophages, Granulocytes, NK cells	ICAM-1	Adhesion of Leukocytes to ECs	
VLA-4 (α4β1)	CD49d/ CD29	Leukocytes except Neutrophils	VCAM-1 CS1 Fibronectin	Cell-Cell and Cell-Matrix adhesion	
Ig superfamily					
ICAM-1	CD54	Leukocytes, ECs	LFA-1 Mac-1	Cell-Cell Adhesion	Enlimomab [98, 99] ISIS 2302 [100]
VCAM-1	CD106	Activated ECs, Dendritic cells	VLA-4	Adhesion of Leukocytes to ECs	

Abbreviations: CD: cluster of differentiation; LFA-1: leukocyte function associated antigen-1; Mac-1: macrophage antigen complex-1; VLA-4: very late antigen-4; ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1; PSGL-1: P-selectin glycoprotein ligand-1; EC: endothelial cell.

LFA-1 is a heterodimer of the $\alpha_L\beta_2$ integrin subunits (CD11a/CD18) and VLA-4 is a heterodimer of the $\alpha_4\beta_1$ integrin subunits (CD49d/CD29). LFA-1 and VLA-4 expression is increased on effector/memory T cells compared to naive T cells. Adhesion mediated by LFA-1 and VLA-4 also plays a role in the extravasation of immune cell through the endothelial cell layer into the underlying tissue. LFA-1 is thought to participate in transendothelial migration via binding to junctional adhesion molecule-1 (JAM-1) which is expressed on endothelial cells [19].

The inflammatory stimuli from IRI and the rejection responses against the transplanted graft tissue increase the expression of adhesion molecules in organ grafts [20, 21]. The activation of endothelial cells and the expression of endothelial cell adhesion molecules are critical for the effective recruitment of leukocytes into inflammatory sites. In the course of an immune response, the vascular endothelial cells located in physiological site interact with all leukocytes in various aspect of the immune response from the initial recruitment of cells into an inflamed site, to the reciprocal activation of leukocytes and endothelial cells and to the angiogenesis of the chronic healing inflammatory reaction [22]. Leukocyte-endothelial cell adhesion and multiple regulated families of adhesion molecules are critical to the above mentioned functions. For this reason, vascular endothelial cell adhesion molecules constitute a highly attractive target for the therapeutic manipulation of immune responses, including therapeutic intervention in organ transplantation.

Therapeutic Targets

Selectins

Selectins mediate the first contact between stimulated endothelial cells and leukocytes. Vascular endothelium activated by stimulation of ischemia or other insults begin to express P-selectin within minutes, and then E-selectin. The blockade of selectin function has been shown to be effective in inhibiting neutrophil extravasation in IRI models [23], and this could be beneficial in organ transplant settings. Brain death or ischemia can induce an inflammatory response of neutrophil binding to the endothelium after reperfusion [24]. The following neutrophil activation amplifies the inflammatory response, which can damage the transplanted organ. Blocking of the selectin function, particularly P-selectin, has reduced IRI in some animal models. It was demonstrated that blockade of P-selectin provided protection equivalent to CD18 blockade after ischemia and reperfusion of the rabbit ear [25]. P-selectin selective blockade and nonselective selectin blockade by Fucoidin (sulfated fucosylated polysaccharide derived from seaweed, which binds to both L- and P-selectin) caused a reduction in endothelial cell injury after cold ischemic preservation of the lung [26].

Integrins

Integrins are heterodimeric cell surface proteins composed of two non-covalently linked polypeptide chains (α and β), themselves consisting of extracellular and transmembrane segments and cytoplasmic tails. The extracellular domains bind to various ligands, including extracellular matrix (ECM) proteins, complement components, and cell surface proteins.

Integrins are subdivided into groups depending upon the β-chain. Eight different β-chains have been identified so far, with β_1 (CD29) and β_2 (CD18) being the most important for cell trafficking and adhesion interactions.

The β_1 integrins include VLA-l to VLA-6. They mediate the binding of different cells to the ECM and control leukocyte localization in the sites of inflammation. For instance, VLA-4 ($\alpha_4\beta_1$, CD49d/CD29) is a lymphocyte receptor for ECM and also a receptor for vascular

endothelium. Therefore, an additional pathway for adhesion and extravasation of leukocyte is established by binding to fibronectin (an ECM protein) and to VCAM-1 expressed on endothelial cells [27−31]. Monotherapy with anti-VLA-4 mAb prolonged the survival of mouse cardiac allografts, and combination therapy of anti-VLA-4 with anti-VCAM-1 resulted in further prolongation of allograft survival [32]. In mouse islet transplantation, treatment with anti-α_4 integrin antibody or with anti-VCAM-1 antibody led to long-term survival of islet allografts [33], but the recipient mice rejected a second donor-type islet allograft, indicating that tolerance was not induced.

CD18 is the common subunit of the β_2 integrin family. The β_2 integrins are essential for adhesion of lymphocytes and other cells. CD18 forms non-covalent heterodimers with four different α subunits: α_L (CD11a) α_M (CD11b) α_X (CD11c) and α_D (CD11d). This group includes $\alpha_L\beta_2$ (CD11a/CD18 or LFA-1), $\alpha_M\beta_2$ (CD11b/CD18 or macrophage antigen complex-1: Mac-1). LFA-1 and Mac-1 bind to ICAM-1 and mediate adhesion to the endothelium and transmigration. LFA-1/ICAM-1 and Mac-1/ICAM-1 interactions may be responsible for changes in leukocyte shape and for the initial invasion of these cells into and through the endothelial layer [34-37]. LFA-1 is expressed on all leukocytes and it is critical for adhesion and de-adhesion of lymphocytes [38]. The expression of Mac-1 is generally limited to the myeloid cells (monocytes, macrophages and granulocytes) and natural killer cells [15]. Mac-1 mediates adhesion of myeloid cells to other cells, fibrinogen and ligands that become insolubilized during activation of the complement and clotting cascades [39−41].

One of the approaches in targeting the CD11CD18/ICAM-1 pathway in allotransplantation has been to block the ICAM-1 ligand CD11CD18 by mAbs directed against CD11, CD18 or LFA-1. Anti-CD18 mAbs inhibit polymorphonuclear leukocytes adhesion to activated endothelial cells *in vivo* [42]. In animal models, Anti-CD18 mAbs have been shown to attenuate IRI [43] and also have immunosuppressive effects [44]. However, chronic inhibition of CD18 function is likely to increase the risk of infection [45, 46]. Patients with a mutated CD18 gene (leukocyte adhesion deficiency-1 [LAD-1]) are characterized by a deficiency in β_2 integrin-mediated adhesion and suffer from recurrent severe infections, abscesses formation and cell-mediated immune deficiencies that lead to chronic illness and death [47, 48].

Since LFA-1 is the only member of the β_2 integrin family expressed on T lymphocytes, inhibition of CD11a function more selectively blocks T cell adhesive interactions. LFA-1 is a key component of the immunological synapse and provides critical co-stimulatory signals during the activation of CD4 and CD8 T cells during interaction with antigen-presenting cells [49−52]. Treatment with anti-LFA-1 mAbs has been very effective in inhibiting acute rejection and prolonging survival of allografts in some animal models. Anti-CD11a mAb monotherapy was shown to induce significant prolongation of cardiac allograft survival in a murine model, but was not sufficient enough for tolerance induction [53−55]. On the other hand, combined anti-LFA-l and anti-ICAM-l mAb therapy induced indefinite graft survival and donor-specific tolerance [55]. In murine corneal transplantation, however, combined treatment with LFA-1 and ICAM-1 mAbs did not reduce the rejection rate any further than anti-LFA-1 mAb alone, whereas anti-LFA-1 mAb treatment resulted in a significant reduction in the incidence of rejection as well as a prolongation of the mean survival time compared to the untreated group [56]. In a mouse islet allograft transplantation model, anti-LFA-l monotherapy was found to be efficacious in inducing significant prolongation of allograft survival and donor-specific tolerance [57, 58]. Sadahiro et al. compared the relative efficacy

of anti-CD18a and anti-VLA-4 in a rabbit heterotopic cardiac transplant model and found that T cellular rejection grade and vascular rejection were significantly lower in animals treated with anti-CD18 than with anti-VLA-4 [59]. This study suggested that anti-VLA-4 mAb may be disproportionate to cellular rejection whereas anti-CD18 mAb is promising therapeutic agent for both cellular and vascular rejection.

Immunoglobulin Superfamily

In this superfamily, the key molecules to promote the adhesion and extravasation of activated immune cells include intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). ICAM-1 is expressed constitutively on leukocytes, endothelial cells and several other cells, and the expression is dramatically upregulated on endothelial cells by inflammatory mediators. Thus, many cell types express ICAM-1 and can use ICAM-1 to deliver signals into T cells through interactions with LFA-1 on the T cell surface. T cell dependent cytotoxicity may be influenced by ICAM-1 and can be inhibited by blocking the binding of ICAM-1 to its receptors including LFA-1 and Mac-1 [60−62]. In a rat small bowel transplant model, Yamataka et al. reported that anti-ICAM-1 mAb was able to delay the onset of acute allograft rejection [63]. In non-human primates with renal allografts, a 12 days induction monotherapy with anti-ICAM-1 mAb significantly prolonged allograft survival, attenuated graft T cell infiltration and reduced arterial endothelial inflammation. In addition, ICAM-1 inhibition in this study successfully reversed pre-existing rejection. Anti-ICAM-1 mAb also bound to circulating recipient T cells, indicating a potential additional role of the antibody in interfering with antigen presentation and/or T cell interactions [64].

The observed beneficial effects of either anti-ICAM-1 or anti-LFA-1 mAb monotherapy in animal allotransplant models have promoted investigation of potential synergistic effects of these treatment modalities, while both agents target the ICAM-l/LFA-l pathway. Several studies have been performed *in vivo* in various animal allotransplant models. In a mouse cardiac allograft model, anti-ICAM-l or LFA-1 mAb alone was insufficient for prolonged tolerance, but combined, these two mAbs therapy induced donor-specific tolerance and indefinite graft survival [55]. In contrast, Brandt et al. reported that treatment with anti-ICAM-l and anti-LFA-l mAbs alone or in combination had rather no effect on allograft survival after heterotopic heart transplantation between fully incompatible rat strains [65]. In another study, a combination treatment of anti-ICAM-l and anti-LFA-l mAbs did not prolong allograft survival in fully mismatched mouse skin transplantation. Skin graft survival, however, was remarkably prolonged when either MHC class I or class II-disparate strains were used, indicating that the strain combination is critically important for the tolerance induction [66]. In the rat hind-limb-cremaster transplantation model, mAbs against LFA-1 or ICAM-1 alone inhibited the activation of leukocytes at the microcirculatory level but did not prolong graft survival. However, the combination of anti-ICAM-1 and anti-LFA-1 mAbs was able to significantly prolong allograft survival [67].

The literature on the use of mAb against anti-adhesion molecules in liver IRI or transplantation is still limited due to the special immunological situation of the liver. In rat partial liver ischemia model, the intraportal injection of anti-ICAM-1 mAb after reperfusion significantly reduced the hepatocellular necrosis, restored the hepatic tissue blood flow and

significantly suppressed the serum levels of liver enzymes during reperfusion [68]. Pre-treatment with mAbs to ICAM-1 plus LFA-1 increased the survival rate, whereas pre-treatment with mAb to ICAM-1 alone failed to influence the survival rate [69]. In rat liver transplantation model, anti-ICAM-1 mAb improved hepatic microvascular perfusion and reduced post-sinusoidal white blood cells adherence, but did not make a major contribution to early graft function [70]. The single administration of anti-ICAM-1 plus anti-LFA-1 mAbs significantly prolonged allograft survival, yet permanent unresponsiveness could not be induced [71].

Another approach in inhibition of ICAM-1 function is the use of antisense oligonucleotides (AS-ODN). AS-ODN are short synthetic oligonucleotides (10 to 25 bases in length) designed to hybridize to the RNA (sense strand) that encodes the protein of interest. On binding to an mRNA, the oligonucleotide may inhibit gene expression of the target protein by Watson-Crick base pairing, depending in part on the target site of the mRNA and the chemical class of oligonucleotide [72−74]. Once the mRNA is bound by the AS-ODN, translational arrest occurs and the mRNA is rapidly degraded by RNAses. Phosphorothioate oligonucleotides have sulphur substituted for one of the nonbridging oxygen in the phosphate backbone, resulting in oligonucleotides with markedly enhanced stability towards cellular and serum nucleases [75, 76]. ICAM-1 deficient mice showed the evidence of protection against IRI [77, 78]. AS-ODN to ICAM-1 has been shown to bind ICAM-1 mRNA in a sequence-specific manner and block protein expression [79]. In a murine heart allograft model, the treatment with AS-ODN alone prolonged allograft survival, and combination therapy with LFA-1 mAb induced donor-specific tolerance [80]. Haller et al. demonstrated that AS-ODN for ICAM-1 protects the kidney against IRI and enhanced immediate graft function in rat renal transplantation [81, 82]. Finally, they succeeded in showing that ICAM-1 suppression with AS-ODN not only obviates delayed graft function, but also improves chronic isograft survival [83].

VCAM-1 is expressed on activated endothelium, dermal and lymph node dendritic cells and bone stromal cells. VCAM-1 mediates the adhesion between lymphocytes and activated endothelium via its ligand VLA-4. The primary ligands of VLA-4 include VCAM-1 and an alternately spliced form of fibronectin, CS1. These two ligands are associated with inflammation. VCAM-1 expression is induced by inflammatory cytokines [17] and the CS1 form of fibronectin is found at sites of inflammation such as graft rejection [84]. The binding of VCAM-1 by VLA-4 on T cells also has co-stimulatory effects, and these effects can be inhibited by mAbs against either VLA-4 on the surface of T cells or VCAM-1 [30, 85, 86]. In a murine cardiac allograft model, treatment with anti-VCAM-1 mAb suppressed allograft rejection and induced long-term graft acceptance associated with inactive infiltrating T cells [87].

Several studies have assessed the treatment targeting both the CD11CD18/ICAM-1 and VLA-4/VCAM-1 adhesion pathways. In a rat heart transplantation model, combined anti-LFA-1 and anti-VLA-4 mAb therapy showed a modest prolongation of graft survival [88]. In a follow-up study, anti-Mac-1 mAb decreased the graft infiltration of macrophages, but no additional impact on graft survival time was found [89].

Human CLINICAL TRIALS with Anti-Adhesion Molecules Agents in Bone Marrow, Kidney and Islet Transplantation

Since the first report on the use of LFA-1 mAb in clinical bone marrow transplantation was published in 1986 [90], there is general interest in the use of anti-adhesion molecules agents in allograft rejection.

Based on previous experimental findings [90, 91], Fischer et al. used anti-CD11a mAb (murine IgG1 antihuman CD11a mAb, 25.3; odulimomab) to prevent graft failure and rejection after HLA nonidentical bone marrow transplantation in children with severe inherited diseases. They found a beneficial efficacy to increase the rate of engraftment and survival in multicenter trial [92].

In solid organ transplantation, odulimomab was first tested in kidney transplant recipients. Odulimomab was used in the treatment of histologically documented first acute rejection in first kidney transplantations, and this study suggested that odulimomab was inefficient in reversing ongoing acute rejection [93]. A randomized multicenter trial comparing odulimomab with rabbit antithymocyte globulin (rATG) as induction treatment in first kidney transplantations was published [94]. This trial demonstrated a better clinical tolerance and a possible beneficial effect on renal function recovery of odulimomab, whereas there was no statistically significant difference in the incidence or severity of acute rejection, nor in the patient or graft survival.

A humanized IgG1 antihuman CD11a mAb (efalizumab) was tested in a renal transplant study. A phase I/II randomized open-label multicenter trial was initiated [95] where thirty-eight patients undergoing their first living donor or deceased renal transplant were randomized to receive efalizumab 0.5 or 2 mg/kg weekly subcutaneously for 12 weeks. The frequency of patient survival, graft survival and acute rejection was similar between combination therapy with efalizumab, cyclosporine, mycophenolate mofetil and steroids compared with half-dose cyclosporine, sirolimus and prednisone. However, 3 of 38 patients (8%) who were treated with higher doses of efalizumab and full dose cyclosporine developed post-transplant lymphoproliferative disease, and efalizumab was withdrawn from clinical use in April 2009.

Efalizumab was used in islet transplantation in type I diabetic patients as well. Posselt et al. reported that all eight patients treated with the efalizumab-based immunosuppressive protocol achieved insulin independence and four out of eight patients became independent after single islet transplantation [96]. In another study, efalizumab-based immunosuppressive protocol was compared with the Edmonton protocol that utilized the combination of daclizumab intravenous administration, sirolimus and low-dose tacrolimus oral administration [97]. In this study, efalizumab-based protocol included subcutaneous administration of efalizumab and oral administration of mycophenolate mofetil and tacrolimus. This pilot study demonstrated that only two out of eight Edmonton protocol treated patients achieved insulin independence after single islet transplantation, while all four patients treated with the efalizumab-based regimen achieved lower morbidity and remained insulin-independent while on efalizumab. However, the continuation of these two trials was terminated by withdrawal of efalizumab from the market due to safety concerns, thus long-term follow up was limited.

A murine IgG2a antihuman ICAM-1 mAb, enlimomab (BIRR1) has been tested in human kidney transplantation. A phase I clinical trial was completed with 18 kidney transplant patients to determine the optimal dose levels [98]. Trough BIRR1 serum levels of > 10 μg/ml was associated with a reduction in a rate of delayed graft function and acute rejection compared to the lower-dose group. These results established a dosing schedule and the clinical safety of enlimomab, and suggested that the addition of anti-ICAM-1 mAb to triple drug therapy (azathioprine, corticosteroid and cyclosporine) may be useful in controlling allograft rejection and possibly in limiting reperfusion injury.

A randomized multicenter, double-blind trial of enlimomab for the prevention of acute rejection and delayed onset of graft function in cadaveric renal transplantation in an unselected population of patients was subsequently performed [99]. There was no significant difference in the incidences of first acute rejection at 3 months between the placebo and enlimomab groups, and enlimomab did not reduce the risk of delayed onset of graft function. Patient and graft survival at 1 year were comparable in both groups. These data suggested that anti-ICAM-1 mAb therapy might be worthwhile to re-evaluate especially in selected patients with higher risk for rejection or delayed graft function, rather than the general renal transplant patients.

Safety and efficacy of ICAM-1 phosphorothioate antisense oligodeoxynucleotide (ISIS 2302) were assessed in renal transplantation. Phase I and phase II placebo-controlled, double-blinded clinical trials of ISIS 2302 were conducted in recipients of deceased donor kidneys [100]. In this study, ISIS 2302 produced slightly improved renal function and had no evident toxicity. However, ISIS 2302 did not further reduce the rate of acute rejection episodes or increase graft survival compared to a concentration-controlled cyclosporine-predonisone regimen in this pilot study.

Conclusion

Adhesion molecules lead leukocytes to extravasation from the circulation and infiltration into transplanted graft during ischemia-reperfusion injury, acute and chronic allograft rejection. The efficacy of various mAbs against adhesion molecules has been assessed in different animal and human organ transplant models; the use of some antibodies has been shown to be promising, including the prolongation of allograft survival and donor-specific tolerance. Based on the results of these animal models, clinical studies have been initiated mainly in renal transplantation that demonstrated a potential usefulness of adhesion molecule inhibition in controlling reperfusion injury and allograft rejection. Nevertheless, there is still uncertainty on the expression, regulation and function of adhesion molecules. Further elucidation of the multiple complex functions and mechanism of adhesion molecules will help us to develop more efficient therapeutic strategies.

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Chapter 15

Agents Targeting B-Cells and the Humoral Responses

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Abstract

The accumulating knowledge on B cell-mediated allograft injury has augmented the interest in the potential of B-cell depletion strategies in solid organ transplantation. Monoclonal antibodies directed at B-cell surface antigens have come to replace other therapies targeting the B-cell pool, such as splenectomy. The chimeric mouse/human anti-CD20 monoclonal antibody, rituximab produces significant and long-lasting B-cell depletion.

Rituximab is currently used in transplantation to inhibit the production of alloreactive antibodies in HLA-sensitized patients, ABO-incompatible transplant recipients and also in the treatment of allograft rejection. Rituximab treatment also seems safe. Alemtuzumab is a humanized monoclonal antibody targeting the cell surface antigen CD52. CD52 is expressed on B-cells as well as T-cells, natural killer cells, monocytes and macrophages. Alemtuzumab induction therapy has been shown to reduce the risk of acute rejection. Alemtuzumab therapy however evokes a transient but profound suppression of most mononuclear cells and is associated with some serious side-effects. Its use so far in solid organ transplantation has been limited. Current

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experience with the B-cell-depleting monoclonal antibodies rituximab and alemtuzumab are herein summarized.

Keywords: rituximab, alemtuzumab, humoral rejection, ABO-incompatible transplantation, CD20

Abbreviations

ABOi: ABO-incompatible
AMR: Antibody- mediated rejection
APRIL: A proliferation-inducing ligand
ATG: Antithymocyte globulin
BAFF: B-cell activating factors
BCMA: B-cell maturation protein
BSA: Body-surface area
BLyS: B-lymphocyte stimulator
CDC: Complement-dependent cytotoxicity
DSAs: Donor-specific antibodies
FCXM: Flow cytometry crossmatch
HLA: Human leukocyte antigen
LD: Living donor
mAB: Monoclonal antibody
MICA/B: MHC-class I-related chain A or B
VEC-antibodies: Vascular endothelial cell antibodies

Introduction

The aim of the immunosuppressive therapy in organ transplantation is to prevent rejection of the allograft. The role of the T-cells in this setting is undisputed and the T-cells have therefore been the primary target of the immunosuppressive therapy after solid organ transplantation. Yet, the involvement of humoral immunity in acute rejection has been recognized since the early days of organ transplantation and several strategies and methods to avoid humoral rejection in organ transplantation such as the complement-dependent cytotoxicity (CDC) crossmatch were being developed already in the 1960's [1−3]. Further advances in the field of immunology have generated an increased knowledge about B cell-mediated allograft injury. However effectiveness of the therapies aimed to suppress the B-cell population has often been low or lacking and the treatment itself has been associated with considerable risks.

With the introduction of desensitization protocols to overcome the immune barriers posed by the ABO-system as well as HLA, therapeutic strategies to avoid and overcome antibody-mediated rejection have been explored.

This chapter will summarize the immunosuppressive approaches directed against the B-cells with particular emphasis on kidney transplantation and the use of rituximab, a chimeric

monoclonal antibody (mAb) against CD20 that induces a profound depletion of B-cells in the peripheral blood and alemtuzumab, an anti-CD52 T-cell and B-cell–depleting monoclonal antibody.

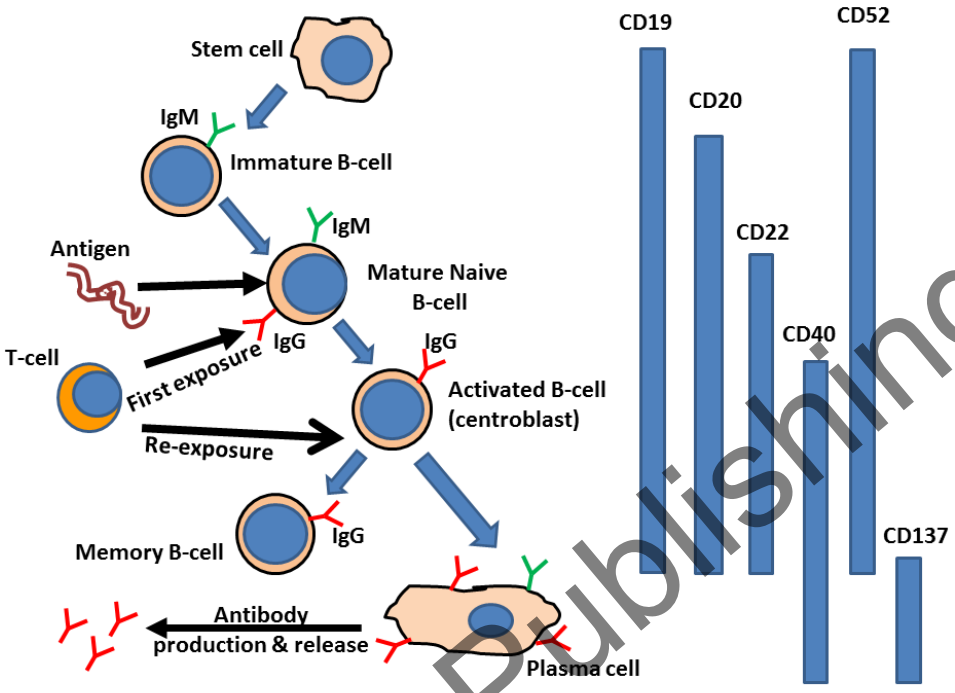

Figure 1. The sequence of B-cell maturation. Specific CD (cluster of differentiation) markers on the cell membrane appear during different transitional phases.

Antibody-mediated rejection has long been a dreaded complication of allograft transplantation. Until the early 2000's, apart from apheresis for antibody removal, therapies that targeted the antibody-producing B-cells were largely limited to splenectomy and the chemotherapeutic agents, cyclophosphamide and deoxyspergualin. In the 1970's, before the introduction of cyclosporine, splenectomy was used in (ABO-compatible) kidney transplantation as an adjunct to the immunosuppression. However, the benefit of this procedure was unclear [4]. Yet in ABO-incompatible transplantation after the first successful transplantations in the 1980's, a general belief was that splenectomy was necessary in order to avoid hyperacute rejection [5, 6]. The therapeutic efficacy however is limited, as a considerable fraction of the B-cell/plasma cell population resides in compartments other than the spleen, such as the bone marrow and lymph nodes. Splenectomy also carries some obvious risks. Extra surgical risk is added to the transplantation and asplenism is known to be associated with an increased risk of infection, especially with encapsulated bacteria [7−10]. Deoxyspergualin (gusperimus) is an antiproliferative agent primarily inhibiting antibody production as well as T-cell costimulation. The drug is a synthetic analogue of spergualin, produced by the bacterium *Bacillus laterosporus*. The first report on the immunosupressive properties of deoxyspergualin was published in 1990. Deoxyspergualin is approved in Japan for the treatment of steroid-resistant rejection in kidney transplantation and it is administered

intravenously, usually given in cycles of 10−14 days. A common side effect is transient leucopenia, but otherwise the known serious side effects are few [11]. A pilot study on tolerance-induction in kidney transplantation was not able to show any additive effect when comparing alemtuzumab + deoxyspergualin with alemtuzumab alone but the number of subjects was small (n=10) [12]. Cyclophosphamide is an alkylating chemotherapeutic agent related to nitrogen mustard. By inhibition of the lymphocyte cell cycle, it suppresses the clonal expansion of both B and T cells. Consequently the drug has a marked immunosuppressive effect.

However side effects such as nausea, bone marrow depression, hemorrhagic cystitis and transient alopecia are common [13]. The drug is also cardiotoxic and requires careful monitoring [14]. In Japan it has been used as a B cell-depleting agent in ABO-incompatible (ABOi) kidney transplantation as induction therapy, from -2 weeks before transplantation to 3 months after transplantation [15]. Cyclophosphamide has not been evaluated in any clinical trial in solid organ transplantation. Today the therapeutic alternatives for B-cell depletion also include the mAbs rituximab and alemtuzumab, directed against two epitopes found on the B cells (Figure 1). Rituximab was registered in 1997 for the treatment of lymphoma and alemtuzumab was in 1999 for the treatment of chronic lymphocytic leukemia. As both agents induce a rapid and sustained elimination of cells expressing the target molecule, they have gained much interest in the field of transplantation.

The Immunologic Response after Organ Transplantation

Allograft rejection affects approximately 20% of solid organ recipients. Recipients of some organs such as intestine and lungs have a higher risk of allograft rejection, possibly owing to the high number of immunocompetent cells within these organs that act as strong triggers of the alloimmune response. Allograft rejection is associated with an impairment of organ function that, if left untreated, will eventually lead to graft loss. The diagnosis is principally made on the histopathology of tissue biopsies together with a clinical assessment of the organ function and the presence or absence of circulating donor-specific antibodies. Grossly, acute allograft rejection is characterized as either cellular or antibody-mediated rejection. In chronic rejection the clinical course is less fulminant without clear signs of ongoing inflammation. Herein we summarize the basic characteristics of these different rejection types. A more detailed description of allograft rejection is found in chapter 2.

Cellular Rejection

The most common type of acute allograft rejection is characterized by an infiltration within the allograft of predominately T-lymphocytes. Such allograft rejections are termed cellular or T-cell mediated rejections. In kidney allografts, most commonly, an inflammation of the tubuli and the interstitium is seen but the inflammation can also engage the endothelium. Strategies for the immunosuppressive therapy of transplant patients have therefore largely been directed at inhibiting the T-cells. The severity of the allograft rejection

is normally graded according to organ-specific criteria, such as the Banff criteria, rejection activity index (RAI) in liver transplantation and the International Society for Heart and Lung Transplantation heart biopsy grading scale. Although cellular rejection is primarily a T-cell event, there is growing evidence to support that B cells play a role in cellular rejection as well (Figure 2).

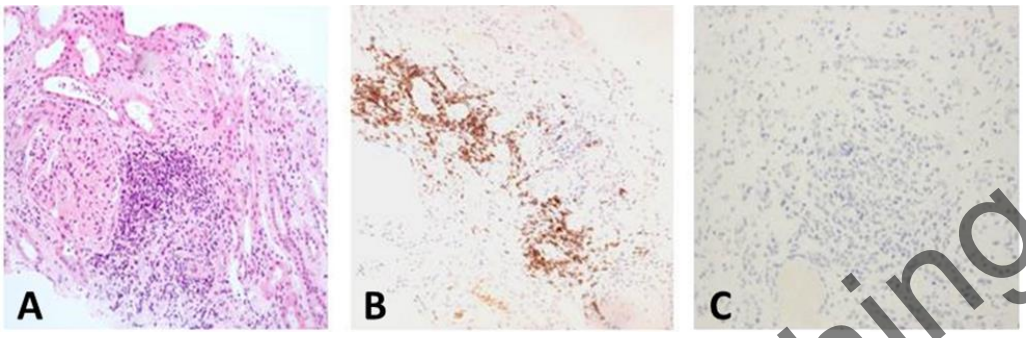

Figure 2. Immunohistochemistry of kidney transplant biopsies obtained from one individual before and after rituximab treatment. A: Hematoxylin and eosin staining before treatment with rituximab during acute cellular rejection Banff grade IIA; B: Immunohistochemistry using CD20 as a B-cell marker before treatment with rituximab (CD20 positive cells in brown staining); C: Immunohistochemical staining using CD20 (brown staining) as a B-cell marker after treatment with rituximab revealing the absence of CD20 positive cells.

Recent studies have revealed an antibody-independent role for the B-cell during acute rejection due to its ability to secrete inflammatory cytokines and chemokines, to regulate T-cells and dendritic cells, as well as its participation in antigen presentation [16]. Furthermore, in kidney transplantation, a subtype of cellular rejection with nodular dense B-cell infiltrates has been observed. In these studies, the B-cell rich rejection episodes were more prone to be steroid-resistant and subsequently associated with an increased risk of graft loss [17, 18].

B-Cells and Antibody-Mediated Rejection

The B-cells and their terminally differentiated successors, the plasma cells have been assigned a central role in both acute antibody-mediated rejection as well as in chronic rejection. Acute allograft injury in the absence of significant cellular infiltrates is termed antibody-mediated rejection. An antibody-mediated rejection arises when recipient antibodies bind to donor tissue antigens, usually found on the endothelial surface of the allograft. The result of the antibody-antigen interaction is an endothelial damage followed by local microvascular inflammation but also platelet activation and microthrombus formation. In severe forms, the endothelial and arterial injury may present as transmural arteritis, interstitial edema and necrosis. Antibody-binding also results in complement activation and local deposition of C4d. C4d is a split product of the complement system. In kidney as well as heart and lung transplantation, C4d is therefore used as a marker of antibody-binding with complement activation. A diffuse C4d-staining in these organs together with circulating donor-specific HLA antibodies is strongly indicative of antibody-mediated rejection. In liver

transplantation, the impact of a positive C4d-staining is less clear. In pancreas and intestinal transplantation, C4d is probably of value but more supporting data is needed.

To further underline the importance of B-cell alloimmunity, a recent population-based study in kidney transplant recipients found that vascular rejection, i.e., rejection with histological signs of endothelitis, accounted for 45% of the biopsy-proven acute rejection episodes. While such rejection episodes can be either T-cell-mediated or antibody-mediated, as outlined above, the risk of graft loss was 9.07 times higher in antibody-mediated vascular rejection than in T cell-mediated rejection [19].

Donor-Specific Antibodies

The antibodies typically involved in antibody-mediated rejection are directed against the donor's human leukocyte antigens (HLA) HLA class I or II. Recipient HLA-sensitization has long been recognized as critical for the early and late transplantation outcome [20]. Antibody status is therefore routinely analyzed before transplantation, using various techniques. The CDC crossmatch remains the gold standard test for the detection of preformed antibodies prior to transplantation but newer techniques such as the flow cytometry crossmatch (FCXM) and the Luminex assays are increasingly used, owing to a higher sensitivity than the CDC assay. Transplantation across a positive crossmatch is associated with an increased risk of early graft loss in kidney as well as heart and lung transplantation and is therefore usually avoided. In liver transplantation, crossmatch status is of lesser importance, probably owing to the liver's intrinsic immunomodulatory qualities. Whereas the CDC crossmatch and flow cytometric crossmatch are used to assess the presence of antibodies to a specific donor, the solid phase assays such as the Luminex assay can be used to characterize the antibody repertoire in an HLA-sensitized individual.

Donor-specific HLA-antibodies may also develop after transplantation. Such antibodies are commonly referred to as *de novo* donor-specific (HLA) antibodies. Information is growing about the impact of *de novo* DSA but there is currently no consensus about optimal treatment. Non-adherence to the immunosuppressive therapy most probably contributes to the development of these antibodies. The appearance of *de novo* DSA is often associated with a reduction in graft function and is often concomitant with histopathological signs of either acute or chronic antibody-mediated rejection [21]. Sometimes these antibodies are however identified without signs of graft function impairment [22]. Today it is not yet possible to accurately distinguish biologically inactive antibodies from those that are harmful. The subclass of C1q-fixing antibodies (IgG_1 and IgG_3) *i.e.*, antibodies capable of activating the classical complement pathway, has received a growing interest and in some studies, have been associated with a particularly poor prognosis [23−25].

The antibodies involved in antibody-mediated rejection may also be represented by the blood group isoagglutinins. The anti-A/B-antibodies are natural antibodies, *i.e.*, they are formed without any known prior antigen stimulation. The anti-A/B antibodies are, however, cross-reactive with epitopes on cell wall components of commensal gut bacteria and arise during the development of the immune system. The importance of the anti-A/B antibodies in organ transplantation is almost exclusively limited to ABO-incompatible transplantation,

further discussed below. Accidental ABO-incompatible transplantation owing to mistyping of the blood type is nowadays very uncommon.

Occasionally non-HLA vascular endothelial cell antibodies (VEC-antibodies), *i.e.*, antibodies against MHC-class I-related chain A or B (MICA/B) antigens, antibodies against platelet-specific antigens and antibodies directed at molecules of the renin-angiotensin pathway, give rise to an antibody-mediated rejection but such antibodies are less well characterized and there is no crossmatch or other test routinely used today to screen for the presence of such antibodies.

Whereas anti-A/B antibodies are natural antibodies and found in virtually all people (apart from small children and blood type AB individuals), HLA antibodies and non-HLA VEC antibodies may develop after exposure to foreign tissue. Such sensitizing events include previous blood transfusions, pregnancies and transplantations.

B-Cell Activation

The role of B cells and its lineage in allograft rejection is increasingly recognized and appears more complex than previously thought. B cells are nowadays known to be activated through three different pathways: One pathway is initiated in response to proteins such as HLA and it is referred to as T cell-dependent. This pathway involves antigen-receptor binding as well as CD40/CD40-ligand interactions. A second pathway is referred to as T-independent type 2 because the B-cell response can be generated in the absence of T cells. This is initiated as a response to mucopolysaccharides in the intestinal flora similar to the blood group antigens A and B. The third pathway is referred to as T-cell independent type 1. This pathway requires signals delivered through Toll-like receptors that bind endotoxin or other substances and it gives rise to a polyclonal B-cell activation.

Thus, the first two pathways result in monoclonal B-cell activation and are antigen-specific while the third pathway is not antigen-specific. The exact mechanism through which anti-donor antibodies and other humoral factors lead to vascular rejection is not entirely known but membrane attack complexes of complement and activated platelets induce the activation of endothelium and give rise to the production and local release of tissue factor, inflammatory cytokines, and cell adhesion molecules [26].

B-Cell Function

The B-cells play an important role at several stages of immune activation, as already mentioned. First, B-cells may differentiate into plasma cells, capable of producing large volumes of soluble antibodies and into $CD27^+$ memory B-cells, important for the long-term immunity. The B-cells also serve as antigen-presenting cells necessary for the activation of $CD4^+$ T-cells and are capable of delivering various co-stimulatory signals needed for the activation of $CD8^+$ T-cells [27]. Furthermore, B-cells secrete cytokines such as interferon-gamma and the interleukin IL-6 [28]. The immunosuppressive effect of the B-cell-depleting agents, rituximab and alemtuzumab is therefore diverse. These therapeutic antibodies do not however target the antibody-production directly since neither of them is directed at molecules

expressed on the antibody-producing plasma cells. A description of agents targeting the plasma cell pool is found elsewhere. Additionally, alemtuzumab also has direct effects on the T-cell population.

B-Cell Depletion in Organ Transplantation

The central roles of the B cells in allograft rejection have provided the rationale for the use of anti–B cell therapies in various clinical settings, including (i) desensitization of crossmatch positive or ABO-incompatible recipients in living donor transplantation, (ii) induction therapy to prevent acute allograft rejection, (iii) the treatment of acute antibody-mediated rejection (together with apheresis), (iv) highly HLA-sensitized patients awaiting deceased-donor kidney transplantation or (v) the treatment of steroid-resistant cellular rejection. In the following section, the current knowledge of the therapeutic antibodies rituximab and alemtuzumab in the abovementioned clinical settings are further discussed.

Rituximab

Since the beginning of the 2000's, rituximab has gained much interest in renal transplantation. Although the approved indication for rituximab treatment is limited to B cell lymphoma and rheumatoid arthritis, rituximab has been explored as a therapeutic option for virtually all autoimmune diseases and for various indications in transplantation. Rituximab is generally well-tolerated and serious side effects are rare, which in part explains its popularity. There are several ongoing randomized controlled trials evaluating rituximab in kidney transplantation [29].

Rituximab is a chimeric mouse/human antibody of the IgG_1 subtype directed at the transmembrane protein CD20 [30]. The target molecule CD20, functions as a calcium-channel and is involved in cell-cycle regulation [31]. CD20 is expressed on all mature B cells but not on hematopoietic stem cells, the antibody-producing plasma cells or on any other cell type [32]. There are two principal hypotheses to explain the function of rituximab. Firstly, CD20 mAb alters Ca^{2+} transportation, leading to a disruption of the cell cycle and apoptosis [33]. Secondly, CD20 mAb activates innate immune system, with strong experimental evidence in support of the latter hypothesis [34]. Following intraperitoneal injection of rituximab in mice naturally devoid of cells belonging to the innate immune system, the B cells within the peritoneal cavity are coated with rituximab but not eliminated. This indicates that B-cell depletion is not mediated solely by the induction of apoptosis [35]. Furthermore, in rituximab-treated macrophage-deficient mice, B-cell depletion is reduced, suggesting that B-cell depletion is mediated principally by monocytes/macrophages via Fc-γ-receptors, so called antibody-dependent cell-mediated cytotoxicity and/or phagocytosis (ADCC) [30, 36]. Furthermore, the Fc-γ-IIIa receptor (CD16) has a particular affinity for IgG_1, the rituximab isotype. The Fc-γ-IIIa receptor is strongly expressed on NK-cells and exists in two genotypes with a difference in the amino acid in position 158. Individuals homozygous for valine in this position have high-affinity Fc-γ-IIIa receptors and a stronger response to rituximab, indicating the effect of rituximab is in part mediated by NK-cells [37]. However, several other

mechanisms may play a role in the activation of the innate immune system, including complement-dependent cytotoxicity. In *in vitro* studies, rituximab binding has initiated the complement cascade, resulting in the formation of a membrane attack complex and cell destruction [38].

Pharmacokinetics and Pharmacodynamics of Rituximab

The use in oncology, transplantation and other medical specialties has provided abundant information on safety, dosage and the pharmacokinetics of rituximab. In general, administration of rituximab results in a rapid and sustained depletion of circulating and tissue-based B cells as early as 24 to 72 hours after administration and the effect is long-term. In kidney allograft recipients, the B-cells remained depleted for 6 to 9 months in 83% of subjects [39]. B-cell recovery began at approximately six months following completion of treatment (Figure 3). Moreover, rituximab effectively reduced $CD19^+$ B-cells in peripheral blood in patients receiving 1000 mg rituximab twice as treatment for rheumatoid arthritis whereas its effect was less pronounced in the bone marrow. Activated antigen-presenting B-cells ($CD19^+$ $HLA\ DR^+$) as well as memory B cells ($CD19^+$ $CD27^+$) were however significantly reduced in both compartments [40].

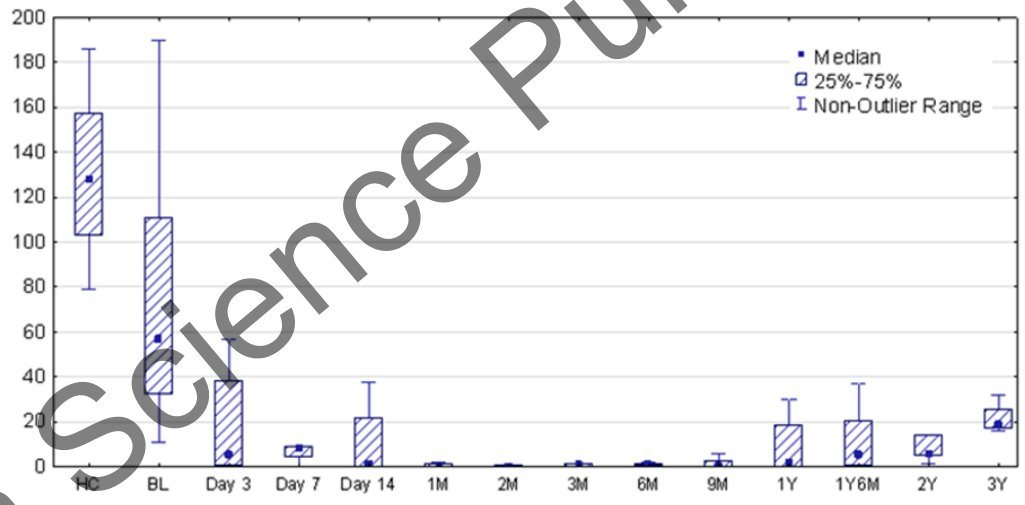

Figure 3. Changes in $CD19^+$ B-cells/µL before and after treatment with rituximab and in healthy controls. Immunophenotyping by flow cytometry. HC = healthy controls. BL = baseline, i.e., before threatment with rituximab. M= months after rituximab treatment. Y = years after rituximab treatment.

It has been demonstrated in lymphoma patients that the serum concentration of rituximab correlates to the level of B-cell depletion and to the clinical effect [41]. Moreover, the clearance and volume of distribution is correlated to body-surface area (BSA) but there is great inter-individual variability in the concentrations of rituximab obtained [42]. Several assays for the measurement of the rituximab concentration in blood have been developed, including enzyme-linked immunosorbent assays (ELISA) using polyclonal and monoclonal

antibodies against the rituximab idiotype or the mouse Fv part, as well as flow cytometry assays, detecting the binding of rituximab to CD20 [41, 43, 44]. However, there are no international standards or recommendations available today for rituximab measurement. In studies determining the concentration of rituximab, methods measuring both free and bound rituximab have been used, making it difficult to compare the results in different studies. Optimal concentration is not known for any patient group today [45]. Furthermore, the elimination (half-life) of rituximab varies in different studies but is generally long (4−20 days) and rituximab has been detectable in the circulation of non-Hodgkin's lymphoma patients for as long as 6 months [41, 42, 46, 47]. Of importance in transplantation is that even very low concentrations (< 1 µg/mL) of rituximab may affect crossmatch results. In two patients without donor-specific HLA antibodies, CDC crossmatches performed on sera obtained between 1 to 10 weeks after rituximab administration, when rituximab was still measurable, were positive while the CDC crossmatches on sera obtained a year after the rituximab administration when rituximab was no longer detectable, turned out negative [48].

The optimal dose of rituximab in kidney transplantation is currently unknown. In a study on the effects of rituximab in uremic patients, the B cells were completely eliminated even in the group receiving the lowest dose of 50 mg/m^2 BSA [47]. However, in lymphoma patients the most commonly used regimen consists of one dose of 375 mg/ m^2 BSA of rituximab per week over a four-week period [35]. In kidney transplantation, most commonly a single-dose of rituximab 375 mg/ m^2 BSA is given [49−51]. A single-dose of rituximab 375 mg/m^2 BSA evokes a long-term elimination of B cells in peripheral blood and within the graft (Figure 2) [52]. In the lymphoid compartment, a single-dose of rituximab 375 mg/ m^2 BSA does not induce at complete B cell depletion but a reduction of the B cells has been observed in studies on lymph nodes and spleen [53] (Figure 4).

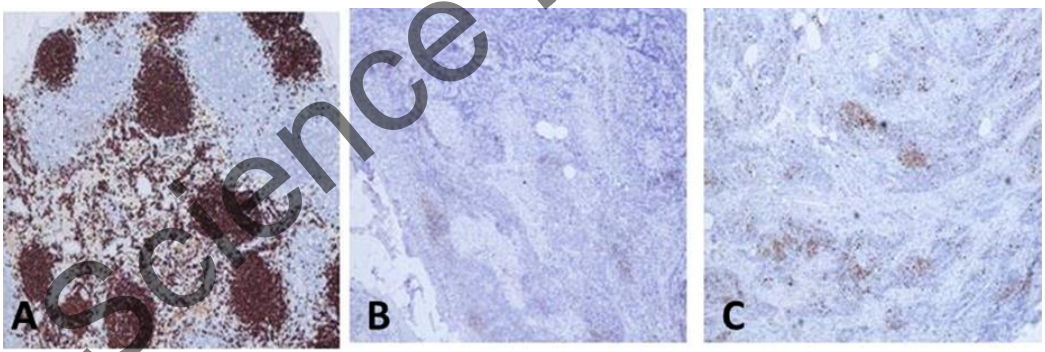

Figure 4. Immunohistochemistry of lymph nodes before and after rituximab treatment. A: Normal lymph node obtained from a uremic patient with no immunosuppressive therapy. CD20 positive cells in brown staining; B: Immunohistochemistry using CD20 as a B-cell marker after treatment with rituximab. CD20 in brown staining; C: Immunohistochemistry using CD79a as a B-cell marker performed on the same lymph node as in figure 5b after treatment with rituximab. CD79a herein stains brown.

Alemtuzumab

The target molecule of the humanized rat monoclonal antibody alemtuzumab is CD52, a cell surface marker expressed on most mononuclear cells, including both B and T lymphocytes. The immunosuppressive effect is profound, as the majority of mononuclear cells are depleted for a long-term period [54, 55]. Alemtuzumab has been used for more than three decades in several hematological malignancies, bone marrow transplantation or some autoimmune disorders [56−60].

The use of alemtuzumab in clinical transplantation is more recent and although its use in transplantation is rapidly increasing, it has so far been almost exclusively limited to kidney transplantation induction [61, 62]. Alemtuzumab is usually administered as a single dose (30 mg) intravenous infusion over at least 2 h after premedication with corticosteroids, antihistamines and antipyretics. Several infusion-related adverse effects have been reported and include respiratory (bronchospasm, acute respiratory distress syndrome, respiratory arrest) and cardiac events (arrhythmias, myocardial infarction, acute cardiac insufficiency) that may be due to the cytokine release syndrome [63]. Several autoimmune diseases (thyroiditis, thrombocytopenia) have been reported after the clinical use of alemtuzumab while other immunosuppression-related complications (i.e., infections, malignancy) did not seem to differ between patients receiving alemtuzumab and those receiving other induction protocols [64].

Pharmacokinetics and Pharmacodynamics of Alemtuzumab

Alemtuzumab binds to the cell surface antigen CD52 present at high levels on T and B lymphocytes, and at lower levels on natural killer cells, monocytes, and macrophages. There is little or no CD52 detected on neutrophils, plasma cells, or bone marrow stem cells. Alemtuzumab acts through antibody-dependent cellular cytolysis and complement-mediated lysis following cell surface binding to B and T lymphocytes.

In a cohort of patients with B-cell chronic lymphocytic leukemia receiving iterative subcutaneous alemtuzumab, a significant and profound depletion occurred within the $CD4^+$ and $CD8^+$ lymphocyte subsets. The $CD4^+$ lymphocytes were reduced from a median cell counts of 1524 cells/μL (range 215–7564 cells/μL) at baseline to 43 cells/ cells/μL (range 5–434 cells/μL) at the end of therapy, and the $CD8^+$ lymphocytes from 1167 cells/μL (range 277–6070) to 20 cells/μL (range 0–1125 cells/μL). NK cells, NK-T cells and normal B cells were also profoundly depleted. In short, the median cell counts at the end of treatment were 4 NK cells/μL (range 1–8 cells/μL), 1 NK-T cell/μL (range 0–11 cells/μL and 1 B cell/μL (range 0–41 cells/μL).

Granulocytes and monocytes were also significantly reduced. The recovery was slow and the number of cells within all the lymphoid subpopulations remained significantly lower than at baseline for up to 18 months. The median time to reach $CD4^+$ and $CD8^+$ blood concentrations of >100 cells/μL was 4 months but the median cell counts remained below 25% of baseline for all lymphocyte subsets for more than 9 months, with the exception of

granulocytes and monocytes. Interestingly, normal B cells remained at a low level 18 months after the treatment [65].

The Clinical Use of Rituximab and Alemtuzumab

We herein give a brief summary of the current status of rituximab and alemtuzumab in organ transplantation and the experience with these therapeutic antibodies in some clinical settings of relevance.

Rituximab and Alemtuzumab for Desensitization Prior to Living Donor Kidney Transplantation

Living donor (LD) kidney transplantation not only offers superior patient and graft survival compared with deceased donor transplantation but it also represents a means to expand the total donor pool [66, 67]. However, immunologic barriers limits the potential use of LD [68]. Preformed antibodies, most commonly anti-A/B antibodies or HLA antibodies, against the donor kidney antigens, has been the most common contraindication to LD donation and accounted for an estimated 30–50% of all LD refusals, as transplantation in the presence of such antibodies can evoke a hyperacute rejection, leading to graft loss within hours to days [69]. Desensitization protocols to overcome these immunologic barriers have therefore been developed and are becoming increasingly popular. It should be noted however, that the classical definition of desensitization is *'the reduction or abolition of reactions to a specific antigen or allergen'* whereas desensitization in organ transplantation usually refers to therapies to allow for transplantation in the presence of donor-specific antibodies with the use of immunosuppressives. With the emergence of these desensitization protocols, the incidence of hyperacute rejection has been significantly reduced [5]. However, in many studies, a high incidence of antibody-mediated rejection has been observed following ABO-incompatible as well as crossmatch-positive kidney transplantation [5, 15, 70−72].

Current desensitization protocols are all based on the same principle of removing existing antibodies and preventing rebound of antibodies in the recipient after transplantation. For the removal of antibodies, there are a number of apheresis techniques available today, including therapeutic plasma exchange and various types of immunoadsorption. Generally several apheresis sessions are needed for efficient antibody removal, both prior to and sometimes after the transplantation. To prevent rebound, apheresis is combined with immunosuppressive therapy. Usually a standard triple immunosuppressive regimen is given, combined with anti-B-cell induction. The most commonly used B-cell depleting agent for this purpose is rituximab [50, 70, 73, 74]. The donor-specific antibody levels are monitored and transplantation performed when the antibodies are sufficiently reduced. This process normally takes 1–2 weeks [68]. For this reason, desensitization is almost exclusively limited to living donor transplantation.

ABO-Incompatible Kidney Transplantation

In 1900, Landsteiner discovered the first and most important blood group system, the ABO system. According to the law he formulated, individuals of blood group A have antibodies against blood group B, while individuals of blood group B have antibodies against blood group A. AB individuals do not produce any ABO antibodies and blood group O subjects have antibodies against both A and B (Figure 5).

As the A and B antigens are expressed on most cell types in the body and not only on erythrocytes, the same principles of ABO-matching generally apply in transplantation, as in the transfusion of red blood cells (Figure 6). Already in 1950's it became obvious that ABO-matching was very important for the success of a transplantation [75]. Underscoring this fact, is the analysis of Cook et al., including 25 deceased donor ABO-incompatible kidney transplantations performed accidentally, as a result of ABO mistyping. In this study, graft survival was only 4% at one year [76]. However in the 1970's it became known that, a blood subgroup with lower tissue antigen expression (blood type A_2) as well as antibody removal through plasmapheresis could allow for a certain decrease of the pretransplant risk. In some centers A_2-incompatible programmes were started using only regular immunosuppression, achieving a 1-year graft survival of around 55% [77]. In 1982, Alexandre et al. conducted the first larger study on living donor non-A_2 ABOi kidney transplantation [5, 78].

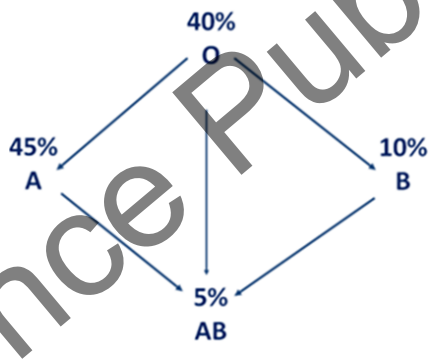

Figure 5. The rules of compatibility and distribution of the ABO-system. The likelihood that two unrelated individuals are ABO-incompatible is approximately 36%.

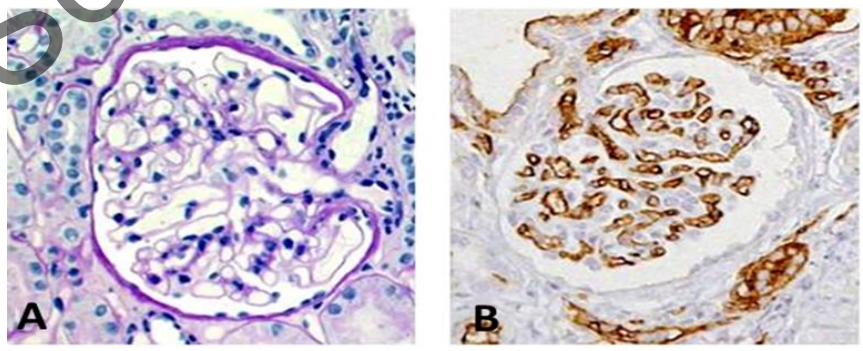

Figure 6. Normal kidney tissue in a blood type A_1 individual. A: Periodic acid Schiff-staining of normal kidney tissue; B: Immunohistochemistry for the detection of A antigen - A antigen staining brown.

By 1989, a total of 39 LD ABOi kidney transplantations had been performed, with a 1-year graft survival of approximately 75%. According to Alexandre et al., splenectomy was necessary for the ABOi transplantation to be successful, as three patients in the very beginning, who were not splenectomized, lost their grafts. The protocol also included repeated plasmapheresis, the use of donor platelet transfusion and the administration of substance A (A trisaccharide) or substance B (B trisaccharide) depending on the A/B incompatibility, antilymphocyte globulin and triple maintenance immunosuppression (corticosteroids, azathioprine and cyclosporine). Based on their success, similar protocols were implemented at a few other centers worldwide, most notably in Japan. Already at an early stage, the Japanese achieved results of the ABOi kidney transplantations similar to those of ABO-compatible kidney transplantation, further supporting the ABOi programs [71, 79]. The ABOi programs in Japan have in fact been very successful and to date > 1000 ABOi kidney transplantations have been performed. Outside Japan, however the risks involved by combining plasmapheresis for antibody removal with splenectomy to reduce the antibody producing B-cell pool, in addition to quadruple immunosuppression was a deterrent. Yet in the beginning of the 2000's, ABOi kidney transplantation gained a renewed interest both in Europe and the USA primarily owing to new therapeutic options that became available, i.e., rituximab and, in Europe, the Glycosorb ABO system allowing for the selective removal of anti- A/B antibodies, but also as a corollary to the success in Japan and an increasing demand. ABO-incompatible kidney transplantation has now become common clinical practice in many centers worldwide. The procedure also seems safe. In an analysis including 1420 ABO-incompatible kidney recipients and their matched controls, there was no significant difference in graft survival at 3 years [80]. The use of rituximab in ABOi transplantation has also become widespread, replacing splenectomy as the key B-cell-depleting therapy. There are reports however of successful ABO-incompatible kidney transplantation without any specific anti-B cell therapy, neither rituximab nor splenectomy [81]. Rituximab has also been used in ABOi living donor liver transplantation. There are currently no studies available evaluating alemtuzumab in ABOi transplantation.

HLA-Incompatible Organ Transplantation

Together with protocols for ABOi transplantation and primarily owing to an increasing demand, protocols to overcome a positive crossmatch in living donor kidney transplantation have also been developed [82−84]. These protocols are similar to the ABOi protocols, aiming at reducing the donor-specific HLA antibodies (Table 1). The transplantation is performed when/if a seroconversion to a negative crossmatch is obtained. Apheresis is often continued after transplantation in the early postoperative period, with the purpose of preventing antibody-mediated rejection during the most sensitive period. The long-term results however are at present not in parity with crossmatch negative transplantation or ABOi transplantation. In a recent study from Harvard, including 39 patients with a positive CDC crossmatch against their living donor prior to desensitization, 61% developed antibody-mediated rejection. Among these 39 patients, 25 received rituximab induction and of those 18 experienced AMR. The overall graft survival at 5 years was 72% [85]. In comparison, the reported 5-year survival in ABO-compatible LD transplantation and in ABOi transplantation is currently

above 80%. Yet, it should be emphasized that the therapeutic options for this group of patients are limited.

Table 1. Various desensitization protocols currently in use; IA-immunoadsorption, ATG – antithymocyte globulin, AMR – antibody- mediated rejection, XM - crossmatch

		John's Hopkins	Mayo Clinic	Tokyo Women's Hospital	Freiburg	Karolinska
ABOi	Postop. pre-emptive IA	Yes	No	No	No	Yes
	Rituximab	No	Yes	Yes	Yes	Yes
	ATG	No	Yes	No	No	No
	Incidence of AMR	17.8%	18%	16%	0%	4.2%
	Apheresis technique	TPE	TPE	Double-filtration plasmapheresis	Glycosorb-ABO IA	Glycosorb-ABO IA
XM+	Crossmatch type	CDC XM+ and/or FACS XM+	CDC XM+ and/or FACS XM+	Luminex+ only		FACS XM+
	Postop. pre-emptive IA	Yes	Yes	No		No
	Rituximab	No	No	Yes		Yes
	ATG	No	Yes	No		No
	Incidence of AMR	n/a	41%	6.8?		18%
	Apheresis technique	TPE	TPE	Double-filtration plasmapheresis		Protein A IA

Often the waiting time on the deceased donor waiting list is long and patient survival following desensitization superior to dialysis [86]. Overcoming the barrier posed by performed donor-specific HLA antibodies at high levels is medically challenging for several reasons. First, the mismatched HLA antigens are highly immunogenic. Second, the HLA-sensitized individuals have an immune defense that is already alerted. They are therefore more prone to develop antibody-mediated rejection and subsequently graft loss compared with kidney recipients without any HLA-antibodies, even in the absence of donor-specific HLA antibodies at transplantation. Protocols for CDC crossmatch positive transplantation have not been broadly implemented and are consequently not part of common clinical practice. Centers that currently perform crossmatch positive transplantations have, for most part, included rituximab in the immunosuppressive regimen [87, 88]. Alemtuzumab has not been evaluated in this setting.

Rituximab and Alemtuzumab As Induction Therapy to Prevent Acute Allograft Rejection

Owing to the positive experience of rituximab induction in HLA- and ABO-incompatible transplantation, rituximab has also been tested as induction therapy in conventional (HLA and ABO-compatible) transplantation. Yet, the first placebo-controlled randomized double-blind trial on rituximab induction, was unable to prove that rituximab induction reduced the risk of acute allograft rejection. This multicenter study extended over a period of six months, included a total of 140 patients and used a composite end-point, combining allograft rejection,

graft loss or death. In this short-term study, the treatment appeared safe, as rituximab did not seem to increase the risk of infections or death [89].

However, the results of a 3-year extension of this study including the same cohort of patients were more alarming as rituximab seemed to be associated with an increased risk of death with 8 out of 70 patients in the rituximab-treated arm being dead compared with no patients in the placebo-arm [90]. In a similar but larger study on rituximab induction, including twice the number of patients (n=280), rituximab was not superior to placebo in preventing allograft rejection either. However, in the group of HLA-sensitized patients with a panel reactive antibody assay (PRA) rituximab might have been of benefit as the rituximab-treated patients had an incidence of allograft rejection of 17.9% compared with 38.2% in the control group [91]. In a group of AOi-incompatible patients, rituximab also seemed to prevent HLA-sensitization [92]. To summarize, safety and efficacy of rituximab induction is yet to be established for patients with an immunological low risk profile undergoing kidney transplantation. Rituximab induction in liver, heart, lung, pancreas or intestinal transplantation has not been evaluated in any randomized trial. Rituximab induction is however part of protocols for intestinal and multivisceral transplantation [93].

Induction with Alemtuzumab has also proved effective in reducing the rate of acute rejection in several clinical trials. Thus, a recently published study reporting the results of a large randomized controlled trial in kidney transplant revealed a lower rate of biopsy proven acute rejection during the first 6 months after transplantation in the kidney recipients receiving alemtuzumab (7%) compared with kidney recipients under basiliximab-based treatment (16%), corresponding to a 58% proportional reduction [94]. These results and the apparently more intense immunosuppression were not associated with an increased rate of serious infections requiring hospitalization. However, BK virus infections (but not CMV infections) were more common in the alemtuzumab-based treatment group. Post-transplant lymphoproliferative disorder (PTLD) occurred in one (< 0.5%) patient assigned to receive alemtuzumab induction and in three (1%) patients receiving basiliximab. A meta-analysis of ten RCT compared the results of kidney transplantation when using alemtuzumab induction versus IL-2r blockers or T-cell depletive regimens. The nearly seven hundred patients receiving alemtuzumab induction had a lower rate of biopsy proven acute rejection (overall and after the first year) compared with those receiving the other two regimens as well as a lower incidence of new onset diabetes mellitus after transplantation. However, these results seem to have come with the cost of a higher rate of serious infectious adverse events in patients treated with alemtuzumab while another study reported an increase in CMV infections in the alemtuzumab-treated group compared with controls receiving no induction therapy (28% *vs*. 12%) [63, 95]. A study performed on the OPTN/UNOS registry did not find an increased risk of malignancy (including PTLD) when using alemtuzumab compared with other induction regimens (albeit after a limited follow-up period) [96]. Unfortunately, the meta-analysis did not provide any information on the antibody-mediated rejection nor did it report on the development of DSA after transplantation. Alemtuzumab induction has also been used in pancreas, intestinal, liver, heart and lung transplantation with virtually all studies showing similar or superior results compared with more established regimens [97−103]. In a retrospective study on alemtuzumab in a cohort of 336 patients undergoing lung transplantation, a lower frequency of acute cellular rejection and a higher 5-year patient and graft survival in patients receiving alemtuzumab (n=127) was observed compared with patients receiving other or no induction therapy (n=209). In addition, the study signaled a

lower frequency of bronchiolitis obliterans in the alemtuzumab-treated group, the hallmark of chronic rejection after lung transplantation. Another study found a significantly lower number of high-grade allograft rejection during the first year after lung transplantation in the patients receiving alemtuzumab induction. The rate of infectious complications in this patient group was similar with that in patients receiving standard immunosuppression and anti-thymoglobulin (ATG) induction but these encouraging early results of this particular study were not followed by a decrease in chronic rejection and allograft dysfunction.

Rituximab and Alemtuzumab As Treatment of Acute Antibody-Mediated Rejection

Rituximab has been used as treatment of antibody-mediated rejection in kidney transplantations as well as in heart, liver and pancreas transplantation [104–107]. In this setting, rituximab has most commonly been given together with apheresis and other immunosuppressive agents. As expected, it is difficult to reverse the damage done by alloantibody in the setting of an established B cell immune response, and the efficacy of targeting B cells with rituximab under these post-transplant circumstances has been difficult to clearly establish. There is however a number of studies indicating a positive effect of rituximab as part of the treatment for antibody-mediated rejection. In one study using historic controls to compare the effect of high-dose IVIG alone, with therapeutic plasma exchange, IVIG and rituximab, graft survival at 3 years in the therapeutic plasma exchange/rituximab group was 91.7% compared with 50% for the IVIG alone-group [108].

Novel B-Cell Directed Therapies with Future Potential Use in Transplantation

Several other compounds aiming at B-cell depletion or blocking B-cell activation are available and currently being tested in various hematological malignancies and autoimmune diseases, yet few have been tested in transplantation (Table 2).

Table 2. Biological agents targeting B-cells

	Type	Mode of action
Rituximab	anti-CD20 mAb	B cell depletion
Ocrelizumab	Humanized anti-CD20 mAb	B cell depletion
Epratuzumab	Humanized anti-CD22 mAb	B cell depletion
Obinutuzumab	Humanized anti-CD20 mAb	B cell depletion
Belimumab	Humanized anti-BlyS mAb	inhibitor of B cell activation
Atacicept	Fusion receptor protein	inhibitor of B cell activation
Alemtuzumab	Humanized anti-CD52 mAb	T and B cell depletion

Ocrelizumab

Ocrelizumab, a humanized anti-CD20 mAb with a lower immunogenicity than rituximab has been tested in a randomized, double-blind, phase III study in patients with lupus nephritis with rather disappointing results. The overall renal response rates after 48 weeks with ocrelizumab were not statistically superior to those with placebo while the ocrelizumab treatment was associated with a higher rate of serious infections [109].

Obinutuzumab

Obinutuzumab is a novel glycoengineered type II anti-CD20 monoclonal antibody, with a higher affinity for CD20 epitope compared with rituximab. A phase III, randomized trial in patients with chronic lymphocytic leukemia comparing chlorambucil monotherapy, chlorambucil and obinutuzumab or rituximab revealed a longer progression-free survival in the patients receiving chlorambucil and obinutuzumab arm on the expense of infusion-related reactions, neutropenia and thrombocytopenia but not an increased risk of infection [110].

Blockers of BAFF and APRIL

Other promising targets for altering the humoral response include the B-cell activating factors (BAFF), also known as B-lymphocyte stimulator (BLyS) and a proliferation-inducing ligand (APRIL). These ligands from the tumor necrosis factor-family act as antiapoptotic survival factors critical for the maturation of the B-cell lineage. BAFF signals through three different receptors: the BAFF-receptor, transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and the B-cell maturation protein (BCMA) [111]. APRIL signals through the latter two. Belimumab is a fully human monoclonal antibody developed to target BAFF. This approach has been shown to be effective in the treatment of systemic lupus erythematosus but a phase II study aiming at the desensitization with belimumab of sensitized patients awaiting kidney transplantation has been terminated due to failure to decrease the antibody levels [112]. Whether belimumab has a role in transplantation, is yet to be established [29]. Atacicept is a fully humanized recombinant fusion protein containing the extracellular ligand-binding portion of the human TACI (transmembrane activator and calcium modulator and cyclophilin-ligand interactor) receptor (also known as TNFRSF13B) linked to a recombinant Fc domain of human IgG. Atacicept binds to the cytokines BAFF and APRIL, involved in B-cell differentiation, maturation, and survival. Atacicept reduces B-lymphocyte survival by inhibiting these B-cell stimulating factors. Experimentally, in a lupus-prone mouse model, atacicept impacted serum levels of IgM and plasma cells [113]. A phase Ib, double-blind, placebo-controlled trial found a dose-dependent reduction in immunoglobulin levels in atacicept-treated patients, most notably affecting IgM levels and a dose-dependent reduction in total and mature B cells [114]. Several clinical trials have explored its efficacy in various conditions [115]. Given its role in lowering B-lymphocyte numbers and immunoglobulin levels, atacicept holds a certain promise as a new agent in the armamentarium for desensitization protocols and the treatment

of AMR in kidney transplant recipients. However no clinical studies in transplantation are currently ongoing.

Epratuzumab

Epratuzumab is a humanized monoclonal antibody targeted against CD22. Studies in animal models have shown that CD22 is central for B-cell maturation and survival [116]. Initially tested in patients with hematological malignancies, epratuzumab induced a rapid disease remission with minimal toxicity and side effects [117]. In addition, several trials conducted in patients with systemic lupus erythematosus, epratuzumab demonstrated a statistically significant and clinically meaningful improvement [118, 119].

Conclusion

B-cells may terminally differentiate into antibody-producing plasma cells, involved in antibody-mediated allograft injury and memory B-cell capable of sustaining an immune response long-term, but the B-cells are also involved at several other stages of immune activation following transplantation. Indeed, the importance of the B-cell immune response in transplantation is becoming more and more recognized. Therapies aiming at suppressing the B-cell population have been used in transplantation for a long time with some success and some considerable risks. With the introduction of the monoclonal antibodies rituximab, targeting the $CD20^+$ B-cells and alemtuzumab, directed at $CD52^+$ B and T cells, long-term B-cell depletion can now be achieved effectively and with rather few side-effects. These antibodies have primarily been used as induction agents in immunological high-risk transplantations together with other immunosuppressive therapies, with promising results. Rituximab has also been used in the treatment of allograft rejection. Small-series studies indicate that rituximab in this setting is of benefit. The effect of rituximab induction in patients with an immunologic low-risk profile or as part of regular anti-rejection therapy is yet however to be established. Alemtuzumab has only been evaluated as an induction agent and primarily in kidney and lung transplantation.

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Chapter 16

Agents Targeting Plasma Cells

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Abstract

The main obstacle in the early days of transplantation was acute cellular rejection. Now, humoral rejection is the main challenge to achieve long-term success. Allo-antibody production by plasma cells (PCs) against HLA antigens can be a significant barrier to successful transplantation. Focus on alloantibody monitoring, removal, and plasma cell-directed therapy may significantly improve long-term allograft survival. The main limitation of preventing humoral rejection is that most of currently available anti-rejection agents do not have significant effect on the terminally differentiated plasma cells which are non-dividing and long lived, which persist to produce donor-specific antibodies (DSAs). Few agents have been shown to affect the function of plasma cells. They are: proteasome inhibitors (bortezomib, carfilzomib); anti-B-lymphocyte stimulating factor therapies (belimumab) and a monoclonal antibody against interleukin-6 receptor (tocilizumab). These agents are relatively new and the only agent that has been widely used in clinical transplant is bortezomib. Most of the clinical experience with bortezomib is with renal transplantation. However, it has been successfully used in liver, pancreas, intestinal, multivisceral, heart and lung transplantation. The most common use is for the treatment of acute humoral rejection, but it has also been used as part of desensitization protocols, induction therapy and even to prevent progression of chronic rejection.

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This review discusses the importance of plasma cell, the role of alloantibody in graft outcomes and the current data supporting anti-plasma cell agents' efficacy in experimental and clinical transplantation.

Keywords: proteasome inhibitor, alloantibodies, plasma cells, bortezomib, belimumab, carfilzomib, transplantation

Abbreviations

APRIL	A proliferation-inducing ligand
AMR	Antibody mediated rejection
BlyS	B-lymphocyte stimulator
DSAs	Donor specific antibodies
HLA	Human leukocyte antigen
IVIG	Intravenous Immunoglobulin
MHC	Major histocompatibility complex
PCs	Plasma cells
rATG	Rabbit antithymocyte globulin
	B-lymphocyte stimulator (BLyS) and

Introduction

The main problem in the early days of transplantation was acute cellular rejection. Now, persistent antibodies against the donor's human leukocyte antigen (HLA), known as donor-specific antibodies (DSAs), is the main challenge. It has been shown that the majority of all transplant recipients will develop DSA before or at the time of graft failure [1]. In addition, approximately 30% of patients awaiting a kidney transplant in the United States have already DSAs [2]. DSAs can be obtained by previous transplants, blood transfusions or pregnancy. The presence of DSAs has been associated with hyperacute rejection, antibody-mediated rejection and high rates of organ loss [3, 4].

In order to prevent graft loss, treating the source of antibody production is crucial. In the last decade, attempts to decrease DSAs focused on removing or blocking circulating antibodies and targeting B-cells [5]. This has been accomplished by using intravenous immunoglobulin (IVIg), plasmapheresis (PP) and monoclonal antibodies against the B-surface marker CD20 (rituximab). Intravenous immune globulin and plasmapheresis desensitization therapy can reduce circulating antibodies in some patients. This reduction of antibodies through desensitization improves transplant rates for highly sensitized patients while promoting acceptable 1-year-allograft survival. However, one complication that remains following desensitization is a high risk of acute rejection, occurring in as many as 62% [6]. In addition, the majority of the desensitized patients will develop chronic alloantibody mediated rejection prematurely [7]. Another agent historically used to address the presence of DSA both as part of desensitization protocols and to treat acute antibody-mediated rejection (AMR) is rituximab. However, the success of this approach was limited.

Although rituximab has been shown to reduce intensity of acute AMR, it had minimal effect on antibody production and lead to poor outcomes [8, 9].

After 2007, because available therapy (rituximab, IVIg, and plasmapheresis) was not eliminating DSAs and preventing graft loss, the focus of investigation turned to find new therapies to target plasma cells, which are the major antibody producing cells. Thus, the anti-plasma cell agent bortezomib, a medication used to treat multiple myeloma (a plasma cell cancer), was tested in transplantation [9].

Bortezomib is the only anti-plasma cell agent widely used in clinical transplant. Most of the clinical experience is with renal transplantation. However, it has also been successfully used in liver, pancreas, intestinal, multivisceral, heart and lung transplantation. The most common use is for the treatment of acute humoral rejection, but it has also been used as part of desensitization protocols, induction therapy, and even to prevent progression of chronic rejection. This review discusses the importance of plasma cell, the role of alloantibody in graft outcomes and the current data supporting use of anti-plasma cell agents in experimental and clinical transplantation.

B-Cells, PLASMA CELLS, and Allo-Antibodies

To understand the effect of anti-plasma cell agents on the production of DSAs, it is important to review basic concepts of B-cell differentiation and antibody production. After contacting with an antigen in the presence of co-stimulatory signals provided by T-cells, small resting B lymphocytes undergo a structural and functional metamorphosis involving proliferation and differentiation. Some B-cells undergo clonal expansion, undergo maturation of receptor affinity and are able to secrete different antibody isotypes to become memory cells, while others become specialized in secretion of antibody, the plasma cells. Most of these plasma cells, particularly those producing IgM, are short-lived and die by apoptosis after a few days of intense Ig secretion to limit the response after the antigen is eliminated. A minority of B-cells become plasma cells and home to the bone marrow where they find conditions for longer survival, which maintains protective Ig titers in the blood [10].

Several characteristics distinguish plasma cells from memory B cells. Memory B cells do not spontaneously secrete antibody but, after antigenic stimulation, elicit rapid secondary responses by proliferating and differentiating into antibody secreting cell as well as generating more memory B cells. There are two types of plasma cells, short-lived and long-lived (terminally differentiated). Short-lived plasma cells/plasmablasts are susceptible to conventional therapy (rituximab, or increase in maintenance immunosuppression). The reason they respond to conventional therapy is because they depend on the inflammatory microenvironment of inflamed tissue and secondary lymphoid tissue [8, 11]. Upon treatment with rituximab or IVIg, the inflammation subsides, destroying the necessary microenvironment for plasma cell to survive. These short-lived plasma cells, once displaced from the microenvironment, undergo apoptosis. However, some plasma cells are terminally differentiated, non-dividing cells that spontaneously secrete antibody and have lost nearly all surface-bound immunoglobulin, and major histocompatibility complex (MHC) class II molecules. They reside in survival niches in the bone marrow and spleen and can survive there for many years [12, 13, 14].

Terminally differentiated long-lived plasma cells are the major source of alloantibody production and present a difficult challenge for many therapies because they are non-dividing cells and, as mentioned above, have lost many surface markers that could be targeted. Long-lived plasma cells express relatively few surface antigens, and do not express common pan-B cell markers such as CD20 making therapies, e.g., a rituximab or antithymocyte globulin ineffective at causing plasma cell apoptosis [15, 16].

Antibodies provide the first line of defense against infections, and from the evolutionary standpoint is important to obtain long-term humoral protective immunity against infections. They also play a critical role in allo-immunity. The level, affinity, and duration of exposure to allo-antibodies determine the degree of graft damage. The half-life of free immunoglobulin (Ig) is less than 3 weeks [17–19], continuous antibody production is necessary to sustain antigen-specific Ig levels in the serum. Although B cells can secrete antibody at different stages of differentiation, antibody responses are typically maintained by terminally differentiated plasma cells that have a high rate of antibody production, providing long-term humoral immunity. Already within 1 week of antigen presentation, long-lived plasma cells can appear. During the lifespan of the plasma cell, which can last for years, antibodies are produced in large volumes at a rate of 10 thousand molecules per second [20–23]. Therefore, allo-antibody production by plasma cells (PCs) against HLA antigens can be a significant barrier to successful transplantation [15]. Terminally differentiated, long-lived PCs (plasma cells) in the bone marrow and other secondary lymphoid tissue are the most likely source of anti-HLA antibody [12, 24].

Focus on alloantibody monitoring, removal, and plasma cell directed therapy may significantly improve long-term allograft survival. As discussed above, currently available anti-rejection agents do not have any effect on the terminally differentiated plasma cells [25]. Several newer drugs, many of which are part of current desensitization protocols, have been suggested to have activity against PCs including rituximab [26, 27], polyclonal rabbit antithymocyte globulin (rATG) [28], and IVIg [29]. However, Perry et al. showed that current desensitization protocols have little impact on antibody production by normal PCs [15]. They showed that rituximab, polyclonal rabbit rATG and IVIg failed to cause apoptosis of PCs, and neither rituximab nor rATG blocked antibody production [15]. Polyclonal rATG has been shown to contain antibodies against CD38 and CD138, both cell surface markers present on PCs, and to cause apoptosis of PCs in vitro [28]. However, it has been demonstrated that in vivo rATG treatment had no effect on PC number or function in the spleen [30] or bone marrow [31]. One of the limitations to measure the effect of immunosuppression therapy on plasma cells and antibody production is that PCs in humans are an extremely rare cell type (especially in peripheral blood), and the existence of assays available to measure their function is scarce. It is also possible that the reduction in DSA production detected with some of those drugs in the initial observations might be due, in part, to changes in the number and function of non-PC cell types [25]. The reduction in serum DSA levels observed in several studies could be due to factors other than treatment including absorption of antibody by the allograft [32], the development of blocking “anti-idiotypic” antibodies [33], or the self-limited nature of an acute alloimmune response during acute cellular rejection [34].

Proteasome Inhibition, the Plasma Cell, and Alloantibodies

The proteasome system constitutes a multi-enzyme complex (26S proteasome) of large size (700 kilo-Dalton) present in the cytosol and in the nucleus of all eukaryotic cells, and it is highly conserved from yeast to man. It is abundant in all cells, both cancer cells and normal cells, representing about 1% of the cellular protein overall. The ubiquitin-proteasome pathway was first described in 1984, and recognized as critical for cellular homeostasis, and the principal cellular pathway responsible for the degradation of intracellular dysfunctional proteins and a rapid turnover of key regulatory proteins [35]. Ubiquitin effectively tags proteins, marks them for presentation to the proteasome, where the protein is digested and ubiquitin is actually recycled in the cell. Ubiquitin is the marking agent to covalently link the protein and present it to the proteasome structure. It plays an essential role in degrading super- numerous, defective or misfolded proteins, which are targeted for proteasomal degradation by ubiquitinylation.

This complex resembles a cylinder and consists of a 20S core and 19S subunits capping each end. The 20S core consists of four heptameric rings, and the proteolytic activity of the proteasome is inside it. This unit has three different proteolytic activities: chymotryptic-like, tryptic-like, and caspase-like hydrolyzing activity. The 19S regulatory complex consists of: an ubiquitin chain receptor that only recognizes ubiquitin-marked proteins; a cleavage enzyme that releases ubiquitin; and ATPases that help unfold the protein and drive the polypeptide chain down the central channel [36, 37]. Proteasomes are needed to degrade a proportion of newly synthesized proteins that fail to obtain their native conformation. In addition, proteasomes are involved in the regulation of many important cellular processes, including cell cycle and apoptosis, by controlling the degradation of key regulatory factors [38, 39]. The ubiquitin-proteasome system is also responsible for the production of MHC molecules (Figure 16.1).

All cells can be affected by proteasome inhibitors. However, because of their high rate of Ig production, both short and long-lived plasma cells are particularly sensitive to inhibition of the proteasome [40, 41]. Proteasome inhibitors cause accumulation of nondegraded, misfolded proteins within the endoplasmic reticulum of plasma cells and, subsequently, lead to activation of the terminal unfolded protein response, ultimately leading to apoptosis and decrease in antibody production [42, 43]. Proteasome inhibitors have been used for the treatment of B cell malignancies, lupus, and antibody-mediated rejection. In transplantation they are used to decrease the production of antibodies. However, proteasome inhibitors do not cause significant decrease in DSA levels in the first few weeks post-treatment, first because of the long serum half-life of IgG (~3 weeks), and second because it does not completely deplete terminally differentiated DSA-PCs [25]. The marrow population of long-lived PCs is rapidly replaced after depletion. Replacement PCs may have new target antigens because they do not originate from cells with specificities similar to that of preexisting PCs [25]. Although incomplete PCs depletion may result in persistence of allo-antibodies, it has been suggested that it may be beneficial because complete interruption of existing antibody production could lead to profound immunodeficiency and increased infection rates in transplant candidates [25].

The first paper using proteasome inhibitors to prolong graft survival was published in 2001. Luo et al. using a proteasome inhibitor (dipeptide boronic acid, same as bortezomib) in a mouse heart transplant model of acute rejection [44, 45], showed that it can prolong survival. Grafts from treated animals (treated from day 3 to 6) survived 19.8 days compared to 7.3 days in the control group (p=0.0005). Another experiment using the same proteasome inhibitor in an islet cell transplant mouse model showed that islets of treated animals (treated for 17 days) survived in 50% of the recipients for up to 60 days, whereas the control group rejected the islet graft in 7 days [46]. The first report using proteasome inhibitors in a human transplant recipient to inhibit antibody-mediated rejection was published in 2008 [15, 47, 48].

Bortezomib

The proteasome inhibitor bortezomib, N-(2, 3-pyrazine) carbonyl-L-phenylalanine-L-leucine boronic acid, is a boronic acid dipeptide derivative, formerly known as PS-341, which binds very selectively and reversibly to the 26S proteasome [49] (Figure 16.2). This drug was initially developed for use in the cases of severe cachexia and inflammation until the establishment of its anti-tumoral activity in 1997 [50]. In 2003 bortezomib (Velcade™) was approved by the FDA for the treatment of multiple myeloma and mantle cell lymphoma, an aggressive form of non-hodgkin lymphoma. Besides of being used for treatment of B cell malignancies, it has been used for lupus, and antibody mediated rejection [41].

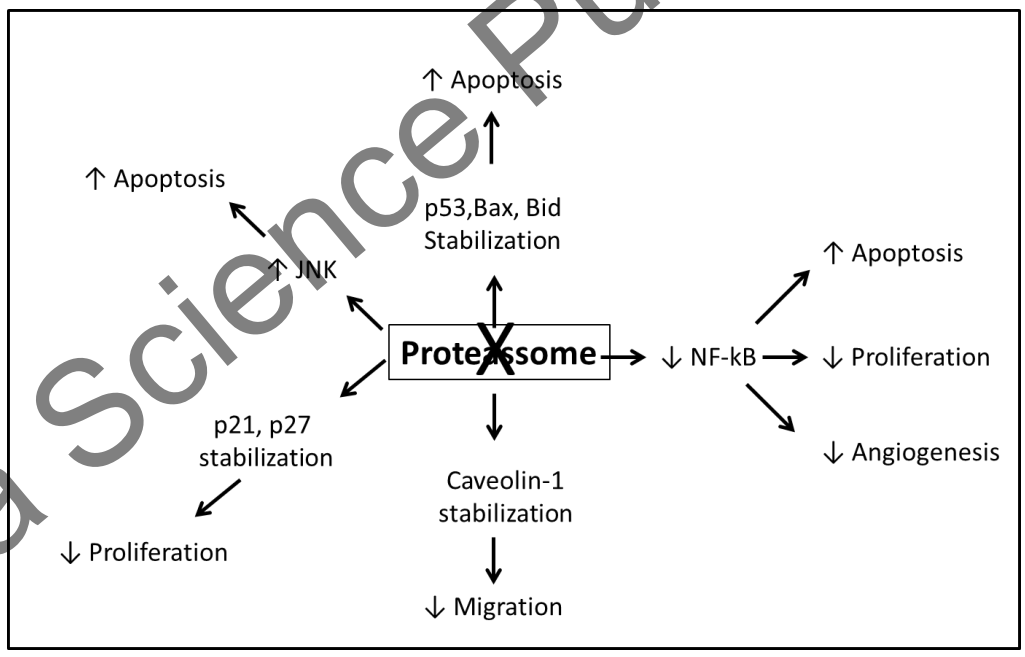

Figure 16.1. The intracellular pathways and proteins affected by proteasome inhibition with bortezomib. Abbreviations: JNK, c-Jun *N*-terminal kinase. Adapted from Wright JJ 2010, ref 80).

Mechanisms of Bortezomib Action

Bortezomib is a peptidomimetic and inhibits by receptor competition. It mimics the proteasome substrate, but has a unique terminal containing a boronic acid, which very specifically interacts with the proteasome. The threonine active site, the threonine hydroxyl group, interacts with the boronic acid to form a very tight complex.

Because the proteasome affects homeostasis of thousands of proteins, it is expected that bortezomib have multiple mechanisms of action and cellular consequences. There are four major effects of proteasome inhibitors that are considered responsible for its immunomodulatory effects: 1) inhibition of nuclear factor-kappa B (NF-kB) activity; 2) inhibition of proliferation and induction of apoptosis via cell cycle arrest; 3) induction of apoptosis via endoplasmic reticulum stress; and 4) inhibition of class I MHC expression via reduction in peptide production [51] (Table 16.1).

Bortezomib's Use in Clinical Transplantation

By 2009, many transplant centers began using proteasome inhibitors. By that time, there were reports of more than 70 patients treated worldwide, which were summarized by Everly et al. [52, 53]. Most clinical studies of bortezomib in transplant patients have involved post-transplant treatment to prevent antibody- mediated rejection (usually combined with cellular rejection) or to deplete antibody that developed de novo after transplantation [15, 47, 54–56], but it has also been used as induction therapy [57] and to prevent/treat chronic rejection [58, 59].

Bortezomib treatment led to PC apoptosis and immediately decreased their numbers in the bone marrow, and thereby blocked anti-HLA IgG secretion in vitro. Two patients treated with bortezomib for humoral rejection after allogenic kidney transplantation demonstrated a transient decrease in bone marrow PCs in vivo and persistent alterations in alloantibody specificities. This suggests that alloantibody-producing PCs undergoing bortezomib-mediated apoptosis likely were replaced by new PCs with other specificities. Total IgG levels were unchanged [15].

Table 16.1. Mechanisms of immunomodulatory effects of bortezomib. Adapted from Walsh RC 2012 (ref 51)

Inhibition of antigen processing for MHC class I presentation
Inhibition of NF-kB and regulation-mediated cytokine production
Induction of cell arrest and apoptosis
Induction of Endoplasmic Reticulum stress apoptosis and terminal unfolded protein response

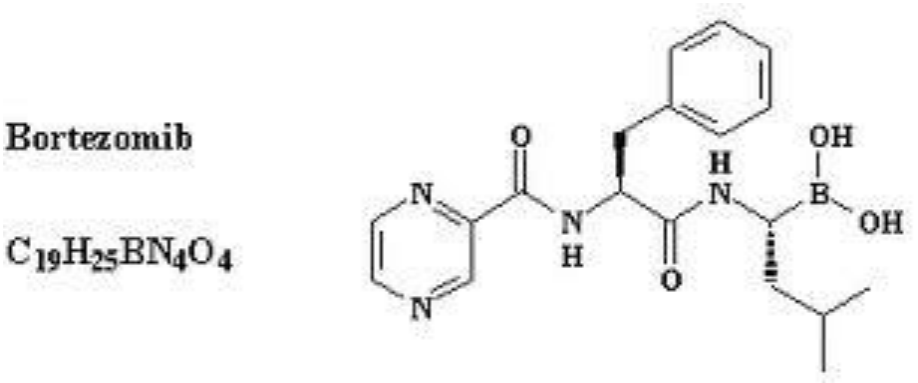

Figure 16.2. Chemical composition of the proteasome inhibitor Bortezomib. N-(2, 3-pyrazine) carbonyl-L-phenylalanine-L-leucine boronic acid, is a boronic acid dipeptide derivative, formerly known as PS-341, which binds very selectively and reversibly to the 26S proteasome.

Bortezomib's Phamacokinetics and Phamacodynamics

The clinical pharmacokinetics of bortezomib is not well documented partly due to analytical difficulties. Bortezomib is administered intravenously and is formulated with mannitol as a lyophilized powder. It has also been used subcutaneously. After injection, bortezomib is distributed widely and quickly to the blood and most tissues [60]. Surface area did not appear to influence bortezomib clearance, suggesting that it could be at a fixed dose.

Overall, the kinetic profile is characterized by a large volume of distribution and a high systemic clearance. The excretion routes remain to be determined as well as the contribution of biotransformation. No drug-drug interactions have been reported but some might be expected, given the metabolic profile of bortezomib [61].

Bortezomib Treatment of Acute Humoral Rejection

Most of the experience of treating antibody- mediated rejection in clinical transplantation derived from renal transplants. However, bortezemib has also been used successfully to treat acute humoral rejection in pancreas, liver, intestinal, multi-visceral, heart and lung transplantation [62–64].

The case reports on the clinical use of bortezomib, summarized by Everly [52, 53], show that over 95% of all patients treated for acute rejection with a bortezomib-based regimen and approximately 50% of patients with a chronic alloantibody- mediated rejection achieved allograft stabilization and/or rejection reversal. In the bortezomib-based therapy cases, approximately 50% of the patients achieved a reduction in DSA, whereas in the rituximab trial no reduction in DSA was seen following therapy. The majority of acute rejection cases were late antibody-mediated rejection and treatment consisted of only one cycle.

Plasmapheresis was used in most of the patients. In these cases, bortezomib treatment led to allograft stabilization in 83% of patients and most patients also had a reduction in alloantibodies. The limitation to assess its efficacy is that most of the experience with bortezomib in transplantation is from non-randomized studies.

In the report by Everly et al., bortezomib plus plasmapheresis was used as a rescue therapy for antibody-mediated rejection and concomitant acute cellular rejection (ACR) in 6 kidney transplant recipients. The rejection was reversed and DSA intensity was reduced in all patients. In the study by Perry et al., significant reduction in the DSA after bortezomib treatment occurred in 2 patients and this reduction was associated with a depletion of allospecific bone marrow plasma cells. Triverdi et al. showed that bortezomib associated to plasmapheresis can prevent humoral rejection by inhibiting production of allo-antibodies [55]. He demonstrated that after 1 month, all of 11 patients treated with bortezomib/plasmapheresis had a DSA reduction of over 50%; and in 76%, complete elimination of DSA. At a mean follow-up of 14 months post-treatment, all 11 patients had stable graft function, in 6 patients the DSA reappeared, and in 3 patients they could not be eliminated [65].

Bortezomib As Induction Therapy

Dunn et al. reported for the first time the use of bortezomib as part of induction therapy in solid organ transplant. They reported one case of high-risk positive crossmatch son-to-mother transplant that was performed after desensitization. The induction immunosuppression was supplemented with bortezomib. Following transplant, donor-specific antibody production remained stable after transplant, with near complete abrogation of class I specificities. There were no bortezomib-related complications, and the patient had excellent early graft function. Serial biopsies did not reveal acute antibody-mediated rejection [57].

Bortezomib's Effect on Chronic Alloantibody-Mediated Rejection

New findings also emerged related to bortezomib's effect on chronic alloantibody-mediated rejection. Schwaiger et al. [66] reported on a patient that had reduction in urinary protein excretion following bortezomib treatment. Additionally, Lubetzky et al. reported on 4 patients with >1 g of proteinuria (mean of 2.6 g/day pre-treatment). The use of bortezomib-based therapy led to a large reduction in proteinuria (mean 0.68 g/day) by 3 months after treatment [67].

Touzot et al. analyzed 28 non-sensitized kidney transplant patients with AMR associated with de novo DSAs. Patients were placed in three groups according to their antirejection treatment: group I: plasma exchange-Rituximab (n=10); group II: bortezomib (n=8); and group III optimization of maintenance immunosuppression (n=10). Anti-class I and anti–DR DSA disappeared after treatment in group I and remained negative during follow-up, whereas anti-DQ DSA persisted without any modulation. In contrast, anti-class I and II HLA-DSA mean fluorescence intensity remained unchanged in groups II and III. One year after the

AMR treatment, the lowest decline in estimated glomerular filtration rate was observed in patients treated with bortezomib [59].

Desensitization with Bortezomib

In addition to treating acute antibody-mediated rejections with proteasome inhibition, many centers have also started using bortezomib in transplant candidates for desensitization. Raghavaiah et al. were the first to report the use bortezomib in desensitization protocols [68]. Their early findings suggest that bortezomib is superior to plasma exchange alone and nearly 67% of the patients treated with bortezomib have reduced their plasma cells in bone marrow, compared to none of the plasma exchange alone patients. Another study by Walsh et al. has shown that 2 cycles (8 doses) of bortezomib can reduce pre-transplant HLA antibodies [54]. On the other hand, a report of two sensitized renal allograft candidates treated pretransplant, showed only a mild reduction in serum DSA levels after one 4-dose cycle [69]. In addition, Diwan et al. showed that Bortezomib alone did not decrease serum DSA levels in highly sensitized patients. However, bortezomib-treated patients who underwent plasmapheresis showed a greater decrease in DSA compared to a historical control group who underwent plasmapheresis alone. Thus, bortezomib depletes DSA-PCs and appears to potentiate DSA removal by plamapheresis in sensitized renal transplant recipients [25].

Toxicity of Bortezomib

Because it affects multiple proteins homeostasis, the cytotoxicity of bortezomib is significant and is mainly associated with induction of apoptosis, anti-angiogenic effects, inhibition of NF-κB activation, and stabilization of cell-cycle regulatory proteins (Table 16.2). Regarding toxicity to bortezomib in transplant patients, the most common toxicities in all case reports are peripheral neuropathy (generally mild), thrombocytopenia/leukopenia, and gastrointestinal (GI) disturbances (especially diarrhea) [52, 70, 71].

In cancer patients, the incidence of side effects is higher than in the transplant setting because the number of doses is usually markedly superior. In the APEX trial, including more than 600 patients, the most significant side effects of all grades were diarrhea (57%), nausea (57%), fatigue (42%), constipation (42%), neuropathy (36%), vomiting (35%), anorexia (35%) and thrombocytopenia (35%). Although the majority of these adverse events were grade 1 or 2, thrombocytopenia, gastrointestinal toxicity and peripheral neuropathy have been more extensively studied because they are probably the most significant, especially peripheral neuropathy.

Thus, the frequency of grade 3–4 thrombocytopenia and gastrointestinal symptoms are approximately of 25% and 20%, respectively. Concerning bortezomib-related peripheral neuropathy, its incidence of grade 3–4 ranged from 8% to 15% [72].

Table 16.2. Bortezomib cytotoxicity mechanisms

Induction of apoptosis
Anti-angiogenic effects
Inhibition of NF-kB activation
Effects on cell-cycle regulatory proteins
Increased cellular sensitivity to hypoxia

In transplantation, a recent report by Schmidt et al. showed that among 51 patients treated for AMR and 19 patients treated for desensitization received 96 bortezomib cycles (1.3 $mg/m^2 \times 4$ doses); mean (SD) follow-up was 16.3 (9.0) months. Patients treated for AMR received a mean (SD) of 4.9 (2.0) bortezomib doses in 1.3 (0.5) cycles; and patients treated for desensitization, a mean of 7.3 (1.6) doses in 1.8 (0.4) cycles. In the AMR cohort, two cases of cytomegalovirus infection, two cases of BK virus infection, and one case of Epstein-Barr virus infection were observed. No cases of viral infection were observed in the desensitization cohort. Malignancies were not observed. Significant bortezomib toxicities included anemia and peripheral neuropathy, which were manageable. Anemia was more common in patients treated for AMR; and peripheral neuropathy, more common in patients treated for desensitization [71]. In addition, bortezomib's safety regarding viral reactivation of cytomegalovirus, Epstein Barr virus, and BK virus are not clearly known, therefore precautions must be taken (especially in combination regimens).

Given that bortezomib leads to apoptosis of plasma cells, one major concern is whether it kills all plasma cells equally thereby eliminating protective immunity to previous vaccination. This fortunately is not the case. Proteasome inhibition (bortezomib) has differential effect on those plasma cells such as myeloma cells that produce high amounts of IgG antibodies (highly active plasma cells) compared to those that produce low amounts of IgG [40].

The treatment with bortezomib still requires a blood count and biochemistry to be performed at least once per cycle (usually on day 1 of each cycle), and some patients develop bortezomib–related hypotension requiring intravenous hydration after bortezomib administration.

Other Biological Inhibitors

Carfilzomib

Carfilzomib irreversibly inhibits the proteasome and has been used so far exclusively in the treatment of multiple myeloma [73]. It has been studied and shown to be effective in treating myeloma refractory to bortezomib and lenalidomide. Additionally, it is associated with a lower rate of peripheral neuropathy than bortezomib [74] (Figure 16.3).

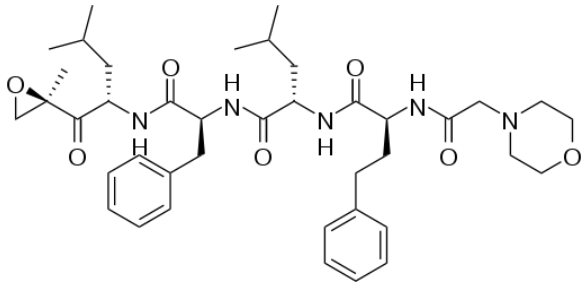

Figure 16.3. Carfilzomib is a selective irreversible inhibitor of proteasome. (*S*)-4-Methyl-*N*-((*S*)-1-(((*S*)-4-methyl-1-((*R*)-2-methyloxiran-2-yl)-1-oxopentan-2-yl) amino)-1-oxo-3-phenylpropan-2-yl)-2-((*S*)-2-(2-morpholinoacetamido)-4-phenylbutanamido) pentanamide. It is an epoxomicin derivate. Carfilzomib irreversibly binds to and inhibits the chymotrypsin-like activity of the 20S proteasome.

Table 16.3. Anti-plasma cell agents and mechanisms of action

Bortezomib	inhibitor of the proteasome (reversible)
Carfilzomib	inhibitor of the proteasome (irreversible)
Belimumab	human monoclonal antibody that inhibits B-cell activating factor (BAFF), also known as B-lymphocyte stimulator (BLyS)
Atacicept	blocks activation of B cells by the tumor necrosis factor by binding to B-lymphocyte stimulator (BLyS) and a proliferation–inducing ligand (APRIL)
Tocilizumab	humanized monoclonal antibody against interleukin-6 receptor
Anti-IL-5 antibody	depletion of eosinophils and PC survival factors

Belimumab

Belimumab is a human monoclonal antibody that inhibits B-cell activating factor (BAFF), also known as B-lymphocyte stimulator (BLyS). It has been approved by FDA to treat autoimmune disorders (lupus) and is being investigated for use in solid organ transplant. This group of compounds may also have the ability to kill the plasma cell and therefore may be effective in decreasing/removing DSA alone or in combination with proteasome inhibitor therapy [75].

Atacicept

Atacicept is a recombinant fusion protein designed to inhibit B cells, thereby suppressing autoimmune disease. This protein combines the binding site for two cytokines that regulate maturation, function, and survival of B cells, B-lymphocyte stimulator (BLyS) and a proliferation-inducing ligand (APRIL), with the constant region of immunoglobulin. Atacicept blocks activation of B cells by the tumor necrosis factor receptor superfamily member 13B (more commonly known as TACI), a transmembrane receptor protein found predominantly on the surface of B cells. Like the monoclonal antibody belimumab, atacicept blocks the binding of BLyS, but it also blocks APRIL. Binding of these TACI ligands induces

proliferation, activation, and longevity of B cells and thus their production of autoantibodies. Atacicept is thought to selectively impair function of mature B cells and plasma cells with less impact on progenitor cells and memory B cells.

Tocilizumab

Tocilizumab is a humanized monoclonal antibody against interleukin-6 receptor, and is FDA-approved for treatment of systemic juvenile idiopathic arthritis [76, 77].

Anti-IL-5 Antibody

Treatment with anti-IL-5 is able to reduce, but not eliminate, alloreactive PCs in the bone marrow. This is because of the targeted reduction of eosinophils leading to a reduction in the PC survival factors as proliferation-inducing ligand and IL-6 [78]. Eosinophils have been shown to govern PC persistence. Interleukin 5 (IL-5) depletion is known to reduce eosinophils [79] (Table 16. 3).

Conclusion

There is growing evidence for the importance of allo-antibodies in both acute and chronic rejection. A new interest in role of plasma cells in solid organ transplant has opened a new avenue for dealing with alloantibodies, one of the major limitations to prolong allograft survival today. The resistance of long-lived plasma cells against immunosuppressive medication creates a serious problem for the treatment of Ab-mediated rejection. Many agents initially believed to have anti-plasma cell effects are not truly effective. Bortezomib, a reversible proteasome inhibitor, has been shown to be the most effective to reduce allo-specific plasma cells. Most of the clinical experience of treating antibody-mediated rejection with bortezomib derived from renal transplants. However, it has also been used successfully to treat acute humoral rejection in pancreas, liver, intestinal, multi-visceral, heart and lung transplantation. Bortezomib has also shown a relatively good safety profile. It will be important to develop newer drugs that are selective, efficient and safe in depleting long-lived plasma cells and eliminating DSAs. Randomized controlled trials will be crucial to compare effectiveness of these drugs.

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Chapter 17

Immunosuppressive Antibodies

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Abstract

Currently, a wide variety of both polyclonal and monoclonal antibodies are being evaluated or utilized to prevent and treat the adverse host-graft interactions in organ transplantation. Although many of these antibody therapies successfully reduced the incidence of acute rejection episodes and improved short and/or long-term graft survival, the complications associated with the use of these agents, such as the incidence of opportunistic infections and neoplasm, are still of concern. In this chapter, a *general* description *of the* immunosuppressive antibodies that have been in clinical trials or have already been *employed in clinical practice* of transplantation is given. Those that have been particularly well-studied and approved by FDA are discussed in greater detail.

Keywords: antibody, transplantation, graft, immunosuppressive agent

Abbreviations

BPAR	Biopsy-proven acute rejection
CNI	Calcineurin inhibitor
CsA	Cyclosporine A
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
FDA	Food and Drug Administration

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GFR	Glomerular filtration rate
GVHD	Graft versus host disease
MLR	Mixed lymphocyte reaction
MMF	Mycophenolate mofetil
MPA	Mycophenolic acid
mTOR	Mammalian target-of-rapamycin
PTLD	Post-transplant lymphoproliferative disease
RATG	Rabbit-derived antithymocyte globulin
Treg	Regulatory T cells

Introduction

The improvement in surgical techniques and the discovery of immunosuppressive agents have made great progress in preventing organ rejection. A large array of immunosuppressive agents provides excellent short- and long-term graft survivals in experimental conditions and clinical practices. However, all immunosuppressive medications demonstrate substantial interactions and toxicities. Immunosuppressive protocols must be balanced to not only minimize graft rejection, but also to avoid unwanted complications. The ideal solution would be the selective suppression of the rejection process without impairing other functions of the immune apparatus. Thus, agents that specifically interact with the rejection-related cellular components of host immune system are required. On account of the high specificity of their effects, antibodies have been selected as the most promising candidates. A variety of antibodies directed against the different components of the immune reaction have therefore been tested as immunosuppressants.

The earliest antibodies tested in transplantation were polyclonal [1]. In general, polyclonal antibodies for therapeutic interventions are obtained from the serum of animals (heterologous) immunized with the human thymocytes or lymphocytes. The targets of polyclonal antibodies are usually not clear. A broad spectrum of cell types, such as T cells, B cells, NK cells and macrophages, might be included [2]. Polyclonal antibodies have some general advantages: the methodology required to produce polyclonal antibody is low-tech; it is inexpensive to produce large amounts of multi-target antibodies; a polyclone can recognize multiple epitopes and thus can help amplify signal from target protein with low expression level and provide more robust detection.

Although polyclonal antibody preparations have been clinically shown to be effective in many cases, problems related to toxicity, specificity and lot-to-lot variation have limited their use. The active antigen-specific antibodies in a serum preparation usually represent a relatively small portion of the total antibodies, and the rest of the antibodies are not only ineffective but can even toxic or immunogenic. Administration of non-human antibodies in patients may result in immune responses against the foreign proteins. Patients may have an acute reaction to a polyclonal antibody treatment, characterized by the symptoms such as fever, rigor, headache, myalgia and arthralgia, and even anaphylaxis. Some patients may develop serum sickness or immune complex glomerulonephritis at later stage of the treatment. Besides, the targets and the working mechanisms of polyclonal antibodies are usually not clear and polyclonal antibodies may cause general immunosuppression, further

leading to post-transplant lymphoproliferative disorders (PTLD) or serious infections, e.g., cytomegalovirus. To reduce their toxicity, the use of highly purified serum fractions and intravenous administration combined with other immunosuppressants, such as calcineurin inhibitors (CNIs), cytostatics and cortisteroids are usually considered.

Due to the problems caused by the polyclonal antibodies in therapeutic practice, many investigated polyclonal antibodies have been superseded. Thus far, two preparations of antithymocyte globulin, atgam and thymoglobuline are still available in the market. Accumulated clinical data suggest that antithymocyte globulin is an effective and generally well-tolerated option for the prevention and treatment of acute renal graft rejection in renal transplant recipients [3–6].

Compared to the polyclonal antibodies, monoclonal antibodies are directed towards exactly defined antigens. Theoretically, they should be more specific and less toxic. Such expectations have been approved by significant performance of several monoclonal antibodies, such as the Muromonab-CD3 that has been shown to be one of the most potent immunosuppressive substances and is administered to control the steroid- and/or polyclonal antibodies-resistant acute rejection episodes. However, although some polyclonal and monoclonal antibodies produce profound immunosuppressive effects, the therapeutic experiences with rodent or other non-human antibodies have been somewhat disappointing. Many animal-derived antibodies directed against cell surface molecules have failed to deliver on their potential advantages for suppressing unwanted immune responses in transplantation settings. The discrepancy between the high promise and poor clinical outcomes of therapeutic non-human antibodies can be explained at least in part by the limiting effect of the ubiquitous human antixenoantibody response and failure of xenoantibody to activate human complement or Fc receptor-expressing immune cells. This knowledge gave the impetus for creating chimeric or humanized mAbs or fully human antibodies to be utilized as immunosuppressive agents. Chimeric antibodies are produced through a gene fusion strategy in which codons for the antigen-binding Fv region from none-human antibody are joined to codons for human constant region sequences. Humanized antibody can be generated either by the creation of a more human-like non-human antibody chimera through the selective alteration of the sequence of amino acids in the Fab portion of the molecule or by a "direct creation" through the insertion of the appropriate complementary-determining region (CDR) codons (responsible for the desired binding properties) into the sequence of the "scaffold" of a human antibody. Fully human monoclonal antibodies can be produced using transgenic mice or phage display libraries. Human immunoglobulin genes are transferred into the murine genome, after which the transgenic mouse is vaccinated against the desired antigen, leading to the production of human monoclonal antibodies [7]. Phage display is a laboratory technique that uses bacteriophages to connect proteins with the genetic information that encodes them. In this technique, a gene encoding human antibody of interest is inserted into a phage coat-protein gene, causing the phage to "display" the protein, allowing the transformation of murine antibodies *in vitro* into fully human antibodies [8].

Currently, a wide variety of antibodies, including full-sized antibodies, fragments, conjugates and fusion proteins are being investigated or routinely utilized in the prevention and treatment of adverse host-graft interaction. The targets of these antibodies include the well-known lymphocyte surface molecules, cytokines, co-stimulatory signals and various B-cell epitopes involved in the process of allograft rejection. In this chapter, general information about the immunosuppressive antibodies that have been in clinical trials or have already been

employed in clinical practice of transplantation is given in a tabular format (Table 17.1) [9]. Those which have been particularly well-studied and approved by Food and Drug Administration (FDA) are discussed in greater detail.

The Well-Studied and FDA Approved Antibodies

Antithymocyte Globulin

Currently, the well-known antithymocyte globulin agents are atgam and thymoglobulin. Both drugs are used as anti-rejection, more often immunosuppression induction agents before and/or during organ transplantation. They were reportedly the most common induction agent primarily used in patients with renal, intestine and pancreas transplantation [10, 11].

The therapeutic preparation of atgam is sterile solution that contains anti-thymocyte globulin. It is the purified, concentrated, and sterile gamma globulin, primarily monomeric IgG, from the serum of horses immunized with human thymus lymphocytes. Atgam Sterile Solution is indicated for the management of allograft rejection in renal transplant patients. The therapeutic thymoglobulin is a purified and pasteurized preparation of polyclonal gamma immunoglobulin raised in rabbits against human thymocytes. It has been indicated for the prevention and/or treatment of renal transplant rejection in several countries worldwide. Accumulated data show that Thymoglobulin induction combined with other immunosuppressive therapy is more effective in preventing episodes of acute renal graft rejection in adult renal transplant recipients than immunosuppressive therapy without induction.

Atgam sterile solution is a lymphocyte-selective immunosuppressant as demonstrated by its ability to reduce the number of circulating thymus-dependent lymphocytes that form rosettes with sheep erythrocytes. In addition to its antilymphocytic activity, Atgam contains low concentrations of antibodies against other formed elements of the blood. In rhesus and cynomolgus monkeys, Atgam reduces lymphocytes in the thymus-dependent areas of the spleen and lymph nodes. It also decreases the circulating sheep-erythrocyte-rosetting lymphocytes that can be detected, but ordinarily atgam does not cause severe lymphopenia. In general, when atgam is given with other immunosuppressive therapy, such as anti-metabolites and corticosteroids, the patient's own antibody response to horse gamma globulin is minimal. A small clinical study showed that atgam administered with other immunosuppressive therapy and measured as horse IgG had a serum half-life of 5.7 ± 3 days [12].

The primary clinical experience with atgam sterile solution has been in renal allograft patients who were also receiving concurrent standard immunosuppressive therapy (azathioprine, corticosteroids). Wechter et al. tested its efficacy and safety in controlled studies in 358 renal allograft recipients and noted that fourteen daily doses of atgam (along with azathioprine and corticosteroids) provided sufficient immunosuppression by delaying early acute rejection [13].

Table 17.1. Polyclonal and monoclonal antibodies-sources and mechanisms of action

INN	Proprietary name	Species	IG class and subclass or IG format	Specificity target name	Company	Clinical indication	Development status	Regulatory agency status and year
Abatacept	ORENCIA®	*Homo sapiens*	CTLA4 (cytotoxic T-lymphocyte-associated protein 4, CD152) - IGHG1 Fc	CD80 (B7-1, CD28LG1) CD86 (B7-2, CD28LG2)	Bristol-Myers Squibb(Princeton NJ USA)	Graft-versus-host disease (GvHD) (solid organ transplant)	Phase I	
Alemtuzu-mab	CAMPATH®(US); MABCAMPATH® (EU) LEMTRADA®	Humanized	IgG1 - kappa	CD52	Berlex Inc. /Genzyme Corp. /Millennium Pharmaceuticals Inc.（USA)	Kidney transplant rejection	Phase I/II	
Basiliximab	SIMULECT®	Chimeric	IgG1 - kappa	IL2RA (interleukin 2 receptor alpha subunit, IL-2RA, TAC, CD25)	Novartis Pharmaceuticals Corp. (East Hanover NJ USA)	Kidney transplant rejection, acute (Prevention)	Phase M	FDA approval January 02, 2003
						Organ transplant rejection, acute (Prevention)	Phase M	FDA approval May 12, 1998
Begelomab	BEGEDINA®	*Mus musculus*	IgG2b - kappa	DPP4 (dipeptidyl peptidase 4, ADCP2, TP103, CD26)	ADIENNE Pharma & Biotech(Caponago MB Italy)	SR-GvHD (Steroid-refractory acute GvHD）; GvHD	Phase I/II	
Belatacept	NULOJIX®	*Homo sapiens*	CTLA4 (cytotoxic T-lymphocyte-associated protein 4, CD152) - IGHG1 Fc	CD80 (CD28LG1) ;CD86 (CD28LG2) ;bispecific	Bristol-Myers Squibb(Princeton NJ USA)	Kidney transplant rejection (Prevention)	Phase M	FDA approval June 15, 2011

Table 17.1. (Continued)

INN	Proprietary name	Species	IG class and subclass or IG format	Specificity target name	Company	Clinical indication	Development status	Regulatory agency status and year
Cedelizumab		Humanized	IgG	CD4 (p55)	Centocor Ortho Biotech Products LP (Raritan NJ USA)	Allograft rejection (Prevention)	Phase II	
Daclizumab	ZENAPAX® (EC withdrawal 2009)	Humanized	IgG1	IL2RA (interleukin 2 receptor alpha subunit, TAC, CD25)	PDL (Protein Design Labs) BioPharma(Fremont CA USA)	Liver transplantation	Phase II	
Daclizumab	ZENAPAX® (EC withdrawal 2009)	Humanized	IgG1	IL2RA (interleukin 2 receptor alpha subunit, IL-2RA, TAC, CD25)	PDL (Protein Design Labs) BioPharma (Fremont CA USA)	Liver transplantation	Phase II	
						GvHD	Phase II	
						Pediatric kidney transplantation	Phase I/II	
					Roche, F. Hoffmann-La Roche Ltd. (Basel Switzerland) (EU)	Kidney transplant rejection, acute (Prevention)	Withdrawn	FDA approval 1997
Mirococept		*Homo sapiens*	CR1 (complement receptor type 1, C3b/C4b receptor, C3BR, Knops blood group, KN, CD35, 41-238) peptidyl-myristoylated	complement C3b, C4b	MRC, King's College(London UK)	Transplantation	Phase II	

INN	Proprietary name	Species	IG class and subclass or IG format	Specificity target name	Company	Clinical indication	Development status	Regulatory agency status and year
Muromonab-CD3	ORTHOCLONE OKT3®	*Mus musculus*	IgG2a - kappa	CD3E (CD3 epsilon)	Centocor Ortho Biotech Products LP (Raritan NJ USA) / Janssen Biotech, Inc(Horsham PA USA)	Acute kidney transplant rejection; Liver / Heart transplant rejection (reversal)	Phase M	FDA approval Sept. 14, 1992
Odulimomab	ANTILFA®	*Mus musculus*	F(ab')2 - G1	ITGAL (integrin alpha L subunit, lymphocyte function associated antigen 1, CD11a)	Pasteur M (Lyon France)	Allograft rejection	Phase III	Not renewed April 01, 2000; Temporary Authorizations for Use Aug. 01, 1994
Ruplizumab	Antova™	Humanized	IgG1 - kappa	CD40L, TNFSF5, TRAP (TNF related activation protein), CD154	Biogen Idec(Cambridge MA USA)	Allograft rejection	Phase I/II	
Siplizumab	ALLOMUNE™	Humanized	IgG1 - kappa	CD2 (lymphocyte function-antigen 2, LFA-2)	AstraZeneca(London UK) / BioTransplant (Charlestown USA) / MedImmune(Gaithersburg MD USA)	GvHD	Phase II	
						Acute kidney transplant rejection	Phase I/II	
Visilizumab	NUVION® SMART™ anti-CD3	Humanized	IgG2	CD3E (CD3 epsilon)	PDL (Protein Design Labs) BioPharma(Fremont CA USA)	Kidney transplantation	Phase II	
						Allograft rejection	Phase I/II	

Table 17.1. (Continued)

INN	Proprietary name	Species	IG class and subclass or IG format	Specificity target name	Company	Clinical indication	Development status	Regulatory agency status and year
Ziralimumab		*Homo sapiens*	IgM - nd	BSG (basigin, Ok blood group, CD147)	Abgenix (Fremont CA USA)	Solid organ transplantation	Preclinical	
						GvHD	Preclinical	
zolimomab aritox	H65-ricin A chain immunotoxin, H65-RTA	*Mus musculus*		CD5 (T1, LEU-1)	Unknown	GvHD	Discontinued	
	MEDI-500, T10B9, T10B9.1A-31	*Mus musculus*	IgM - kappa	T cell receptor (TR, TR alpha_beta)	AstraZeneca (UK) /MedImmune (Gaithersburg MD USA)	GvHD	Phase III	
	HuMax-TAC™	*Homo sapiens*		IL2RA (IL-2R alpha subunit, TAC, CD25)	Genmab A/S (Copenhagen Denmark)	Transplant rejection	Preclinical	

(Modified tables of the IMGT Web resources > IMGT Repertoire (IG and TR) > Genes and clinical entities [internet] available at: http://www.imgt.org/mAb-DB/index#top)

INN: International Nonproprietary Name;

Discontinued: stopped testing before marketing

Phase I: safety testing and pharmaceutical profiling in humans. In Phase I clinical trials, researchers test a new drug or treatment in a small group of people (20–80) for the first time to evaluate its safety, determine a safe dosage range, and identify side effects.

Phase II: effectiveness in humans (dose-ranging studies in target population). In Phase II clinical trials, the study drug or treatment is given to a larger group of people (100–300) to see if it is effective and to further evaluate its safety.

Phase III: extensive clinical trial in humans (late-stage clinical trials in humans). In Phase III studies, the study drug or treatment is given to large groups of people (1,000–3,000) to confirm its effectiveness, monitor side effects, compare it to commonly used treatments, and collect information that will allow the drug or treatment to be used safely.

Phase M: on the market.

Withdrawn: withdrawn from marketing

These phases are defined by the FDA in the Code of Federal Regulations.

When administered with conventional therapy at the time of rejection, atgam increases the frequency of resolution of the acute rejection episode. The drug has also been administered as an adjunct to other immunosuppressive therapy to delay the onset of the first rejection episode [14]. However, accumulated date have not consistently demonstrated it efficacy in the improvement of functional graft survival associated with therapy to delay the onset of the first rejection episode. In controlled trials, investigators frequently reported the adverse reactions such as fever, chills, leukopenia, thrombocytopenia and dermatologic reactions [15]. Because of its unsatisfactory performance in the therapeutic use in transplantation settings, atgam was gradually replaced by thymoglobulin.

Thymoglobulin is a rabbit-derived antithymocyte globulin product (RATG) approved earlier in Europe in 1984, but it was not available in the U.S. until 1998. Although its elimination half-life is measured in 2–3 days, RATG can cause persistent lymphopenia that extends beyond its presence in the body and thus give sustained immunosuppression. RATG has been well accepted for medical use, and according to Annual Data Reports of Scientific Registry of Transplant Recipients (USA) [16], more transplant patients have been administering T cell depletion drugs as an induction agent in the last decade. In a double blind study, Brennan et al. compared RATG and atgam in renal transplant recipients for induction. 72 transplant patients were randomized to receive daily induction doses of 1.5 mg/kg of RATG (n=48) or 15 mg/kg of atgam [n=24) for at least 7 days. All patients received maintenance therapy with corticosteroids, azathioprine or mycophenolate mofetil (MMF), and cyclosporine A (CsA). At 6 and 12 months, acute rejection rates were significantly lower among patients receiving RATG (RATG *vs*. atgam: 4% *vs*. 17%, $p = 0.038$ and 4% *vs*. 25%, $p=0.014$, respectively) (17]. The efficacy of RATG on patients with preexisting kidney dysfunction was estimated by Bajjoka and colleagues. They utilized daily RATG dosing in order to delay CNI introduction. Tacrolimus was initiated when serum creatinine fell below 1.3 mg/dL or after a maximum of five doses of RATG was given. The patients were compared to eighty patients initiated on tacrolimus therapy within 48 hours post-transplant, while both groups received three months of steroids and MMF. Although graft and patient survival were not different between the groups after twelve months, the RATG group did have a higher glomerular filtration rate (GFR). Moreover, 16% of patients in the RATG group who were dialysis-dependent prior to transplant regained renal function [18].

The evaluation of RATG treatment is not limited to renal transplantation. According to 2011 Annual Data Reports of Scientific Registry of Transplant Recipients (USA) data, 50.1% of heart transplant patients received induction therapy, with RATG being the most commonly used agent (18.1%) [10]. Universal induction therapy has been shown to reduce rejection rates, however, it also predisposes patients to a higher incidence of infectious complications and malignancies. In a retrospective review of 5,897 heart transplant patients, Higgins et al. showed that survival benefits of induction therapy was only associated with patients with the highest risk for rejection, typically in young adults with 4 or more HLA mismatches. Patients considered at a lower risk of rejection who also received induction therapy, were found to have worse survival as a result of infectious and neoplastic complications [19]. However, lung transplant patients receiving induction therapy showed better graft survival compared to the patients receiving no induction. In a prospective study of 1999 lung transplant patients, RATG reduced rejection episodes when added to a triple immunosuppression [20]. On the other hand, the effects of RATG on graft versus host disease (GVHD) were also investigated. In two independent clinical trials, RATG has been used to reduce acute GVHD in recipients

getting progenitor cell transplants [21]. Although it showed that higher doses reduced GVHD but the benefit was counteracted by increased infections; a long-term follow up proved that at both high (15 mg/kg) and low [7.5 mg/kg) doses, chronic GVHD was reduced [22]. However, a similar trial of RATG presented a trend in reduction of chronic GVHD that was not statistically significant [23]. The Canadian Blood and Marrow Transplant Group is currently conducting the first randomized trial in chronic GVHD using an even lower dose of RATG (4.5 mg/kg) in an attempt to confirm these observations. The endpoint is the reduction in the proportion of patients with chronic GVHD at 1 year, off immunosuppressants [24].

Although various organ transplant patients benefit from the use of antithymocyte globulin as induction agents and anti-rejection drugs, the safety issue of RATG and atgam does cause concern. These agents have been associated with reaction such as fever, chills, thrombocytopenia, diarrhea, vomiting, transient neutropenia, syncope with a generalized seizure and other systemic effects [25, 26]. Serum sickness, although reported to be variable, has also been documented in patients receiving either atgam or RATG. Patients receiving repeated therapy showed a higher incidence of opportunistic infections (viral, fungal and parasitic) and malignancies [27–29].

Muromonab-CD3

Muromonab-CD3, also known as orthoclone OKT3, is a mouse IgG2a antibody that was first introduced in the setting of renal transplant induction therapy in the early 1980s, when it was shown to effectively treat acute allograft rejection [30,31]. OKT3 experienced considerable success in reversing acute rejection episodes for a variety of solid organ transplants, especially in high-risk rejection patients [32]. Its popularity continued to grow when it was unequivocally shown to significantly decrease the percentage of acute rejections of cadaveric renal transplants as compared to conventional high-dose steroid therapy [33].

The principal mechanism of OKT3 is its specific interaction with the epsilon chain of the CD3 on T cells (Figure 17.1). This interaction transiently activates T cells, resulting in a "cytokine release syndrome"- the release of cytokines such as tumor necrosis factor, interferon gamma, IL-2, IL-3 and IL-6 [34, 35]. In such activation, T cells are unable to proliferate or differentiate, instead, they subsequently disappear from circulation through massive lysis [36].

Although OKT3 has been administered in transplantation with remarkable early clinical success, its adverse effects have drawn many concerns. The first dose of OKT3 could cause short-term physiologic changes resembling a systemic inflammatory response (secondary to cytokine release syndrome). This could manifest as high fever, hypotension, chills, nausea, vomiting, diarrhea, dyspnea and even pulmonary edema [37, 38]. More rarely, OKT3 treatment led to aseptic meningitis or intragraft thrombosis [39, 40]. As a murine monoclonal antibody, the induction of anti-murine or anti-idiotype antibodies after OKT3 administrations seems hardly avoidable.

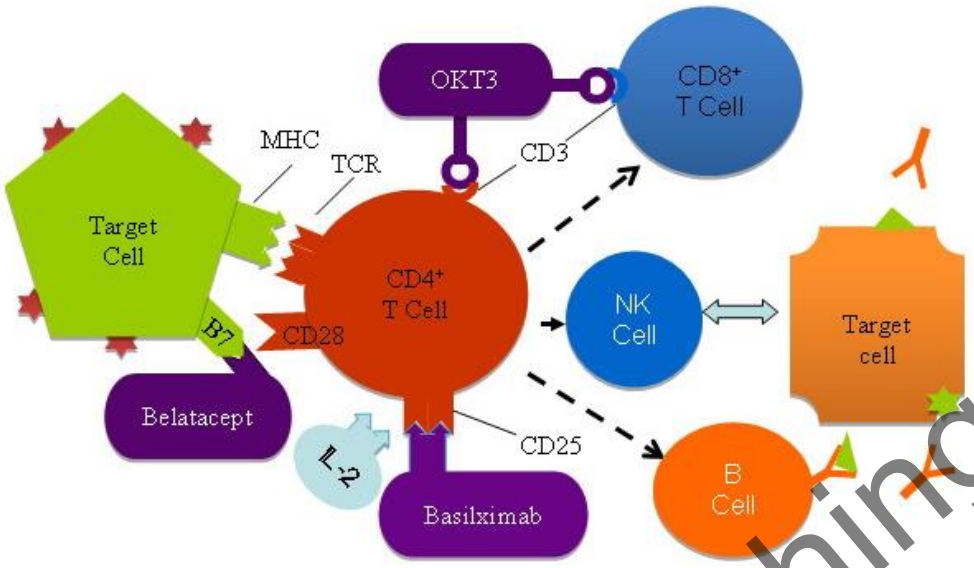

Figure 17.1. Cells and molecules targeted by the *FDA-approved* therapeutic monoclonal antibodies in transplantation. The OKT3 binds to CD3 inducing selective modulation and inactivation of T cells. The belatacept and basilximab block the signal transduction of B7 to CD28 and IL-2 to CD25, respectively.

Comparative studies have revealed further disadvantages of OKT3 treatment relative to other antibody therapies. In kidney transplants, OKT3 was most notably outperformed by RATG treatment, which demonstrated roughly equivalent 1-year graft survival rate (89% RATG *vs.* 81% OKT3) with fewer side-effects [41, 42]. A long-term associational study showed that patients treated with RATG had more than twice mean graft survival rates compared to those of OKT3-treated patients (9.5 *vs.* 4.6 years) [43]. In other comparative studies, RATG produced fewer side-effects, a lower incidence of bronchiolitis obliterans syndrome, and superior 5-year survival outcomes (52% *vs.* 34%) in lung transplant patients than that of OKT3 [44, 45]. Additionally, OKT3 has been shown to be less effective than RATG in reducing infectious episodes, delaying rejection and promoting survival [46].

Because of its numerous side-effects and declining usage, along with the development of better-tolerated alternatives, OKT3 was gradually removed from the market. So far, a number of modified OKT3 Fc variants have been explored to avoid the issue of the antigenicity and other disadvantages, however, the efficacy and toxicity of these alternatives is still under investigation [47–49].

Basiliximab

Basiliximab (trade name Simulect) is a chimeric mouse-human monoclonal antibody to the α-chain of the interleukin-2 (IL-2) receptor (CD25). It is a Novartis Pharmaceuticals product [50] and was approved by the FDA for prevention of acute organ transplant rejection in 1998 and of acute kidney transplant rejection in 2003 [51] (Table 17.1).

Basiliximab specifically binds the CD25 on activated T lymphocytes (Figure 17.1). Through competitive antagonism of IL-2, basiliximab supplements standard immune-suppressive therapy after organ transplantation. It is given in two doses, the first within 2

hours of the start of the transplant operation and the second 4 days after the transplant. Minimum serum level should be 0.2 μg/ml (ELISA). 24 Hours after a single intravenous dose of basiliximab 2.5 to 25 mg, approximately 90% of available IL-2 receptors on T lymphocytes were complexed with the drug. This level of Basiliximab binding was maintained for 4 to 6 weeks when renal transplant patients received basiliximab 20 mg 2 hours before and then 4 days after transplantation surgery [52].

The efficacy and complication of basiliximab have been extensively evaluated in various transplant models, especially in renal allotransplantation. Nashan et al. published results of a randomized prospective trial with basiliximab induction (20 mg dose on day 0 and day 4 post-transplant) early in 1997. Compared to placebo on a double therapy background (CsA and steroids), the 6-month incidence of biopsy-proven acute rejection (BPAR) was lower in the basiliximab group (29.8% *vs*. 44%), as was the incidence of antibody-requiring steroid-resistant first rejection episodes (10% *vs.* 23.1%). In addition, there was no evidence of cytokine release syndrome, nor was there any significant difference in rates of infection or PTLD [53]. This result was independently verified in phase 3 trials at a number of other institutes, in the setting of triple therapy (CsA, azathioprine and prednisone; 6-month acute rejection rate 20.8% *vs*. 34.9%) compared to the placebo [54], and with deceased donor and living donor transplants [55–57]. Early evidence argued that basiliximab with triple therapy drastically improved GFR in pediatric renal transplant patients, (98 mL/min vs. 75 mL/min) and reduced BPAR (7.1% *vs*. 26.1%) compared to the placebo [58], however, such significance was not supported by the subsequent study from Offner et al. [59]. In addition to the evaluation of efficacy, a number of studies have examined different dosing and supplementary immunosuppressive regimes. Matl et al. used one dose of basiliximab (40 mg administered on the day after surgery) and showed slightly improved BPAR rates (17.0% *vs.* 19.6%) as well as lower acute rejection incidence (20% *vs*. 22.5%) compared to the conventional two-dose course, with no increase in morbidity [60,61]. A similar outcome was observed in the later study by Baquero et al., further supporting the cost-saving single-dose application of basiliximab [62]. In two large, well-designed trials, the percentage of patients with BPAR after renal transplantation was significantly lower with basiliximab 20 mg (administered 2 hours before and then 4 days after transplantation surgery; 30 or 33%, respectively) than placebo (44 or 46%) at 6 months after surgery. Basiliximab was well tolerated during clinical trials. Cytokine release syndrome was not observed in patients who received basiliximab, and in comparison with placebo, the incidence of infections (including active cytomegalovirus infection) and PTLD was similar [63]. Because both basiliximab and RATG had been widely used in renal transplant patients, a number of studies compared their outcomes in a variety of settings. In a one-year follow up pilot study, Mariat et al. demonstrated that renal transplant patients treated with basiliximab had a significantly higher BPAR rate than those treated with RATG (50% *vs.* 19%) [64]. However, their observations were not supported by the large prospective studies. Reported by Lebranchu et al., in randomized 100 patients to two-dose basiliximab with CsA or RATG with delayed CsA, all on a background of MMF and steroids, the BPAR rates (8% in both), patient survival (98% *vs*. 94%), and graft survival rates (100% *vs.* 96%) were all comparable (RATG *vs*. basiliximab) in both groups [65].

In addition to renal applications, basiliximab has also been used in liver, heart, and to a lesser extent, lung transplants. In a two-dose basiliximab induction regime in liver transplant patients, the antibody was well-tolerated and effective in reducing BPAR rates (35.1%

basiliximab *vs.* 43.5% placebo at six-month follow up), although to a lesser extent in HCV-positive cohorts [66–68]. In small group of lung transplant patients, a comparative evaluation of basiliximab induction was conducted. 15 patients receiving basiliximab plus triple therapy demonstrated lower rates of acute and chronic rejection in high-risk recipients, compared to 13 controls receiving triple therapy alone (13.3% *vs.* 38.5% acute and 20% *vs.* 38% chronic rejection incidence) [69]. As CNI-sparing or CNI-free regimes have been shown to be well-tolerated without compromising acute rejection outcomes [58, 70, 71], efforts were also made to eliminate steroids or CNIs from immunosuppressive regimens in the context of basiliximab induction. Lin et al. demonstrated that by allowing for delayed tacrolimus administration, basiliximab induction preserved lower renal insufficiency incidence than that of the conventional therapy (of 26% *vs.* 67%) at three-month follow up [72]. In the setting of steroid-withdrawal protocols, Filipponi et al. demonstrated that HCV-seropositive individuals maintained lower BPAR rates with normal steroid supplementation (24.3% *vs.* 39.4%) compared to those without, but better patient survival than the without group (84.3% *vs.* 61.0%) [73]. The short-term HCV recurrence was less prevalent in those with steroid-free treatment [74, 75]. In heart transplant patients, basiliximab did not give consistent results. Rosenberg et al. showed that basiliximab treatment could safely allow for delayed CsA administration and thereby minimize renal toxicity post-heart transplant [76]. However, Mehra et al. demonstrated only a non-significant increase in time to first BPAR with Basiliximab induction relative to controls (73.7 *vs.* 40.6 days at six-month follow up) [77]. Comparative studies with anti-thymocyte globulins also showed controversial data. Decreased rates of infection and similar efficacy with basiliximab relative to RATG were reported by Mattei et al., but two other groups demonstrated that RATG is superior to basiliximab in both biopsy scores and freedom from rejection [78, 79].

The side effects of basiliximab injection have been extensively investigated. The incidence of adverse events for basiliximab was determined in four randomized, double-blind, placebo-controlled clinical trials for the prevention of renal allograft rejection. Two of the studies used a dual maintenance immunosuppressive regimen comprised of cyclosporine, USP (MODIFIED) and corticosteroids, whereas the other two studies (Study 3 and Study 4) used a triple-immunosuppressive regimen comprised of cyclosporine, USP (MODIFIED), corticosteroids, and either azathioprine or MMF. Because clinical trials were conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice. The adverse reaction information from clinical trials does, however, provide a basis for identifying the adverse events that appear to be related to drug use and for approximating rates [50]. Adverse events were reported by 96% of the patients in the placebo-treated group and by 96% of the patients in the basiliximab-treated group. In the four placebo-controlled studies, the pattern of adverse events in 590 patients treated with the recommended dose of basiliximab was similar to that in 594 patients treated with placebo. Basiliximab did not increase the incidence of serious adverse events compared with placebo. The most frequently described adverse events were gastrointestinal disorders, reported in 69% of basiliximab-treated patients and in 67% of placebo-treated patients. Patients with basiliximab treatment may experience hives, itching, cough, wheezing, hard breathing or swallowing, sore throat, fever, chills, fast heartbeat, muscle aches, dizziness, or fainting, difficult or painful urination, decreased urination, swelling all over the body, or other signs of

infection. In some cases, basiliximab injection may increase the risk of developing an infection or cancer [63].

Belatacept

Belatacept (trade name Nulojix) is a fusion protein composed of the Fc fragment of a human IgG1 immunoglobulin linked to the extracellular domain of cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) [80], which is a molecule crucial for T-cell co-stimulation, selectively blocking the process of T-cell activation (figure 17.1). It is intended to provide extended graft survival while limiting the toxicity generated by standard immune suppressing regimens, such as CNIs. It differs from the abatacept (Orencia), which failed to sustain graft survival in non-human primates due to weaker affinity for CD86 compared to CD80, by only 2 amino acids. Belatacept was developed by Bristol-Myers Squibb, Princeton, NJ, USA and approved by the U.S. FDA in 2011 [81, 82].

The function of belatacept in transplantation settings has been extensively investigated. The goal of research on co-stimulatory blockades is not only to keep acute rejection rates low, but also to improve long-term outcomes through the induction of tolerance. Before FDA approval, a 2-year result from the BENEFIT and BENEFIT-EXT studies reported that belatacept-based regimens sustained better renal function, similar patient/graft survival, and an improved cardiovascular/metabolic risk profile versus CsA [83]. In the belatacept-based regimens versus a CsA-based regimen in kidney transplant recipients, belatacept was associated with similar patient/graft survival, better renal function, and an improved cardiovascular/metabolic risk profile relative to CsA at 1 year. Acute rejection and PTLD was observed more frequently in belatacept-treated patients. In a 2-year evaluation program, patients received a more intensive (MI) or a less intensive (LI) regimen of belatacept or a CsA-based regimen. Total 493 of 666 patients (74%) in BENEFIT and 347 of 543 (64%) in BENEFIT-EXT trial completed the 2 years of treatment. The proportion of patients who survived with a functioning graft was similar across groups (BENEFIT: 94% MI, 95% LI, and 91% CsA; BENEFIT-EXT: 83% MI, 84% LI, and 83% CsA). Belatacept's renal benefits were sustained, as evidenced by a 16 to 17 mL/min (BENEFIT) and an 8 to 10 mL/min (BENEFIT-EXT) higher calculated glomerular filtration rate in the belatacept groups versus CsA. There were few new acute rejection episodes in either study between years 1 and 2. Because PTLD risk was highest in EBV-negative patients, an efficacy analysis of EBV (+) patients was performed and was consistent with the overall population results. There were two previously reported cases of PTLD in each study between years 1 and 2 in the belatacept groups. The overall balance of safety and efficacy favored the LI over the MI regimen. Later, in a Phase 3 trial from Kidney Transplant Service, belatacept was compared to CsA as a component of maintenance immunosuppression in kidney transplant recipients. Vincenti et al. studied whether co-stimulatory pathway blockade could be used as maintenance immunosuppression instead of CsA without adverse effects on survival, cardiovascular or renal profiles. Although the BENEFIT showed a slightly higher incidence of early acute rejection and more common PTLD in the belatacept group, the belatacept patients had superior renal, metabolic and cardiovascular function profiles, and these patients had similar graft and patient survival rates at one year post-transplant compared to the CsA group [84]. However, two important issues were raised [85]. The 3-year data of the BENEFIT-EXT study

showed a significant advantage regarding cardiovascular risk factors in belatacept-treated patients, but this advantage was not sustained. Besides, the incidence of PTLD was significant. Van den Hoogen and Pipeleers argued that the balance between benefits and risks of belatacept as a substitute for CsA might be less optimistic than suggested earlier, especially since the advantages with respect to cardiovascular risk factors seem to diminish with longer follow-up and the incidence of PTLD seems to increase. Later on, a long-term belatacept exposure to maintain efficacy and safety at 5 years was reported in 2013 (University Hospital, Toulouse, France) [86]. In the 5-year results of the long-term extension (LTE) cohort, a total of 456 (68.5% of intent-to-treat) patients entered the LTE at 36 months; of these, 406 patients (89%) completed 60 months. The Trial named Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression randomized patients receiving a living or standard criteria deceased donor kidney transplant to the same MI or LI regimen of belatacept or CsA-based regimen as that administered in the BENEFIT and BENEFIT-EXT trial reported by Larsen et al. [83]. Between months 36 and 60, death occurred in 2%, 1% and 5% of belatacept MI, belatacept LI and CsA patients, respectively; graft loss occurred in 0% belatacept and 2% of CsA patients. Acute rejection between months 36 and 60 was rare: zero belatacept MI, one belatacept LI and one CsA. Rates for infections and malignancies for months 36–60 were generally similar across belatacept groups and CsA: fungal infections (14%, 15%, 12%), viral infections (21%, 18%, 16%) and malignancies (6%, 6%, 9%), respectively. No new PTLD cases occurred after 36 months. Mean calculated GFR at month 60 was 74 for belatacept MI, 76 for Belatacept LI and 53 for CsA. These results further confirmed that the belatacept LI regimen can provide a consistent safety profile and a sustained renal function benefit. More benefits of belatacept have been appreciated based on recently reported cases. Koppula et al. [87] reported two cases of thrombotic microangiopathy (a *de novo* disease that is usually associated with immunosuppressive drugs or can be seen as a part of endothelial damage that accompanies antibody-mediated rejection), that were successfully treated by converting to belatacept for maintenance immunosuppression. Most recently, a study reported the use of belatacept as a rescue therapy for two kidney-transplant patients presenting with severe adverse events after treatment with CNIs and mammalian target-of-rapamycin (mTOR) inhibitors [88]. Two kidney-transplant patients developed severely impaired kidney function after receiving CNIs. The use of everolimus was associated with severe angioedema. Belatacept was then successfully used to improve kidney function in both cases, even though estimated GFR before conversion was <20 mL/min. Their data showed that belatacept can be used as a rescue therapy, even if kidney function is very low in kidney-transplant patients who cannot tolerate CNIs and/or mTOR inhibitors. In addition to the kidney, a belatacept-based immunosuppression in liver transplant recipients was reported by Klintmalm et al. [89]. In the 1-year experience from a phase-II randomized study, they evaluated the safety and efficacy of Belatacept in *de novo* adult liver transplant recipients. Patients were randomized (N=260) to five immunosuppressive regimens: basiliximab + high dose (HD) belatacept + MMF, belatacept HD + MMF, low dose (LD) belatacept + MMF, tacrolimus + MMF, or tacrolimus alone. All received corticosteroids. The proportion of patients who met the primary end point (composite of acute rejection, graft loss, death by month 6) was higher in the belatacept groups (42–48%) versus tacrolimus groups (15–38%), with the highest number of deaths and grafts losses in the belatacept LD group. By month 12 the proportion surviving with a functioning graft was higher with tacrolimus + MMF (93%) and lower with belatacept LD (67%) compared to other groups (90% with basiliximab +

belatacept HD; 83% with belatacept HD; 88% with tacrolimus). Mean calculated GFR was 15–34 ml/min higher in belatacept-treated patients at 1 year. Two cases of PTLD and one case of progressive multifocal leukoencephalopathy occurred in belatacept-treated patients. Follow-up beyond month 12 revealed an increase in death and graft loss in another belatacept HD group. These data, again, raised the safety issue of belatacept utilization in liver transplantation that was further addressed by LaMattina et al. [90]. A retrospective review of adult liver transplant recipients with hepatitis C receiving belatacept was conducted. All patients were EBV IgG seropositive. The primary endpoint was patient and graft survival, with secondary endpoints including the incidence of acute rejection, degree of renal function recovery, and occurrence of major side effects. Seven liver transplant recipients with hepatitis C received belatacept in the perioperative period. Belatacept was initiated between 2 and 90 days post-transplant and the duration of therapy ranged from 19 to 89 days. Patients were transitioned onto CNI therapy when they reached chronic kidney disease stage 2 or better. Six-month patient and graft survival was 86%. There was one episode of graft rejection on belatacept therapy in a patient who also had early rejection before initiation of belatacept. These results suggest that belatacept with mycophenolic acid (MPA) may be a safe maintenance immuno- suppression regimen in hepatitis C-positive liver transplant recipients with renal dysfunction, and that this regimen can serve as an effective bridge to CNI therapy.

Blockade of signal 2 by belatacept raised question regarding its effects on regulatory T cell (Treg) function, as these cells depend on the CD28-CD80/86 interaction in addition to the IL-2-mediated pathway. Because Tregs play a crucial role in immune tolerance, long-term belatacept usage could have a negative impact on chronic rejection rates. Bluestone et al. studied the concomitant effects of belatacept and basiliximab in renal transplant recipients. Their result, however, showed that Belatacept in combination with basiliximab (IL-2 blockade) did not affect Tregs in the long-term compared to CNI regimens [91]. Recently, the inhibitory effects of belatacept on allospecific regulatory T-cell generation in humans were explored by Levitsky et al. [92]. They tested the effects of belatacept on human Tregs in mixed lymphocyte reactions and in combination with maintenance agents used in transplant recipients. Belatacept, MPA, and sirolimus, either alone or in combination, were added to healthy volunteer Treg-MLR (mixed lymphocyte reaction), followed by testing H-TdR (tritiated thymidine) incorporation for inhibition of lymphoproliferation and flow cytometry to analyze for newly-generated $CD4^{+}$ $CD25^{high}$ $FOXP3^{+}$ Tregs in carboxyfluorescein succinimidyl ester (CFSE)-labeled MLR responders. In addition, the modulatory effects of putative Tregs generated in the presence of these drugs were also tested using the lymphoproliferation and flow cytometric assays. In comparison with medium controls, belatacept dose-dependently inhibited both lymphoproliferation and Treg generation in HLA-DR matched and mismatched MLRs either alone or in combination with MPA or sirolimus. Belatacept alone and in combination with agents used in transplant recipients inhibited the *in vitro* generation of human Tregs. Their observation further demonstrated that belatacept might be a negative factor to Treg and less optimal agent for tolerance induction in human organ transplantation.

Conclusion

Because of their remarkable outcomes, antibody therapies have become an attractive approach in the treatment of transplant rejection. Recent advances in biotechnology and understanding of transplant immunology have allowed emergence of numerous new types/forms of mAbs, targeting different co-stimulatory signals and novel protein constructs. Theoretically, the newly-developed antibodies would be more effective, specific and less toxic. However, under extensive testing, the performance of many such agents is not as satisfactory as expected. In most of the cases, a given antibody protocol should be implemented with calculated, evidence-based considerations of the potential risks and complications involved. As trials proceed and the influx of new agents persists, antibody-based therapies are likely to continue evolving. In the future, transplant professionals will increasingly require knowledge of the mechanisms and pharmacological characteristics of these novel therapeutic agents.

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Chapter 18

Complement Inhibitors

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Abstract

The complement system is a major player in the innate immune response and can also modulate the adaptive immune response including antigen processing and presentation, B cell activation, and T cell proliferation and differentiation. The mechanism of rejection in solid organ transplantation is influenced by the initial inflammatory response and subsequent adaptive alloimmune response, both of which have been shown to be modulated by diverse complement components. The complement plays a critical role in ischemia reperfusion injury, hyperacute rejection, acute antibody-mediated rejection, and accommodation. The central role of complement in the pathophysiology of many diseases turned it in the spotlight of the pharmaceutical industry; however there are many obstacles to use it in the clinical setting. Currently, only two complement-targeting drugs, a recombinant human C1-INH (cinryze) and an anti-C5 monoclonal antibody (eculizumab) have gained FDA approval (but its use in transplantation still not approved). However, a large number of other complement inhibitors are currently in advanced pre-clinical development or in clinical trials for various indications. The further development of potential complement therapies have significant promise for further improving outcomes in clinic transplantation. Here, we review the literature on the role of complement in graft ischemia-reperfusion injury, antibody-mediated rejection, cell-mediated rejection, and accommodation. In addition, we discuss the current status of complement intervention therapies in clinical transplantation.

Keywords: complement system**,** complement inhibitor, eculizumab, ischemia reperfusion injury, rejection, transplantation, antibody mediated rejection

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Abbreviations

aHUS	Atypical hemolytic uremic syndrome
AMR	Antibody mediated rejection
APC	Antigen-presenting cells
CR	Complement receptor
CRP	C-reactive protein
CVF	Cobra venom factor
DAMPs	Damage-associated molecular patterns
DAF	Decay accelerating factor
DSAs	Donor specific antibodies
ICAM	intercellular adhesion molecule-1
IRI	Ischemia reperfusion injury
IVIG	Intravenous immunoglobulin
LPS	Lipopolysaccharide
MAC	Membrane attack complex
MCP	Complement Membrane Cofactor Protein
MBL	Mannose-binding lectin
MASPs	MBL-associated serine proteases
PMN	Polymorph nuclear cells
rhC1-INH	Recombinant human C1 inhibitor
SiRNA	Small interfering RNA
tPA	Tissue plasminogen activator
VCP	Vaccinia virus complement control protein

Introduction

The complement system provides an efficient protection against microorganisms. The term “complement” was first used by Paul Ehrlich to describe a supplementary role to the cellular immune system against microorganisms [1]. However, current research supports that the complement system bridges the innate and adaptive immune responses. Because inappropriate activation or regulation can cause cellular damage, it plays a significant role in the pathology of many conditions. A large body of literature is now available that supports the role of complement in various aspects of immune response following organ transplantation [2]. For many years, the view of complement in organ transplantation was largely limited to the effector functions of complement in antibody-mediated rejection, but now it is known that it plays a critical role in ischemia reperfusion injury, in adaptive immunity and consequently in cellular rejection and tolerance [3]. Here, we review recent findings concerning the role of complement in graft ischemia-reperfusion injury (IRI), antibody-mediated rejection and accommodation, and cell-mediated rejection. We also discuss the current status of complement-based therapies in clinical transplantation and prospects for the future.

Description of the Complement System

The complement system is a major element of the innate immune system. Its main biological function is to recognize "foreign" particles and microorganisms, and to promote their elimination either by opsonization or lysis [3]. In addition to defense functions, it contributes to maintenance of homeostasis by recognizing and eliminating apoptotic and necrotic cells. The complement system is composed of more than 35–40 proteins or glycoproteins consisting of recognition molecules C1–C9, factor B, factor D, mannose-binding lectin (MBL) and MBL-associated serine proteases (MASPs), proteolytic enzymes, effector products and receptors (e.g., CR1, CR2, CR3, CR4, C3aR, and C5aR); and regulators (factor I (fI), factor H (fH), properdin (fP), CD46, CD55, and CD59) which are widely distributed in the circulation (circulating complement) or in tissues (local complement) [3]. Its activation leads to the generation of products with immunoprotective, immunoregulatory, proinflammatory and cytolytic properties.

Activation of Complement

The complement cascade is activated by three different pathways [4].

1) The classical pathway is initiated by the binding of C1q to tissue-adherent surveillance molecules such as IgG, IgM, and C-reactive protein (CRP); this leads to activation of the C4, C2, C3 components to create the C3 convertase complex (C4bC2a).
2) The lectin pathway is similar to the classical pathway in that its activation also leads to the formation of the C4b, C2a, C3 convertase complex. However, instead of relying on antibodies to recognize pathogenic components, the lectin pathway uses mannose-binding lectins and ficolins to identify patterns of carbohydrate ligands that are found on the surface of a wide variety of microorganisms.
3) The alternative pathway is activated by direct binding of C3b to the activated surface: this acts as an amplification step and accounts for about 80% of all complement activation, regardless of the initial trigger [5]. Spontaneous hydrolysis of C3 to $C3H_2O$ (C3w) leads to conformational change that enables binding to Factor B and formation of the initial alternative pathway C3 convertase (C3wBb). The alternative pathway (which involves complement factor B) provides a means to amplify the amount of C3b deposited on cells by the classical or lectin pathway, and as such may not have a specific damage-associated molecular trigger. Nevertheless, the involvement of this pathway is suggested by the phenotype of factor B-deficient mice, which are protected from renal ischemia-reperfusion injury [6].

All three pathways result in the cleavage of C3 into its active fragments C3a and C3b, by the C3 convertases. All these pathways lead to the rapid initiation of a proteolytic complement cascade involving three major types of effectors. 1) The anaphylatoxins (C3a and C5a) are potent pro–inflammatory molecules that attract and activate leukocytes through interaction with their cognate C3a and C5a receptors (C3aR, C5aR), respectively. 2) The

opsonins (C3b, iC3b and C3d) which label targeted surfaces through covalent binding to facilitate transport and favor the removal of target cells or immune complexes. 3) The terminal membrane-attack complex (MAC, C5b–9) directly lyses targeted (opsonized) pathogens or damaged self-cells by becoming integrated to the phospholipid membranes and creating holes, which cause cell death and local coagulation [5]. Activation of the complement cascade by three major pathways − classical, lectin and alternative − leads to the deposition of complement component C3 on the surface of the pathogen. This marks the pathogen for removal by the mononuclear phagocyte system or for destruction by the membrane attack complex, which is comprised of complement components C5b, C6, C7, C8 and C9 (referred to as C5b–C9) [7].

Activation of the classical and lectin pathways depends largely on foreign material, but under certain situations (e.g., tissue ischemia and reperfusion) both pathways can be activated and cause autologous injury. Similarly, deposition of C3b via the alternative pathway of activation and amplification is non-discriminatory, and can damage host cells if not properly regulated. The combination of time, location, intensity of activation, and concentration of regulatory molecules prevents the complement cascade to injure host cells. C3b and C3 have short-half-lives which limit their effect on the site of activation. In addition, whereas the convertases are actively stabilized on foreign cells by properdin, soluble and membrane bound regulator molecules inactivate and degrade active complement molecules on host cells [2].

The complement components can be divided into circulating complement (central) and locally-synthesized complement components (peripheral). Circulating complement components are mainly synthesized by liver and are in the circulating blood, whereas locally-synthesized complement (extravascular) is produced by tissue-resident and migratory cells. The parenchymal cells of numerous organs can synthesize complement and the production of complement is enhanced by stress-related factors such as lipopolysaccharide (LPS), IL-1, interferon-γ (IFN-γ) and TNF [7]. It has also become clear that peripheral synthesis of complement contributes to T cell priming and the shaping of the adaptive immune response that determines transplant rejection. Indeed, the peptides C3a and C5a, which are released by local complement activation, are now understood to act as cofactors for stimulating antigen presentation and enhancing the activation of naive alloreactive T cells [8–10]. The cells targeted by complement vary with the type of organ. For example, in post–ischemic heart and intestine, the principal targets of complement include endothelial cells and myocytes [11, 12], whereas in the kidneys tubular epithelial cells are the main target [13]. This unusual sensitivity of proximal tubular epithelial cells to complement has been attributed to the expression of various complement components by these cells and to the lack of complement regulator expression [7].

Complement Regulation

Damage to autologous tissues (human cells) by complement activation is prevented by soluble and surface-bound natural regulators that inhibit the enzyme steps at several stages leading to the cleavage of C3 and C5, or that prevent the formation of the membrane attack complex. They maintain homeostasis by preventing uncontrolled activation of complement in

tissues. However, when these controls are exhausted, it can result in tissue injury and this is associated with many autoimmune diseases [7]. Similar to complement components, some complement regulatory proteins are synthesized by parenchymal cells outside the liver, including Decay accelerating factor (DAF), Complement Membrane Cofactor Protein (MCP), CR1 and CD59 [3].

Regulators of complement play a critical role in the "accommodation" phenomenon, the development of endothelial cell resistance to destruction in the presence of a pathogenic antibody and complement. It was first described in ABO-incompatible transplantation, [14] and is very important to prolong graft survival in xenotransplantation. Endothelial resistance to the deleterious effects of alloantibodies and complement may be acquired through the upregulation of complement regulatory proteins and other cell-survival factors. Accommodation of cardiac and renal allografts is related to enhanced expression of CD55, CD59, HO-1, BCL-2 and BCL-XL [15, 16]. Indeed, genetic manipulation of complement regulators that are expressed by donor organs is a main strategy to prevent antibody-mediated destruction of xenotransplant organs [17].

Several mechanisms are involved in regulation of complement, which include the following: 1) Factors that accelerate the decay of the C3 and C5 convertases (complex molecules that once assembled cleave C3 into C3b), such as CD35, CD55, CD46, and CFH related protein-1. 2) Factors that regulate the alternative pathway through the degradation of C3b and C4b, such as CFH and CFI, secreted by the liver. 3) Factors that inhibit the terminal pathway, i.e., CD59 (also known as Protectin), inhibit C9 association with C5b-8 to prevent C5b-9 formation (MAC) [3].

CD35 (complement receptor 1, CR-1), CD46, CD55, C4b-binding protein (C4BP), and CD59 are from the same family of proteins and function by accelerating the decay of the C3 and C5 convertases or by acting as a cofactor for the proteolysis (by factor I) of these convertases inhibiting both the classical and alternative pathways. CD35 combines decay-accelerating activity and cofactor activity for Factor I in a single molecule. Because it has both properties, it has advantages over other inhibitors, but it has limited tissue distribution. CD55 (decay-accelerating factor-DAF) is present on the cell surface and has only decay-accelerating activity. It destabilizes C3 convertase and accelerates the dissociation of C3bBb and inhibits just the classical activation pathway. CD46 (Membrane Cofactor Protein-MCP) has only cofactor activity. It binds to C3b and serves as a cofactor for complement factor-I (CFI, a soluble complement factor) mediated cleavage of C3b into inactive C3b (iC3b) and inhibits just the alternative pathway [18, 19].

The soluble regulators C4BP and factor H have both decay-accelerating activity and cofactor activity, but C4BP only affects the classical pathway and factor H only targets the alternative pathway. If complement activation proceeds as far as C5b–C8, the terminal pathway regulator CD59 binds to this complex and prevents the formation of C5b–C9. The complement factor I-CFH-related protein 1 (CFHR-1) inhibits C5 convertase and binds to C5 and C5b-6, preventing the formation of MAC [7, 20].

Experimental and clinical data support the importance of complement regulators in prolonging graft survival. Pavlov et al. showed that DAF knockout allografts were rejected faster than wild type grafts [21]. Diffuse endothelial expression of CD55 was seen in patients with no sign of allograft dysfunction whereas in patients who had developed allograft dysfunction, they could not detect CD55 expression [16]. Brodsky et al. showed in renal biopsies that there was an inverse relationship between C4d and CD55 staining, and this was

associated with better graft function and longer graft survival in patients with high CD55 expression [22]. Using a wild-type miniature swine-to-α-1,3-galactosyltransferase knockout (GalT-KO) swine renal transplant model, Griesemer et al. showed that the expression of CD59 in the accommodated kidney was elevated and deposition of C5b-9 was reduced [23]. Tan et al. demonstrated that patients without AMR showed an increased expression of CD55 and CD59 in their allograft biopsy samples, but those patients with AMR grafts lost the expression of the two proteins during the acute phase, suggesting that the presence of these proteins could be protective [15]. In addition, Brodsky et al. [22], in a clinical renal transplant study, found that CD55 expression had a protective effect on peritubular capillary C4d-negative renal allografts, indicating that the CD55 expression may be used as a potential biomarker of renal allograft in patients without evidence of AMR.

Impact of Complement in Cell-Mediated Immunity

The complement system, as a main component of the innate immunity, has been shown to be an essential participant in adaptive immune responses, including antigen processing and presentation, B cell activation, and T cell proliferation and differentiation [7, 24–29]. The crosstalk between the complement system and adaptive immunity was summarized by Asgari et al. [19].

Studies have shown that complement interacts with antigen-presenting cells (APC). T cells and APCs express complement receptors, including C3a and C5a receptors (C3aR and C5aR), that allow them to sense the products of complement activation, both soluble and membrane–bound [30]. In turn, APCs produce autonomously C3 and C5 [7, 28, 29]. Locally produced C3a and C5a interact with their receptors on antigen-presenting cells and T cells and act via the expression of costimulatory molecules CD28 and CD40L to induce T cell proliferation and differentiation [10]. Peng et al. observed that APCs from C5aR-deficient mice, or wild type DCs treated with C5aR antagonist express less MHC class II and B7.2 which leads to their reduced capacity to stimulate allospecific T cells [30]. Other evidence of the influence of complement on adaptive immunity was shown by Csomor et al. They found that C1q can induce the maturation of monocytic DCs and increase their secretion of IL-12 and TNF-α as well as enhancing their T-cell stimulating property [31]. Baruah et al. noticed that DC from C1q-deficient mice could not stimulate the proliferation of MHC class II restricted antigen-specific T cells or support the differentiation of these cells to effector Th1 cells [32].

More recently, Weaver et al. [33] have shown that development of regulatory T cells and Th17 cells is affected by signaling of C5aR in APCs. They demonstrated that in the absence of C5aR, there is an increase in production of TGF-beta which directs the $CD4^+$ T cells to differentiate to $Foxp3^+$ Tregs. Kemper et al. had previously shown the effect of the regulatory complement component, CD46, in the development of Tregs [34]. Raedler et al. have shown that the effect of complement on adaptive immunity is not limited to APCs by demonstrating that following stimulation with proinflammatory cytokines, endothelial cells activate the alternative pathway of complement leading to production of C5a [35].

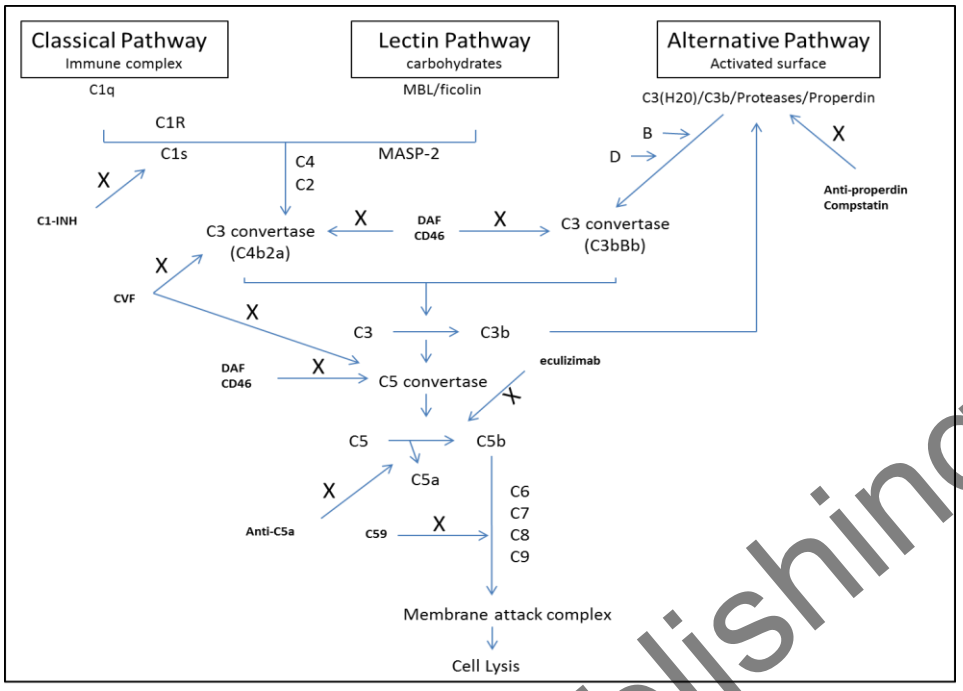

Figure 18.1. Complement system and its modulation. Activation of all three complement pathways results in cleavage of C3, leading to formation of C3a and C5a and ultimately membrane attack complex. DAF, CD46 and CD59 are complement regulatory molecules.

Table 18.1. Complement therapeutics in the market or in clinical trials. Adapted from Ricklin D 2007 (ref. 2)

Agent	Action mechanism	Use/stage of development
Protease inhibitors		
• C1-INH	Inhibits C1r/C1s, kallikrein and other proteases	Market, phase 1
• rhC1INH (Rhucin)		Clinical phase 3
Soluble complement regulators		
• sCR1/TP10	Factor I cofactor (decay accelerator)	Clinical phase 2 (CABG)
• CAB-2/MLN-2222	Chymera of DAF and MCO (factor I cofactor, decay accelerator)	Clinical phase 1 (CABG)
Therapeutic antibodies		
• Eculizumab	Humanized long- acting mAb against C5	Marketed
• Pexelizumab	Humanized short- acting mAb against C5	Clinical phase 3 (AMI, CABG)
Complement component inhibitors		
• Compstatin	Peptidic C3 inhibitor	Clinical phase 1
Receptor Antagonists		
• PMX-53	Peptidic C5aR antagonist	Clinical phase 2 (RA, psoriasis)

Table 18.2. Complement therapeutics in pre-clinical development. Adapted from Ricklin D 2007 (ref. 2)

Agent	Action mechanism
Protease inhibitors	
• Factor D inhibitors (BCX1470)	Serine inhibitors
Soluble complement regulators	
• sCR1-sLe^x (TP-20)	sCR1 with sLe^x-rich glycosilation to target sites of inflammation
• sCR1 (Mirococept)	sCR1 with lipopeptide membrane linker
Therapeutic antibodies	
• TNX-234	Humanized antibody against Factor D
• TNX-558	Humanized antibody against C5a
• TA106	Antibody against Factor B
• Neutrazumab	Antibody blocking the C5a receptor
• Anti-properdin	Antibody against properdin
Complement component inhibitors	
• ARC1905	Aptamer-based C5 inhibitor (PEG-ylated)
Receptor antagonists	
• JPE-1375, JSM-7717	Small molecule/peptidimimetic antagonists for C5a receptor

The interactions of activated complement with the adaptive system reflect clinically in increased incidence of rejection. Naesens et al. examined the whole genome expression profile of 53 human kidney allografts using microarrays. They found a significant difference in the expression of complement genes C1 (including C1q, C1r and C1s), C2, C3, C4, C6, and complement factor B between the live and deceased donor kidneys before implantation. They also demonstrated that the complement gene expression from biopsy samples at the time of implantation had significant correlation with both early and late graft survival, suggesting that an increase in the expression of complement components could be related to immune activation responsible for future rejection episodes leading to shorter graft survival [36].

Complement and the Coagulation System

The interplay between the complement and coagulation cascades has an important negative effect on the outcome of antibody-mediated rejection. Both of these pathways are serine protease cascades with multiple potential links. C5a and C5b–C9 mediate the expression of tissue factor by endothelial cells, enabling endothelial cells to initiate the coagulation system by releasing thrombin from pro-thrombin. In addition, thrombin, factor XIa, factor Xa, factor IXa and plasmin, which are members of the coagulation cascade, were all found to cleave complement components C3 and C5, indicating the potential of the

coagulation cascade to initiate complement activation independently of the traditional complement activation pathways [37, 38]. Inhibition of coagulation may have a beneficial effect in organ transplantation. When applied to islet allograft transplantation (in which graft acceptance is profoundly limited by activation of the complement and coagulation cascades), monotherapy with α1-antitrypsin, which is a serine protease inhibitor of both complement and coagulation, induced transplant tolerance in mice [39].

Complement Inhibitors

The specific targets of complement-based therapeutics are illustrated in Figure 18.1. The most common complement inhibitors used in clinical trials and in pre-clinical studies are summarized in Tables 18.1 and 18.2, respectively. Below, we will describe the mechanism of action and other characteristics of some of them.

Eculizumab

Eculizumab is the most investigated and clinically used complement inhibitor (FDA approved). Its discovery and development have been reviewed by Rother et al. [40]. It has been clinically used successfully in atypical hemolytic uremic syndrome [41–45], thrombotic microangiopathy [46], dense-deposit disease and C3 glomerulonephritis [47] in kidney disease, as well as their recurrence after transplantation [48]. Eculizumab (Soliris, Alexion Pharmaceuticals) is a humanized monoclonal antibody that is specific for human complement, with a hybrid human Ig-G2/Ig-G4 Fc heavy chain and a human kappa light chain. The presence of a hinge region from human Ig-G2, which does not bind to Fc receptors (and therefore does activate complement and bind to immune cells leading to an inflammatory response), and the CH2-CH3 from human Ig-G4 that does not activate complement, is essential for its activity [40]. Eculizumab, by binding to a cryptic epitope of C5 with very high affinity, prevents its entry into C5 convertase [49]. No nonspecific or unexpected binding of eculizumab to any human tissue was observed [41]. C5 is an attractive target because, as it is common to all pathways of complement activation, its blockade stops the progression of the cascade regardless of the triggering stimuli. On the other hand, C5 blockade preserves better critical immunoprotective and immunoregulatory functions of upstream components that result in C3b-mediated opsonization and immune complex clearance. This effect prevents C5 cleavage and the formation of C5a and the membrane attack complex (C5b-9). C5a is a potent anaphylatoxin that mediates leukocyte chemotaxis, increases vascular permeability, alters smooth muscle tone and induces secondary inflammatory mediators such as hydrolytic enzymes, reactive oxygen species, arachidonic acid metabolites and cytokines [40]. In addition, eculizumab acts by blocking the activation of the terminal complement components to form MAC and can potentially prevent the pathogenic effects of complement on graft endothelium. When administered intravenously, eculizumab's half-life is ~11 ± 3 days, and it is distributed in the vascular space. Its serum concentration reaches a steady-state after 150 days [3, 41]. Eculizumab was first approved by the FDA and European Medicines Agency for the treatment of paroxysmal nocturnal

hemoglobinuria in 2007. Its major adverse effect is an increased risk of infection, especially of encapsulated bacteria. Preventive vaccination against B Meningococcus or pretreatment with antibiotics is recommended before the initiation of this treatment. Regarding eculizumab use, optimal dose and length of treatment remain to be defined [3].

Eculizumab in prevention of Antibody-mediated Rejection (AMR): Because of its capability to inhibit complement activation and the identification of a signature of complement activation (C4d deposition) in AMR, eculizumab has been considered for the prevention and/or treatment of AMR. The non-randomized, proof-of-concept study by Stegall et al. in 2011 remains the largest series published so far [50]. Twenty-six highly sensitized recipients with a positive crossmatch received eculizumab on the postoperative day and then weekly for the first 3 months. In this series, a remarkably low incidence of AMR (7.7%) was observed compared to a historical control group (41.2%). The percentage of patients who developed high levels of donor specific antibodies (DSAs) after transplantation was similar in both groups. However, all patients in the control group who developed a high level of de novo DSAs also developed acute AMR as compared to 15% of eculizumab-treated patients. An important finding was that all biopsy samples taken from both groups with high DSA levels were positive for C4d deposits in the graft. Altogether, these data suggest that the clinical phenotype in eculizumab-treated patients resembles accommodation.

Eculizumab in treatment of acute AMR: Although eculizumab is helpful in preventing AMR in sensitized patients, its efficacy to treat acute AMR remains controversial. A few studies have reported some efficacy of eculizumab as a salvage treatment for refractory AMR in ABOi or in highly sensitized kidney recipients [51–56].

Eculizumab in chronic AMR: There is indirect evidence that complement activation is involved in the pathogenesis of chronic antibody-mediated rejection (cAMR). Diagnosis of cAMR relies on the presence, in a graft biopsy, of transplant glomerulopathy [3, 57]. However, it is not clear if cAMR plays any role in its pathophysiology.

Recombinant Human C1 Inhibitor (rhC1-INH)

C1 inhibitor (C1-INH) is a serine protease inhibitor (serpins) that inactivates several different proteases in the complement (C1r, C1s, and MASPs), coagulation (kallikrein, Factor XI, Factor XII and thrombin), and fibrinolytic systems (tPA and plasmin) [58].The heterozygous deficiency of C1-INH results in hereditary angioedema, and recombinant C1-INH has recently been used for its treatment. C1-INH is a single-chain, heavily glycosylated protein [60] with a protuberant active loop-containing residues that bind specifically to the reactive center of the target protease. C1-INH is the only known inhibitor of the C1r and C1s subunits; it is a major suppressor of Factors XIIa and XIa, inhibiting plasmin; and also inhibits MBL-associated serine proteases (MASP)-1 and -2 [61, 62]. Thus, C1-INH regulates the fibrinolytic, coagulation, and MBL pathways. C1-INH also binds to C3b, which, together with factor B, initiates the alternative pathway [62]; CI-INH binding prevents the formation of the C3b/factor B complex, thereby inhibiting the alternative pathway activation. C1-INH can also bind to and inhibit kallikrein in the contact system, and thus control the release of the vasodilator and pro–inflammatory mediator bradykinin [61, 64]. By binding E-selectin

present in endothelial cells, C1-INH can concentrate at sites of inflammation and can also regulate leukocyte adhesion and transmigration across the endothelial surface [65].

sCR1 and Recombinant CR1

Complement receptor type 1 (CR1, CD35, C3b/C4b receptor) is a transmembrane glycoprotein regulator that not only accelerates C3 and C5 convertase dissociation but also acts as a cofactor for the cleavage of C3b and C4b, blocking the generation of both C3a and C5a and the MAC. Its extra-cellular domain is composed of 30 repeating units referred to as short consensus repeats each consisting of 60 ± 70 amino acid residues [66]. Recombinant sCR1 is a large molecule (230 kDa) with a plasma half-life of 55 hours and is one of the most applied complement inhibitors. Soluble complement receptor 1 (sCR1), which lacks the transmembrane and cytoplasmic regions of CR1, effectively inhibits the complement system by inactivating the C3 and C5 convertases in all three complement signaling pathways [67–69]. Perfusion of rat donor kidneys with a membrane-localizing complement regulator derived from human CR1 (APT070) has been shown to significantly improve the outcome of syngeneic renal transplantation after prolonged cold storage, suggesting that this strategy could increase the feasibility of using severely ischemic donor organs in clinical transplantation [69]. sCR1 has been shown to ameliorate hyperacute rejection in both *ex vivo* and *in vivo* xenotransplant models [71–73]. Delivery of sCR1 to the endothelial cells of a transplanted organ has been done via gene therapy [20]. Studies indicate that sCR1 is nontoxic and has low immunogenicity [20]. One limitation is its short half-live. To increase its half-life and improve efficiency of sCR1 therapies, different strategies were used: 1) Combining sCR1 with a serum albumin-binding peptide derived from Streptococcal Protein G, significantly extends the half-life of sCR1 [73]; 2) attaching a tail consisting of a basic peptide and a myristoyl fatty acid group which localizes CR1 to graft endothelial and epithelial cells (mirococet) [70, 75].

CR2

CR2 binds the complement fragment C3d, and has been used as a potential therapeutic agent. CR2 is expressed primarily on B cells [20]. A recombinant soluble form of CR2, an IgG-(CR2) 2 dimer, has been shown to inhibit the humoral immune response in mice, presumably by binding to all available C3d molecules and preventing ligation of CR2 on B cells[20].

Cobra Venom Factor (CVF)

CVF is an acidic glycoprotein with a molecular mass of about 140–150 kDa isolated and characterized from the venom of several cobra species. CVF triggers the alternative pathway cascade to create the C3/C5 convertase by forming complexes with factors B and D, and then C3, in a manner similar to that of C3b. The C3/C5 convertase formed with the participation of

CVF is much more stable than that produced by C3b and acts independently of complement restriction factors that cause the consumption of C3 and its eventual depletion [75]. CVF has frequently been used to inhibit complement activation in xenotransplantation, but this agent is too toxic for administration to humans. Furthermore, because CVF is a xenogenetic protein, its repeated injection can potentially invoke an antibody response and become less effective. In addition, CVF treatment can potentially activate both C3 and C5 to yield C3a and C5a, which may provoke an excessive inflammatory response [30].

Compstatin

Compstatin is a synthetic polypeptide (Figure 18.2) that binds C3 and putatively inhibits its association to the C3 convertases. Because of its small size, it has a promising drug potential. It was originally identified as a 13-residue disulfide-bridged peptide, which can selectively bind to human and other primate forms of the central complement component C3 and its active fragment, C3b [77]. Because it binds to the central component of all three complement cascades, preventing the conversion of C3–C3b, compstatin simultaneously impairs the initiation, amplification, and terminal pathways of complement [77]. Tjernberg et al. cultured isolated human islets in human ABO-compatible hirudin plasma and found that complement activation resulted in lysis of the pancreatic islet cells in the absence of compstatin, but addition of compstatin effectively inhibited C3b/iC3b binding and the generation of C3a and sC5b-9, thereby protecting the islets against ischemia reperfusion injury that often occurs following clinical islet transplantation [78].

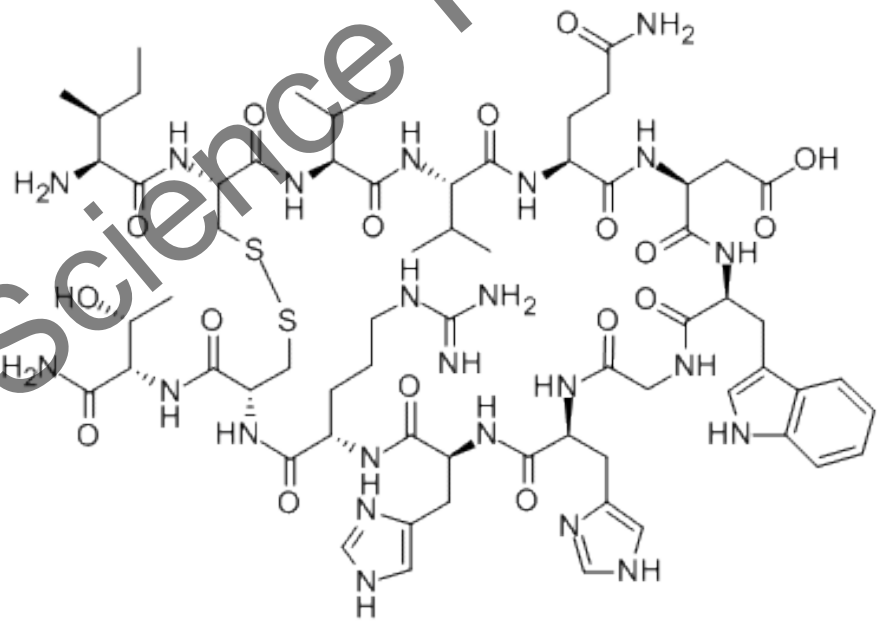

Figure 18.2. Compstatin chemical structure. H-Ile-Cys-Val-Val-Gln-Asp-Trp-Gly-His–His-Arg-Cys-Thr-NH2;H-Ile-Cys-Val-Val-Gln-Asp-Trp-Gly-His-His-Arg-Cys-Thr-NH2,(Disulfide bond).

Vaccinia Virus Complement Control Protein (VCP)

VCP, the 35-kDa vaccinia virus complement control protein, is a complement inhibitor isolated from cells infected with vaccinia virus. This protein has structural and/or functional homology to members of the regulators of complement activation family. VCP regulates the classical and the alternative pathways of complement activation by preventing the activated C3 (C3b) and C4 (C4b) from triggering further steps in the cascade, including the formation of the MAC [79]. VCP has been shown to significantly reduce cardiac xenograft damage in a mouse-to-sensitized rat model [80] as well as a guinea pig-to-rat xenotransplant [81] model. Its ability to inhibit complement activation in baboons has already been demonstrated [82]. It has further been shown to improve kidney structure and function in rats after IRI by inhibiting C3 biosynthesis [80]. Its small size and its molecular mimicry of host proteins may make VCP a good option as a complement regulator for preventing both AMR and early cellular graft rejection [30].

Specific siRNA

Specific double-stranded small interfering RNAs (siRNAs) can be used for gene silencing to reduce the local expression of complement components transcribed in donor organs. Specific siRNA molecules have been used to selectively silence the expression of C3 or C5aR in a mouse renal ischemia/reperfusion injury model [83, 84]. Vector-delivered C3-or C5aR-specific siRNA molecules offered substantial protection against post-ischemic acute kidney injury when administered intravenously 2 days before the induction of injury. Moreover, Zheng et al. have evaluated the usefulness of *ex vivo* treatment with siRNA as part of the graft preservation procedure. Preservation of mouse cardiac grafts in University of Wisconsin solution containing mixed siRNAs targeting C3, TNF-α, and Fas at 4°C for 48 h before transplantation into syngeneic recipients significantly improved the survival rate and histology of the grafts [85].

The limitation of siRNAs is that they have generally a short half-life in vivo and can cause ubiquitous gene silencing, since they lack a tissue-specific homing mechanism. Therefore, at present, the rate-limiting step in the adoption of siRNA as an effective therapeutic agent is the lack of safe, tissue- or cell-specific in vivo delivery systems [86].

Other Complement Therapies

Intravenous Immunoglobulin (IVIG)

Although not a typical complement inhibitor, it has been shown to have anti-complement properties. It is prepared from pooled human plasma (from approximately 50,000–100,000 healthy donors) and contains >90% intact IgG. IVIG is known to affect complement activation by absorbing C3a and C5a, binding activated C3b and C4b, accelerating the conversion of C3b to its inactive form iC3b, and reducing MAC formation [30].

Anaphylatoxin Receptor Antagonists (C5aR Antibodies and Antagonists)

Selective inhibition of the binding of C5a to its receptors offers a very promising opportunity for inhibiting the inflammatory response without depleting the defensive potential of complement [2, 88–91]. PMX-53 is cyclic peptidomimetic of a molecular size under 1kDa with a high oral bioavailability (Figure 3). Although it was safe and well tolerated, its half-life is only 70 min. Neutrazumab binds to C5aR and inhibits the binding of C5a to its receptor. TNX-558 neutralizes C5a by binding to the C5a itself [2].

Other Complement Inhibitors

- Anti-Factor B antibody (TA-106)
- Anti-Factor D antibody (TNX-234)
- Anti-properdin antibody
- Fusion protein CR2-CRRY
- Fusion protein CR2-factor H
- Serine protease inhibitor α1‑antitrypsin
- Soluble chimeric fusion protein, CD59-Ig

Importance of Complement for Transplantation

Complement plays a crucial role in the inflammatory response after transplantation. There are many potential applications of complement modulation in clinical transplantation including: (A) reduction in ischemia reperfusion injury before or after transplant; (B) prevention or treatment of acute AMR in cross-match positive or ABO-incompatible transplantation; (C) prevention or treatment of complement-related renal diseases that recur after renal transplantation (e.g., C3 glomerulonephritis, dense deposit disease, renal thrombotic microangiopathy, and atypical hemolytic uremic syndrome (aHUS); and (D) prolongation of graft survival in xenotransplantation (Table 18.3).

There are several possible mechanisms that complement activation may lead to direct graft injury, including: 1) The formation of MAC (C5b9) via classical pathway activation causes direct cell lysis. 2) The attraction of inflammatory cells (e.g., neutrophils and macrophages) by chemoattractants C3a and C5a. 3) The activation of endothelial cells via C3a and C5a, leading to expression of adhesion molecules (E-selectin, VCAM-1, ICAM-1), pro-inflammatory cytokines (IL-1α, IL-6) and chemokines (CXCL-8, CCL5) [3]. 4) The promotion of the expression of adhesion molecules by parenchymal and endothelial cells in response to soluble C5b-9, and the synthesis of pro-inflammatory molecules (CCL2, CXCL8) and tissue factors (which contribute to thrombotic injury) [3].

Table 18.3. Potential indications for complement modulation in transplantation

Reduction in ischemia reperfusion injury
Prevention or treatment of AMR
Prevention or treatment of complement-related renal diseases that recur after renal transplantation
Tolerance induction
prolongation of graft survival in xenotransplantation

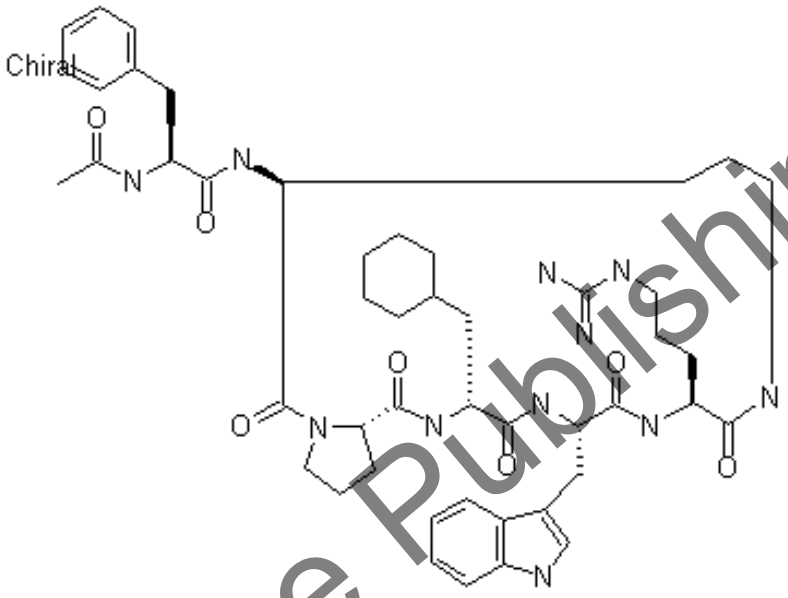

Figure 18.3. PMX-53 molecular structure.

Therapeutic Approaches to Regulate the Complement System

New complement-targeted treatments can be categorized into two types: those that are introduced into the donor or organ before it is transplanted to prevent or reduce complement-mediated damage; and those that are administered to the transplant recipient to induce systemic complement inhibition. In addition, strategies for intervention in the complement cascade can be split into two approaches: A) Inhibiting the activation of C3, which is common to all pathways of complement activation (classical, alternative and lectin), B) inhibiting C5 or downstream components of the terminal cascade. The first strategy is exemplified by using the naturally occurring cell surface and soluble complement inhibitors of the regulators of complement activation.

Since the ligands of such molecules are only the fragments of activated complement C3 and C4, these molecules are specific in preventing progression of the complement cascade only at sites of complement activation [18].

Genetic Manipulation of the Graft

This has been widely tested in xenotransplantation. Transgenic pigs expressing decay accelerating factor (DAF) and membrane cofactor protein (MCP) have been generated, and these human inhibitors have been shown to be abundantly expressed on porcine vascular endothelium. *Ex vivo* perfusion of hearts from control animals with human blood caused complement-mediated destruction of the organ within minutes, whereas hearts from transgenic animals were refractory to complement and survived for hours [20].

Delivery of Therapeutic Agents into the Graft through Donor or Graft Treatment

This is based on the treatment of donor or graft with complement inhibitors or regulators. Treating the donor organ itself with complement inhibitors may be a less toxic approach. Systemic inhibition of complement activation at the time of transplantation is avoided for reducing the risk of infectious complications. Gene silencing can be used to reduce the expression of complement components that are transcribed in the donor organ. This technique builds on the phenomenon of RNA interference, in which gene expression is effectively silenced using specific double-stranded small interfering RNAs (siRNAs). A set of siRNA molecules that selectively silence the expression of C3 or C5aR has been assessed in ischemia/reperfusion injury in mouse native kidneys [84, 85]. An alternative to gene silencing is to directly perfuse the donor organ with preservation fluid containing a complement inhibitor, Lewis et al. used a C5aR antagonist to reduce ischemia/reperfusion injury and prolonged survival of kidney isografts in mice [91].

Another approach is to target a systemically delivered complement regulator to the site of complement activation. Targeted complement inhibition has two aims: firstly, to inhibit specific points of the cascade as appropriate, and secondly, targeting the inhibitor to the site of complement activation in disease. Such strategies have the potential to avoid the need to inhibit complement totally and systemically, and to increase the bioavailability of the inhibitor used, thus reducing the required doses and toxicity [18]. This method involves fusing the complement receptor CR2 − which detects membrane-bound C3b, inactive C3b (iC3b) and C3d − to the complement regulator CRRY or factor H, which inhibit complement activation at the targeted site. In a syngeneic heart transplant model, a single systemic injection of CR2-CRRY (which inhibits all complement pathways) or CR2-factor H (which inhibits the alternative complement pathway) has been shown to reduce post-ischemic myocardial damage [92]. A novel development has been to derive a soluble chimeric fusion protein, CD59-Ig [87] that inhibits MAC formation in a site-specific manner, thus introducing the concept of site-directed complement inhibition using the specificity of the immunoglobulin to target the location of desired complement inhibition.

Other approach uses complement-regulatory proteins that have been engineered to remain in the donor organ after transplantation. The prototype of this approach is an analogue of the terminal three domains of the human complement-regulatory protein CD35 (also known as CR1), which inhibits all pathways of complement activation by destabilizing the C3 and C5 convertases. The active fragment is conjugated to a tail consisting of a basic peptide and a

myristoyl fatty acid group, permitting two-step binding to and insertion into the lipid bilayer of the cell membrane. When introduced via the renal artery, the construct (named mirococept) was shown to localize to endothelial and epithelial surfaces of rat and human donor kidneys. Mirococept has a terminal three domains of CD35 that are attached to a tail consisting of a basic peptide and a myristoyl fatty acid group. Following intra-arterial administration, the 23 kDa construct localizes on graft endothelial and epithelial cells. The cells of treated donor organs become resistant to complement-mediated damage owing to inhibition of C3 [70, 75]. Using similar approach, Smith et al. prepared a set of water-soluble complement regulatory molecules bound to addressin that bind with high affinity to outer cell membranes in vitro. Assay of a construct (APT070), as an inhibitor of antibody-mediated hemolysis in vitro, showed large (>100-fold) increases in potency compared to constructs with no membrane addressin [93], indicating that cell surface binding of APT070 could greatly enhance cytoprotection. The addressin membrane targeting approach is therefore capable of localizing biopharmaceuticals (in this case, complement inhibitors) to cell membranes [18]

Systemic Delivery of Complement Inhibitors or Regulators to the Recipient

This is the most used approach and has as advantage the possibility of long-term effect by providing multiple doses of medications. However, the inhibition of the complement is systemic and the risk of infection is increased.

Complement-Mediated Ischemia Reperfusion Injury

The complement system plays an important role in mediating inflammation and apoptosis during the IRI [95–98]. IRI is associated with the production of non-selfantigens leading to complement activation and inflammatory response [98]. Damage-associated molecular patterns (DAMPs) derived from endogenous tissue injury can activate complement via one or more pathways, generating the potent complement factors C3a and C5a and the MAC [100]. Ischemia/reperfusion injury in rodent kidneys induces the loss of the complement regulators and a local increase in the synthesis of complement component C3 by epithelial cells of the proximal tubule. Cleavage of the secreted C3 by the lectin or alternative pathways is followed by C3b deposition on the basolateral surface of epithelial cells. Epithelial cell activation and/or cell death is induced by terminal pathway effectors acting on the renal tubule. These effectors are C5a, which signals through the C5a receptor (C5aR), and the membrane attack complex (C5b–C9).

Ischemia/reperfusion-induced complement activation can produce direct tissue injury through formation of the MAC [100]. Tubular epithelial cells stimulated by C5b–C9 release pro-inflammatory molecules, such as interleukin-6 (IL-6) and prostaglandins, and pro-fibrotic products, such as collagen [7]. In humans, complement activation leading to deposition of

MAC is common during liver transplantation and probably contributes to cell injury and leukocyte sequestration to the allograft [84]. Apart from the direct cytotoxic effect of MAC, there are various indirect mechanisms of complement activation leading to ischemia/reperfusion injury. When the complement pathways are activated, chemokines are induced, and neutrophil activation and infiltration occur, leading to cell injury, apoptosis, and necrosis [30]. Neutrophils are a major source of pro-inflammatory mediators and characteristically infiltrate tissues exposed to ischemia and reperfusion [101]. C5a can cause rapid expression of P-selectin, facilitating the earliest steps in the adhesion process; C5a stimulates neutrophils to upregulate their expression of CD11a/CD18, facilitating neutrophil adhesion to endothelial ICAM-1 as well as being a key chemoattractant for the transmigration of neutrophils [102].

The activation of complement initiates even before reperfusion. It has been shown that changes after donor brain death are associated with complement activation and contributes to allograft dysfunction [103]. Likewise, the inflammatory environment created by IRI can lead to complement activation [37]. Lewis et al. showed that the expression of C5a receptor is significantly higher in deceased donors than in living related donors [39]. In addition, the level of expression of C3 in the donor organ is strongly dependent on the cold ischemic time [104]. Experimental studies demonstrates that the lectin pathway is a common trigger mechanism that initiates complement-mediated ischemia-reperfusion damage in several organ types, and suggest that the alternative pathway is a major amplifier of the injury [7]. The role of the classical pathway in ischemia-reperfusion injury is controversial. C4 and natural IgM were shown to be required for ischemia-reperfusion damage of cardiac and skeletal muscle and intestinal tissue − an observation that suggests a role for the classical pathway [97, 106].

Experimental data suggest that transplant survival may be improved if complement is inhibited in the immediate post-transplant period. Abrogation of renal IRI by treatment with complement inhibitors such as anti-C5 antibodies and C5a receptor antagonists has been demonstrated in animal models [40, 95, 106]. Specific siRNA-mediated silencing of the C5aR gene has also demonstrated significant protective effects in renal IRI [85]. It has been shown that silencing the C3 gene with specific siRNA can significantly protect against renal IRI [84].

Complement Regulators Used in Ischemia Reperfusion Injury

Complement regulators have been used in many animal experiments to reduce IRI. One of the most used regulators of complement in IRI is sCR1. Administration of sCR1 in a rat cardiac model of ischemia/reperfusion injury reduced myocardial infarct size by 44%, as assessed at 7 days post dosage, and minimized the accumulation of neutrophils within the infarcted areas [12]. In addition, sCR1 attenuated the deposition of the MAC and protected rat kidney allografts from vascular injury and cellular infiltration [108]. Complement-depleted animals, and animals pretreated with sCR1, accumulated significantly fewer PMNs in livers and sustained significantly less injury after reperfusion [109]. Soluble derivatives of these natural complement inhibitors are, however not ideal inhibitors of complement activation even in model systems, suffering from short half-lives and inappropriate biodistribution

[110]. To improve efficiency of complement inhibitors, different strategies were used: 1) Combining sCR1 with a serum albumin-binding peptide derived from Streptococcal Protein G, significantly extends the half-life of sCR1 [74]; 2) formation of a soluble chimeric molecule (combined activation blocker-2; CAB-2) consisting of functional moieties derived from CD46 and CD55 [111]. Other regulators have been used to prevent ischemia/reperfusion injury. A naturally-occurring complement inhibitor, CD59 (Protectin) which interrupts assembly of polymers of C9 that form the MAC, has been proposed as a modulator of IRI [112]. The polysaccharide pentosan polysulphate represents another class of polyanionic complement inhibitors whose actions are thought to be exerted through a blocking of the Factor B binding site on C3b, thus preferentially inhibiting the alternative pathway [113].

Complement Inhibitors in Ischemia Reperfusion Injury

In a syngeneic mouse model of kidney transplantation, Lewis et al. recently demonstrated that preservation of kidney grafts in University of Wisconsin solution in the presence of C5a receptor antagonist can significantly increase the graft survival [40]. An alternative strategy reported by Zheng et al. involved the use of C5aR siRNA which was injected into mice 2 days before induction of renal ischemia. The authors observed a protective effect by silencing of C5aR gene expression [85]. Another approach used an Anti-C5 monoclonal antibody therapy which significantly inhibited cell apoptosis, necrosis, and PMN infiltration in myocardial ischemia/reperfusion injury [113]. Grafts of transgenic animals overexpressing C1-INH are protected from endothelial cell damage [114]. C1-INH has been tested as potential therapy for I/R injury of ischemic muscle and brain [116, 117]. In addition, C1-INH treatment following reperfusion therapy in 22 patients with acute myocardial infarction, proved to be safe and effective in reducing complement activation [118]. C1-INH has also been circulated into the organ before implant [119, 120] or added to the preservation solution [120]. Exposure of organs to be transplanted to high concentrations of C1-INH significantly reduces such complications due to the capacity of C1-INH to bind endothelial cells maintaining intact functional capacity [122]. Ghebremariam et al. reported the effect of vaccinia virus complement control protein (VCP) in a rat model of ischemia reperfusion injury. VCP is known to inhibit the activation of C3 (thereby preventing the formation of C3b) and hence down regulate the alternative, classical and lectin pathways of complement. They observed reduced injury in the VCP-treated animals compared to the PBS control-treated group [80].

Complement and Rejection

During rejection, the discovery of C4d deposits in peritubular capillaritis, by Feucht in the early 1990s, represented a major breakthrough in the physiopathology of rejection, and provided indirect evidence for binding of DSAs within the graft and activation of the classical complement pathway [123]. Pratt et al. showed that allograft rejection was strictly dependent on the production of complement in the donor organ. Although all kidneys from wild-type

C57BL/6 donor mice were rejected by fully MHC-mismatched (B10.BR) recipient mice within 2 weeks, kidneys from C3-deficient donors survived for long periods without additional immunosuppressive treatment, indicating a role for donor-derived C3 in transplant rejection [124]. The deficiency of complement regulators led to rejection in a model of heart transplantation in which hearts from CD55-deficient donor mice led to the uncontrolled activation of complement in the transplant organ [21]. In human kidney allografts undergoing rejection, tubular epithelial cells are the main source of complement and are the primary target of the products of complement activation. In addition, smaller amounts of complement are expressed by graft-infiltrating cells [37, 125] Complement can damage the renal parenchyma directly, and complement can mediate indirect damage by enhancing the T cell response against the donor organ [124]. T cells express a variety of complement receptors that allow them to sense the soluble and membrane-bound products of complement activation. C3aR and C5aR signaling on $CD4^+$ T cells was found to enhance the immune response against major histocompatibility antigens and minor histocompatibility antigens [126]. Complement modulates T-reg induction and function [10, 127, 128]. In addition, the maturation of B cells that are primed by alloantigens requires the activation of complement. C3 acts as an opsonin, leading to more efficient retention of antigens in the B cell areas of lymphoid tissue and to a reduced threshold for B cell stimulation by antigens [26]. In a pre-sensitized monkey renal-allotransplant model, intra-graft expression of CD59 and CD46, on sequential biopsies, was associated with accommodation [129]. Stegall et al. reported their experience of cross-match positive recipients treated with eculizumab in comparison with a historical group who received conventional immunosuppression plus plasma exchange based on the DSA titres. After a month, the control group showed 36% incidence of AMR, but the eculizumab group had no sign of rejection with stable graft function despite persistence of DSA titres. In addition, protocol biopsies at 1 year showed a significant reduction in chronic AMR in the eculizumab-treated patients [51]. The major roles of complement in the pathogenesis of acute AMR include: (a) causing MAC formation via classical pathway activation, leading to direct cell lysis; (b) mediating acute graft injury by producing the chemoattractants C3a and C5a, which attract inflammatory cells (e.g., neutrophils and macrophages); (c) activating endothelial cells via C3a and C5a, and thereby increasing the expression of adhesion molecules, cytokines, and chemokines, including E-selectin, VCAM-1, ICAM-1, IL-1, IL-6, CCL5, and CXCL8; and (d) promoting the expression of adhesion molecules by endothelial cells in response to soluble C5b-9 and the synthesis of pro-inflammatory molecules (e.g., CCL2 and CXCL8) and tissue factor (which contributes to thrombotic injury) [30].

Table 18.4. Limitations of complement-based therapies

Limitations of Complement therapies
Many drugs have limited activity due to short-half lives
High susceptibility to bacterial infection and autoimmune diseases
Inhibition of a single pathway may be insufficient to provide effective response
Lack of tissue specificity.
Lack of a quantitative method to measure complement activity inside the graft
High price

Limitations

Therapeutic inhibition of the complement system presents a clinical challenge. Despite multiple drug candidates, only very few underwent clinical trials. There are many limitations to the development of drugs that affect the complement: A) Many drugs have limited activity. Many critical steps in the complement cascade are dependent on large protein-protein interactions, which are challenging to be modulated with small molecules, which are preferred drugs in traditional drug discovery due to potential for oral administration. For example, soluble derivatives of natural complement regulators are not ideal inhibitors of complement activation because of their short half-lives and inappropriate biodistribution [110]. In addition, small–drug molecules and peptide drugs have some potential drawbacks in terms of lower stability and shorter plasma half-lives [30, 130]. B) The complement system exerts important protective effects which are related to the ability of C3b to label invasive microorganisms and immune complexes leading to clearance by cells of the reticulo-endothelial system. Prevention of complement activation can reduce both innate and acquired immune responses, but if this inhibition is uncontrolled and long-term, this may lead to a susceptibility to bacterial infection and autoimmune diseases [2, 7, 131, 132]. C) Inhibition of a single pathway may be insufficient to provide effective response [2]. D) Lack of tissue specificity. In most cases, there is systemic inhibition of complement and not at the graft. For example, siRNA therapy can cause ubiquitous gene silencing, since it lacks a tissue-specific homing mechanism [30, 87]. Treating the donor organ itself with complement inhibitors may be a less toxic approach. This way, systemic inhibition of complement activation at the time of transplantation, which might be associated with higher risk of infectious complications, is avoided. E) Measuring response to treatment is another challenge because of the lack of a quantitative method to measure complement activity inside the graft. Development of new ligands case (where CR2 was conjugated with super-paramagnetic iron oxide nanoparticles for MRI) for imaging studies that are suitable for the detection of activated complement fragments (split fragments of C3 that are covalently bound to the activating tissue) by external body imaging [132] (Table 18.4).

Conclusion

The complement system is a major player in the innate immune response and can also modulate the adaptive immune response including antigen processing and presentation, B cell activation, and T cell proliferation and differentiation. Therefore, it plays a crucial role in graft ischemia/reperfusion injury, antibody-mediated rejection and accommodation, and cell-mediated rejection [133]. Despite testing many complement modulating agents, only few have therapeutic potential, and only two complement inhibitors have been licensed by the FDA: a humanized anti-C5 monoclonal antibody, eculizumab for treating paroxysmal nocturnal hemoglobinuria; and a recombinant C1 esterase inhibitor, cinryze, for treating hereditary angioedema [30]. Although the current use of complement inhibitors and regulators in the clinical setting is very limited, and there is only weak evidence due to the lack of randomized trials, they are promising approaches to improve outcomes in clinical transplantation. The main limitation is that modulation of the complement system is

extremely complex and may impair the ability to defend against infections, to achieve tolerance, and to control the clotting system. In addition, it is not clearly known which step of the complement cascade should be targeted. One potential approach in the future is to genotype patients for complement before transplantation that could provide a basis for stratifying risks and therapy. Future clinical studies should focus on identifying those patients that will benefit most from this therapy and will optimize the protocols for administration. In addition, randomized controlled trials will be crucial to compare effectiveness of these drugs.

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Chapter 19

Agents Targeting Ischemia-Reperfusion Injury

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Abstract

Ischemia-reperfusion injury (IRI) remains one of the most challenging fields of transplantation. IRI is a multicausal and multifactorial phenomenon and is highly associated with the use of expanded criteria donors (ECD) that might result in delayed graft function (DGF) and/or early graft loss or late graft failure. On the other hand, there is a need to accept ECDs in the age of donor-organ shortage. Therefore, magnitude of the problem has increased since IRI became a regular clinical issue, instead of remaining a marginal field of interest. The complex pathophysiology of the IRI has not been clarified yet in all the details. During ischemia, numerous metabolic and ultrastructural changes occur, depending on the duration of the ischemia. The core process is decrease in cellular oxidative phosphorylation. The drop in cellular ATP leads to dysfunction of ATP-dependent membrane ion-pumps, resulting in calcium, sodium and water influx to the cells. The swelling results in disruption of the cell membrane and intracellular membranes. Hypoxia leads to microcirculatory, micro-rheological, inflammatory, innate and adaptive immune responses, and other alterations.

IRI is a main therapeutical target. The better organization, logistics for organ retrieval and allocation are aimed to reduce the cold ischemic time. Since the shipping of organs covers even far distances, development of new preservation solutions and launching of machine perfusion has taken important place in the armamentarium. Different types of preservation solutions cover the abovementioned pathways of IRI; they protect against cell swelling, oxidative stress, acidosis, and apoptosis and block enzymatic activity, while maintaining cell energy level. The hypothermic perfusion

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system opens the possibility to apply pharmacological maneuvres on hypothermically perfused organs. Therapeutical agents targeting IRI includes metabolic strategies, antioxidant strategies, reducing inflammatory responses, factors modulating immune response as well as antiapoptotic possibilities. MicroRNAs and gene transfer is a promising therapeutic option for solid organ transplantation and in the prevention of IRI in transplant recipients. Cell therapy, particularly with stem cells may provide an immediate source of numerous biologically active molecules. Organ bioengineering and regeneration have shown the potential to revolutionize the field of organ transplantation. However, in case of more complex organs, the investigations are still in very early stages.

Keywords: Transplantation, ischemia-reperfusion, preservation

Abbreviations

ALDH-2	Aldehyde dehydrogenase 2
ANXA5	Annexin A5
ATN	Acute tubular necrosis
BMI	Body mass index
BUN	Blood urea nitrogen
CAT	Catalase
CCR	C-C chemokine receptor
CIT	Cold ischemic time
CMV	Cytomegalovirus
CXCL10	C-X-C motif chemokine 10
DAMPs	Damage-associated molecular patterns’
DCD	Donors after cardiac death
DGF	Delayed graft function
ECD	Expanded criteria donors
ECMO	Extending extracorporeal membrane oxygenation
ER	Endoplasmatic reticulum
ERK	Extracellular signal-regulated kinase
ET-Kyoto	Extracellular type Kyoto
ET-1	Endothelin-1
FDA	Food and Drug Administration
GFs	Growth factors
GSH	Glutathion
GSH-Px	Glutathion peroxidase
HES	Hydroxyethyl-starch
HIF1α	Hypoxia-inducible factor
HMGB	High-mobility group box 1
HMP	Hypothermic machine perfusion
HOC	Hyperosmolar citrate
HOPE	Hypothermic oxygenated perfusion
HO-1	Heme oxygenase
HTK	Histidine-tryptophan-kethoglutarate
ICAM-1	Intercellular adhesion molecule 1

IGL-1	Institute Georges Lopez-1
IRI	Ischemia-reperfusion injury
IT-Kyoto	intracellular type Kyoto
JNK	C-Jun N-terminal kinase
LFA-1	Lymphocyte function-associated antigen
LTB4	Leukotriene B4
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic protein-1
MHC	Histocompatibility complex antigens
MMP	Matrix metalloproteinase
mPT	Mitochondrial permeability transition
MyD88	Myeloid differentiation factor 88
NF-κB	Nuclear-factor-κB
NADH	Nicotinamide adenine dinucleotide-H
NAC	*N*-acetylcysteine
NHB	Non-heart beating
NMP	Normothermic machine perfusion
PAF	Platelet-activating factor
PARP-1	Poly (ADP-ribose) polymerase 1
PEG	Polyethylene-glycol
PFC	Perflourocarbon
PFS	Persufflation
PHD	Prolyl hydroxylase domain
PSGL-1	P-selectin glycoprotein ligand-1
RAGE	Receptor for advanced glycation end products
RANTES	rRegulated on activation, normal T cell expressed and secreted
r-ATG	Rabbit delivered polyclonal antibody
ROS	Reactive oxygen species
TNF-α	Tumor necrosis factor-α
shRNA	Short hairpin RNA
SCS	Static cold storage method
SOD	Superoxide dismutase
TLM	Two-layer method
tNKA-β	Truncated Na^+/K^+-ATPase β
TRAM	TRIF-related adaptor molecule,
TRIF	Toll-IL-1 receptor domain-containing adaptor inducing interferon-β
Unisol-CV	Phosphate-free cryoprotectant vehicle version
UNOS	United Network for Organ Sharing
UW	University of Wisconsin (solution)
VAD	Ventricular assist device
VLA-4	Very late antigen-4
VSL-MPS	Vasosol machine perfusion solution
VSOP	Vascular system oxygenated persufflation
WIT	Warm ischemic time
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase

Introduction

Ischemia-reperfusion injury (IRI) remains one of the most challenging fields of transplantation. IRI is a multicausal and multifactorial phenomenon which is highly associated with the use of expanded criteria donors (ECD), that might lead to delayed graft function (DGF) and/or early graft loss, or late graft failure. On the other hand, there is a need to accept ECDs in the age of donor-organ shortage. Therefore, the importance of the problem has increased since IRI became a regular clinical issue [1-9].

The Food and Drug Administration (FDA) held an open public workshop in September 2011 to discuss the current state of science related to the effects of IRI on outcomes in kidney transplantation. Clinical trials of various drug products administered in the peritransplant period were summarized; a few demonstrated early improvements in DGF, but none demonstrated an improvement in late graft function. One viewpoint is that acute kidney injury due to ischemia-reperfusion causes transient renal dysfunction followed by healing and recovery of function and that the non-modifiable donor and recipient factors, such as age and comorbidities determine long-term outcome. The FDA urged that medical products are urgently needed to minimize the effects of IRI and DGF and this may allow for a reduced discard rate of donated organs and improve long-term allograft survival. The development of such medical products will rely on both clinical and non-clinical research [10].

The pathomechanism of ischemia-reperfusion injury is quite complex and affects numerous systems and processes in the subjected organ, in the circulating blood as well as in remote organs. However, the complex pathophysiology has not been clarified yet in all the details. Since a tissue or organ is subjected to cessation of blood supply for many reasons and to restoring its circulation afterward, it suffers from IRI. During transplantation process, it is unavoidable. The magnitude of the damage depends on several factors that include the time factor (duration of the ischemia), the temperature, the ischemic tolerance of the affected tissue/organ, the dynamics of the reperfusion, the structural-functional state of the tissue/organ (e.g., condition of the microvessels), and the presence of pre- or postconditioning factors.

Although the pathomechanism of ischemia and reperfusion is widely investigated, transplantation creates a special situation for IRI. At the donor site, the organ often suffers from circulatory insufficiency, haemodynamic instability or even shock (leading to brain death), this goes on during the organ procurement, storage and transport (e.g., cold preservation). Finally, it suffers from the warm ischemia during the implantation surgery. Here, the reperfusion occurs from the recipient's body, with the recipient's blood.

This chapter is aimed to provide an overview on the pathophysiology of IRI as well as on the organizational and therapeutical possibilities for reducing its effect (Figure 19.1.).

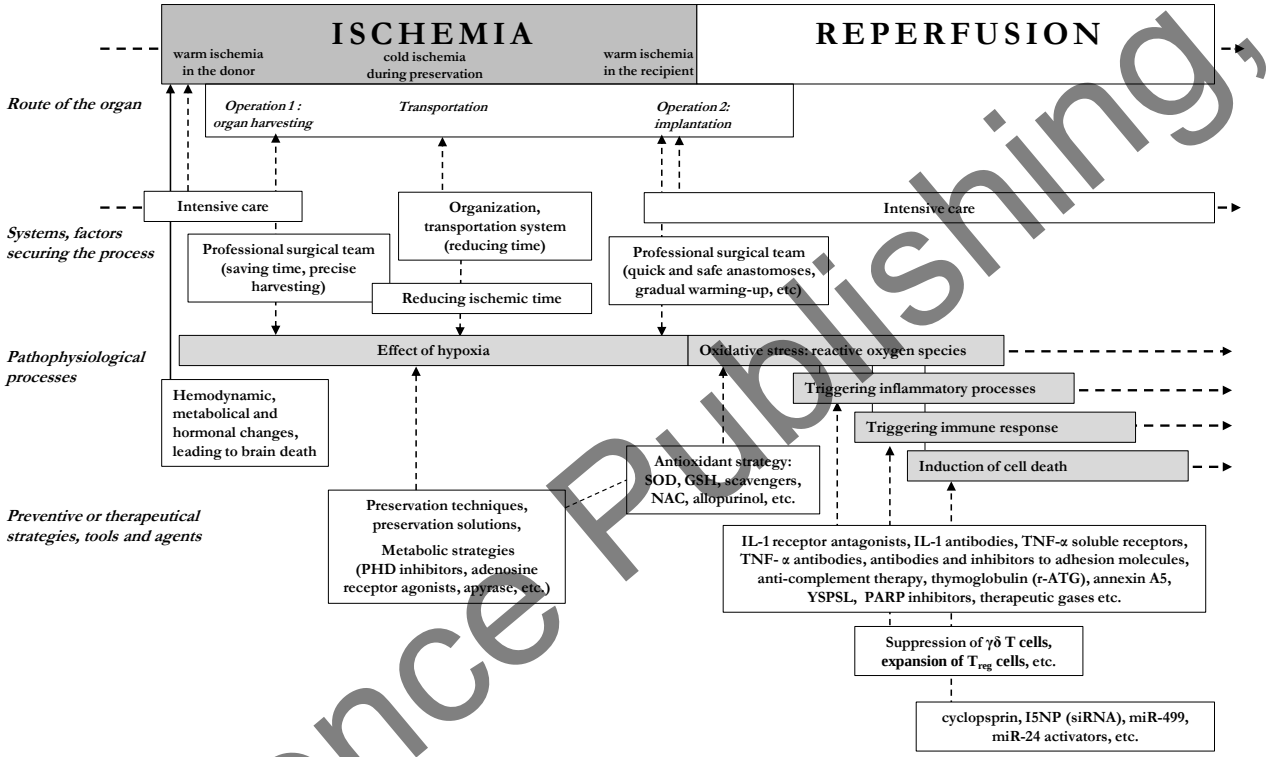

Figure 19.1. Elements of the transplantation-related ischemia-reperfusion: route of the organ, systems and factors securing and supporting the processes, the sequence of pathophysiological processes, preventive or therapeutical tools and agents.

The Time Factor for Transplantation-Related Ischemia-Reperfusion in the Clinical Practice

IRI is a target of ongoing debate and research in transplantation surgery. Every organ has a specific time-frame, within that period the reperfusion must be reestablished. This organ-specific ischemic time is called as cold ischemic time (CIT) that is specified by two landmarks: the onset of CIT is the moment when the aorta is clamped in the donor together with the immediate start of perfusion by cold preservation fluid, and the endpoint is the moment when the organ is implanted to the recipient and the blood-stream starts to flow into the organ by opening up the newly set anastomosis of the organ-specific artery. One of the first experiences was published by Koyama et al. [11] in 1985. They perfused the removed kidney with Eurocollins solution, stored for 24 hours and then transplanted. It worked well [11].

This time-period looks like to be a passive one, but it is not true. Time was always a limiting factor since the beginning, as it was already discussed by Starzl et al. [12]. When the aorta is clamped in the donor, and the preservation fluid starts to run into the organs, the abdominal cavity will also be cooled down with ice-cold sterile saline. In the next few minutes, the organs are taken out by the harvesting surgeon team.

The sequence of organs for harvesting is always the same: thoracic organs first, then liver, then pancreas and finally the kidneys [13, 14]. This is easy to understand that inspite of all efforts to keep the abdominal cavity cooled, the duration of this process has a relatively "warm ischemic" character. Since kidneys are the last, they suffer the most. After harvesting, the organs are packed separately in sterile perfusion fluid, and then placed in ice for shipping. Priority of organs reflects the tolerance for ischemia: heart goes first, then lungs, then the liver, the pancreas, and finally the kidneys. That means that the different organ harvesting teams will arrive and depart to the operating room in a different time. The abdominal team arrives first and departs last. They perform the basic preparation for the abdominal aorta, together with sternotomy.

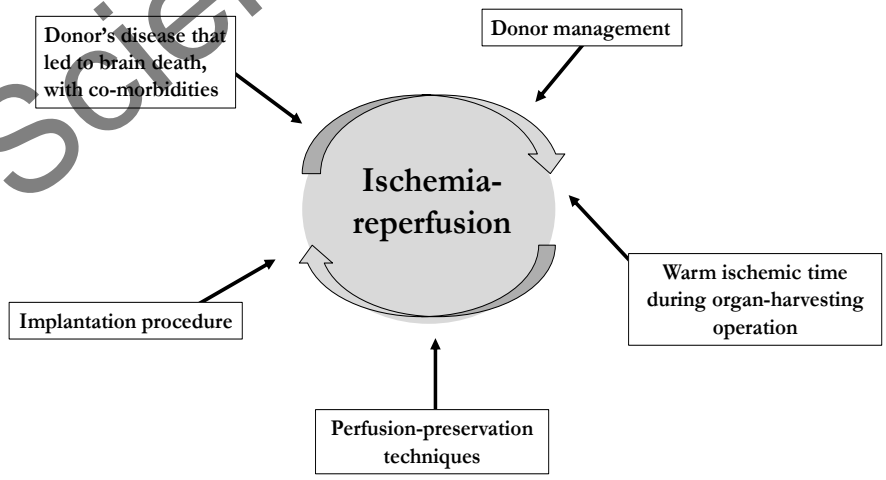

Figure 19.2. Factors and conditions that have impacted on the transplantation-related organ ischemia-reperfusion.

The thorax team will arrive at this time and will complete the preparation for the heart and lungs. Installation of perfusion fluids and cannulation follows. Compartments are divided by clamping the abdominal aorta right below and above the diaphragm. When the CIT starts, both teams perfuse the organs with their separate fluid. In these few minutes, the organs receive the first damage that correlates with the speed of cooling down: if this process lasts long, the ischemic injury might be moderate as well. When perfusion is completed, the thorax team takes the heart and the lungs out and immediately departs. Abdominal team will complete the liver (pancreas) and kidney preparation, then close the cadaver's sterno-laparotomy, and depart thereafter [15]. That kind of co-operation makes possible to cut the travelling-time as short as possible for the thoracic organs.

As mentioned earlier, CIT is organ-specific. It is about 4 hours for the heart, 4−7 hours for the lungs, 8−12 hours for the liver and maximum 24−28 hours for the kidney. These technical approaches were developed in the last decades beginning with the first attempts by Thomas Starzl [12], and summarized by de Graauw et al. [16] in 2014.

There is great potential for sharing the best practices and for direct transfer of expertise at the European level, and especially to export this standardized training in organ retrieval to other European countries and even further. The ACCORD-programme is a best example for this [16].

Multiple factors have an impact or might prevent or diminish ischemia-reperfusion damage (Figure 19.2):

1. Baseline disease led to brain death, donor age together with the co-mobidities of the donor: the presence of diabetes mellitus, severe atherosclerosis, hypertension or other systemic diseases [17]. The cause of brain death also counts: a skull trauma with consecutive bleeding in an otherwise healthy person will result in a superior organ quality over a subarachnoid bleeding in a hypertensive, and obese donor [18]. These factors will limit the absolute quality of the organs. All further factors listed later diminish the quality [19].
2. The donor management. It is obvious that intensive care doctors put all efforts to save the patient's life. However when brain death is determined, the quality of care might change. Proper donor management will determine the quality of perfusion and therefore the quality of the organs. Standardized set of critical care end-points and the goal of donor management also have an impact on the number of organs transplanted per donor in ECDs [20]. The immediate consequence of brain death will be an "endocrine storm" with extreme peaks in the mean blood pressure, severe diabetes insipidus and fever, causing significant perfusion problems to the organs [21]. Hypotension needs active drug administration and sometimes a complex cardiopulmonar resuscitation is necessary. The maintainance of organ perfusion is often a high challenge to the intensivist. The overdose of cardiotonics like dopamine, nor-adrenaline can have an ischemic effect on microcirculation. The use of hydroxyethyl-starch (HES) in high doses might result in Kuppfer's cells crisis [22]. The duration for which cadaver is exposed to these factors before harvesting is also important. In the majority of the brain death cadavers, the situation is stable, however, that might change in minutes. Antibiotics and blood transfusion are needed in some cases.

3. An extreme example is the non-heart beating (NHB) donors, or in other terms, the donors after cardiac death (DCD) [23]. The use of DCDs is different country by country and strongly correlates with the beliefs and acceptance of euthanasia, and depends on the general legal and ethical background of the country. Wherever it is permitted, the so called Maastricht-criteria are used to categorize, and allow DCDs [24]. In case of a DCD, after stopping the ventilator in the ICU, the heart stops functioning first. Time is recorded, and when the isoelectronic sign appears on the ECG, other organs can be taken out really quick. This is usually performed by using a double-balloon catheter that is inserted into the aorta, through the femoral artery, in seconds. The two balloons are then inflated, creating a closed section of the abdominal aorta (below the diaphragm, and over the femoral) that consists of celiac trunk, mesenteric superior and inferior, and renal arteries [25, 26]. Cold perfusion starts usually 3−4 minutes after cardiac death, and then the organ harvesting operation continues the usual way. DCDs are maximally exposed to ischemic injury, due to the so called agonal time [27]. Organs from a DCD have a shorter half-life compared to a normal brain death donor, and at least 15% less 5-year survival compared to a live-donor. NHB donors carry the potential of expanding the cadaveric organ (e.g., kidney) pool. Although, transplants from NHB donors are associated with a greater incidence of early adverse events, long-term outcomes appear comparable with transplants from heart beating donors [28].
4. An important factor is the harvesting operation itself. The more rapid the cooling down, the speedier and more professional is the harvesting of the organs, and the more complete the perfusion is, the better the results are.
5. Perfusion fluid is an independent factor itself. In the early ages, the first solution was named after Belzer. Subsequently, the histidine-tryptophan-kethoglutarate (HTK) and the solution of University Wisconsin (UW) were developed [29]. The main target of these solutions was the Na^+/K^+ ATPase, and the transmembrane traffic of K^+. Therefore all of them are rich in K^+, and poor in Na^+. Many additives have been tried in the last decades. The preservation solutions are discussed in details later in this chapter.
6. A prolonged CIT will also have an impact on organ injury [30]. Transportation in ice with a passive stabilization fluid was always a challenge to improve. The earliest attempts to create a perfusion machine were dedicated to Sir Roy Calne in UK. Besides being the pioneer in liver transplantation in UK, he also dealt with perfusion techniques [31, 32]. This is well-known that other “big names”, like Joseph Edward Murray who performed the first living related kidney transplantation in Boston in 1952, also contributed to develop this machine, together with the well-known pilot Charles Lindbergh [33]. Machine perfusion is based on the idea of imitating the blood circulation in the organ. In contrast to passive cold storage, small cannulas are inserted to the relevant artery and veins of the organ that is closed into an organ-shaped plastic box, and permanent or intermittent flow is maintained for hours. The difference between the cold and warm perfusion is mainly that the former is executed by a special solution, while warm means blood-flow, which was previously filled up into the machine [34]. These investments meant a revolution in organ storage and also in ischemia-injury. Machine-perfusion revolutionized the research and development of perfusion fluids.

7. A last factor is the implantation procedure. Here we have to mention the warm ischemic time (WIT) [4, 35]. This time-period starts at the moment when the cold-stored organ is placed into the recipient body, still without a circulation, and finishes when the blood flows into the organ (declamping of the relevant artery after the anastomosis). The WIT ends up together with CIT by definition. However, we also mentioned the early WIT that is the duration of the harvesting when the cooling is not perfect in the donor body. Implantation procedure can be a standard operation, but it also can be a nightmare. In some difficult cases, mostly in liver transplantation, revascularisation is difficult, the arteries are tiny, and the circulation will not be optimal. That can be an additional factor for ischemia-reperfusion. A prolonged biliary warm ischemia time results in aggravated injury of the bile duct and the surrounding vascular plexus [36]. The general state of the recipient at the time of implantation might also be a relevant factor; age, cardiac co-morbidities, anaemia and decompensated circulation counts among others. In case of liver transplantation, severe decompensated liver cirrhosis can easily be a cause of polytransfusion during the explantation of the liver from the recipient. In these cases, some ischemia may be present for hours even after an uneventful implantation [37]. The ischemia will last until the cause is eliminated (the pump function of the heart normalizes, the anaemia corrected, etc.). Thus, anaesthesia and intensive care teams have crucial importance.

Pathophysiology of Ischemia-Reperfusion Injury

The limitations of this chapter do not allow discussing the complex pathophysiology of the ischemia and the consecutive reperfusion in details. Huge number of papers deals with various aspects and components of these richly orchestrated processes, their balance and imbalance. In this sub-chapter, we aim to give a concentrated overview of the processes involved and presenting possible therapeutical targets.

The Effect of Hypoxia on Cells

During ischemia, numerous metabolic and ultrastructural changes occur, depending on the duration of the ischemia. These changes include decreased adenosine 5'-triphosohate (ATP), decreased phosphocreatine, decreased glutathione, accumulation of inorganic phosphate, protons, creatine and glycolysis products, increased hypoxanthine, altered ion distribution, altered membrane potential, cellular swelling, cellular acidosis, as well as cytoskeletal disorganization [2, 6, 38–44].

The core process is the decrease in cellular oxidative phosphorylation. ATP is further dephosphorylated to ADP, AMP and purines, leading to accumulation of hypoxanthine. The drop in cellular ATP leads to dysfunction of ATP-dependent membrane ion-pumps, resulting in calcium, sodium and water influx into the cells. The osmotic load is exacerbated by the accumulated metabolic intermediates and products during anaerobic glycolysis (glucose-6-phosphate, glucose-1-phosphatem α-glycerol phosphate, lactate, nicotinamide adenine dinucleotide-H, H^+), forming cellular edema [38, 40, 41, 43, 45].

The swelling results in disruption of the cell membrane (opening stretch-activated channels, further modulating the conductance) and intracellular membranes, such as of the endoplasmatic reticulum, the Golgi-apparatus, mitochondrial membrane and the cytoskeletal microtubule [41, 42, 46]. Besides the swelling, acidosis also decreases the stability of the membranes, including the lysosomal ones. The implicated lysosomal enzymes disrupt ultrastructural components. Disruption of carrier proteins leads to free iron (Fe^{2+}) in the cytosol [6, 38, 40, 41].

The intracellular calcium overload is characteristic, which is originated from the endoplasmic reticulum (ER) stores by redistribution (early in ischemia), and from extracellular space (in prolonged ischemia and during the reperfusion) [41, 47, 48]. Calcium overload blocks the function of the mitochondrial NADH-coenzyme Q oxidoreductase, affecting further mitochondrial complexes. Cytochrome c releases, and mitochondrial permeability transition (mPT) pores open. Due to the increased intracellular calcium, phospholipases and proteases are activated. Activation of calpain and caspase proteases results in translocation of Na^+/K^+ ATP-hydrolase to the cytoplasm and xanthine dehydrogenase (XDH) conversion to xanthine oxidase (XO) [2, 38, 49, 50]. Normally, more than 90% of the XO in tissues exists in XDH isomeric form, which cannot transfer electrons to molecular oxygen to form superoxide. The XDH-XO content and conversion rate may vary among tissues and organs [50].

During lack of oxygen, the oxygen-sensing prolylhydroxylase dysfunction leads to post-translational activation of signaling cascades and transcription factors, including hypoxia-inducible factor (HIF1α) and nuclear-factor-κB (NF-κB) 40–42]. Depending on the ATP loss, necrosis (all ATP stores are lost) or programmed cell death (in the presence of partially preserved ATP cytochrome c releases with caspase 3 activation) may occur. The latter cell death form happens mostly in reperfusion [41, 51]. Reduced mitochondrial activity, calpain activation (by increased intracellular Ca^{2+}), free radical generation, Fas ligand overexpression, increased bcl-2-like protein 4 (BAX) and decreased bcl-2 proteins and other triggers lead to apoptosis [42, 44].

The Oxidative Stress

During reperfusion, when oxygen is supplied to the tissue, an oxidative burst strikes up due to increased amount of hypoxanthine, xanthine oxidase enzyme and free iron anions due to the processes occurring during ischemia. Since the studies of McCord's and his co-workers, it is known that the major pathway by which toxic oxygen metabolites are produced during the reperfusion of a previously ischemic tissue, is a xanthine-oxidase (XO)-dependent process [49]. As discussed above, the xanthine-dehydrogenase isoform is converted to oxidase isoform by a calcium- and protease-dependent process during the ischemia. Ischemia leads to degradation of ATP to hypoxanthine, a substrate for XO. When reperfusion starts, oxygen appears in the tissue which acts as an electron acceptor during the XO activity, generating superoxide anion [49, 52].

In addition to the role of XO, there are further sources of the superoxide radical ($\bullet O_2^-$) production during ischemia-reperfusion that include activated neutrophils and the disturbed mitochondrial electron transport chain [38, 43, 49, 52]. As a remote effect, it was shown that

XO released from ischemic muscle can be taken up by the liver where it mediates Kupffer-cells and polymorphonuclear neutrophil activation [53].

The production of reactive oxygen-centered free radicals (reactive oxygen species, ROS) is an early process; their level increases within minutes after starting the reperfusion. During this rapid burst, the nascent superoxide ($\bullet O_2^-$) is metabolized to hydrogen peroxide (H_2O_2) by the catalase enzyme. According to the Haber-Weiss reaction (its second step by Fenton reaction in the presence of ferrous ion), from superoxide anion and hydrogen peroxide, further oxygen-centered reactive species are generated. These are the hydroxyl anion (OH^-) and the harmful hydroxyl radical ($\bullet OH$):

$$\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + OH^- + O_2$$

Reactive oxygen-centered free radicals cause lipid peroxidation (on membrane phospholipids and on polyunsuturated fatty acids) leading to structural and functional cell damage. Transmembrane and structural proteins are damaged or functionally altered by sulfhydryl cross-linkings [49, 54–58], ROS activate mitogen-activated protein kinase (MAPK) pathway, induce oxidative DNA damage and a consequent activation of poly (ADP-ribose) polymerase 1 (PARP-1) [40, 56].

Inflammatory Reaction

Ischemia-reperfusion is known to cause "sterile inflammation", in which the innate and adaptive immune responses and complement and platelet activation also takes part.

The endothelial damage caused by ROS leads to increased expression of adhesion molecules such as E- and P-selectins, intercellular adhesion molecule 1 (ICAM-1) for leukocyte recruitment: at rolling step, the P-selectin-leukocyte P-selectin glycoprotein 1 (PGSL-1) interaction, during adhesion, the ICAM-1-CD11a/CD18 and CD11b/CD18 interactions, while during transmigration, the platelet-endothelial cell adhesion molecules (PECAM-1) have important role. Neutrophils are attracted by numerous cytokines, including interleukin-1 (IL-1), IL-6, IL-17, tumor necrosis factor-α (TNF-α), monocyte chemotactic protein-1 (MCP-1), C-X-C motif chemokine 10 (CXCL10) and the RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted; member of IL-8 superfamily). Leukocyte migration is facilitated by matrix proteins, matrix metallopeptidase 9 (MMP-9), laminin and fibronectin as well [2, 8, 38, 39, 41, 43, 44, 56]. CXC and RANTES pathways also contribute to macrophage accumulation [59].

Activated neutrophils generate ROS (by NADPH oxidase) and release cytokines, causing cytotoxicity, and promoting platelet aggregation. After reacing the extravascular compartment, neutrophils release ROS, elastases, proteases. Neutrophils (and Kupffer cells in the liver) produce IL-1ß and TNF-α. These cytokines further activate leukocytes and $CD4^+$ lymphocytes. TNF-α binds to TNF-R1 and TNF-R2. Nuclear factor kappa B (NF-κB), MAPK and c-Jun N-terminal kinase (JNK) become activated. NF-κB up-regulates expression of TNF-α, ICAM-1 and vascular cell adhesion protein 1 (VCAM-1). Macrophages secrete cytokines such as IL-1ß, IL-12, IL-18, TNF-α and INF-γ. Besides these, the complement system is also involved. C3a, C5a and complemet components, iC3b and C5b-9 play

important role. C5a is a potent proinflammatory mediator, inducing production of IL-1, IL-6, TNF-α and monocyte chemoattractant protein 1. C5b-9 promotes leukocyte activation and inhibits endothelium-dependent relaxation. The inflammatory process is further exacerbated [2, 38, 40, 41, 43, 44, 59, 60, 61].

From the endothelium, numerous vasoactive agents are originated. Nitric oxide is an important vasodilatator, however, paradoxically, the early increase in NO release may exacerbate the reperfusion because of the higher oxygen concentration coming with the increased blood flow [38, 40, 43]. The above discussed superoxide anion reacts with NO forming peroxynitrite ($ONOO^-$), which is a harmful free radical and reduces the bioavailability of the NO. Vasoconstriction is resulted not only from the decreased bioavailability of the NO, but from the effect of endothelin 1 (ET-1). Besides these, thromboxane A_2 as well as vasoconstrictor and -dilatator prostaglandins take part in the vasoregulatory conditions [39, 40, 43, 57, 62, 63]. The platelet-activating factor (PAF) produced in the postischemic endothelium increases vascular permeability, and leukocyte adhesion. Leukotriens, such as leukotriene B4 (LTB4) and thromboxanes act as chemoattractants for granulocytes [2, 9, 40].

In the damaged endothelium, major histocompatibility complex antigens (MHC class I and II) become up-regulated, thus increasing the immunogenicity in the allograft [2, 40, 44].

Innate and Adaptive Immune Responses

By cell/tissue destruction, 'damage-associated molecular patterns' (DAMPs) are released to the extracellular compartment. These DAMP ligands (e.g., high-mobility group box 1 protein, HMGB1; cytoplasmatic Ca^{2+} regulator S100, hyaluronic acid, DNA or ATP) bind to pattern recognition receptors, such as toll-like receptors (mainly TLR-4) and receptor for advanced glycation end products (RAGE). Toll-like receptors are expressed on antigen-presenting cells (macrophages, dendritic cells, B cells), besides other cells of the innate immune system (mast cells, neutrophil, basophil and esoinophil leukocytes) [8, 40, 64, 65]. Ligand binding to TLRs leads to interaction with intracellular adaptors (e.g., myeloid differentiation factor 88, MyD88; Toll-IL-1 receptor domain-containing adaptor inducing interferon-β, TRIF; TRIF-related adaptor molecule, TRAM), activating transcription factors NF-κB, MAPK pathway, including Ras, Raf-1, extracellular signal-regulated kinase (ERK), p38 MAPK, and JNK [2, 8, 39, 40, 42, 44].

In the sterile inflammation of ischemia-reperfusion, T lymphocytes (both $CD4^+$ and $CD8^+$) and other cells of the adaptive immune system are involved both via antigen-specific and antigen-independent mechanisms. In IRI γδ T cells producing IL-17 play role [40, 66].

Microcirculatory and Micro-Rheological Alterations

Determining microcirculatory flow pattern and the blood rheological factors have a pivotal role, including the so called micro-rheological parameters, such as red blood cell deformability and red blood cell aggregation. These all can be altered during ischemia and by

reperfusion [55, 67]. Impaired red blood cell deformability may contribute to and increase blood viscosity and cause perfusion problems in the microcapillaries, because the erythrocytes have to be deformed while passing through them [55, 68, 69]. The enhanced red blood cell aggregation elevates blood viscosity and increases the flow resistance in the microcirculation [55, 70]. Altered blood rheology has an impact on the shear stress profile on the endothelial surface and thus modulates numerous vascular endothelial functions [71, 72]. The enhanced aggregation also results in a more expressed axial migration of the red blood cells in the vessels (along the axis of the flow, mostly due to the parabolic flow pattern), leading to a widening cell-poor plasmatic zone at the vicinity of the endothelium (Poiseuille zone). The widening Poiseuille zone gives room for the leukocyte tethering, margination and affects adhering. Furthermore, the wide cell-poor zone slows down the rolling and enables the leukocytes to stick more easily on the endothelial surface [73].

Ischemia leads to local metabolic changes (decrease in pH, increase of H^+ and lactate), altering the mechanical properties of blood cells. The pH of stagnant blood decreases in the area excluded from the circulation during the ischemic process, which turns the red bloods cells' discocyte shape into a stomacyte or sphero-stomacyte form [74–78]. The echinocyte and sphero-echinocyte forms may appear when the ATP depletion and calcium accumulation are dominant [74, 76]. Both morphological transformations mean worsening of micro-rheological characteristics: deterioration of red blood cells' deformability and disturbed aggregation [55, 74, 76, 77].

During ischemia the rheological properties of blood significantly worsen in the excluded region and they can be impaired further with the prolongation of the ischemic time-period. During stasis, hematocrit increases locally and altered fluid distribution results in elevated protein concentration (or plasma loss), leading to increased plasma viscosity. These factors, together with the less deformable erythrocytes and aggregates, result in an increased local blood viscosity [55, 67]. Furthermore, from the pathophysiological viewpoint, the mechanical trauma to red blood cells could not be neglected [79]. Additionally, the hypoxia leads to impaired endothelial cell barrier function [40, 72]. By revascularization, the metabolites flushing into the systemic circulation together with the damaged erythrocytes may cause further changes in the microcirculation and even in remote organs [80–82].

During reperfusion, newly generated oxygen-centered free radicals initiate chain reactions in the red blood cells; damaging the cell membrane (lipid peroxidation), the transmembrane proteins (receptors, ion pumps) with the formation of sulfhydryl cross-links, altering the hemoglobin molecules (methemoglobin, Heinz-body formation), as well as the structural proteins [43, 49, 54, 55, 58, 67, 83]. Red blood cells are rich in iron, which catalyzes the free radical reactions through the Fenton-reaction; making these cells highly sensitive to oxidative stress [54].

During the complex hemodynamical changes under ischemia-reperfusion, NO plays an important role in the local flow regulation [38, 40, 43, 63]. The NO has a beneficial effect on red blood cells, improving their deformability, and erythrocytes also act as an enzymatic source of NO [62, 84]. However, in the presence of NO and $\bullet O_2^-$, peroxynitrite anion formation can jeopardize the red blood cells [55].

In systemic circulation, the hemorheological changes are also non-specific and accompany the acute phase reactions: increase of plasma viscosity by elevated fibrinogen concentration and α_2-macroglobulin, increase in immunoglobulin levels, decrease in albumin

level, rise in leukocyte count, increase or decrease of platelet count, as well as hemoconcentration and erythrocytes' micro-rheological changes [67, 85–87].

In the microcirculatory bed, the formation of "no-reflow" phenomenon is characteristic for tissue ischemia-reperfusion. It means that despite restarting the circulation in large-caliber vessels, there is a slow down or total arrest in the microcirculation [88–90]. The phenomenon is caused by microvascular spasm, swelling of endothelial cells, increase of capillary permeability, interstitial edema, micro-thrombi, neutrophil adhesion and plugging, and local acidosis. Additionally, the presence of red blood cells with impaired deformability and enhanced aggregation contributes to the microcirculatory disturbance [55, 68, 69].

Some Organ-Specific Alterations

In case of a kidney, the most vulnerable part is the tubular system. Acute tubular necrosis (ATN) is a well-known phenomenon that is caused mainly by ischemic-reperfusion injury. In case of a delayed kidney graft function, the ATN is in the background. It depends on a number of factors, including activation of transcriptional factors, endothelial injury of peritubular small vessels, immune responses, and inflammatory processes associated with necrosis and apoptosis of renal tubular epithelium. Fortunately, tubular epithelium may regenerate in weeks [65, 91].

The transplant clinician will see a perfect kidney graft with intact circulation on the ultrasound image, however the serum creatinine / blood urea nitrogen (BUN) will not decrease, and/or there is a complete anuria. The patient will be dependent on a kidney replacement therapy (dialysis), sometimes for weeks. ATN unfortunately can serve as background to an acute cellular rejection; therefore a transcutan biopsy will certainly be taken from all kidney transplant patients having an ATN after the first week of transplantation. Glomeruli will also suffer from ischemia, however, the result will come months later. Since there is a relative "redundance" in glomeruli in the normal human body, loss of some percentage will not give an immediate clinical symptom. If the "glomerular mass" (the summarized capacity of all working glomeruli) is enough for the recipient, a light increase of the serum creatinine / BUN will only appear but proteinuria might occur. It is a consequence of inadequate reabsorption of small proteins from proximal tubular cells damaged by IRI, rejection, toxic agents (tubular proteinuria), increased passage of albumin and/or protein with higher molecular weight. Disruption of glomerular barrier caused by recurrent or *de novo* glomerulonephritis, transplant glomerulopathy, chronic rejection, glomerular proteinuria can be the reason for that [92].

However, if any additional damage will injure the graft-kidney (acute rejection, cytomegalovirus or other severe infection) in the long-term, the remnant part will not be enough any more, and rapid deterioration may occur. Early after transplantation, tubulointerstitial damage is predominantly related to ischemia reperfusion injury, acute tubular necrosis, acute and subclinical rejection and/or calcineurin inhibitor nephrotoxicity, superimposed on preexisting donor disease [93]. Therefore, ATN kidneys are often exposed to an early chronic rejection. That means a massive fibrosis without an active immunological impact. The half-life of these kidneys might be shorter [94, 95].

In contrast with kidney, liver can suffer from an irreversible injury during arterial ischemic period. The explanation is dual blood supply of the liver. Portal vein will collect the blood from the GI tract and transfer them to the hepatocytes through the portal triad (Rappaport acinus). Detoxication and synthetic function will be executed here. Arterial blood however will always be parallel to the larger and smaller bile ducts. Anoxia in the arterial system will be associated with biliary damage. Biliary damage means a death of biliary epithelium that is very weak in restoration [96, 97].

After the devastation of the biliary epithelium, the remodeling will never be complete, causing fibrosis, stenosis, and occlusion in the biliary system. Ischemic type biliary lesions are the main source of obstructive jaundice, requiring repeated interventional radiological procedures, dilatation, stenting, and sometimes even an indication for retransplantation of the liver [97]. The findings of Farid et al. also suggested a possible pathophysiological role of portal blood flow in the oxygenation of the biliary tree after liver transplantation [98].

Ischemic-reperfusionic injury will cause severe arrhythmias to the implanted heart that might easily convert to a fatal ventricular fibrillation. Impaired recovery of cardiac function due to IR injury is considered to be a consequence of oxidative stress and intracellular Ca^{2+} overload in the myocardium. These changes in the ischemic myocardium appear to activate both extracellular and intracellular proteases which are responsible for the cleavage of extracellular matrix and subcellular structures involved in the maintenance of cardiac function [99].

It has been observed that hypercapnic ventilation during reperfusion reduces the development of pulmonary oedema and has a protective effect on the oxygen transport ability of the lungs after warm ischemia [100]. It was also observed in animal models that methylene blue was able to attenuate IRI in lung transplant [101].

Therapeutical Issues

Considering the complex pathophysiology of ischemia-reperfusion injury, numerous targets and agents are involved. Several of them have been already discussed in details in previous chapters, such as cell trafficking and adhesion, protein kinase-C inhibitors, JAK-STAT inhibitors, anti-adhesion molecular-agents or complement inhibitory agents.

Here we focus on organizational issues, preservation techniques and special agents for metabolic, antioxidant, anti-inflammatory and immune-modulating approaches.

Organization - Reducing Ischemic Time

The extension of the criteria for donor organ acceptance, especially for organs that have suffered from periods of warm ischemic injury prior to graft retrieval, results in even higher demands on preserving these ischemia-sensitive grafts.

The shortage of donors led to the introduction of several organ sharing networks to optimize the allocation of organs. That was designed, in the belief that logistics and organization have a positive impact on reducing the cold ischemic time (CIT) and optimizing the procurement technique, and the used solutions [102].

It is clear that the standardized technique for organ retrievals has a positive effect on IRI [16]. On the contrary, some heart transplant centers are not particularly pleased with a Eurotransplant allocation system. It seems that due to extension of CIT, networking for organ allocation also has its opponents. Haneya et al. published that the new allocation system decreased the proportion of local and regional organ harvesting. It generates a higher ischemia time without increasing the number of transplantations and without improving the clinical outcome after heart transplantation [103]. Donor age and CIT together might be significant factors for a suboptimal graft-survival. The Eurotransplant Senior Program executes the allocation by age-matching. Bahde et al. published that in the face of rapidly aging population, the main risk factors for graft survival were CIT >16 hours, body mass index (BMI) $\geq$25 kg/m^2, and kidney re-transplantation [1]. Networking systems, like United Network for Organ Sharing (UNOS) or Eurotransplant face the problems of having more expanded criteria donors (ECD) and a demand for shipping the organs to far distances. Rescue allocation means that the retrieved organ can not be allocated to the best-matching recipient for logistic reasons, and have to be transplanted as soon as possible. Mossdorf et al. found that rescue allocation procedure did no have any impact on graft survival [104].

Preservation Techniques

The development of optimal preservation solutions and perfusion systems is still a developing field with many successes and challenges and with numerous comparative experimental studies and in the clinical practice (5, 105–123]. In 1969, Collins et al. [124] developed a simple cold storage organ preservation method. Collins developed a preservation solution based on previous work concerning organ preservation at hypothermic conditions. It was rapidly adopted by many clinical transplant centers throughout the world. For the next two decades, very little was added to clinical preservation [124].

Mechanical perfusion, as discussed later, has a benefitial effect on the organ shipping. Since the last 15 years, a renewed interest for machine perfusion has emerged based on studies performed on preclinical models and seems to make consensus in case of ECD or deceased after cardiac death donations [125, 126]. Normothermic perfusion restores energy levels in the kidney allowing renal function to be restored *ex vivo*. This has several advantages: cold ischaemic injury can be avoided or minimized, the kidney can be maintained in a stable state allowing close observation and assessment of viability, and lastly, it provides the ideal opportunity to add therapies to directly manipulate and improve the condition of the kidney.[105] New perfusion methods and solutions are also available. Lindell et al. [127] reported the difference between a pulsatile QRS LifePort and Waters RM3 protocols. After 24 hours, the oxygen partial pressure and oxygen delivery in the LifePort perfusate were significantly lower than those in the RM3 but not low enough to change lactate production. TheLifePort ran significantly colder than RM3 (2° versus 5 °C). The arterial pressure waveform of the RM3 was qualitatively different from the waveform of the LifePort. Preservation injury after transplantation was not different between the two devices. When the LifePort was changed to nonpulsatile flow, kidneys displayed significantly greater preservation injury compared to RM3 [127]. The use of DCD (or NHB) donors in liver transplantation was an initiative to use machine perfusion in this field. The hypothermic

oxygenated perfusion (HOPE) system is one of the several competing rescue strategies against biliary injury. Using an animal model, Schlegel et al. [128] published that reperfusion injury was significantly decreased by HOPE treatment as tested by hepatocyte injury, Kupffer cell activation, and endothelial cell activation. Recipients receiving non-perfused DCD livers displayed less body weight gain, increased bilirubin, and severe intrahepatic biliary fibrosis. In contrast, HOPE-treated DCD livers were protected from biliary injury, as detected by cholestasis parameter and histology [128, 129].

A new development for the heart-recipients waiting for a transplant is the ventricular assist device (VAD). VAD provides long-term support especially for children with end-stage heart failure before a suitable heart becomes available. A thromboembolic event remains a major complication influencing their survival [130]. Also extending extracorporeal membrane oxygenation (ECMO) into postoperative period provides early, continuous and effective support for donor hearts with long CIT and maximizes the use of such marginal organs [131].

Hypothermia

After circulation stops, tissues metabolism keeps generating severe metabolic problems. The reduction of enzyme activity and metabolic rate is dependent on the reduction of temperature. Each 10 °C temperature fall results in halved metabolism and reduced oxygen need. Consequently, during cold storage (4 °C), the cellular metabolism does not stop, the inhibition of metabolic products and toxic substances is not completely blocked but suppressed to 10% by hypothermia. In the early 1960s it was already proved that simple ice cooling improved organ preservation [32, 132].

Preservation Solutions

These solutions include a number of specific compounds to reduce the side effects of hypothermic storage and to reverse injuries [133, 134].

- **Maintaining energy and control acidosis:** As the metabolism persists at 4 °C and ATP is depleted, anaerobic metabolism generates acidosis faster. If the acidosis is severe, it causes lysosomal damage and cell death. However, mild acidosis may have a protective effect. Therefore the preservation solution has an important role in ensuring adequate pH using a buffer [134].
- **Avoiding cell swelling (impermeants, colloids):** Under these conditions, the activity of ATP-dependent Na^+/K^+ pump is inadequate. To prevent cellular edema, impermeants and colloids are added to the preservation solutions. Impermeants such as saccharides and non-saccharides are effective at the level of cell membranes and the interstitial compartment. There are solutions with glucose (MW 180), mannitol (MW 182), sucrose (MW 342) or raffinose (MW 504) saccharides. Their effectiveness depends on the molecular weight: saccharides with larger size are more effective in limiting cell swelling than those with lower molecular weight agents. Non-saccharide, such as gluconate, citrate or lactobionate is included in some

solution to further improve counteracting the edema by electrochemical forces [133, 134]. The large molecules of hydroxyethyl starch (HES), dextran or polyethylene-glycol (PEG) colloids are used as intravascular compartment fluids and appear to be useful especially for organs with prolonged preservation time [135].

- **Electrolytes:** based upon the potassium and sodium concentrations, solutions can be intracellular (high potassium/low sodium) or extracellular type (low potassium) [133]. Ca^{2+} plays an important role in regulating enzyme activity. Under hypothermic condition, the cellular ATP concentration is depleted and intracellular concentration of Ca^{2+} is elevated, playing an important key role in IR injuries [136].

Additives to preservation solutions. Those are added to improve the results and to prolong preservation times.

- **Agents against reactive oxygen species:** Anti-oxidants such as allopurinol, glutathione, mannitol or tryptophan are needed to prevent formation of ROS and to inhibit xanthine oxidase, thus protecting the organs against ROS-mediated damage [133, 134].
  - **Superoxide dismutase (SOD).** SOD, an enzymatic free radical scavenger, specific for superoxide, has been used successfully to protect organs from structural damage during reoxygenation of ischemic tissue. It reduced the acute and chronic rejections without impact on the incidence or duration of delayed graft function [137].
  - **Alfa-tocopherol (Vitamin E)** is an anti-oxidant and anti-apoptotic agent. When added to the Vasosol, it could improve liver function (reduced aspartate aminotransferase and decreased cytokines) during reperfusion in hypothermic machine perfusion [138]. Additionally, its water-soluble analogue in UW solution improved endothelial viability and decreased reperfusion injuries of lungs [139]. Campbell et al. [140] indicated that supplementation of Belzer's Machine Perfusion Solution or Unisol with α-tocopherol or a caspase inhibitor (Q-VD-OPH,) could promote survival of βt3 pancreatic murine cell line [140].
  - **Lazaroids.** They can reduce iron-mediated lipid peroxidation. Although the beneficial effect has been proved in several experimental studies showing that lazaroids prevent early but not late stages of oxidant-induced cell injury, they have not been effective in clinical trials [141, 142].
- **Enzymes:** When intracellular proteases are activated they decrease the level of intracellular proteins. To reduce the effect of activated matrix metalloproteinase (MMP) leading to a detachment of endothelial cells from underlying matrix, hydroxyethyl starch (HES) is added in some solutions. Apoptosis-related caspases are also activated during cold preservation [143]. It has been proven that endoplasmic reticulum stress is involved in cold IRI [144].
- **Anti-apoptotic agents.** Prolonged ischemic time increases the level of apoptosis [145]. Adding specific apopototic protease inhibitors for blocking the activation of apoptotic proteins into the preservation media improved the survival of various cells and tissues. Inhibition of caspases could be a drug target in ischemic reperfusion injuries [146]. For example, p35 or Q-VD-OPH is able to inhibit the intrinsic and/or

extrinsic pathways [140]. It has been shown that in kidney, p53 inhibitor, pifithrin-alpha can block the transcriptional activation of Bax and p21 pro-apoptotic proteins [147].

- **Pharmacological manipulation of poly (ADP-ribose) polymerase-1 (PARP-1) activity** may have salutary effects in cold-stored organs in transplantation [148].
- **Nifedipine and diltiazem** (calcium channel blockers) were shown to be protective in renal transplantation [149, 150].
- The protective effects of truncated Na^+/K^+-ATPase β (tNKA-β) against renal IR injury have been evaluated by Gong et al. They published that these protective effects may occur via stimulation of PKC-ε pathways [26].

Preservation solutions target the above mentioned harmful pathways, composed to counteract these processes thus increasing chance to longer storage time and improved preservation quality. Below the clinically used preservation solutions are briefly summarized.

Static Hypothermic Storage - Intracellular Solutions

These solutions are hypertonic, with high potassium concentration and reduced sodium in order to restrict the passive water and ion fluxes, so mimicking the intracellular space.

- **EuroCollins Solution:** As the first preservation solution, it was developed by G.M. Collins [124]. The solution has a high potassium ion content (115 mM), phosphates for pH buffering (15 mM K_2HPO_4, 43 mM KH_2PO_4,) and glucose (195 mM) served as the osmotic agent besides chloride (15 mM), sodium (10 mM) and $NAHCO_3$ (10 mM). In 1976, Eurotransplant Foundation eliminated magnesium, and from that time, the name of solution is EuroCollins (EC).
- **Citrate solution (Marshall/Ross, hypertonic citrate solution, hyperosmolar citrate - HOC):** It was developed in Australia in the late 1970s [151]. The electrolytic composition was characterized by high potassium (79 mM), high sodium (84mM) and magnesium-sulphate (40 mM) content. To maintain intracellular pH, citrate was added (80 mM) replacing phosphate and other buffer agents. Citrate can be replaced with HEPES. Instead of glucose, mannitol was added (185 mM) providing better osmotic conditions, scavenging ROS and lowering the viscosity [151].
- **Phosphate-buffered sucrose (Sucrose solution):** developed in the early 1980s. The impermeant saccharide sucrose (140 mM) sustains the hypothermic cell swelling. Other components are NaH_2PO_4 (13 mM), Na_2HPO_4 (56 mM) and sodium (125 mM) [116].
- **University of Wisconsin solution:** the gold standard for preservation was introduced in the late 1980s by F.O. Belzer's group [152]. Besides an intracellular-typer electrolyte content (120 mM potassium, 25 mM sodium, 5 mM magnesium-sulphate, 20 mM chloride) and 25 mM KH_2PO_4 buffer, 100 mM lactibionate and 30 mM raffinose, as impermeant were added. It contains 50 g/l HES for improving oncotic

support. Furthermore, as antioxidant defense, glutathione (3 mM) and allopurinol (1 mM) were also added, besides the ATP precursor adenosine (5 mM) [152, 153].

Static Hypothermic Storage – Extracellular Solutions

These solutions are isotonic, mimicking the normal extracellular environment.

- **Histidine-trpytophan-ketoglutarate solution (HTK, Custodiol, Bretschneider's):** It was introduced by Bretschneider originally as a cardioplegic solution [154]. Later this solution has been used for kidney, liver and pancreas transplantation. The electrolyte composition is quite different from the previously discussed ones: 9 mM potassium, 15 mM sodium, 0.0015 mM calcium, 32 mM chloride and 4 mM magnesium-sulphate. As impermeant, 198 mM histidine and 38 mM raffinose were added. Histidine also acts as a buffer. Additives: tryptophan (2 mM) serves as membrane stabilizer, ketoglutarate (1 mM) acts as a substrate for anaerobic metabolism. It contains 38 mM mannitol. This solution has a low viscosity leading to a rapid flow rate and cooling [154].
  Modified HTK solution (Custodiol-N) contains glycine to further increase the preserved organs' quality, alanine to inhibit hypoxic cell injury, as well as the iron chelator deferoxamine and membrane-permeable hydroxamic acid derivative LK 614 to inhibit free radical-mediated injury. Further components are N-acetyl-L-histidine (against the potentially toxic effect of histidine) and L-Arginine (N-donor, facilitatoing NO formation after reperfusion) [155−157].
- **Celsior Solution:** It was developed by Menasche et al. in 1994 [158]. The electrolyte composition is: 15 mM potassium, 100 mM sodium, 0.25 mM calcium, 42 mM chloride and 13 mM magnesium. As impermeant, 30 mM histidine, 80 mM lactibionate and 60 mM mannitol were added. Additives: gluthatione (3 mM) and glutamic acid (20 mM) for the antioxidant defense [158].
- **IGL-1 Solution (Institute Georges Lopez):** A kind of combination of the extracellular composition of the Celsior solution and the colloidal content of the UW solution, in which polyethylene glycol (PEG-35, 1 g/l) is used instead of HES. The electrolyte composition is: 25 mM potassium, 120 mM sodium, 0.5 mM calcium, 5 mM magnesium-sulphate. As buffer 25 mM KH_2PO_4, as impermeant, 100 mM lactibionate and 50 mM raffinose were added. Additives: adenosine (5 mM) [159, 160, 161].
- **Unisol:** Taylor et al. [162] formulated a new hypothermic preservation solution which has a phosphate-free cryoprotectant vehicle version (Unisol-CV). The role of the cryoprotective agent (DMSO) was studied using Unisol-CV compared to Euro-Collins solution. Without freezing the cells, there was no significant difference in the viability of cells but was higher after treatment with Unisol-CV [162]. Unisol has been evaluated for the preservation of a variety of cell, tissues, organs, and large mammals [163]. Lastly, survival and proliferation of βt3 pancreatic cells were examined comparing Unisol to Belzer Solution. In the absence of any supplements,

Unisol maintained cells better than Belzer Solution. Although the number of survived cells was similar after 24 h hypothermic exposure but the cell proliferation was significantly better and the delayed cell death was lower using Unisol [140].

- **Vasosol:** Vasosol machine perfusion solution (VSL-MPS) was developed by Guarrera et al. in 2004 based on Belzer machine perfusion solution with additional compounds which was designed to enhance vasodilatory and antioxidant capacity of solution (N-acetylcystein, L-arginine, nitroglycerine, prostaglandin-E1, α-ketoglutarate) [164].
- **Polysol:** developed at the University of Amsterdam in 2005 as a classic preservation solution enriched with amino acids, vitamins, and antioxidants [165]. It has been tested both experimentally and clinically as CS solution and as HMP solution [166–168].
- **Kyoto Solution:** An extracellular type Kyoto (ET-Kyoto) solution was initially developed for lung preservation but later its effectiveness was proved in various tissues and organs [169]. It contains trehalose as non-reducing trisaccharide which was proved to protect cell membrane under hypotermic and ischemic condition making it superior to UW, to the intracellular type Kyoto (IT-Kyoto) and to EC solutions. Membrane permeable cAMP analogue and nitroglycerin was added to further improve the effects of ET-Kyoto solution protecting especially the vascular endothelium, called new-ET-Kyoto solution [170].

Static and Dynamic Preservation

Organs for transplantation can be preserved by static and dynamic ways. During static storage, the inevitable, slowed down cellular and molecular changes are attempted to make positive effects, while during dynamic storage, the reactivatibility of the still operational remaining metabolism is utilized [171, 172].

Most transplant centers apply the simplest and cheaper static cold storage method (SCS) [173]. Hypothermia decrease tissue metabolism which, in turn, decreases the quantity of toxic substances produced. The positive effects of preservation solutions specially developed to preserve specific tissues and organs can be further enhanced by the addition of substances that block the formation of harmful substances, thus promoting the viability of the transplant [122, 174–177].

Dynamic preservation methods include the hypothermic machine perfusion (HMP) and normothermic machine perfusion (NMP). These methods use machinery to pump the preservation solution through the organ providing necessary nutrients to all tissue layers and also pushing any toxic metabolites away. The first HMP system had been developed in the 1960s allowing longer distance transportation of organs [178]. **A**nother big advantage of this system is that it allows for organ viability measurements on-the-fly and also provides feedback about the effect of preservations added. Main HMP options are: with or without oxygen, pulsatile or non-pulsatile, pressure and the perfusion solution selection [129, 163, 171, 172, 179]. As increased oxygen supply during storage reduce the likelihood of ischemic damage, but promotes the formation of free radicals [180, 181], oxygen enrichment is one of the most studied methods (see next section).

We have insight that hypothermic preservation caused several injuries [182]. Increasing the temperature of machine perfusion systems opened a new way in the evolution of preservation [183]. Normothermic (37 °C) (NMP) and subnormothermic (25−32 °C) machine perfusion systems also have proven positive effects in a number of experimental and clinical applications, especially in case of marginal and older transplants [171]. NMP applied for 18 hours after a 2 hour ischemia led to normalized kidney functions in canine kidney, while applying cold storage for the same amount of time rendered the kidneys non-functional [184]. NMP use resulted in stable metabolic liver function which was less pronounced than using cold storage method [183, 185]. Non-heart beating donor human kidneys' primary graft nonfunction and delayed graft function rates showed significant reduction using normothermic recirculation protocol compared to those where traditional preservation techniques were used [186].

Applying SCS is technically simpler and also a cheaper solution in short term. In the long term, HMP seems more cost-effective as implant organs' DGF rate becomes lower, patients are less likely to need dialysis again, hospital stay of patients was shorter, and graft survival rate has been superior compared to the cheaper and easier SCS methods [173, 187]. Since both NMP and HMP results have proven superior in comparison with the conventional static storage, it should become part of everyday practice by developing as an easy-to-carry and easy-to-use unit, helping patients' quality of life and reducing transplant costs and increasing the number of transplantable organs.

Perfusion of Gas

Medical gases are a decent possibility to avoid transplants' ischemic-reperfusionic damage [188].

Oxygen (O_2). Different methods are available to provide adequate oxygen to the organ being preserved. Preoxygenating the perfusion solution in pressure chamber increases the solution's oxygen level ensuring that more oxygen flows to the organ. Solutions have a different ability of maintaining this increased oxygen level in the solution: Perfadex keeps the level the longest, followed by Celsior and UW [189]. Oxygen enriched hypothermic machine perfusion (HOPE) can provide beneficial effects even after hours of SCS [128]. Oxygen enrichment also works with NMP although not as effectively as with HMP [129].

Persufflation (PFS, Isselhard's term) is also known as vascular system oxygenated persufflation (VSOP) made humidified pure oxygen venous insufflation possible (13−18 Hgmm) that can be performed anterograde or retrograde [190].

All of above mentioned techniques of tissue aerobiosis can reduce ischemic damage, improve mitochondrial integrity and the functional recovery of damaged organs compared to non-oxygenated or SCS, proving that oxygenated perfusion provides a shelter from injuries [191–193]. Dual-layer perflourocarbon technique a.k.a. the two-layer method (TLM) is another possible way for providing organs the adequate oxygen [194]. Perflourocarbon (PFC) liquid is capable of storing high levels of oxygen. During TLM preservation, PFC and UW preservation solution are applied together. As they have different density, they form layers where the organ is located between the layers taking oxygen from the PFC side and adenosine for ATP generation from the UW side [195, 196]. Increased oxygen supply during storage

reduces the likelihood of ischemic damage, improves mitochondrial integrity and the functional recovery of organs [144]. At the same time, it promotes the formation of free radicals [180, 181], thus other types of therapeutic bioactive gases such as carbon monoxide (CO), nitric oxide (NO) and hydrogen sulphide (H_2S) are under examination.

Carbon monoxide (CO). Even though high levels of CO are toxic, low levels introduced by exogen means provide protection against IRI having anti-inflammatory, vasodilating, and antiapoptotic functions [197, 198]. After inhalating CO-enriched air with experimental animals' liver, kidney, and small intestine, transplants showed improved survival and functions, reduced inflammatory factors compared to animals exposed to open air [197, 199, 200].

Nitric oxide (NO). NO's role is already described above. Topically administered sodium nitrite protects the rat kidney against IR injury and dysfunction [201]. NO being a vasodilator also helps the perfusion solution flow through the organ [202].

Hydrogen sulphide (H_2S). Supplemental H_2S can mitigate renal graft IRI incurred during transplantation and prolonged cold storage, improving early graft function and recipient survival in a clinically applicable model of transplantations [203–205].

Table 19.1. Some agents targeting ischemia-reperfusion [2, 6, 9, 39, 40–42, 44, 45, 65, 211–214]

Main focus	Agent, intervention	Process, target
Metabolic strategy	apyrase nucleotidase regadenoson, ATL146e, Bay 60-6583 PHD inhibitors	ATP breakdown, AMP conversion to adenosine adenosine receptor agonists, inhibition of oxygen sensing PHD, HIF stabilization
Antioxidant strategy	Allopurinol SOD CAT GSH-Px NAC Vitamin E Vitamin C lazaroids biofalvionoids bendavia	reducing free-radical production, scavenging free radicals, protecting cell integrity, inhibiting lipid peroxidation
Agents reducing inflammatory responses	anti-ICAM-1 antobodies soluble PSGL-1, CTLA4Ig, IL-1 receptor antagonists antibodies against TNF-α PAF, LTB4 antagonists IL-8 inhibitors thymoglobulin MMP inhibizot Bay 12-9566 diannexin YSPSL TAK-242 anticomplement agensts (sCR1, h5G1.1-scFv), endothelin receptor antagonists	down regulation of adhesion molecules, reducing the cell-to-cell interactions by down-regulation or blocking adhesion molecules, reducing cytokin effects inhibition of TLR4 sustaining the complement pathway modulating vascular response
Agents modulating immune response	T-cell based approaches	modulating innate immune response, expansion of T_{reg} cells, suppression of γδ T cells
Antiapoptotic possibilities	cyclosporine miR-499 or miR-24 activators	inhibition of apoptosis

Cryopreservation

The limitation of the chapter allows just a brief note, however, it is important to mention here in the context of preservation. During cryopreservation, the tissues are frozen at ultra-low temperature (below -100 °C) avoiding cellular damage by the ice formation (damage from the extra- end intracellular ice crystals, dehydration and increase in concentration of solutes). Methods includes controlled-rate and slow freezing (slow programmable freezing), when the typical cooling rate is about 1 °C/minute after treatment with cryoprotectants (glycerol, dimethyl sulphoxide, 1, 2-propanediol). Other method is the vitrification, in which after incubation with cryoprotectants an ultra-rapid freezing technique is applied. The cryopreservation has been succesfully applied for oocytes, sperm, stem cells, skin, blood products, embryo and vascular grafts. However, for whole organs, this method is still not practical [206–210].

Agents against IRI

Along the transplantation process (from donor to recipient), there are numerous strategies, tools and agents diminishing the ischemic-reperfusioninc damage, including metabolic and antioxidant strategies, agent targeting various pathways of the inflammatory processes and immunological approaches (Table 19.1). Concerning the preservation techniques, couple of them has been already discussed.

Metabolic Strategy

The HIF is a key factor in metabolic switch from fatty acid oxidation to glycolysis, by modulating transcription of glycolytic enzymes [2, 39, 40, 215]. Prolyl hydroxylase domain (PHD) enzymes, that are oxygen-sensing (PHD1−3), regulate the stability of HIF. Pharmacological PHD inhibitors thus may increase ischemic tolerance resulting in HIF stabilization [40].

Nucleotide and nucleoside signaling is another potential therapeutical target. During reperfusion, ATP is released to the extracellular compartment (from necrotic, apoptotic and inflammatory cells). ATP binding to P2 receptors (P2Y6, P2X7) aggravates inflammation and tissue injury. Blocking ATP release or ATP receptor signaling may attenuate sterile inflammation: apyrase (ATP breakdown), nucleotidase, specific adenosine A2A receptor agonists on Adora2a (regamedason, ATL146e) and Adora2B receptors (Bay 60-6583). Most of these studies are in preclinical phase. Further targets for metabolic adaptation were presented related to the mitochondrial aldehyde dehydrogenase 2 (ALDH-2) and AMP-activated protein kinase (AMPK) [40].

Antioxidant Strategy

Classical antioxidant agents, including allopurinol (XO inhibitor), glutathion (GSH), enzymes such as superoxide dismutases (SOD), catalase (CAT), gluthation peroxidase (GSH-Px), and small molecular scavengers, vitamin E, vitamin C, *N*-acetylcysteine (NAC) and α-lipoid acid have non-specific but important role [6, 44, 65]. Antioxidant tools include lazaroids, which are lipid peroxidation inhibitors, bioflavinoids (quercetin, curcumin) preserving tissue integrity, and mycophenolate mofetil [6]. An example for the mitochondria-targeting cytoprotective peptide is the bendavia (Szeto-Schiller peptide SS-O2 and SS-31 analogue), that has been experimentally shown to reduce ROS level in mitochondria [212].

Further details on the antioxidant agents are discussed above in the sub-heading dealing with preservation solutions and techniques.

Agents Targeting Inflammatory Processes

Agents modulating cell trafficking, reducing adhesion and other cell-to-cell interactions and complement inhibitor agents have been discussed in other chapters in more details. Here, their ischemia-reperfusion-related aspects are briefly summarized.

Intercellular adhesion molecule-1 (ICAM-1), facilitates adhesion of leukocytes to endothelial cells and is up-regulated during IR injuries. Administering anti-ICAM-1 antibody to recipients revealed that ICAM-1 blockade can reduce the injuries at the reperfusion stage in experimental animals and in clinical trials [216]. Soluble P-selectin glycoprotein ligand-1 (PSGL-1), costimulatory molecule CTLA4 Ig, IL-1 receptor antagonists, antibodies against TNF-α, as well as PAF and LTB-4 antagonists, and IL-8 inhibitors are part of the antileukocyte strategy [39, 217]. Anticomplement therapy includes C3 convertase inhibitor, soluble complement receptor 1, recombinant antibody h5G1.1-scFv for C5 [6, 39, 40].

Thymoglobulin, a rabbit delivered polyclonal antibody (r-ATG) has a blocking effect on cell-to-cell interaction by down modulation of adhesion molecules (E-selectin, ICAM-1, PECAM-1) and specific receptors (lymphocyte function-associated antigen – LFA-1, very late Antigen-4, VLA-4, *C*-C chemokine receptor type 5 and 7, CCR5, CCR7), so reducing the leukocyte rolling and adherence. It can diminish the inflammatory mediator release and induce T-cell depletion (by complement-related lysis and apoptosis) and so reduce the lymphocyte number [218, 219]. However, it is not specific for T-cells as it contains antibodies against B-cells, NK cells, monocytes, neutrophils, platelets and even against erythyrocytes. However, in an experimenal and clinical setting, thymoglobulin has been successfully used, reducing acute rejection and improving early graft function [211, 218, 219].

Early administration (within 10 days) of matrix metalloproteinase inhibitor, Bay 12-9566 (MMPs has role in leukocyte transmigration too) could reduce tissue damage in rat kidney transplantation [213, 214].

Novel therapies include diannexin, which is a recombinant annexin A5 (ANXA5) homodimer, and binds to phosphatidylserin. Phosphatidylserin is translocated to the endothelial surface in ischemia-reperfusion, binds to leukocytes and platelets contributing to the prothrombotic and inflammatory process [44, 220].

The agent called YSPSL is a recombinant fusion protein of PSGL-1. It binds to P-selectin on the endithelial cells and thus prevents neutrophil rolling and adhesion [44, 221].

The role of heme oxygenase (HO-1) is to convert heme into biliverdin, CO and free ferrous ion. The CO has anti-inflammatory and cytoprotective effect involving HIF stabilization and via activation of HIF-dependent responses [40, 41, 188, 222]. Furthermore, CO has anti-platelet, anti-apoptotic, anti-inflammatory (decreasing mRNA expression of adhesions molecules) and vasodilatatory effects (increasing the cGMP production) as well [2, 40, 197, 223, 224]. So induction of HO-1, e.g., by gene therapy or protoporphyrins or curcumin, can be cytoprotective [2, 225].

Immunological Approaches

Activation of the innate immune response is a critical mechanism in IRI [2]. T cell-based approaches, such as suppression of γδ T cells and expansion of T_{reg} cells are still in experimental, preclinical phase. Immunosuppressive therapy, that has been discussed in other chapters, thus has indirect links with ischemia-reperfusion-related common responses [2, 40, 226].

MicroRNAs

MicroRNAs (miRNAs) are short, non-coding RNA molecules [21−23 nucleotides length) that take part in regulatory mechanisms of gene expression [227–229]. Dozens out of known more than 1500 miRNAs have been described to be related to IRI: miR-1, miR-21, miR-24, miR-29a, miR-29c, miR-92a, miR-133a, miR-144/451, miR-320, miR-494, miR-499, among others [229–232]. These can be also therapeutic targets, such as oligonucleotide inhibitors of miR-92a may promote angiogenesis [40, 229, 233], or administration or activation of miR-499 results in inhibition of apoptosis [231], and miR-24 was reported to be protective in murine cardiac IRI [230]. However, miRNAs are currently under intensive investigation with promising therapeutical applications.

Ischemic Pre- and Postconditioning, and Trophic Factors in Organ Preservation

Concerning transplantation, the ischemic preconditioning and postconditioning have been widely investigated in experimental models on several pathways (induction of NOS, HO-1 or inhibition of apoptotic pathways), opening the door to interventions decreasing injuries and increasing protections [2, 234–236]. The clinical studies having more complex conditions and raising ethical and legal issues are needed to be strengthened.

Catecholamines are important graft-modifying agents. Pretreatment of brain-dead donors with low-dose dopamine reduces the need for dialysis after kidney transplantation [237], and improves the clinical course of recipient with heart allograft without negative

effects to the donors [238]. As the induction of protective enzymes like HO-1 could be stimulated by dopamine, this agent is capable of making the organ more resistant to the insults of IR and inflammation. Retrospective studies proved that treatment of brain-dead organ donors with catecholamines is associated with less rejection and a better long-term graft survival of kidneys transplanted from these donors. Catecholamines can also modulate cytokine production and prevent cold-induced damage [239].

Desmopressin, artificial form of the normal human hormone arginine vasopressin (the antidiuretic hormone, or ADH), was associated with improved renal graft survival [240].

Dexamethasone: Pre-treatment with glucocorticoid receptor agonist dexamethasone decreased damaging and pathological changes after renal IRI and promoted the activation of glucocorticoid receptors resulting in upregulating the ratio of eNOS/iNOS [241].

Insulin induces protection of tissues via an antiapoptotic effect. Preconditioning with insulin showed better cardioprotective effect than administration only at reperfusion [242].

Donor pretreatment has great advantages for the recipient as an improved long-term graft survival could thus be achieved cost-efficiently and without great effort or side effects.

Gene therapy

Gene transfer can be the key for preserving transplantable organ viability and preventing ischemic reperfusion injuries. Concerning the pathophysiology of the IRI, numerous points can be identified where gene therapy may be useful, including induction of antiapoptotic or protective genes, or blockade of pro-apoptotic and pro-inflammatory signal transduction, besides others [2, 243]. Anti-inflammatory cytokines (IL-3, IL-10) are used for donor pre-treatment with single substances or by gene transfer. Results are better state of liver after cold exposure by the modulation of immunocompetent cells and induction of stress proteins like HO-1, anti-apoptotic Bcl-2/Bcl-x, among others [244–246].

For delivering therapeutic genes into cells, tissues and organs two kind of genes are used: recombinant and non-viral. Non-viral genes transfer fall into two categories by their physical or chemical nature (e.g., ribozymes, lipid- or liposomally-mediated gene transfer, antisense oligodeoxynucleotides, synthetic short interfering RNA-siRNA, vector-expressing short hairpin RNA - shRNA or electroporation) [247, 248]. Non-viral vectors do not activate the immunological system of recipients but have low effectiveness *in vivo* [249]. Although viral gene transfers have higher transfection, their effectiveness is less than non-viral transfers. Further, different virus families used as gene therapy vectors activate immune response of recipient [250].

The gene therapy could be used in three phases of transplantation: during donor preconditioning, after donor retrieval called *ex vivo* gene transfer and treatment of the organ recipient. It was proved that under normothermic preservation and reperfusion, the effectiveness of this therapy could be improved compared to hypothermic perfusion [251]. As we are getting to know the pathophysiology of IRI, there are several points where gene therapy could be applied. Preclinical studies showed that transferring gene of anti-oxidative, anti-apoptotic, HO-1-inducing molecules or gene transfer of cytokines, cytoprotective (heat shock) proteins and blockade of T cell co-stimulation are able to reduce the IRI [2, 3, 248, 252].

Cell Therapy

Organ storage requires numerous biologically active molecules such as from stem cells but independently from the cell source, a wide spectrum of cytokines and growth factors (GFs) is worth considering. Supplementing the preservation solution with mesenchymal cells [253–255] or various growth factors [256, 257] enhance the recovery of organs by increasing the post transplantation survival rate, improving mitochondrial respiratory activity and depressing free radical processes [3].

Research about this type of therapy can be categorized into two main categories: supplementation of the storage solutions with the test compound and donor pre-treatment. Problems with the first option are the slowing-down of substances penetration or uptake in the tissues by the low temperatures and the difficulties controlling the process. The latter option is limited by ethical reasons, being a treatment with no real benefits to the donor.

Bioengineering

The interventions mentioned above enhance the number and viability of transplantable organs. Nevertheless, the number of donors suitable for implantation is still not sufficient and immunosuppressive therapy is an additional factor that might lead to several complications. These two most critical points of organ and tissue transplantation can be overcome by the development of regenerative medicine [258]. During organ regeneration and bioengineering, viable tissues and organs are produced artificially.

Cells of heterologous, allogeneic or autogenic origin are cultivated *in vitro* to reach necessary volume. In case of extensive organ failure or due to exact tissue properties, it is difficult to extract and then produce functional and long-time viable expansions. In these special cases, stem cells have an outstanding importance as such cells have the ability to proliferate in an undifferentiated but pluripotent state and the ability to differentiate into specific cell types of all three embryonic germ layers *in vitro*.

Although cell transplantation is not so invasive and it is a low-risk operation, the transplant efficiency is low [259]. Covering the cell-sheet with omentum flap or cultivating cells on amniotic membrane showed better results as these meshes induce angiogenesis, promote revascularization and healing of injured organs or tissues having positive effect to the attachment and cell differentiation [260, 261].

For best results, cultured cells need a three-dimensional scaffold to form tissue or organs. Possible solutions are naturally derived materials (collagen, alginate), synthetic polymers (polyglycolic acid, polylactic acid, polylactic-coglycolic acid) and acellular tissue scaffolds [262, 263]. The natural organ structure and vasculature can be preserved by a sublimation of their antigenic cellular components via mechanical and chemical manipulation leaving only acellular extracellular components [264]. These acellular scaffolds provide microstructures and extracellular cues for cell engraftment, differentiation, transplantation and repopulation. Under biomimetic conditions which simulate the human body environment, the recellularized scaffold can form a functional tissue.

Experimental and clinical applications have been made when the urethra, urinary bladder, vaginal tissue, heart valve or engineered vascular graft using autologous cells have been

generated [265]. For now, the more complex bioengineered small animal and human organs showed viability only for a short time when transplanted *in vivo* [266–269]. For example, in the case of artificial liver tissue, the critical factor is the construction of sinusoids which have an important role in the transport of oxygen to all cells and nutrients and in the elimination of toxic secretions of the cells. By cocultivation of three different hepatic-origin cells on micro-liver tissue scaffolds, No et al. generated xenogeneic bioartificial liver with sinusoids which was able to recover the liver function of 90% hepatectomized mice [270].

In case of semi-transplantation, animal organs are prepared as an acellular scaffold, which then could be repopulated with human stem cells to generate immunosuppression-free transplantable human organs [271].

The encapsulation methods could generate a new source of organs without the need of immunosuppression and without the need of viable human donor organs. During this method, xenograft scaffold is repopulated with autologous, heterogeneic or xenogeneic cells and then encapsulated by hydrogel developing an immune-protected xenogeneic system [270].

All these results are encouraging in terms of ultimate human clinical use.

Conclusion

The complex pathophysiology of the IRI has not been clarified yet in all the details. During ischemia numerous metabolic and ultrastructural changes occur, depending on the duration of the ischemia. The core process is the decrease in cellular oxidative phosphorylation. The drop in cellular ATP leads to dysfunction of ATP-dependent membrane ion-pumps, resulting in calcium, sodium and water influx to the cells. The swelling results in disruption of the cell membrane and intracellular membranes. Hypoxia leads to microcirculatory, micro-rheological, inflammatory, innate and adaptive immune responses, and other alterations.

IRI is a main therapeutical target. The better organization, logistics for organ retrieval and allocation is aimed to reduce the cold ischemic time considering other donor and recipient factors as well. Since the shipping of organs will cover more distances, the development of new preservation solutions, and the launching of machine perfusion are new tools in the armature. Pulsatile, hypothermic perfusion seems to be the best option so far. Different types of preservation solutions cover the abovementioned pathways of IRI: they protect against cell swelling, oxidative stress, acidosis, apoptosis, block enzymatic activity, while maintaining cell energy level. Main components of these solutions are therefore, saccharides (glucose, raffinose, sucrose), phosphate or histidine buffer, hydroxyethyle-starch, potassium, and citrate, and sometimes gluthation, or allopurinol. Some additives, like superoxide dismutase and lazaroids were used as well. As anti-apoptotic agents, p53-inhibitor, pifithrin-alpha, as well as nifedipine were also administered. Since the beginning of organ transplantation, the machine perfusion was a challenging field, in contrast to static perfusion.

The hypothermic perfusion system opens the possibility of applying pharmacological maneuvres on hypothermically-perfused organs. Ideally, good preservation should facilitate the use of marginal and older organs and provide real-time viability assessment before transplantation. Normothermic (37 °C) or sub-normothermic (25−32 °C) perfusions are becoming popular as preservation alternatives that may indeed achieve these goals. An

attractive technique, called persufflation, is aimed to resuscitate damaged organs by the perfusion of gas through their vasculature. Gaseous oxygen during cold storage was highly effective in improving liver graft. Another, more static way to deliver O_2 to grafts is the dual-layer perfluorocarbon technique.

Therapeutical agents targeting IRI includes metabolic strategy (ATP breakdown, AMP conversion to adenosine, adenosine receptor agonists, inhibition of oxygen sensing PHD, HIF stabilization), antioxidant stratregy (reducing free-radical production, scavenging free radicals, protecting cell integrity, inhibiting lipid peroxidation), agents reducing inflammatory responses (down regulation of adhesion molecules, reducing the cell-to-cell interactions by down-regulation or blocking adhesion molecules, reducing cytokine effects, inhibition of TLR4, sustaining the complement pathway, modulating vascular response), factors modulating immune response (modulating innate immune response, expansion of T_{reg} cells, suppression of γδ T cells) as well as antiapoptotic possibilities.

MicroRNAs can be also theraputic targets, such as oligonucleotide inhibitors of miR-92a may promote angiogenesis. Gene transfer is a promising therapeutic option for solid organ transplantation and in the prevention of IRI in transplant recipients. Cells of different origin, particularly stem cells, may be considered as immediate sources of numerous biologically active molecules for application in organ storage. Organ bioengineering and regeneration have shown the potential to revolutionize the field of organ transplantation by successfully addressing two of the most critical issues in the field: the need for new, inexhaustible source of organs and the achievement of an immunosuppression-free state. In case of more complex organs, the investigations are still in very early stages.

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Part III: Cell Therapy in Induction of Transplant Tolerance

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Chapter 20

Cellular Therapies in Induction of Transplantation Tolerance

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Abstract

Myeloid-derived suppressor cells (MDSCs), a heterogenous population of innate immune cells, are gaining attention for their ability to regulate innate and adaptive immune responses. The exacerbation of cancer, infection, and autoimmunity has been associated with MDSC expansion. Increased frequencies of MDSCs have also been observed in tolerized transplant recipients. Over the past decade, methods have been devised to enhance the generation and expansion of MDSCs for therapeutic applications in transplantation. Propagated MDSCs significantly varied in the mechanisms they employed to inhibit T cells and in their abilities to modulate the immune response and promote graft survival, most likely due to differences in methodologies used to generate the MDSCs. Here we examine the factors contributing to MDSC generation and the mechanisms utilized by MDSCs to effectively regulate T cells. Furthermore, we review the reports correlating MDSCs with improved allograft survival and the achievement of transplantation tolerance.

Keywords: myeloid-derived suppressor cells, immunoregulatory, transplantation, tolerance

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Abbreviations

1-MT: *1-methyl-tryptophan*
Abs: Antibodies
Ag: Antigen
Arg-1: Arginase-1
BM: Bone marrow
DCs: Dendritic cells
DCFDA: Dichlorofluorescein acetate
ES: Embryonic stem
Flt3L: Fms-like tyrosine kinase 3 ligand
GCN2: General control non-depressible 2
G-CSF: Granulocyte-colony stimulating factor
GM-CSF: Granulocyte-macrophage colony-stimulating factor
GVHD: Graft-versus-host disease
HM: Hematopoietic stem
HPSCs: Hematopoietic stem and progenitor cells
HSCs: Hepatic stellate cells
IDO: Indoleamine 2,3-dioxygenase
ILT2: Immunoglobulin-like transcript 2
iNOS: Inducible nitric oxide synthase
JAK: Janus kinase
Lin: Lineage
MDSCs: Myeloid-derived suppressor cells
NO: Nitric oxide
PBMCs: Peripheral blood mononuclear cells
PD-L1: Programmed death ligand 1
$PGE_{2:}$ Prostaglandin E_2
Rh: Recombinant human
ROS: Reactive oxygen species
SCF: Stem-cell factor
STAT3: Signal transducer and activator of transcription 3
TCR: T cell receptor
Tregs: Regulatory T cells
TSA: Trichostatin A
VEGF: Vascular endothelial growth factor
VLP: Virus-like particles

Introduction

Origin of MDSCs and Phenotype

In the steady state, myeloid precursors originating from the bone marrow (BM) migrate to secondary lymphoid tissues and differentiate into granulocytes, macrophages, and dendritic

cells (DCs). During pathogenesis, these myeloid cells can adopt suppressive features. In healthy individuals, myeloid suppressor cells are present in reduced numbers, however, an expansion of these cells is observed in cancer, infections, autoimmune diseases, and in transplantation [1]. It was proposed that the name of this population of suppressive cells be altered to myeloid-derived suppressor cells (MDSCs) to avoid confusion with the abbreviation of mesenchymal stem cells [2]. The remarkable ability of MDSCs to inhibit innate and adaptive immune responses has evoked interest for the potential use of these cells for therapeutic applications in transplantation. MDSCs display a wide variety of mechanisms through which they can regulate T cells, including the expression of immunoregulatory enzymes arginase-1 (Arg-1) [3, 4], inducible nitric oxide synthase (iNOS) [5], and indoleamine 2,3-dioxygenase (IDO) [6], production of reactive oxygen species (ROS) [7, 8] and regulatory cytokines (IL-10 and TGF-β) [9], and promotion of regulatory T cells (Tregs) [10, 11].

MDSCs consist of a heterogenous population of cells of myeloid origin and lack expression of molecules of fully differentiated, mature myeloid cells. MDSCs are mainly comprised of cells bearing morphology of granulocytes and monocytes. In mice, MDSCs are identified based on expression of CD11b, Gr1 (Ly6C and Ly6G), and IL-4Rα and lack of lineage (Lin) markers of DCs and other mature myeloid cells[1]. In humans, a molecule analogous to Gr1 does not exist. However, human MDSCs exhibit their own unique molecular profile, including expression of CD33, CD34, CD14 (monocytic), and CD15 (granulocytic) and downregulate expression of HLA-DR [12, 13].

MDSC Activation and Expansion

Based on findings from cancer studies, it is evident that the activation and expansion of MDSCs can be influenced by factors produced by the tumor cells themselves as well as factors produced by T cells and tumor stromal cells [14]. The examination of factors produced in the local tumor microenvironment has demonstrated roles for prostaglandins, granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), and stem-cell factor (SCF) [14, 15]. Many of these factors share common signaling pathways, including Janus kinase (JAK) and signal transducer and activator of transcription 3 (STAT3), which have been associated with maintaining normal cellular processes and regulating cell survival, differentiation, and apoptosis [16, 17]. A reduction in MDSC expansion was observed in $STAT3^{-/-}$ tumor-bearing mice along with a coinciding increase in anti-tumor T cell responses [18]. In addition to growth factors, various cytokines produced by activated T cells and tumor stromal cells, including IFN-γ, IL-6, IL-4, and TGF-β are critical to the activation of MDSCs. Of particular relevance to transplantation, which results in significant production of IFN-γ, blockade of IFN-γ or disruption of STAT1 compromised NO-mediated suppression by monocytic MDSCs [19]. Similarly, the combined actions of IL-13 and IFN-γ were shown to induce concomitant expression of Arg-1 and iNOS in $CD11b^{+}$ cells sorted from the spleens of tumor-bearing mice [20].

Mechanisms of Suppression

Immunoregulatory Enzymes

Despite the heterogenic composition of MDSCs, they share the ability to inhibit T cell responses. Suppression by MDSCs is largely mediated through restriction of the semi-essential amino acid L-arginine, which is necessary for maintaining T cell homeostasis, and regulating proliferation and differentiation [3, 21, 22]. Immunoregulatory enzymes Arg-1 and iNOS utilize L-arginine as a substrate and can effectively mediate the conversion of L-arginine into L-ornithine and urea or nitric oxide (NO) and L-citrulline, respectively. Decreased availability of L-arginine has been shown to downregulate CD3 ζ chain expression and impair T cell receptor (TCR) signaling [23, 24]. Furthermore, the reduction of L-arginine in the extracellular microenvironment can activate the amino acid stress response pathway general control non-depressible 2 (GCN2) within T cells. Activation of GCN2 induces T cell proliferative arrest through inhibition of protein translation, including those involved in regulating the cell cycle (cyclin-dependent kinase 4, cyclin-dependent kinase 6, cyclin D3) [22]. Interestingly, Arg-1 activity has also been linked to the induction of natural Tregs [25]. In addition to these mechanisms, NO generated through the catalytic activities of iNOS can suppress T cells through a number of mechanisms, such as the inhibition of JAK/STAT signaling and MHC class II expression and induction of T cell apoptosis [1, 26]. Therefore, the combined activities of Arg-1 and iNOS can contribute to MDSC-mediated suppression [4]. The inhibition of one or both of these enzyme can impair the ability of MDSCs to inhibit T cells [27].

Similarly, studies examining the suppressive mechanisms of MDSCs in human cancers have revealed a correlation between expression of IDO, the rate limiting enzyme that mediates the catabolism of L-tryptophan, in the local tumor microenvironment and tumor progression and metastasis [28, 29]. IDO has been considered to be a critical immunosuppressive factor in maintaining maternal tolerance [30] and CTLA-4 Ig-mediated tolerance in islet transplantation [31]. Mechanisms through which MDSC-derived IDO inhibited T cells include depletion of local L-tryptophan and production of cytotoxic kynurenine metabolites, which can initiate the GCN2 pathway. Inhibition of IDO activity through *1-methyl-tryptophan* (1-MT) attenuated tumor-derived MDSC-mediated T cell suppression, restored Th1 polarization, and increased apoptosis of T cells [6]. IDO has been shown to correlate with Treg induction; higher frequencies of Tregs were detected in IDO^+ cancer tissues [29]. Recently, it was demonstrated that direct contact between activated T cells and MDSCs is necessary for IDO-mediated suppression. The MDSCs, which were generated from human umbilical cord blood cells and recombinant human (rh) G-CSF and GM-CSF, failed to express IDO in the absence of contact or in the presence of 1-MT [32].

Reactive Oxygen Species

Accumulating evidence suggests that production of ROS by MDSCs contributes to the differentiation of myeloid cells and is critical for mediating T cell suppression [1]. ROS has been shown to inhibit the maturation of DCs and promote the expansion of MDSCs. $Gr1^+$

cells that were isolated from C3 tumor-free C57BL/6 mice and transplanted into naïve congenic recipients differentiated into $CD11c^+IAb^+B7\text{-}2^+$ DC or $F4/80^+$ macrophages within five days of transfer. In contrast, the differentiation of $Gr1^+$ cells was delayed when transplanted into C3 tumor-bearing mice. Furthermore, analysis of ROS accumulation in $Gr1^+$ cells through the oxidant-sensitive dye dichlorofluorescein acetate (DCFDA) revealed a significant increase in DCFDA levels in $Gr1^+$ cells isolated from tumor-bearing mice, indicating that elevated production of ROS within these cells was preventing immature myeloid cell differentiation [8]. In a subsequent study by the same group, it was determined that ROS-mediated mechanisms were responsible for the inhibition of Ag-specific $CD8^+$ T cells by MDSCs [7]. Integrin proteins (CD11b, CD18, and CD29) expressed on the surface of MDSCs regulated the expression of ROS.

Peroxynitrites

Peroxynitrites, the product of the reaction between NO and superoxide anion, has emerged as a key player in MDSC-mediated T cell tolerance. Peroxynitrites are responsible for the nitration of amino acids cysteine, methionine, tryptophan, and tyrosine [33]. Nitration of tyrosine residues in the TCR can alter signaling and interfere with the ability of T cells to interact with peptide-MHC complexes on antigen-presenting cells (APCs). Direct interactions between tumor-derived MDSCs and T cells resulted in increased nitrosylation of tyrosine residues present on TCR and CD8 molecules and Ag-specific T cell unresponsiveness. The neutralization of peroxynitrites in vivo, through the administration of the peroxynitrite scavenger uric acid, abrogated the suppressive effects of MDSCs and restored T cell reactivity [34].

Regulation through B7-H1

Although the role of the inhibitory molecule programmed death ligand 1 (PD-L1, B7-H1) in MDSC-mediated suppression is still under investigation, studies from our group as well as others suggest that B7-H1 expression on MDSCs is necessary for the induction of T cell hyporesponsiveness and the expansion of Tregs. The expression of B7-H1 has been shown to be upregulated in $CD11b^+Gr1^+$ MDSCs isolated from a variety of cancer models [35, 36]. Inhibition of B7-H1 in co-cultures consisting of MDSCs, syngeneic $CD4^+$ T cells, and allogeneic BM DCs abrogated the suppressive capacity of MDSCs. Interestingly, the depletion of Tregs through CD25-specific antibodies (Abs) in tumor-bearing mice resulted in the downregulation of B7-H1 expression in tumor-derived MDSCs. IL-10 levels were also significantly decreased in the supernatants of tumor-derived MDSC cultures following Treg depletion and a coinciding increase in IFN-γ was observed. In our mouse model of islet transplantation, the frequency of antigen-specific $CD25^+Foxp3^+$ cells was substantially increased in the graft, blood, and lymphoid organs in MDSC/islet co-transplanted recipients. However, cotransplantation with $B7\text{-}H1^{-/-}$ MDSCs resulted in a significant reduction in Treg frequencies. B7-H1 deficiency also reduced the ability of MDSCs to prolong islet allograft survival [37].

Induction of Regulatory T Cells

Apart from direct effects on T cells, it has been suggested that MDSCs alter T cell functions through indirect mechanisms, such as induction of $CD4^+CD25^+Foxp3^+$ T regulatory cells. While arginine metabolism by MDSCs has been primarily linked to the development of Tregs, locally induced Tregs have been reported in the presence of immunoregulatory cytokines IL-10 and TGF-β [9]. Elevated numbers of $Gr\text{-}1^+CD115^+$ cells have been described in tumor-bearing mice. These cells produced significantly high levels of IL-10, NO, TGF-β, and IL-2. Co-culture of $Gr\text{-}1^+CD115^+$ cells with T cells showed significant induction of Foxp3 expression in T cells. $Thy1.1^+$ mice bearing HA-MCA26 tumors that were simultaneously transferred with $Gr\text{-}1^+CD115^+$ cells and $Thy1.2^+CD4^+$ HA-TCR^+ T cells had reduced anti-tumor T cell responses and greater tumor growth compared to the control group. Neutralization of IL-10 enhanced T cell responses and suppressed tumor growth [9]. In a related study, it was shown that CD80 expression on $CD11b^+Gr\text{-}1^+$ cells modulates Treg activity through interactions with CD152 (CTLA4) on Tregs. The dependency of Treg-mediated suppression on MDSCs was investigated by culturing splenocytes isolated from mice immunized with the heterologous antigen human papillomavirus type 16 virus-like particles (VLP) with $CD4^+CD25^+$ T cells alone or in combination with $CD11b^+$ $Gr\text{-}1^+$ cells isolated from mice bearing the spontaneously transformed syngeneic mouse ovarian surface epithelial cell line, 1D8. Transfer of isolated $CD4^+CD25^+$ T cells alone did not induce significant antigen-specific T cell inhibition. Co-administration of $CD11b^+$ $Gr\text{-}1^+$ cells was required to achieve maximal suppression [38]. Gene expression profiling of MDSCs show that Treg-inducing capabilities are not only limited to immunoregulatory cytokine production but include chemokine production, such as CCL3, CCL4, and CCL5, which serve as ligands for the chemokine receptor CCR5. Secretion of these chemokines facilitated the recruitment of Tregs, which were shown to preferentially upregulate CCR5 [39].

MDSCs in Transplantation

Given that MDSCs are activated under inflammatory settings, such as those that accompany transplantation, MDSCs could be potentially used as an immunotherapeutic agent for achieving transplantation tolerance. Indeed, an accumulation of MDSCs has been observed in renal allografts in rat and human recipients. Elevated $CD3^-$class II^-CD11b^+ cells were detected in the blood and kidney allografts of rat recipients administered anti-CD28 Abs. These MDSCs exhibited T cell inhibitory properties and could induce apoptosis of alloreactive T cells through NO-dependent mechanisms [40]. Inhibition of iNOS led to the rejection of grafts. Similarly, development of $CD11b^+CD33^+HLA\text{-}DR^-$ cells was observed in human renal transplant recipients. Monocytic $CD14^+$ cells were capable of inhibiting $CD4^+$ T cell proliferation. The expansion of $CD11b^+CD33^+HLA\text{-}DR^-$ expressing myeloid cells over time correlated with increased frequencies of Tregs in vivo [41].

The role of MDSCs in transplantation has also been demonstrated in transgenic mice overexpressing the inhibitory receptor immunoglobulin-like transcript 2 (ILT2). Engagement of ILT2, which is expressed on T and B lymphocytes and APCs, with HLA-G, a human immunosuppressive non-classical MHC molecule present on allografts, results in the

modification of cellular functions. Additionally, these interactions have also been shown to promote the development and expansion of $CD11b^+/Gr\text{-}1^+$ cells. Naïve ILT2 transgenic mice exhibited greater frequencies of $CD11b^+/Gr\text{-}1^+$ cells in the blood and spleen compared to wild-type mice. Following allogenic skin transplantation, the frequency of MDSCs in ILT2 mice and the suppressive function of these cells increased. The MDSCs displayed a unique transcriptional profile, with significant upregulation of Arg-1. Adoptive transfer of MDSCs isolated from ILT2 recipients of major MHC class II disparate skin allografts into wild-type recipients of MHC class II disparate skin allografts, prolonged allograft survival and inhibited the development of antigen-specific cytotoxic T cells [42].

$CD11b^+CD115^+Gr\text{-}1^+$ monocytic cells with suppressive functions have also been identified in a mouse cardiac transplant model, in which tolerance was induced through administration of anti-CD40L mAb treatment and donor-specific transfusion. The contribution of MDSCs was evaluated by selectively depleting $CD11b^+Gr1^+$ monocytes, which prevented the induction of tolerance. Furthermore, it was demonstrated that in tolerized recipients, $CD115^+CD11b^+Gr1^+$ monocytes were generated in the BM, mobilized into the blood, and then migrated to the graft. These cells were shown to mediate T cell suppression in an antigen non-specific manner and promote the development of Tregs. However, tolerance could not be achieved in $CCR2^{-/-}$ recipients since monocytes were sequestered in the BM. Likewise, the use of P/E $selectin^{-/-}$ recipients also prevented tolerance induction. Monocytes and immune cells were not able to traffic to the allograft. Adoptive transfer of BM common macrophage/DC precursor, but not common DC precursor, into CCR2-/- cardiac allograft recipients promoted long-term graft survival, suggesting that MDSCs can serve as therapeutic agents for promoting transplantation tolerance [Garcia, 2010].

Generation of MDSCs

On account of these promising findings, strategies are being developed to generate and expand MDSCs as cellular therapeutic agents to promote long-term graft survival and establish tolerance. Examination of immune modulatory factors produced in the tumor microenvironment and by tumor cell lines suggests an important role for IL-6 [43−45]. Amongst the numerous factors tested, the minimal factor combination of GM-CSF and IL-6 has been shown to be optimal for the generation of MDSCs. The addition of GM-CSF and IL-6 to mouse BM cultures induced cells with enhanced expression of CD11b, Gr-1, and IL-4Rα. These cells proved to be fully competent MDSCs, displaying significant inhibitory activity against antigen-specific T lymphocyte cytotoxic responses both in vitro and in vivo. Pharmacologically-induced diabetic mice administered GM-CSF and IL-6 BM-derived MDSCs at the time of allogeneic islet transplantation displayed marked potentiation of graft survival compared to control mice, which did not receive MDSCs. Ex vivo analysis of splenic and lymph node T cells restimulated by donor and unrelated islet alloantigens showed inhibition of antigen-specific $CD8^+$ T cell responses [46]. A similar outcome has been reported with human peripheral blood mononuclear cells (PBMCs) isolated from healthy donors and cultured in the presence of GM-CSF and IL-6. The $CD33^+$ cells generated displayed morphology, phenotype, and suppressive functions comparable to human MDSCs [15].

The addition of the Th2-associated cytokine IL-13 to BM cultures has been shown to produce an MDSC subset with potent T cell suppressive properties. IL-13 upregulated expression of Arg-1 in MDSCs. T cell suppression by MDSCs was dependent on Arg-1-mediated mechanisms, as evidenced by the addition of the arginase inhibitor nor-NOHA or excess L-arginine to MDSC-T cell co-cultures. The transfer of IL-13-induced MDSCs during allogeneic BM and purified T cell transplant did not prevent the onset of graft-versus-host disease (GVHD), however, IL-13-induced MDSC-treated mice exhibited significantly improved survival [47].

Prostaglandin E_2 (PGE_2) is one factor amongst many that is elevated in the tumor microenvironment [14]. PGE_2 is recognized for its ability to suppress the differentiation of human monocytes into functional DCs [48]. It was recently demonstrated that the addition of PGE_2 to GM-CSF and IL-4 human PBMC cultures redirected the differentiation of $CD1a^+CD14^-$ DCs and promoted the development of $CD1a^-CD14^+CD80^-CD83^-$ cells. The phenotype and suppressive function of PGE_2-induced MDSCs were similar to that of MDSCs isolated from cancer patients. PGE_2 induced the expression of COX2, a key regulator of PGE_2 synthesis. Factors associated with MDSC suppression (iNOS, IL-10, IDO, ILT2, PD-L1) were also upregulated. The physiological relevance of PGE_2 was determined by measuring the frequency of MDSCs in cancer ascites samples from individual patients. The frequency of infiltrating $CD11b^+CD33^+$ cells was directly proportional to the concentration of PGE_2. Inhibition of PGE_2 by selective COX inhibitors abolished MDSC induction [49].

Several studies have reported that histone deacetylase inhibitors can enhance hematopoietic stem and progenitor cells (HPSCs) and block DC differentiation, favoring a population of $CD11c^-CD11b^+$ cells [50−52]. Inhibition of histone deacetylase by trichostatin A (TSA) increased the frequency of MDSCs in $GM\text{-}CSF^+$ BM cultures. Furthermore, the in vivo administration of TSA and GM-CSF enhanced the frequency and absolute numbers of functional $CD11b^+Gr1^+$ cells in the BM. Similar findings were reported in the spleen, in which TSA was shown to significantly increase the absolute numbers and frequency of splenic $CD11b^+Gr1^+$ cells with allogeneic T cell suppressive properties in animals given GM-CSF [53]. In a subsequent study by the same group, it was demonstrated that MDSCs could be mobilized in mice through administration of recombinant human Fms-like tyrosine kinase 3 ligand (Flt3L). The adoptive transfer of $CD11b^+Gr1^+$ cells, isolated from the spleens of BALB/c Flt3L-treated mice, into BALB/c recipients of C57BL/6 cardiac allografts significantly prolonged allograft survival [54].

Immunoregulatory cell types, such as hepatic stellate cells (HSCs), have also been shown to promote MDSCs. HSCs use various mechanisms, such as production of complement component 3 [55] and retinoic acid [56] and upregulation of B7-H1 expression [57], to modulate T cell responses and promote regulatory T cells. We recently described a method utilizing HSCs for the generation and expansion of MDSCs. Cotransplantation of allogeneic islets with HSCs resulted in an accumulation of $CD11b^+CD11c^-$ cells in islet allografts within 7 days of transplantation. These $CD11b^+CD11c^-$ cells exhibited reduced expression of costimulatory molecules (CD40, CD80 and CD86) and increased expression of Arg-1, iNOS, and B7-H1. $CD11b^+CD11c^-$ cells proved to be weak APCs and could inhibit proliferation of T cells stimulated by anti-CD3/CD28 mAbs. A positive correlation between $CD11b^+CD11c^-$ cell frequencies and Tregs was observed in the grafts. In vitro, the co-culture of HSCs with BM cells, referred to as hepatic stellate cell-induced myeloid cells (H-MCs), was effective in yielding large numbers of $CD11b^+CD11c^-$ cells. The phenotype and function of in vitro

generated H-MCs was similar to $CD11b^{+}CD11c^{-}$ cells generated by HSC co-transplantation in vivo. Co-transplantation of H-MCs with allogeneic islets in diabetic mice promoted allograft survival. iNOS appeared to be a contributing factor since MDSCs propagated from $iNOS^{-/-}$ mice had reduced T cell suppressive properties and were compromised in their ability to protect islet allografts following co-transplantation [58]. The induction of Tregs by MDSCs was shown to be dependent on the expression of B7-H1 on MDSCs. The frequency of Tregs was significantly reduced in islet/$B7\text{-}H1^{-/-}$ MDSC recipients compared to islet/WT MDSC recipients. Comparisons of in vitro co-cultures of WT or $B7\text{-}H1^{-/-}$ MDSCs with BALB/c T cells showed expansion of $Foxp3^{+}$ cells in the WT MDSC-T cell group [37].

The generation of functional MDSCs has also been demonstrated using mouse embryonic stem (ES) cells and BM-derived hematopoietic stem (HM) cells. ES and HS-derived MDSCs could be categorized into two subpopulations of monocytic $CD115^{+}Ly\text{-}6C^{+}$ and granulocytic $CD115^{+}Ly\text{-}6C^{-}$ cells. In vitro, the ES and HS-induced MDSCs displayed T cell inhibitory properties through production of NO and IL-10. Adoptive transfer of ES-MDSCs prevented GVHD following allogeneic BM transplantation [59].

Conclusion

Current findings suggest that MDSCs have the potential to be utilized for cellular immunotherapy in transplantation and may even promote Ag-specific tolerance. MDSCs are equipped to regulate innate and adaptive immune responses and utilize various mechanisms, including expression of immunoregulatory enzymes, production of ROS and peroxynitrites, upregulation of inhibitory molecules, and induction of Tregs. However, due to the heterogenous nature of these cells, generation of stable and functional MDSCs can be challenging. Furthermore, there is limited information on the migration and kinetics of MDSCs after adoptive transfer. While there has been a great deal of progress in characterizing MDSCs in rodent models, human MDSCs remain poorly defined. The factors that contribute to the induction of human MDSCs can be complex. With further study, the influence of MDSCs on transplant outcomes can be elucidated and methodologies to generate potent MDSCs in sufficient numbers can be identified.

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Chapter 21

Stem Cell-Derived Immunosuppressive Cells Therapy in Organ Transplantation

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Abstract

In the field of organ transplantation, the idea of harnessing the tolerance inducible capacity of immunosuppressive cells has become attractive. Although the evidence suggesting clinical efficacy has been accumulated, there also remain several obstacles limiting their practical application, such as sufficiency and homogeneity of cells for therapy. Pluripotent stem cells, especially embryonic stem cells and induced pluripotent stem cells, may provide answers to these problems by their pluripotency and unlimited propagation capacity. In theory, pluripotent stem cells have the capacity to differentiate into any kind of cells, including immunosuppressive cells, as much as needed. In this chapter, the technologies to generate immunosuppressive cells derived from pluripotent stem cells and their clinical potential are discussed.

Keywords: embryonic stem cells, Induced pluripotent stem cells, regeneration, Immunosuppressive cells, cell therapy

Abbreviations

AGM:	Aorta-gonad-mesonephros
BMP-4:	Bone morphogenetic protein 4

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DCreg cells:	Regulatory dendritic cells
EB:	Embryoid body
EC:	Embryonal carcinoma
ES cells:	Embryonic stem cells
Flt-3L:	Fms-related tyrosine kinase 3 ligand
G-CSF:	Granulocyte colony-stimulating factor
GM-CSF:	Granulocyte and macrophage colony-stimulating factor
GMP:	Good Manufacturing Practice
GvHD:	Graft-versus-host disease
HPC:	Hematopoietic progenitor cell
HSC:	Hematopoietic stem cell
IL-2:	Interleukin-2
IL-3:	Iinterleukin-3
IL-6:	Interleukin-6
IL-10:	Interleukin-10
iNOS:	Inducible nitric-oxide synthase
iPS cells:	Induced pluripotent stem cells
LIF:	Leukemia inhibitory factor
MDSC:	Myeloid-derived suppressor cell
MEFs:	Mouse embryonic fibroblasts
MLR:	Mixed lymphocyte reaction
MSC:	Mesenchymal stem cell
PBMC:	Peripheral blood mononuclear cell
PSC:	Pluripotent stem cell
SCF:	Stem cell factor
TGF-β:	Transforming growth factor-β
TNF-α:	Tumor necrosis factor-α
TPO:	Thrombopoietin
Treg cells:	Regulatory T cells
VEGF:	Vascular endothelial growth factor

Introduction

To date, many types of immunosuppressive cells have been discovered, and the idea of harnessing their tolerance inducing capacity to overcome transplant rejections has become attractive. Indeed, several clinical trials incorporate immunosuppressive cells as part of the immunosuppression protocol [1]. One of the causes limiting the usage of immunosuppressive cell therapy is limited number of available cells. The difficulty in isolating and expanding the cells polyclonally is also a major issue. To overcome these problems, pluripotent stem cells (PSCs) have attracted attentions.

Pluripotent Stem Cells as the Potential Source of Regenerative Cell Medicine

PSCs are characterized by pluripotency and unlimited propagation capacity. Pluripotency means the potential to differentiate into any of three germ lineages, that is, endoderm, ectoderm and mesoderm. By these characteristics, PSCs can yield us any cells of interest and as many as necessary in theory. First reported PSCs were embryonal carcinoma (EC) cells [2], which are isolated and cloned from teratocarcinoma of mice. EC cells have pluripotency and can contribute to mouse chimeras when injected into early embryo. However EC cells often harbor genetic mutations, and abnormal karyotypes accumulated during the development of the teratocarcinoma are also observed frequently. For the clinical use, PSCs avoiding these genetic aberrations were strongly needed.

Embryonic stem cells (ES cells) had been developed in this context. ES cells are established cell lines derived from the inner cell mass of a mammalian blastocyst-stage embryos [3]. ES cells exhibit pluripotency and also have unlimited propagation capacity. In addition, the well-established methods for genetic modification make it easy to impart ES cells with various preferable characters for investigation or clinical use. Therefore, ES cells can differentiate into any of somatic cells, with desired characteristics if necessary. ES cells also avoid genetic mutations because such cells are derived from normal embryo. By these characteristics, ES cells have attracted attention not only as research tool of embryology but also as resource for cell therapy. After human ES cells were first reported by Thomson et al. in 1998 [4], ES cells have been proposed as the potent source of regenerative cell medicine and the tissue transplant, that is, regenerative medicine. However, for the purpose of clinical application of human ES cells, there are two major obstacles that need to be overcome, that is, ethical concern and immunological rejection. Establishment of ES cells results in destruction of the embryo, which faces ethical controversies. On the other hands, because ES cells-derived transplants are allografts for host, there is the risk that the transplants are immunologically rejected by host and result in failure of engraftment. To avoid the latter problem, additional manipulations, such as somatic-cell nuclear transfer, can be employed. However, induced pluripotent stem cells (iPS cells) provided an approach to the fundamental solution for these problems from a different angle.

iPS cells technology researchers have made groundbreaking discovery. iPS cells can be generated from various types of somatic cells by simultaneous introduction of reprogramming factors. In 2006, Takahashi and Yamanaka first reported the established cell lines which were very similar to ES cells in many respects including morphology, gene expression, unlimited propagation and the capacity to differentiate into any of the three germ layer, or pluripotency. These cell lines were generated from mice somatic fibroblast cells by introduction of four transgenes, Oct3/4, Sox2, Klf4 and c-Myc [5]. Takahashi and Yamanaka named these cell lines "induced pluripotent stem cells". Since iPS cells can be derived from adult somatic cells, they can avoid the ethical issues of ES cells. In addition, since the cells of interest can be differentiated from the patient's own cells, iPS cells can reduce the risk of immune-rejection. Originally, retroviruses were used to deliver the genes of the four reprogramming factors, also known as "Yamanaka factors", into fibroblasts of mice. Although advantage of using retroviruses as gene transfer vehicles is high transfection efficiency, there is the risk of destruction of host genome because transgenes are integrated into host genes by this method.

To reduce this risk, non-viral generation methods which allow us to establish integration-free iPS cells have been developed, for example, by the employment of episomal vectors [6,7]. The first report of human iPS cells [8], which were generated from adult human dermal fibroblasts, allows us to extend the possibility of iPS cells for clinical application. To date, ES cells and iPS cells together have become potential and promising resources for regenerative medicine.

Hematopoietic Differentiation in Embryogenesis

Models for hematopoietic development during embryogenesis *in vivo* have been well established. Studies of *in vitro* hematopoietic differentiation of ES cells and iPS cells (PSCs) are based on their accumulated knowledge. Following is the simplified outline of embryonic hematopoiesis [9,10]. Embryonic hematopoiesis of mammals consists of primitive and definitive hematopoiesis. In mouse, the former begins to develop primitive erythrocyte, megakaryocyte and macrophage in yolk sac blood islands at 7.0 days post coitum (dpc). Primitive hematopoiesis occurs transiently, and is replaced soon by definitive hematopoiesis, which starts in the aorta-gonad-mesonephros (AGM) region at 10.5 dpc. AGM region is derived from mesoderm layer of the embryo. The definitive hematopoietic stem cells (HSCs) are produced from the ventral endothelium of the dorsal aorta in AGM region, that is, hemogenic endothelium. These definitive HSCs can differentiate into multi-lineages of mature hematopoietic cells. They migrate to fetal liver followed by bone-marrow, and form the blood cells during rest of the life. Altogether, the mesoderm layer is the origin of hematopoietic cells. In definitive hematopoiesis, the mesoderm layer derives hemogenic endothelium in AGM region, which produces the definitive HSCs resulting in mature hematopoietic cells. Although, as for primitive hematopoiesis, the detailed route of differentiation is still discussed, it is suggested that primitive HSCs are derived from hemangioblast which is common precursor of hematopoietic and vascular endothelial lineages. Hemangioblast is also derived from the mesoderm layer.

Generation of Hematopoietic Lineage Cells from Pluripotent Stem Cells

To maintain and propagate mouse ES cells/iPS cells under undifferentiated states, they should be cultured on a layer of mouse embryonic fibroblasts (MEFs) and leukemia inhibitory factor (LIF). In the case of human ES cells/iPS cells, MEFs and basic fibroblast growth factor (bFGF) are required. Once LIF or bFGF are removed from the culture, PSCs differentiate into any kind of cell *in vitro* depending on the culture conditions. To guide the *in vitro* differentiation toward hematopoietic lineages, there have been several approaches proposed by applying the knowledge of embryonic hematopoiesis. To date, these approaches can be roughly classified into the following two, embryoid bodies (EBs) formation and co-culture system with stroma cell lines.

EBs are three-dimensional aggregates of PSCs. When cultured in non-adhesive environment as cell suspension without anti-differentiation agents, such as LIF or bFGF,

PSCs spontaneously aggregate to form cystic sphere structure. This structure, called as embryoid bodies (EBs), mimic the spatial organization of the embryo through enhancing cell-cell interactions, resulting in differentiation into three germ layers [11]. Several EBs formation methods have been developed, such as suspension culture in the low-adherent container or the hanging drop method. Mouse EBs cultured with human cord serum develop blood islands, which indicates the hematopoiesis capacity of EBs [11]. Without additional manipulation, PSCs randomly differentiate during EB development. In contrast, Chadwick et al. reported that the differentiation fate can be guided towards hematopoietic cells by the combination of bone morphogenetic protein 4 (BMP-4) and several hematopoietic cytokines, such as stem cell factor (SCF), fms-related tyrosine kinase 3 ligand (Flt-3L), interleukin-3 (IL-3), interleukin-6 (IL-6) and granulocyte colony-stimulating factor (G-CSF) [12]. To date, multiple methods to differentiate PSCs into hematopoietic cells through EB formation have been developed. The following is one example. Activin A and BMP-4 promotes the differentiation toward mesodermal lineage from EB. Application of vascular endothelial growth factor (VEGF) induces the transition into $CD34^+$ $CD43^-$ endothelial cells (ECs). During this step, *Brachyury*, which is indispensable for mesodermal differentiation, expresses transiently. ECs give rise to $CD34^+$ $CD43^+$ $CD45^-$ pre-hematopoietic progenitor cells (pre-HPCs) supported by hematopoiesis cytokines such as VEGF, SCF and thrombopoietin (TPO). Around the same period, RUNX1 expresses increasingly, which is one of the hematopoietic regulatory genes and indispensable for definitive hematopoiesis. After that, pre-HPCs mature into $CD34^+$ $CD43^+$ $CD45^+$ hematopoietic progenitor cells (HPCs) [13]. This process seems to mimic embryonic hematopoiesis.

In co-culture system, PSCs are cultured together with a layer of feeder cells. Feeder cells give support to the PSCs in differentiation towards hematopoietic lineages via providing functional extracellular matrix and secreting known and unknown cytokines. For inducing hematopoiesis, stromal cells are commonly employed. Stromal cells are derived from connective tissues. Connective tissues are located between tissues and found everywhere. They have diverse functions. For example, bone marrow connective tissues are described to be involved in hematopoiesis. As for *in vitro* hematopoiesis, well-known feeder stromal cells are OP9. When cultured together with some stromal cell lines, such as ST2, PSCs almost exclusively differentiate into macrophages. In hematopoiesis, one of the growth factors responsible for differentiation towards monocyte-macrophage lineages is macrophage colony-stimulating factor (M-CSF). OP9 stromal cell line was established from newborn calvaria of op/op mouse, which is one of the osteoporosis mouse models and does not produce M-CSF due to mutation in the gene encoding *M-CSF*. Nakano and his colleague examined the hematopoiesis supporting activity of OP9, and first reported that mouse ES cells gave rise to multiple hematopoietic lineages by co-culture together with layer of OP9 cells [14]. After 14 days of co-culture, mouse ES cells were differentiated into c-kit^+ HPCs, TER-119^+ erythroid lineage cells, Mac-1^+ granulocyte-macrophage lineage cells, and $B220^+$ B lineage cells, suggesting that OP9 stromal cell line supports hematopoietic differentiation without restriction by M-CSF. Human PSCs, cultured together with a layer of OP9 cells for 8 days, give rise to $CD31^-$ $CD34^+$ mesenchymal cells, $CD31^+$ $CD34^+$ $CD43^-$ ECs and $CD31^+$ $CD34^+$ $CD43^+$ pre-HPCs [15]. Other than OP9, some stromal cell lines are also used for *in vitro* hematopoiesis from PSCs. For example, C3H10T1/2 cell lines, which was established from C3H mouse embryo, are employed in the generation of platelets from human ES cells [16].

Generation of Mature Hematopoietic Cells from Pluripotent Stem Cells

The development schemes of HSCs towards different types of mature hematopoietic cells have been developed, and the knowledge about key growth factors and signal cascades has been accumulated. By applying them together with hematopoiesis method of PSCs, *in vitro* differentiations of PSCs into several types of mature hematopoietic cells have been achieved, for example, T cells [17,18], B cells [19], NK cells [20], NKT cells [21], erythrocytes [22], platelets [16], macrophages [23–25], and dendritic cells [23–28]. Today, thanks to these achievements, PSCs can be a logical alternative source of hematopoietic cells. Taken together with appropriate cytokines and/or additional feeder cells, PSCs-derived hematopoietic lineage cells are differentiated into mature hematopoietic cells of interests. If necessary, specific progenitor cells of intermediate stages can be purified by cell sorter or magnetic-activated cell sorting.

Following is the example of T cell differentiation [18]. Stromal cell lines, including OP9, can support differentiation to B cell-lineages and myeloid-lineages, but not to T cell-lineages. By contrast, OP9 harboring *Delta-like 1* (*DLL1*) gains the capacity to induce T cell differentiation from embryo-derived HPCs [29]. DLL1 is a ligand of Notch signaling, which is indispensable for T cell differentiation in thymus. Combination of OP9 and OP9 harboring DLL1 [OP9/DLL1) provides T cell-lineages differentiation from PSCs. Human PSCs cultured on a layer of OP9 differentiate into $CD34^{low}$ $CD43^{+}$ HPCs after 13 days of co-culture. These HPCs are transferred on a layer of OP9/DLL1, and then they give rise to $CD4^{+}$ $CD8^{+}$ double positive immature T cells [DP T cells) at day 40 [30]. To mature DP T cells, anti-CD3 monoclonal antibodies are added into culture, followed by the increase of $CD3^{+}$ $CD8^{+}$ single positive cytotoxic T cells.

Followings are the other cases; generation methods for dendritic cells (DCs). Senju et al. reported a method to generate functional DCs from mouse ES cells [31]. In this method, mouse ES cells cultured on a layer of OP9 cells differentiate into hematopoietic lineages, and are subsequently stimulated by exogeneous granulocyte and macrophage colony-stimulating factor (GM-CSF) resulting into immature DCs. Based on this method, this group developed three-step method to generate human ES cells-derived DCs [23] and human iPS cells-derived DCs [24]. For the differentiation of human PSCs-derived DCs, being different from mouse ES cells, stimulation by only GM-CSF is insufficient. Human PSCs are cultured on a layer of OP9 cells for 14−18 days resulting in myeloid-lineage hematopoietic cells, like mouse ES cells, and other adhesive cells. To remove these adhesive cells, harvested cells are incubated in tissue culture dish, and only floating (hematopoietic lineage cells) cells are recovered. These hematopoietic lineage cells are cultured for 8 days in the medium containing GM-CSF and M-CSF, and give rise to precursor cells of DCs and macrophages. These precursor cells can be differentiated into immature DCs by the stimulation of GM-CSF and interleukin 4 (IL-4) in non-adhesive culture environment. On the other hand, Slukvin et al. proposed a slightly different approach employing human ES cells/OP9 co-culture system [26]. In this method, human ES cells are cultured on OP9 cells for 9−10 days to promote initial hematopoietic differentiation, and then transferred to suspension culture in low-adhesive container. In this second period, floating cells (hematopoietic-lineage cells) are expanded and matured to precursor cells of DCs by the stimulation of GM-CSF for 8−10 days. Then, harvested and

purified precursor cells are cultured in medium containing GM-CSF and IL-4 to generate immature DCs. These two approaches are almost identical as a whole, however, there are some minute differences, especially in the period of co-culture of PSCs/OP9 and culture conditions in second step. Interestingly, in human PSCs/OP9 co-culture system, late-appeared hematopoietic progenitor cells ($CD43^+$ $CD45^+$) tend to differentiate to myeloid lineages more than early-appeared progenitor cells ($CD43^+CD45^-$) [32,33]. These subtle differences of the cell stage may influence the whole strategy of differentiation method.

For the generation of DCs, the approaches via EB formation are also developed. In the method developed by Su et al. [34], human ES cells cultured in ultra-low attachment containers aggregate to form EBs. Harvested EBs are disassociated into single cell suspensions, and then cultured in a medium containing SCF, Flt3-L, GM-CSF, IL-3 and TPO for 10 days, resulting in monocyte precursor cells. After second step, the harvested monocyte precursor cells are stimulated by GM-CSF, IL-4 and tumor necrosis factor-α (TNF-α) to give rise to immature DCs. In addition, application of BMP-4 to culture medium during EBs formation has significant effect in hematopoiesis. The methods through EB formation is free from feeder cells, and this is advantageous for clinical application, because the cell medicine for therapeutic use is desired to avoid animal products, such as feeder cells, from the manufacturing processes of regenerative medicine. Also, from the point of view of Good Manufacturing Practice (GMP), restricted conditions and defined reagents should be used. Therefore, hematopoietic differentiation through EB formation seems to be more suitable for the clinical uses than stromal cell co-culture system. However, it is more preferred to avoid other animal-derived component, such as serum. In this context, the demand for culture/differentiation of PSCs under xeno-free conditions has been increased.

Research on feeder-free culture system of PSCs has flourished in recent years [35]. To remove such animal products, various matrices, which can be used to replace feeder cells, have been developed, such as Matrigel and laminin-511 E8 fragment. In addition, xeno-free media are also available today, for example mTeSR1. Recently, xeno-free hematopoietic differentiation methods have been reported [24, 25, 28]. In these protocols, PSCs are maintained with xeno-free matrices and media, and then hematopoiesis cytokines, which are employed in differentiation methods via EB formation, are used together to guide PSCs towards hematopoietic lineages.

Yanagimachi et al. developed the differentiation methods of human PSCs-derived DCs under serum- and feeder-free conditions [25]. This method consists of five steps: induction of mesodermal lineages by BMP-4 (step 1); generation of KDR; $CD34^+$ hemangioblast-like cells with VEGF, bFGF and SCF (step 2); generation of hematopoietic lineages with SCF, Flt-3L, IL-3, TPO and M-CSF (step 3); generation of monocyte lineages with Flt-3L, M-CSF and GM-CSF (step 4); generation of immature DCs with GM-CSF and IL-4 (step 5). Throughout all of the differentiation period except for step 5, the cells are cultured and maintained with serum-free medium and defined matrices. On the other hand, Silk et al. reported another xeno-free method to generate human iPS cells-derived DCs [28]. This method, developed based on xeno-free generation of human ES cells-derived DCs [36], also consists of five steps. Step 1 is culturing of human iPS cells on Matrigel in serum-free medium containing BMP-4, VEGF, SCF and GM-CSF. BMP-4 is removed 5 days after (step 2), VEGF is removed after another 5 days (step 3), and SCF is removed after additional 5 days (step 4). Finally, resulting monocyte lineage cells differentiate to immature DCs with GM-CSF and IL-4 (step 5). Interestingly, a subset of DCs generated in the method of Silk et al. expresses

CD141 and XCR1, which is human equivalent of mouse CD8α$^+$ DC. Mouse CD8α$^+$ DC is known by their capacity of cross-presentation, which is very low in monocyte-derived DC.

Thus, the methods to generate many kinds of mature hematopoietic cells and their sub-populations from PSCs have been developed to date. Improvements towards clinical uses have also been intensified.

Generation of Immunosuppressive Cells from Pluripotent Stem Cells

In this section, we review current progress in the development of immunosuppressive cells from PSCs differentiation in culture. To our knowledge, following cell-types with potent immunomodulation activities are generated from PSCs, and are described below: regulatory T cells, myeloid-derived suppressor cells, regulatory dendritic cells, and mesenchymal stem cells (Table 21.1).

Regulatory T cells

Regulatory T cells (Treg cells) are a subset of T cells, and essential for maintaining peripheral tolerance, preventing autoimmune diseases, and limiting chronic inflammatory diseases. Despite the therapeutic efficacies indicated in animal-model studies [37], there are some obstacles to clinical application. One of the major problems is Treg cells dose [38]. In addition, the lack of specific surface markers of Treg cells makes it difficult to purify Treg cells from cell sources.

Haque et al. proposed *in vitro* generation of Treg cells from PSCs [39]. In this method, mouse iPS cells are transduced with *Foxp3* gene using retrovirus-mediated technique, which express *Foxp3* constitutively. When co-cultured in adult thymic organ cultures, Foxp3-transduced iPS cells give rise to CD4$^+$ CD25$^+$ Foxp3$^+$ cell population, suggesting that Foxp3-transduced iPS cells have potential for differentiating into Treg cells. As mentioned above, OP9/DLL1 is capable of supporting differentiation into T cell lineages via Notch signaling pathway. When co-cultured on a layer of OP9/DLL1 cells, Foxp3-transduced iPS cells become non-adherent multicellular clusters including CD3$^+$ TCRβ$^+$ CD4$^+$ CD25$^+$ CTLA-4$^+$ CD127$^-$ population. Together with the constitutive expression of Foxp3, these populations exhibit typical expression profile of Treg cells. These mouse iPS cells-derived CD4$^+$ CD25$^+$ Foxp3$^+$ cells produce immunosuppressive cytokines, such as transforming growth factor-β (TGF-β) and interleukin 10 (IL-10), but don't produce inflammatory cytokines including interleukin 2 (IL-2) and interferon γ (IFN-γ). In addition, CD4$^+$ CD25$^+$ Foxp3$^+$ cells have ability to suppress the secretion of IL-2 and IFN-γ by CD4$^+$ T cells stimulated with anti-CD3 antibody and anti-CD28 antibody. These results indicate that these CD4$^+$ CD25$^+$ Foxp3$^+$ cells are iPS cells-derived Treg cells (iPS-Treg cells).

To assess the regulatory functions of iPS-Treg cells *in vivo*, collagen-induced arthritis mouse model was employed, in which arthritis is induced by intradermal immunization with chicken type II collagen (CII). Adoptive transfer of major histocompatibility complex (MHC)-matched iPS-Treg cells reduced the incidence of arthritis. In addition, iPS-Treg cells

established from C57BL/6 mice suppressed the arthritis development induced in DBA/1 mice by CII, indicating that application of allogenetic iPS-Treg cells can suppress autoimmunity.

Mouse iPS-Treg cells show the immunosuppressive potential both *in vitro* and *in vivo*. For the application to clinical use, their generation without Foxp3 gene transduction is an attractive approach. Despite that the mechanisms underlying Treg cell development are not fully understood, retinoic acid and TGF-β are reported to enhance the development of Treg cells [40]. In this work, Lu et al. reported that iPS cells cultured with Notch ligands, retinoic acid and TGF-βgive rise to Treg cells, however the number was much lower than the approach of Foxp3 gene transduction. Together with the differentiation of human PSCs-derived Treg cells, efficient integration-free generation method may be a future challenge.

Table 21.1. Immunosuppressive cell-lineages generated from pluripotent stem cells

Cell-lineage	Cell source	Generation method	Immunosuppressive functions	Reference
Regulatory T cells	Mouse ES cells	Gene transfer (Foxp3) Feeder cells (OP9, OP9/DLL1)	Suppress inflammatory cytokines secretion by T cells (*in vitro*) Suppress collagen-induced arthritis (*in vivo*)	Haque et al. 2012 (39)
Myeloid-Derived Suppressor Cells	Mouse ES cells	EB formation, Feeder cells (OP9), Cytokines (SCF, IL-3, IL-6, TPO, VEGF, Flt-3L, M-CSF)	Stimulate Treg cells (*in vitro*) Protect host mice from GvHD induced by allogeneic T cells infusion (*in vivo*)	Zhou et al. 2010 (45)
Regulatory Dendritic Cells	Mouse iPS cells	Feeder cells (OP9), Cytokines (GM-CSF, IL-10)	Suppress proliferation of T cells stimulated by conventional DCs (*in vitro, in vivo*) Stimulate Treg cells (*in vitro*)	Zhang et al. 2014 (52)
Mesenchymal Stem Cells	Human ES cells	Feeder cells (OP9)	(not tested)	Barberi et al. 2005 (58)
	Human ES cells, Human iPS cells	EB formation	(not tested)	Brown et al. 2009 (59) Villa-Diaz et al. 2012 (60)
	Human ES cells	Differentiation medium, Manually cell-picking	Suppress proliferation of responder T cell in MLR assays (*in vitro*)	Trivedi *et al*. 2008 (61)
	Human ES cells	EB formation, Hemangioblast formation (supported by VEGF, Flt-3L, TPO, bFGF), Differentiation medium	Suppress proliferation of responder T cell in MLR assays (*in vitro*) Suppress maturation of DCs (*in vitro*) Reduce symptoms of autoimmune diseases model mice (lupus nephritis, EAU) (*in vivo*)	Kimbrel et al. 2014 (62)

Myeloid-Derived Suppressor Cells

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of myeloid-lineage cells with immunosuppressive activity [41,42]. In animal model, many evidences showing the efficacy of MDSCs have been accumulated [43,44].

Zhou et al. developed the method to generate functionally active MDSCs from mouse ES cells [45]. This method consists of three steps. First, mouse ES cells differentiate towards hematopoietic lineages thorough EBs formation technique (step 1). After 6 days of EB induction, EBs are harvested and disaggregated. Then, EB-derived cells are cultured on a layer of OP9 cells in the medium containing SCF, IL-3, and IL-6 (step 2). After two days of culturing, the medium is removed and replaced with new medium containing SCF, TPO, VEGF, Flt-3L and M-CSF (step 3). After another 10 days of culturing, myeloid-lineage cells start appearing, which contain the cells showing typical phenotype of mouse MDSCs ($CD11b^+$ $Gr\text{-}1^+$) [41]. These MDSC-like cells consist of $CD115^+$ and $CD115^-$ populations. $CD115^+$ population inhibits atni-CD3 / anti-CD28 antibodies-stimulated T-cell proliferation, and also alloantigen-stimulated T cell proliferation, while $CD115^-$ population does not exhibit such immunosuppressive abilities, suggesting that ES cells-derived $CD115^+$ cells are functional MDSCs (ESC-MDSCs). ESC-MDSCs could induce the proliferation of Treg cells, produced NO, and expressed iNOS, arginase-1, IL-10 and TGF-β, suggesting that ESC-MDSCs have same pathway to suppress proliferation of T-cells as *in vivo* MDSCs.

To assess *in vivo* immunosuppressive activity of ESC-MDSCs, GvHD model experiment was performed. Lethally irradiated BALB/c mice without any treatment died within 15 days, whereas mice injected with T-cell-depleted bone marrow cells were rescued and survived for more than 100 days. Additional injection of allogeneic T cells isolated from 129SvEv mice caused severe symptoms of GvHD (loss of hair, hunched posture, diarrhea, and weight loss), and died within 40 days. However, co-grafting of ESC-MDSCs protected the host mice from GvHD lethality, and 81.8% of hosts survived for more than 100 days. This result strongly suggests the immunosuppressive activity of ESC-MDSCs, and their therapeutic efficacy against GvHD.

Interestingly, ESC-MDSCs can be split into two populations by expression of Ly-6C. $CD115^+$ $Ly\text{-}6C^-$ population shows slightly higher suppressive activity than $CD115^+$ $Ly\text{-}6C^+$ population. Recent study indicated that MDSCs isolated from tumor-bearing mice consist of two subsets; granulocytic MDSCs and monocytic MDSCs [46]. The differences in phenotype and suppressive activity suggest that $CD115^+$ $Ly\text{-}6C^-$ ESC-MDSCs are granulocytic, and $CD115^+$ $Ly\text{-}6C^+$ ESC-MDSCs are monocytic. Indeed, colony forming unit assay showed that $CD115^+$ $Ly\text{-}6C^-$ population has potential to differentiate into granulocyte lineage and macrophage lineage, while $CD115^+$ $Ly\text{-}6C^+$ population can differentiate into only macrophage lineage. These results suggest that ESC-MDSCs not only have the potential for therapeutic use, but also are useful to examine the sub-populations of MDSCs.

Regulatory Dendritic Cells

Regulatory dendritic cells (DCreg cells) are sub-population of DCs characterized by a weak expression of MHC and co-stimulatory molecules and a weak ability to activate effector

T cells, although they are able to induce the proliferation of Treg cells and the anergy of autoreactive T cells to induce immune tolerance [47–49]. Therefore, DCreg cells have been tested for the treatment of GvHD [50] and various antoimmune diseases [51].

Recently, our group reported generation of DCreg cells from mouse iPS cells, and demonstrated immune regulatory functions *in vitro* and *in vivo* [52]. Our method consists of three steps. First, iPS cells were cultured on a layer of OP9 cells, and differentiated into hematopoietic lineages (step 1). Resulting hematopoietic progenitor cells and OP9 cells were harvested together, and suspended in medium containing GM-CSF. The cell suspension were re-seeded on new cell culture dish, and cultured for additional 2 days (step 2). During the step 2 process, DC progenitor-like cellular aggregates gradually arose from the iPS cells-derived cells mixed with OP9 cells, accompanied by the increased expressions of CD11b and CD11c in these floating cells. Finally, the DC progenitor-like cells were transferred into low cell adhesion plates, and cultured in medium containing GM-CSF, IL-10 and TGF-β (step 3). Through these three steps, mouse iPS cells gave rise to DCs, which exhibited much lower expression of CD40, CD80 and MHC class II than conventional DCs generated from bone marrow (BM-DCcon cells). In terms of morphology and surface marker, our iPS cells-derived DCs (iPS-DCreg cells) were similar to bone marrow-derived DCreg cells (BM-DCreg cells).

iPS-DCreg cells also demonstrated immunosuppressive capacities. While BM-DCcon cells stimulate the proliferation of allogeneic T cells, iPS-DCreg cells exhibited almost no stimulatory effect, like BM-DCreg cells. In addition, iPS-DCreg cells suppressed the T-cell activation by BM-DCcon cells. In the co-culture, the total number of T cells were not elevated, while $Foxp3^+$ Treg cells were up-regulated, suggesting that iPS-DCreg cells suppressed the proliferation of T cells via the stimulation of Treg cells. Moreover, the number of popliteal lymphocytes was increased in the mice injected with BM-DCcon cells. When the mice were treated with iPS-DCreg cells and BM-DCcon cells together, the proliferation of lymphocytes was suppressed. Taken together, these data show that iPS-DCreg cells effectively inhibit allo-immune lymphocyte proliferation *in vitro* and *in vivo*.

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are fibroblast-like multipotent cells existing in connective tissues [53–55]. MSCs have potent suppressive effects on immune cells through various pathways [56,57]

Various methods to generate PSCs-derived MSCs have been reported. Barberi et al. reported that human ES cells co-cultured on a layer of OP9 cells for 40 days gave rise to $CD73^+$ MSC like cells [58]. These cells were negative for hematopoietic-lineage markers, while expressed typical surface marker identical with MSCs. However, only 5% of differentiated cells showed the phenotype of MSCs, and needed to be sorted for further applications. EBs formation could also help the differentiation toward MSCs [59,60]. Human ES cells-derived EBs were plated onto gelatin-coated plates, and cultured for 2 weeks, resulting in $CD73^+$ $STRO\text{-}1^+$ $CD45^-$ MSCs. Trivedi et al. proposed different approach [61]. In this protocol, human ES cells maintained on Matrigel were differentiated into spindle-shaped fibroblast-looking cells by increasing the interval of culture media changes. During this period, undifferentiated state of ES cells should be removed manually, and remaining semi-

differentiated cells were harvested and cultured with MSC medium (αMEM medium + 10% FCS). Through the two steps of culturing, human ES cells gave rise to MSCs (hESC-MSCs). hESC-MSCs suppressed the proliferation of T cells stimulated with allogeneic PBMC in mixed lymphocyte reaction (MLR), suggesting the immunosuppressive capacity. Recently, Kimbrel et al. reported the improved method to generate hESC-MSCs and their therapeutic efficacy in autoimmune disorder models [62]. In this method, human ES cells were once guided toward EBs, followed by differentiation into hemangioblast by culturing in the methylcellulose-based medium containing VEGF, Flt-3L, TPO, and bFGF. Hemangioblast formation from EBs took 7−12 days. Harvested hemangioblast cells were plated on Matrigel-coated dish, and cultured in MSC medium, resulting into MSCs. Going through a hemangioblast intermediate can contribute to avoid possible carry-over of residual undifferentiated cells, and also helps to expand the progenitor cells of MSCs before further differentiation, resulting in elimination of non-MSC-lineage cells. Indeed, ≥ 95% of living cells generated in this method showed the phenotype of MSCs ($CD73^+$ $CD90^+$ $CD105^+$), suggesting the high purification efficacy.

To assess the immunomodulatory capacity of hESC-MSCs, the effects on DC maturation and proliferation of T cells in MLR were examined. In the former examination, DCs were generated from PBMC monocytes, and the maturation was triggered by IFN-γ and lipopolysaccharide (LPS). Without hESC-MSCs in culture, the expression of maturation marker (CD83) was elevated, while up-regulation of CD83 was suppressed by co-culture with hESC-MSCs. hESC-MSCs also suppressed secretion of IL-12. In the later assessment, the proliferation of PBMCs stimulated by allogeneic immature DCs was significantly suppressed in co-culture with hESC-MSCs. These results indicate potent and broad spectrum of immunosuppressive abilities of hESC-MSCs *in vitro*.

In addition, hESC-MSCs showed therapeutic activity in autoimmune disease animal models. Intravenously injected hESC-MSCs were found to prolong the survival period of BWF1 mice, which are prone to lupus nephritis. hESC-MSCs were efficient against experimental autoimmune uveitis (EAU), which is also a commonly used autoimmune disease model. The symptoms of uveitis in C57BL/6 induced by immunization with retinal protein IRBP were significantly reduced by administration of hESC-MSCs. These results demonstrate that hESC-MSCs have potential to work as immunosuppressive cell medicine.

Conclusion

For clinical use of immunosuppressive cells derived from PSCs, the balance between benefits and risks for patients should be considered. ES /iPS cell technologies can address the major obstacles of previous cell medicine, especially the number and homogeneous property of the available cells. On the other hand, there still remain many problems hindering the practical application. One major technical issue is contamination of animal products, such as feeder cells and serum. A xeno-free method to maintain PSCs to differentiate into several kinds of mature cells has been developed as mentioned above. As for immunosuppressive cell medicine, we should make considerable efforts toward the same kind of technical progresses.

Time and costs of production are another problem. Ordinarily, it takes a long time to establish PSCs and differentiate them into mature cells of interest. Recently, Haruta et al.

established myeloid-lineage progenitor cell line from human iPS cells, which has long-term proliferation capacity and abilities to differentiate into DCs and macrophages [63]. Preparing such proliferative intermediates may contribute to saving time and costs. The quality evaluation of PSCs is also important. The undifferentiated state and differentiation tendency are considered to widely differ depending on cell source, establishment condition, culture method and other factors. The way to evaluate is to do so in one of the active centers in pluripotent stem cell research. Taken together, it will be necessary to combine appropriate cell source and differentiation methods to improve the benefits and reduce the risks in future regenerative medicine.

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Chapter 22

Does MSCs Have a Place in Transplantation?*

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Abstract

The solution to a lot of patients diagnosed with organ-failure is transplantation. The administration of immunosuppressive drugs to the transplant recipient has made solid organ transplantation a practical therapeutic option, as rejection of the engraftment by the recipient immune system can be belated or thwarted. Transplant recipients are more predisposed to complications such as renal failure, heart diseases and opportunistic infections due to the long-term use of these broad-based immunosuppressive drugs. This has created the need for alternative immunosuppressive therapeutic strategies that are specific and prolongs the survival of the transplanted foreign tissue. The ultimate goal of transplantation is to achieve a state of immune acceptance or tolerance to the transplanted organ and emerging evidence in the literature suggest that Mesenchymal stem cells (MSCs), may be the solution due to the their inherent ability to modulate immune responses via direct and indirect interaction with a broad range of different cells that make up the innate and adaptive immune system. Given the potential of MSCs to modulate the immune response of different cells that play a critical role in allograft rejection, these has led to the investigation of these cells to prolong allograft survival in a number of animal transplant models and clinical trials.

Keywords: Mesenchymal stem cells (MSCs); transplantation; immune cells; clinical trials; solid organs; complement; immunogenicity; autoimmune disease; wound healing

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Abbreviations

bFGF: Basic fibroblast growth factor
BM: Bone marrow
BLIMP-1: B lymphocyte-induced maturation protein 1
CCL2: Chemokine (C-C motif) ligand 2
CCR2: C-C chemokine receptor 2
CsA: Cyclosprorine A
DC: Dendritic cells
GvHD: Graft versus host disease
hDMSC: Human dermal mesenchymal stem cell line
HGF: Hepatocyte growth factor
HSCs: Haploidentical hematopoietic stem cells
IDO: Indoleamine 2, 3-dioxygenase
IGF-I: Insulin-like growth factor
LPS: Lipopolysaccharide
MMPs: Matrix metalloproteinases
MSCs: Mesenchymal stem cells
NK: Natural Killer
PAX5: Paired box protein 5
PGE_2: Prostaglandin E2
STAT3: Signal transducer and activator of transcription 3
TGF: Transforming growth factor
Tregs: T regulatory cells

Introduction

Mesenchymal stem cells (MSCs) are multipotent plastic-adherent progenitors that are capable of self-renewing with the inherent potential to differentiate into other cell types such as adipocytes, skeletal myocytes, osteocytes and chondrocytes [1, 2]. Recently, MSCs have been reported to differentiate into non-mesodermal origin such as neural cells, and hepatocytes [3]. They can be identified based on the surface expression of the following markers CD62L, CD50, CD54, CD166, CD106 and CD49. Additionally, they are negative for the co-stimulatory molecules CD86, CD80, CD40 and CD40L [4]. MSCs express the chemokine receptors CXCR4, CXCR5 and CCR1, which have been implicated in the localization of MSCs within transplanted allograft or sites of injury post-infusion [5]. Although, MSCs are positive and negative for certain markers, there is currently no single definite marker that distinguishes MSCs from other cell types. MSCs can also secrete cytokine and growth factors within localized trauma site, which contributes to the therapeutic interest and clinical application in various disease models. The bone marrow (BM) is the most common source from which MSCs are isolated. However, there are many other sources from which MSCs have been isolated such as the dermis, placental, umbilical cord, dental tissue, adipose and skeletal muscle. MSCs from these different tissue sources behave in a similar

manner, in regards to their ability to differentiate, undergo self-renewal, and modulate immune response.

MSCs Differentiation

MSCs that has been isolated from different human sources such as the BM and umbilical cord has been demonstrated to have the capability to differentiate into functional hepatocyte-like cells in vitro. Lee et al. observed that four weeks post-in vitro culture with hepatocyte growth factor (HGF) and oncostatin M, this isolated MSCs displayed features that are characteristic of hepatocytes such as a cuboidal morphology and expression of liver specific cell markers. These differentiated cells were observed to also function similarly to endogenous liver cells in vitro, by examination its ability to produce albumin, secrete urea, store glycogen and its uptake of low-density lipoprotein [6]. The differentiation potential into hepatocyte-like cells was also validated in a different study using MSCs that was derived from adipose tissue acquired from lipectomy. Adipose-derived MSCs was observed to have similar differentiation ability as BM derived MSCs when cultured in vitro in the presence of growth factors and cytokines. However the investigators also reported that adipose-derived MSCs required a longer in vitro culture period to fully differentiate and these differentiated hepatocyte-like cells also had a higher proliferative capacity compared to hepatocyte-like cells that were differentiated from BM MSCs [7]. The ability of MSCs to differentiate into hepatocyte-like cells highlights their clinical potential for the treatment of liver diseases. The administration of MSCs has been demonstrated to alleviate CCL4-induced liver injury in mice [8−10].

MSCs also have the potential to undergo myogenic differentiation into cardiomyocyte-like cells. Toma et al. investigated the ability of lacZ-labeled human-derived BM MSCs to undergo in vivo myogenic differentiation after transplantation into the left ventricle of adult mice. The investigators observed that only a small fraction of the infused BM MSCs survived a week post-infusion; however over-time these cells acquired similar morphology of neighboring cardiomyocytes. The newly differentiated cardiomyocytes also expressed similar desmin, beta-myosin heavy chain, and cardiac topronin T expression levels as surrounding host cardiomyocytes by immunohistochemistry staining [2]. In a different study, Potdar et al., validated the cardiomyocte differentiation potential of MSCs using skin-derived MSCs from humans [11]. The ability to use MSCs from the skin provides the best minimally non-invasive source, given that the skin covers more than 90% of the human body. The investigators used a human-scalp biopsy from androgenic alopecia patient to generate human dermal mesenchymal stem cell line (hDMSC). This hDMSCs cell line was further treated with 5-Azacytidine to undergo differentiation into cardiomyocytes. In a different study, Xie et al., observed that hypoxia/reoxygenation-conditioned medium has the ability to induce BM MSCs differentiation into cardiomyocytes-like cells. The expression of myosin heavy chain and troponin T were detected in the MSCs that were cultured in the hypoxia-conditioned medium and not in the MSCs cultured with normal cardiomyoctes medium. This was the first study indicating the cardiomyocte culture medium have the ability to induce MSCs differentiation into cardiomyocytes-like cells [12]. Given that MSCs is able differentiate into

cardiomyocyte-like cells, it makes it an ideal candidate to replenish injured myocardium following ischemic injury that can result in heart failure.

MSCs have also been reported to differentiate into neuronal-like cells in vitro. Jang et al., demonstrated that MSCs derived from human adipose tissue have the potential to differentiate into neural cells in vitro in the presence of basic fibroblast growth factor (bFGF) and forskolin [13]. The mechanisms by which MSCs differentiate into neuronal like-cells are poorly understood. Jing et al., reported that decreased Notch signaling by microRNA-9 could be one of the mechanisms by which MSCs can differentiate into neurons [14]. In a different study, epidermal growth factor receptor signaling was also reported to promote the differentiation of MSCs from the umbilical cord into neuronal like cells [15]. The *in vivo* potential of MSCs to differentiate into neuron like-cells in mice was investigated in a study by Kopen et al. The investigators observed that 12 days post-injection of MSCs into the lateral ventricle of neonatal mice, these injected cells migrated into the cerebellum and forebrain. A few cells within the striatum were thought to have differentiated into mature astrocytes based on the expression of glial fibrillary acidic protein. Migrated cells within the brain stem were positive for neutrofilament suggesting that they had differentiated into neurons [16]. The therapeutic potential of differentiated neuronal-like cells from MSCs that were isolated from Wharton jelly of the umbilical cord was examined in an Alzeheimer disease mouse model [17]. Yang et al., observed an improvement of memory, enhanced levels of synapsin 1 level and significant decreased in amyloid β-peptides deposition after transplantation of differentiated neuronal-like cells into an AβPP/PS1 transgenic Alzheimer disease mouse model. The investigators also observed increased expression of anti-inflammatory cytokines associated with M2-like microglia activation compared to pro-inflammatory cytokines. The result from this study was an indication that the transplantation of differentiated neuronal-like cells from MSCs may be a therapeutic option for the treatment of Alzheimer disease.

MSCs have been reported to have the potential to differentiate into chondrogenic and osteogenic like-cells. Longobardi et al., observed that insulin-like growth factor (IGF-I) and TGF-β1 had a synergistic effect on BM-MSCs differentiation into chondrocytes. However, in the absence of TGF-β1 signaling, IGF-1 could still induce chrondrogenesis. The differentiated chrondrocytes had chondrogenic gene profiles similar to human primary chondrocytes that have been cultured in vitro [18]. Kon et al., reported that the transplantation of autologous MSCs were able to repair large bone defects in sheep [19]. In an in vivo human study, it was reported that in five of six children with osteogenesis impercata that received BM-derived MSCs, displayed significant growth six months post-infusion.. In a different study, purified CD105 positive human MSCs exhibited the potential to form bone *in vivo* [20].

The clinical possibility that differentiated MSCs could be used for tissue repair purpose was validated in a study by Horwitz. The effect of autologous BM stromal cell transplantation for the repairment of articular cartilage defects was examined in two patients, whose knee pain prevented them from walking normally. BM cells from the illac crest of each patient were passaged in vitro and clinical symptoms significantly improved six months post-transplantation [21].

MSCs Modulation of Immune Cells

MSCs and T Cells

The activation of T cells and subsequent differentiation into different effector subsets are critical early events that results ultimately in transplant rejection [22]. The presentation of alloantigen to T cells by donors or recipient antigen presenting cells following solid transplantation results in T cell proliferation, differentiation into different subsets that mediate rejection [23, 24]. In vitro studies have shown repeatedly that MSCs can suppress T cell immune response, independent of the stimuli (mitogen or alloantigen) [25, 26]. The inflammatory milieu when MSCs interacts with T cells is vital for MSCs induced suppression of T cell immune response. Comoli et al., reported that under anti-inflammatory milieu, MSCs is unable to suppress T cell proliferation, except under proinflammatory conditions [27]. These results correlate with other studies that indicated that proinflammatory cytokines such as IFN-γ, TNF-a and IL-1β are required for MSCs activation [28]. Additionally, IFN-γ was shown to be required for MSCs mediated suppression of graft versus host disease (GvHD) disease progression *in vivo* [29].

T cell differentiation is preferentially skewed towards T regulatory cells (Tregs) and Th2 cells compared to Th1 and Th17 cells when cocultured with MSCs. This preferential skewness in differentiation was observed in several mouse allograft transplant models and autoimmune diseases [30−32]. The increase in Tregs population in the presence of MSCs is very beneficial in a transplant setting. Tregs can promote tolerance by inhibiting alloreactive T cell response. However, in a different study, MSCs was shown to suppress T cell proliferation independent of Treg presence, indicating multiple other mechanisms of T cell immune suppression. The mechanism of MSCs mediated T cell suppression is dependent on several factors such as direct cell contact, as well as the secretion of soluble factors such as prostaglandin E2 (PGE_2) and Transforming growth factor β (TGF β) and hepatocyte growth factor [33−35].

MSCs and B Cells

B cell mediated immune responses have been implicated in both acute and chronic rejection [36]. B cell proliferation and antibody secretion in response to lipopolysaccharide (LPS) stimulation has shown to be suppressed by MSCs in a dose-dependent manner [37]. The secretion of matrix metalloproteinases (MMPs) and chemokine (C-C motif) ligand 2 (CCL2) was implicated as a potential mechanism by which MSCs can inhibit antibody secretion in vitro. It was demonstrated that MMP-cleaved CCL2, binds to the C-C chemokine receptor 2 (CCR2) receptor on the B cell surface inducing signals that inhibit AKT and Signal transducer and activator of transcription 3 (STAT3) phosphorylation, while inducing expression of paired box protein 5 (PAX5), thereby inhibiting antibody secretion in vitro [38]. PAX5 is an antagonist of B lymphocyte-induced maturation protein 1 (BLIMP-1) expression, which is important for immunoglobulin synthesis and secretion by plasma cells. This observation was further validated in a different study, in which the authors demonstrated that MSCs could suppress B cell differentiation and immunoglobulin synthesis via inhibition of

BLIMP-1 expression [37, 39]. However the soluble factors mediating this suppression was not identified. Some studies are contradictory to the suppressive function of MSCs on B cell differentiation and immunoglobulin secretion [40, 41].

MSCs and DC

Dendritic cells (DC) upon presentation of capture antigen from the peripheral can rapidly activate lymphocytes. Immune response against transplanted allograft resulting in rejection can be initiated in a similar manner upon presentation of alloantigen to B or T cells [24]. The differentiations of precursor cells into DC and maturation process upon activation have been reported to be suppressed by MSCs [42]. Co-cultured DCs with MSCs, are unable to fully induce T cells to proliferate and secrete cytokines [43]. The ability of MSCs to modulate antigen-presenting cell function such as DC, allows it regulate the recipient immune system ability to mount an immune response against the transplanted allograft, therebye promoting survival and function.

One of the ways that MSCs can modulate DC immune response is to induce an immature and tolerogenic phenotype of DC after interaction in coculture [44, 45]. This newly induced tolerogenic phenotype is correlated with a decrease in the expression of CD80, CD86 and CD40 with an increased in CD11b expression [45]. The ability of DC to induce T cell proliferation and differentiation into a Th-1 phenotype is impaired in the tolerogenic state. This induced toleregenic state cannot be reversed with LPS stimulation [45]. The tolerogenic DC secreted elevated levels of anti-inflammatory cytokines compared to normal mature DCs [45]. T cells already undergoing proliferation were rapidly suppressed in the presence of tolerogenic DC cells [45]. An increase in the frequency of Treg cells within the overall T cell population has also been observed in presence of tolerogenic DCs [31].

The down-regulation of tissue adhesion molecules and up-regulation of chemokine receptors facilitates the migration of DC from the peripheral to secondary lymphoid tissues such as the spleen and lymph node, followed by subsequent antigen presentation to T and B cells. English et al., demonstrated that MSCs can suppress the up-regulation of CCR7 on DC following stimulation and reduce the ability of DC to migrate toward chemokines typically found in the lymph node, while anchoring proteins expression were not affected. Additionally, it was also shown that DC antigen presentation to T cells was also impaired in the presence of MSCs [46]. The secretion of soluble factors such as IL-6 and PGE_2 by MSCs has been implicated in MSCs-induced tolerogenic DC phenotype. In a transplantation setting, the infusion of MSCs would retain DCs within the peripheral and for those DCs that were able to escape retention; their ability to present alloantigen to T cell would be impaired. Therefore, alloreactive T cell activation and immune response against the transplanted allograft would be significantly suppressed.

MSCs and NK Cells

The activation of Natural Killer (NK) cells can significantly affect the outcome of the transplanted allograft. NK cells can be activated by allogeneic cells due to the lack of self-MHC molecules on the cell surface, which results in the secretion of proinflammatory

cytokines and induction of cytototixicity response [24]. Spaggiari et al., reported that MSCs impaired IL-2 induced NK cell activation, proliferation and proinflammatory cytokine secretion [47]. The result from this study was also validated in a different study by sotiropoulou et al. The authors showed that the phenotype of NK cells were altered in the presence of MSCs and the ability of NK cells to proliferate, secrete cytokines and lyse target cells were also suppressed. The mechanisms of MSCs mediated suppression of NK function were attributed to cell-to-cell contact and the effect of soluble factors such as TGF-β and PGE_2 [48].

MSCs and Complement

Complement as an important part of the innate immunity plays critical roles in transplant rejection [49, 50]. It is well-established that hyperacute rejection is primarily mediated by complement, which has been induced by natural antibodies [51]. Besides, in chronic humoral rejections, complement also serves as one of the effector mechanisms to attack the allograft after alloantibodies are developed [50, 52, 53]. Emerging evidence also suggest that complement plays a directly role in regulating graft-specific T cell responses. It has been reported at least in animal studies that complement deficient kidney allografts are spontaneously tolerated while complement sufficient renal grafts are rejected [54]. It is also observed that complement activation products C3a and C5a promote $CD4^+$ and $CD8^+$ T cell responses in murine models of heart and renal transplantation while augmenting or inhibiting their signaling significantly enhances or reduces graft rejections [55−57]. Taken together, complement could be another target for transplantation rejection prevention. MSCs have been shown to constitutively produce factor H, the most potent complement inhibitor in the fluid phase, which could contribute to their broad immunoregulatory activity in both the adaptive and the innate immune systems [58, 59].

MSCs Immunogenicity

Because of the potent activity of MSCs to inhibit both the innate and the adaptive immune system, and because MSCs express low levels of MHC I/MHC II, these cells are believed to be immunoprivileged [60]. Because of this belief, and other advantages such as convenience and cost-effectiveness, allogeneic MSCs are widely used in clinical trials. However, it is also observed, both in animal and human studies that infused MSCs do not last long after infusion, suggesting that MSCs could be recognized and attacked by the host immune system after infusion, leading to quick clearance from the body [61]. Indeed, it has been reported that immediately after their contact with blood, MSCs activate the complement system in the innate immunity. Despite the presence of cell surface complement regulators and their production of the complement inhibitor factor H, MSCs are attacked and damaged by the activated complement, leading to reduced cell viability and impaired function [62]. In addition to be recognized and attacked by the complement in the innate immune system, MSCs are found to be able to up-regulate levels of MHC I and MHC II under inflammatory conditions, and subject to T and B cell-mediated attack [60]. Given that MSCs are being developed as a new therapy for many inflammatory diseases, the issue of MSCs

immunogenicity needs to be addressed to improve the outcome of MSCs-based therapy for transplantation, and other disease conditions.

The ability of MSCs to modulate the functions of various cells that are critical in mediating allograft rejection highlights its potential to promote the survival and function of transplanted organ. One of earliest experimental studies demonstrating the *in vivo* immunological suppressive role of MSCs was done using a baboon skin allograft model. The authors reported that systemic administration of MSCs extended skin allograft survival (11 days) in baboon compared to controls (7 days). The addition of MSCs to a mix lymphocyte reaction (MLR) or mitogen-stimulated lymphocytes significantly reduced their proliferative activity by more 50% in vitro [63]. In another study, the immunosuppressive property of MSCs was examined in vivo in a murine allogeneic BM transplantation models. The timing and amount of number of times MSCs were infused was deemed critical in controlling GvHD in mice transplanted with haploidentical hematopoietic stem cells (HSCs) [64]. Using a murine melanoma tumor model, Djouad et al. showed that subcutaneous injection of B16 melanoma cells resulted in tumor growth in allogeneic recipients only when co-transplanted with MSCs. The infusion of MSCs via IV also resulted in tumor growth indicating that cell contact isn't the only mechanism of promoting tumor growth but also the secretion of soluble factors can also promote the tolerance of the transplanted melanoma cells [65].

MSCs and Sold Organ Transplantation in Animal Models

Kidney

For patients with end-stage renal disease, kidney transplantation has dramatically improved their way of life. Unfortunately for these patients, the long-term survival graft survival has not improved over the last decade and each year in the U.S. and abroad, there are thousands of kidney failures. Alloreactivity response mediated at the cellular and humoral level is critical for the loss of kidney allograft despite administration of immunosuppressive drugs [66]. The administration of MSCs was reported to prevent acute renal allograft rejection via the induction of Tregs in a life supporting kidney allograft model [67]. In the same study, it was observed that the secretion of indoleamine 2, 3-dioxygenase (IDO) by MSCs was essential for the generation of Tregs and graft tolerance. B cells can also contribute to allograft rejection via the production of anti-donor antibodies and antigen presentation to T cells [36]. Several in vitro studies have indicated that MSCs can also suppress B cell proliferation, cytokine secretion and antibody production when stimulated in vitro with CpG. Since B cell humoral responses are mostly dependent on T cell help, MSCs suppressive effect on T cell function may indirectly impair B cell functions. The investigators also reported that MSCs suppression of B cell function occurred only in the presence of T cells. To determine if B cell immune response was also suppressed following infusion of MSCs, the levels of circulating levels of antidonor antibodies in the serum was measured and found to be lowered in the MSCs treated group. These results validated previous observed in vitro studies demonstrating that MSCs express IDO, which has been reported to suppress T cell function [68, 69]. Data from this study highlighted the ability of MSCs to modulate the immune

response of multiple immune cell types. In a different study, it was shown that infusion of MSCs attenuated acute cellular renal rejection in Lewis rat via reduction of CD8 T cells infiltration into the transplanted organ [70]. In a recent study, the mechanism of infused MSCs migration to the transplanted kidney allograft was examined in acute rat renal allograft rejection model. The investigators observed that modulation of CXCR4 expression on MSCs using lentiviral overexpression or knockdown impaired or enhanced MSCs localization to the transplanted kidney allograft resulting in acute rejection or tolerance. MSCs proliferation and cytokine secretion were impaired when CXCR4 expression was reduced. Conversely, the localization, proliferation, cytokine secretion and graft tolerance was enhanced when CXCR4 was overexpressed on infused MSCs [71]. Majority of the kidney transplant animal models mentioned have primarily focused on acute allograft rejection, which is very manageable with immunosuppressants. This raises the question if delayed administration of MSCs can also reverse ongoing rejection inflammatory processes and prolong renal allograft function and tolerance. Interestingly, Franquesa et al., reported that a single administration of MSCs at 11 weeks post- kidney transplantation was sufficient to provide immediate protection against the inflammatory and fibrotic processes that lead to kidney allograft rejection in Lewis rats [72]. The MSCs treated group reportedly had up-regulated expression levels of IDO, which was correlated with decreased inflammatory genes within the engraft tissue. This result indicates that MSCs based therapy may be effective in preventing anti-donor reactivity as well as reducing active rejection in kidney organ transplantation.

Islet

An appealing noninvasive therapy for patients with type-1 diabetes is islet transplantation. While, the Edmonton protocol and its derivatives have shown promising results in restoring normal glucose levels, they fail to provide long-term insulin independence resulting in most patients requiring exogenous insulin within two years of post-transplantation [73, 74]. The co-transplantation of MSCs and pancreatic islet under the kidney capsule has been demonstrated to promote allogeneic islet allograft tolerance and survival in BALB/c mice via metalloproteinases dependent mechanisms. It was observed that MSCs can secrete MMP-2 and MMP-9 in vitro and the inhibition of these metalloproteinases both *in vitro* and *in vivo* can impair MSCs mediated suppression of T cell proliferation and cytokine responses [75]. The ability of MSCs to modulate immune cell response has been determined to be via either cell-to-cell contact or through an indirect mechanism that includes secretion of soluble factors that have suppressive effect. Using a rat model of streptozotocin-induced diabetes, the author investigated the effect of systemic administration of MSCs on preventing acute syngeneic and allogeneic islet rejection and improving glucose homeostasis. It was observed that systemic administration could also prolong islet graft survival in a dose-dependent manner, which was also coupled with reduced levels of the pro inflammatory cytokines IFN-y and GM-CSF in blood circulation [76].

In a different study, the route of co-transplantation of MSCs and pancreatic islet was examined more extensively using a marginal islet mass diabetic mouse transplantation model. The co-transplantation underneath the kidney capsule, anterior chamber under the eye, and in the hepatic vein improved glucose homeostasis, reduced islet apoptosis but did not increase beta-cell proliferation [77]. A lot of the islet transplantation models have focused on the

immunosuppressive aspect of MSCs on prolonging graft survival after co-transplantation, neglecting the effect of MSCs on revascularization and graft morphology which are also likely to contribute to graft survival and function giving that MSCs secrete trophic factors. This issue was addressed by transplantation of islet alone or co-transplantation with MSCs underneath the kidney capsule and monitoring glucose levels, graft morphology and vascularization [78]. Greater than 90% of the mice co-transplanted with MSCs had normal glucose levels compared to 42% of mice transplanted with islet alone, highlighting the ability of MSCs to improve islet graft function as previously reported. The morphology and vascularization of the islet co-transplanted with MSCs was similar to endogenous pancreas. Highlighting another mechanism by which MSCs can prolong islet-graft survival. In a different study using co-transplantation of MSCs and islet within an encapsulated alginate, Kerby et al., were able to eliminate revascularization and preservation of graft morphology as mechanism of MSCs mediated islet graft survival and tolerance [79].

Heart

MSCs can prolong the survival and function of semi-allogeniec heart transplant in unconditioned recipient mice by increasing Treg frequency and reducing pro-inflammatory cytokines within the graft of the MSCs treated-group [30]. These observation was further collaborated in a different study in which, inbred wistar rat heart were transplanted onto recipient Fisher344 rats followed by intravenous infusion of donor MSCs [80]. The survival of the transplanted allograft was strikingly extended after infusion of MSCs with a mean survival day of 12d compared to 6d of the control group. Additionally, MSCs also suppressed T cell responses in vitro and in vivo. The heart allograft from the MSCs-treated group displayed a significant reduction in Th-1 like cytokines and up-regulated expression of Th-2 like cytokines. These data indicated that MSCs may promote heart allograft tolerance by skewing T cell immune response towards an anti-inflammatory phenotype. Majority of the studies addressing the effect of MSCs on heart transplantation has been done via intravenous (IV) infusion of MSCs and considered very effective. However the route of intrathymic (IT) infusion of MSCs has remained largely unexplored. The effect of IT infusion of MSCs on cardiac allograft was addressed in a rat heart allograft transplant model [81]. The survival of the cardiac allograft was significantly extended in the IT infusion group compared to IV infusion group with a mean survival of 17.4 days compared to 11.5 days of the IV group. A synergistic effect was also observed with a combination of both IV and IT infusion with a mean survival of 32 day. The potential mechanism of MSCs mediated extended survival was also reported to involve Treg expansion and Th-2 mediated anti-inflammatory cytokines also previously reported in other cardiac allograft transplant models. In contrast to previous studies supporting a role for MSCs in prolonging cardiac allograft survival and function, Wu et al., reported that MSCs after infusion rapidly migrated to the engrafted tissue. These MSCs infused group displayed shortened graft survival compared to control group that received lactacted ringer solution alone. This difference could be due to in vitro retro-viral manipulation of the infused MSCs prior to infusion [82].

Liver

The immunosuppressive properties and ability to differentiate into hepatocyte-like cells has generated clinical interest in examining the effect of MSCs on liver injury and potential role in prolonging liver graft survival. Yu et al., reported that implantation of HGF over-expressing MSCs via the portal vein in a rat liver transplantation model prevented liver failure. HGF has been reported to have a protective effect on hepatocytes and promote the differentiation of activated hepatic stem cells into the liver parenchyma. They also demonstrated that HGF-expressing MSCs treated group had reduced I/R-induced injury and accelerated organ regeneration during early post-transplantation. The infused HGF-expressing MSCs migrated into the liver graft and subsequently differentiated into hepatocyte-like cells. This was probably instrumental in prolonging the survival of the graft and preservation of the normal liver morphology [83]. A lot studies have focus on the role of MSCs to treat different liver injuries, which may occur after transplantation. Kuo et al., reported that MSCs infused either via IV or IT route migrated into the recipient liver and rescue liver failure possibly after differentiating into functional hepatocytes [84]. The infusion of MSCs via the IV route was found to be more effective in rescuing liver failure compared to IT route. In a different study, Kanazawa et al., reported that MSCs could inhibit hepatic ischemia reperfusion injury in rat via inhibition of hepatocellular apoptosis and enhancement of liver regeneration [85]. These studies as well as others have established the proof of principle that co-transplantation of MSCs and liver allograft may aid in prolonging liver graft survival and tolerance. However, animal studies specifically looking at the effect on MSCs on liver allograft are lacking.

MSCs and Immunosuppressive Regimen

Immunosuppressive drugs are administered to majority of the patients that have undergone transplantation; therefore it would be interesting to investigate the effect of these drugs on MSCs function. Several studies have explored the effect of using both MSCs and immunosuppressive drugs on prolonging survival and function of transplanted graft. Ge et al., reported that the administration of MSCs and rapamycin had a synergistic effect on long-term survival and function of a heart allograft from a B6 donor to a BALB/c recipient mice compared to MSCs treated group alone [31]. The synergistic effect of MSCs and conventional suppressive drugs was also reported in several other studies. In a swine hind-limb model, Kuo et al., reported that the combination of MSCs and cyclosporine A prolonged composite tissue allotransplant survival [86]. In acute skin allograft rejection rat model, the IV infusion of MSCs in cyclosporine treated group prolonged allograft survival compared to cyclosprorine A (CsA) alone treated group [87]. In a clinical transplantation setting, the combination of MSCs and immunosuppressive drug would be beneficial to the transplant patient by reducing the amount of workload needed by the drugs to achieve tolerance.

MSCs and Solid Organ Clinical Trials

Majority of the clinical studies in humans have been primarily on the role of MSCs in acute kidney allograft rejection. The general consensuses from these studies are that the infusion of MSCs is safe and clinically possible, although the time point at which MSCs is administered is critically important [88−90]. The early phase infusion was observed to be beneficial to the allograft function, compared to when it was administered after transplantation [91]. Recently in a clinical pilot study, the effect of MSCs in combination with low dose tacrolimus was evaluated in six-kidney transplant patient. None of the patients that received infusion of MSCs experienced any long-term side effects associated with MSCs administration. The MSCs infusion recipients received a lower dose of tacrolimus compared to those that received tacrolimus, indicating that MSCs infusion in renal patients may result in this patient receiving lower dose of immunosuppressive agents. Majority of the trials examining the effect of MSCs on acute renal allograft rejection are promising, however long-term observation of recipients are essential to ensure that there are no adverse reaction to infusion to MSCs.

MSCs Role in Wound Healing

The inherent in vivo ability of MSCs to differentiate into multiple tissues, migrate to the site of injury after infusion and modulate immune responses via secretion of soluble factors are potential mechanisms by which MSCs can stimulate wound healing. Enhanced angiogenesis, accelerated epithelialization and accelerated wound closure have been reported in rodents with acute or diabetic wounds after administration of MSCs. The co-administration of human MSCs and fibroblast growth factor was reported to accelerate cutaneous wound healing in a skin defect model [92]. In a study by Shumakov et al., the investigators investigated the regeneration of deep wounds in 40 Wistar rats after MSCs infusion directly on the burn surface and reported accelerated formation of new vessels and granulation of tissue in the wound after MSCs treatment compared to control. Collectively, these data demonstrate that MSCs can promote wound healing *in vivo*.

Human clinical studies using MSCs to promote wound healing have been promising. The direct application of BM-derived MSCs was reported to induce dermal rebuilding and complete closure of non-healing chronic wounds in three patients [93]. These patients were previously unresponsive to standard and advanced therapies. A study designed by Vojtassak et al., examined a novel technique for the treatment of chronic non-healing wound using autologous MSCs from the patient's BM and skin fibroblasts on biodegradable collagen membrane. The patients received cultured MSCs injection around the edges of the wound on day 7 and 17 [94]. Decreased wound size as well as an increased vascularization of the dermis was observed at 29 days post injections. Falanga et al., reported that the direct application of *in vitro* cultured autologous MSCs using a fibrin spray system onto the acute wounds of patients that underwent skin cancer surgery could significantly decrease wound size within two months [95]. Similar results were also observed in patients that had long-standing non-healing lower extremity wounds five months post-treatment. A correlation between the amount of MSCs applied on the wound surface and reduction in wound size was also

observed. In a cohort of 20 patients with skin wounds, Yoshikawa et al., reported that the administration of autologous MSCs that has been placed in an artificial dermis made of collagen sponge healed the wound in 90 percent of the patients [96].

Systemic administration of MSCs has also been reported to promote wound healing in patients with diabetes and other diseases. The intramuscular administrations of autologous BM in 24 patients with diabetic non-healing foot ulcers was reported to accelerate the wound healing process and improve pain-free walking distance. In the same study, the patient were administered cultured autologous MSCs along with standard wound dressing while the control group only received standard wound dressing and were followed for 3 months. The size of the wound, the ability to walk without any pain, and other biochemical parameters were measure before and at every two-week interval during the 3-month time span of the study. In a randomized double-blind controlled trial study of 41 patients with diabetic critical limb ischemia and foot ulcers by Debin et al., the investigators administered BM derived MSCs, BM-derived mononuclear cells or normal saline intramuscularly into the patients affected limbs. Interestingly, within 6 weeks post administration, significant increase in the healing rate of the ulcer was observed in the MSCs treated group compared to the mononuclear treated group. At 24 weeks of follow-up, there was a significant improvement in pain-free walking in the MSCs treated group compared to the mononuclear treated group [97]. Collectively, these clinical studies suggest that MSCs has clinical benefits when treating non-healing chronic wounds regardless of the route of how it's administered. Although results from these studies are promising, more studies examining the effect the source of MSCs, timing of administration, benefits of MSCs alone or combination with other factors, and numbers of MSCs needs to be thoroughly investigated.

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Chapter 23

Donor Hematopoietic Stem Cells

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Abstract

Inducing chimerism by infusing donor bone marrow cells (DBMC) into transplant recipients can improve clinical outcome. Two strategies have been identified for utilizing DBMC. The first involves the infusion of DBMC along with conventional immunosuppression without intention of engrafting hematopoietic stem cells (HSC). The aim of this strategy is to produce a state of microchimerism, where a small number of donor hematopoietic cells continue to survive in the host after transplantation. Although reciprocal clonal exhaustion has been postulated as an effect of microchimerism, it remains controversial whether microchimerism, often detectable after DBMC infusion, is directly responsible for improved transplant outcome. The second approach couples DBMC infusion with a conditioning regimen to achieve engraftment of donor HSC. The aim of this approach is to produce a state of macrochimerism, where various levels of multilineage donor hematopoietic cells survive in large numbers easily detectable by flow cytometry. These donor cells are presumed to induce clonal deletion of anti-donor progenitor T cells in the thymus. However, since renal allograft tolerance has been achieved even with induction of transient macrochimerism, it has been postulated that some other regulatory mechanism may be involved in DBMC-induced tolerance. More recently, successful induction of renal allograft tolerance has been reported by inducing full donor chimerism, in which recipient hematopoietic cells were totally replaced with donor hematopoietic cells. The consistency and safety of tolerance induction through DBMC infusion needs to be improved before wider clinical application.

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Keywords: Organ transplantation, chimerism, tolerance, donor bone marrow transplantation, hematopoietic stem cells, polymerase chain reaction (PCR) analysis

Abbreviations

AKI: Acute kidney injury
APC: Antigen-presenting cells
ATG: Anti-thymocyte globulin
BMT: Bone marrow transplantation
CKBMT: Combined kidney and donor bone marrow transplantation
CMV: Cytomegalovirus
CNIs: Calcineurin inhibitors
CSA: Cyclosporine
CTL: Cytotoxic T lymphocytes
DBMC: Donor bone marrow cells
DBMT: Donor bone marrow transplantation
GVHD: Graft-versus-host disease
HLA: Human leukocyte antigen
HSC: Hematopoietic stem cells
MHC: Major histocompatibility complex
MMF: Mycophenolate mofetil
NHP: Nonhuman primates
PVP: *Pneumocystis jirovecii* pneumonia
PCR: Polymerase chain reaction
TBI: Total body irradiation
TLI: Total lymphoid irradiation
T_{reg}: Regulatory T cells

Introduction

With the advent of calcineurin inhibitors (CNIs) and other potent immunosuppressive medications over the past three decades, a significant improvement has been achieved in short-term survival rates following organ transplantation. The incidence of serious opportunistic infections, such as cytomegalovirus (CMV) or *Pneumocystis jirovecii* (PJP), also has decreased since the development of effective prophylactic therapy [1]. However, chronic use of immunosuppressive drugs is still associated with a significant risk of cardiovascular disease [2−4], infection [5−7], and malignancy [8−11], which may cause patient death with a functioning graft. In fact, death with a functioning graft occurs in 15% of recipients in the first 10 years after kidney transplantation [12]. In addition to these toxic effects, current immunosuppresive medications fail to consistently prevent the development of chronic rejection, which remains an irreversible process for which there is no treatment.

Therefore, induction of specific immunologic tolerance remains an important goal of organ transplantation, and numerous strategies for inducing tolerance have been developed in

rodent models [13−17]. However, only a limited number of these approaches have been successfully translated to nonhuman primates (NHP) [18−22]. The discrepancy in the ease of tolerance induction between rodents and primate models may be attributed to specific immunological characteristics observed in primates, such as expression of major histocompatibility complex (MHC) class II antigens on endothelial cells [23], the presence of heterologous memory T cells [24−26], and the existence of a more robust inflammatory response [27]. Despite the immunological complexity of primates, donor hematopoietic stem cells (HSC) have been used successfully to induce tolerance in both human and nonhuman primates. Strategies that rely on donor HSC can be categorized as those attempting to achieve microchimerism and those striving for macrochimerism. This chapter reviews the various approaches to tolerance by HSC, which appear to operate through different immunologic mechanisms.

Hematopoietic chimerism is the coexistence of hematopoietic cells derived from two distinct genetic backgrounds in an individual. The definitions vary, but the difference between microchimerism and macrochimerism comes down to the number of detectable circulating cells. Microchimerism involves a very small number of cells (typically < 0.1%), detectable only by polymerase chain reaction (PCR), and the phenomenon has been observed even in transplant recipients receiving conventional immunosuppression. Macrochimerism, on the other hand, is typically induced by HSC transplantation, and the chimerism should be confidently detectable by flow cytometry (typically > 0.1%). Mixed chimerism is defined as a state in which host and donor bone marrow-derived elements coexist in the recipient. Full donor chimerism indicates a state in which the host hematopoietic cells are completely replaced by donor-derived hematopoietic cells. Mixed chimerism has been shown to effectively induce robust allograft tolerance in rodent models [28]. Full chimerism for induction of allograft tolerance in humans has, until recently, been considered too risky for clinical application because of the risk of graft-versus-host disease (GVHD) [29].

Discovery of Chimerism and "Natural Tolerance"

In 1945, Ray Owen reported the presence of red blood cell chimerism in certain dizygotic cattle twins (Freemartin cattle) [30]. He speculated in his report that cross-circulation between twins through placental fusion resulted in a bidirectional, acquired immunologic tolerance that allowed for the continued presence of allogeneic red blood cells in both twins. Around the same time, Peter Medawar found that dizygotic cattle twins were reciprocally tolerant of each other's skin [31] (Figure 23.1). This finding led him to execute his seminal studies on neonatal tolerance, in which tolerance of skin allograft was induced by injecting donor cells shortly after birth of the eventual graft recipient [32].

Reminiscent of Owen's discovery in Freemartin cattle, maternal cells are known to engraft and persist in infants with immunodeficiency even in humans [33]. Maloney et al. later found that microchimerism of maternal origin can persist to adult life in immunocompetent individuals [34]. On the other hand, fetal cells also may persist in maternal blood for decades after pregnancy [35]. However, if reciprocal tolerance is not induced, pre-existing maternal or fetal microchimerism potentially elicits immunological responses. From

clinical similarities between scleroderma and chronic graft-versus-host disease (GVHD), Maloney hypothesized that a maternal microchimerism that persists through childhood and into adult life could play a role in some autoimmune diseases, such as scleroderma [34].

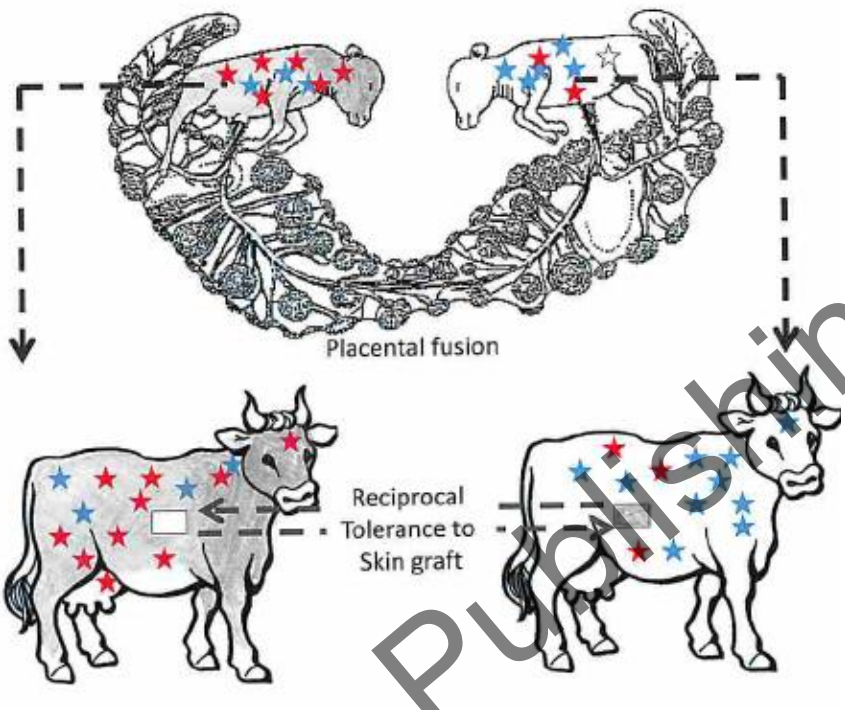

Figure 23.1. The "natural tolerance" of Freemartin cattle. Placental fusion allows cross circulation between two fetuses, which results in continued presence of allogeneic cells (chimerism) after birth [30]. Peter Medawar found that dizygotic cattle twins were reciprocally tolerant of each other's skin [31].

In clinical kidney transplantation, Burlingham et al. evaluated the effect of non-inherited maternal antigen (NIMA) and non-inherited paternal antigen (NIPA) on one haplotype mismatched kidney transplantation [36]. They found that kidney transplant recipients who received NIMA-matched allografts displayed significantly better allograft survival compared with those who received NIPA-matched allografts. These findings support the hypothesis that cells and antigens of the mother modulate the antigen-specific reactivity of the fetal immune system and thereby induce a long-lasting form of tolerance to later antigen challenge in the adult.

Tolerance Through Induction of Microchimerism

Medawar inferred in his 1960 Nobel Prize lecture that "antigens must continue to be present, even though in quantities below the threshold of direct estimation, if a fully non-reactive state is to be maintained [37]." Medawar's theory could not be validated until the advent of PCR technology. In 2003, using sensitive cytostaining and PCR techniques, Starzl

et al. discovered small numbers of donor leukocytes (microchimerism) in 30 recipients of livers or kidneys with long-term stable graft function after all immunosuppression had been discontinued. Starzl concluded that microchimerism must play an essential role in tolerance in these patients [38]. Specifically, he hypothesized that responses of coexisting donor and recipient cells lead to reciprocal clonal exhaustion, followed by peripheral donor-reactive clonal deletion [39]. Starzl's hypothesis was supported by Zinkernagel's study which showed that microchimerism actively maintains cytotoxic T lymphocyte (CTL) unresponsiveness toward a minor histocompatibility antigen by deleting the specific repertoire in the thymus [40].

To enhance microchimerism**,** researchers from Miami subsequently evaluated the effects of DBMC infusion together with conventional immunosuppression in various organ transplants. In cadaver kidney transplantation, they showed a significantly lower incidence of chronic rejection and significantly superior death-censored actuarial graft survival in recipients who received DBMC [41]. Total chimerism levels detected in recipient peripheral blood and bone marrow was 0.074−0.226% and 0.421−1.024%, respectively. However, immunosuppression was not completely withdrawn in these patients.

Other reports have contradicted Starzl's clinical observations. In one study of liver transplantation with DBMC infusion, there was no difference in overall incidence of rejection in patients who received DBMC versus those who did not. Nor was there any increase in the likelihood of successful withdrawal from immunosuppression [42]. Caillat-Zucman et al. looked for evidence of microchimerism in 12 long-surviving (20−30 years) and eight short-term (2 years) cadaveric kidney transplant recipients, but microchimerism was detectable in only 40% of long-term and 14% of short-term recipients [43]. Moreover, in several reports, microchimerism associated with blood transfusion, organ transplantation, and pregnancy has been correlated with allo-sensitization and rejection [44−47]. Thus, to date, clinical analyses have failed to show an unequivocal link between microchimerism and donor-specific hyporesponsiveness.

Although the role of microchimerism in the induction of tolerance remains unclear, DBMC may have other immunological properties that potentially improve allograft survival, and many clinical trials with DBMC have considered this possibility. Monaco et al. were the first to apply infusion of donor bone marrow with anti-lymphocyte serum (ALS) in clinical kidney transplantation [48]. At the time, techniques for detecting microchimerism were not available, and tolerogenic effects by some cellular antigens from bone marrow cells were hypothesized [49]. Mathew et al. demonstrated that while bone marrow cells are immunologically poor stimulators, they have other properties that inhibit CTL generation and induce suppressor (regulatory) T cells [50]. The "veto cells" hypothesis, first described in 1979 by Miller et al. [51], could provide an explanation for the immunological effects of bone marrow cells. There is no distinct cell lineage that defines "veto cells," but they have been described as deletional antigen-presenting cells (APC) which can delete the T cells that recognize them [52, 53]. Attempts to induce clinical renal allograft tolerance by DBMC and anti-thymocyte globulin (ATG) were later tested by Barber et al. [54]. However, in this clinical trial, chimerism was not evaluated and immunosuppression was not successfully discontinued in any of the 57 participants who received DBMC infusion with their kidney allografts [54]. Although complete withdrawal of immunosuppression has never been achieved by DBMC infusion in HLA-mismatched kidney transplantation, a group at Northwestern recently reported successful withdrawal of immunosuppression in half of

recipients of HLA identical kidney allografts when DBMC were administered after alemutuzumab treatment. In this study microchimerism disappeared after 1 year and the regulatory function of T or B regulatory cells and/or the immunodeficiency created by DBMC have been postulated as mechanisms of tolerance [55].

Tolerance Through Induction of Macrochimerism

To induce multi-lineage macrochimerism, HSC engraftment is required. To achieve engraftment of HSC, a treatment such as total body irradiation or cyclophosphamide is necessary to render the host environment permissive for HSC engraftment. Since at least 1.5 Gy of total body irradiation is necessary to achieve engraftment even in syngeneic HSC transplantation, the purpose of the treatment is not to achieve immunosuppression but to create a physiologic space for the expansion of infused hematopoietic stem cells [56].

Preclinical Studies

In the 1980s, tolerance induction by total lymphoid irradiation (TLI) was extensively investigated in large animal models (canines and NHP) and humans. In these studies, DBMC were also infused with TLI, but clear evidence of induction of allograft tolerance through chimerism induction was not reported [57–59]. Based on rodent studies established by Sharabi et al. [60], we have developed a non-myeloablative, clinically applicable preparative regimen to induce mixed chimerism and renal allograft tolerance in fully MHC-mismatched combined kidney and donor bone marrow transplantation (CKBMT) in NHP [19, 61]. This initial regimen included low dose total body irradiation (TBI) (150 cGy x2) plus local thymic irradiation (700 cGy), horse antithymocyte-globulin (hATG), splenectomy, DBMC, and cyclosporine for one month postoperatively. Although the majority of monkeys [11/13] developed multilineage chimerism after conditioning and donor bone marrow transplantation (DBMT), chimerism was not stable and lasted only one month. Nevertheless, 9 recipients achieved renal allograft tolerance with the longest allograft survival exceeding 14 years [19, 62]. This allograft tolerance appeared to be kidney specific, since the same regimen failed to induce heart allograft tolerance [63]. More recent studies have shown that chimerism and renal allograft tolerance can be significantly improved without splenectomy by adding a costimulatory blockade [62]. Nevertheless, it remains challenging to induce stable mixed chimerism in NHP, even with increased numbers of hematopoietic stem cells [64], better MHC matching [65], or multiple costimulatory blockades [65, 66]. It is clear that permanent mixed chimerism is not necessary for long-term renal allograft tolerance, and continued survival of the kidney allograft despite the loss of chimerism suggests involvement of peripheral mechanisms. However, more robust chimerism induction may be necessary to induce non-renal allograft tolerance.

Another problem in our original conditioning regimen was its inapplicability to deceased donor transplantation, as the conditioning regimen must be initiated 6 days in advance of the planned organ transplant. Compressing the regimen into a 24-hour period not only failed to induce chimerism but also caused unacceptable toxicities. An alternative approach, “delayed

tolerance," therefore has been developed to induce tolerance for deceased donor transplantation. In this approach, kidney transplantation is performed with conventional immunosuppression first and then conditioning and DBMT are administered sometime later. However, the kidney allograft can potentially activate memory T cells by the time of DBMT and our study has shown that additional treatment, especially against CD8 memory T cells, was necessary to successfully induce mixed chimerism and renal allograft tolerance [67]. The timing of DBMT is also critical in this approach as recipients who received DBMT shortly after (1 month) kidney transplantation consistently failed to achieve tolerance despite successful induction of chimerism [68]. A detailed analysis revealed significantly higher inflammatory responses at 1 month vs. 4 months after transplantation, which might have prevented tolerance induction.

Clinical Studies

Mixed Chimerism

The first clinical attempt to induce renal allograft tolerance was reported by Murray et al., in 1960 [29]. In this clinical trial, high dose total body irradiation was administered with or without DBMT to induce allogeneic chimerism. However, only one of six patients who received the protocol survived long-term, while others died as a consequence of either infection or hemorrhagic complication. After this report, clinical trials for operational tolerance were not pursued for the subsequent two decades.

In the 1980s, on the basis of large animal studies [57, 58], the Stanford group attempted to induce renal allograft tolerance using a TLI-based regimen with or without donor bone marrow infusion. Although successful discontinuation of immunosuppression was initially reported in three patients [69], two of them eventually lost graft function owing to ureteral stenosis and chronic rejection [70]. One-year graft survival in patients receiving this regimen was 76% [71], which is inferior to graft survival with conventional immunosuppression. The complexity of the regimen, as well as failure to extend graft survival over conventional immunosuppression, hampered further studies using this approach. By the late 1980s, the concept of mixed chimerism as a means to induce allograft tolerance was established [60, 72]. In contrast to the myeloablative regimens for HSC transplantation used to treat malignancies, the conditioning regimens used to induce mixed chimerism are generally nonmyeloablative, which implies that the recipient will recover from the therapy-induced pancytopenia even without engraftment of donor HSC. The advantage of the mixed chimerism approach is that the recipient remains more immunocompetent and is less likely to develop GVHD [72, 73−75]. The Stanford group then revised their TLI-based regimen by adding $CD34^+$ and $CD3^+$ cells to the conditioning regimen in an attempt to induce stable mixed chimerism for renal allograft tolerance. Their current protocol consists of TLI (80−120 cGy, 10 doses total on days 0−9), rabbit ATG (1.5 mg/kg, 5 days total on days 0−4), followed by HLA-matched peripheral blood $CD34^+$ HSC and $CD3^+$ cell infusion on day 11. Mycophenolate mofetil (MMF) and cyclosporine (CSA) are initiated on day 0 and are tapered off over 6 months (Table 23.1). The potential advantage of this approach lies in its applicability to deceased

donor transplantation, since all treatments in the conditioning regimen start after transplantation.

Table 23.1 Conditioning regimens

	MGH	**Stanford University**	**Northwestern University**
Irradiation	Thymic irradiation: 700 cGy day -1	Total lymphoid irradiation: 80-120 cGy days 1-10	Total body irradiation: 200 cGy day -1
Chemotherapy	Cyclophosphamide (60 mg/kg, days -5 and -4) MEDI 507 (0.1 mg/kg test dose on day -2, then 0.6 mg/kg on days -1, 0, and 1) Rituximab (375 mg/m^2 BSA on days -7, -2, 5, and 12)	Thymoglobulin (1.5 mg/kg, days 0-4)	Fludarabine (30 mg/kg) on days -4, -3, and -2 Cyclophosphamide (50 mg/kg) on days -3 and +3
Kidney transplant	HLA mismatch, Living donor (day 0)	HLA match, Living or deceased donor (day 0)	HLA mismatch, Living donor (day 0)
Donor bone marrow transplant	Day 0	Day +11	Day +1 (combined with "facilitating" T cells)
Maintenance immunosuppression	Calcineurin inhibitor (to 8-14 months)	Calcineurin inhibitor (at least 6 months) Mycophenolate mofetil (for 1 month)	Mycophenolate mofetil (at least 1 year) Tacrolimus (at least 1 year)
Chimerism	Mixed, transient	Mixed transient or stable	Full donor

cGy, centigray. BSA, body surface area.

In HLA-matched kidney transplantation, the majority of patients [19/22] who received the above-described protocol successfully developed persistent mixed chimerism, and 16 graft recipients were weaned off immunosuppression with the longest renal allograft survival noted to be 8 years. However, in HLA-mismatched kidney transplantation, the initial attempt to induce stable chimerism was not successful. Unlike HLA-matched kidney transplantation, the patients who received HLA-mismatched kidney plus HSC failed to develop persistent mixed chimerism and renal allograft tolerance [76, 77]. The revised regimen with increased $CD34^+$ cell and $CD3^+$ cell doses has currently been tested to improve engraftment of HLA-mismatched donor HSC.

At MGH, based on decades-long basic and preclinical studies, a non-myeloablative regimen has been developed to induce mixed chimerism and renal allograft tolerance in HLA-mismatched kidney transplantation. Based on clinical experiences in HLA-matched bone marrow transplants used for myeloma or lymphoma [78], the NHP protocols have been modified by replacing TBI and hATG with cyclophosphamide and an anti-CD2 mAb (MEDI-507), respectively [79, 80]. Calcineurin inhibitor is also tapered more slowly (one month in NHP *vs*. 9 months in humans) (Table 23.1). The protocol has been further modified by adding rituximab to suppress the acute humoral rejection observed in the initial protocol [81, 82].

A total of ten subjects were enrolled in the initial studies, and all recipients who received the protocol developed transient mixed chimerism up to day 21. Seven of them successfully discontinued their immunosuppression by 14 months, while four remained immunosuppression-free for 5−12 years. However, three resumed immunosuppression at 5, 7, and 8 years after kidney transplantation as a result of chronic rejection or recurrence of the original disease [82, 83]. Another adverse event observed in these clinical trials was acute kidney injury (AKI), which was observed in most recipients [84]. This event was observed after day 10 and associated with loss of chimerism and recipient hematopoietic cell recovery.

Since AKI was not observed in the NHP studies that utilized TBI rather than cyclophosphamide, we recently tested a revised regimen in which cyclophosphamide was replaced with low dose TBI. Two patients who received this revised regimen have done well, without AKI, and their immunosuppression is currently being tapered (manuscript in preparation). Further clinical trials are planned using a new regimen with belatacept which has been developed in the NHP study [85].

On the basis of the tolerance observed in our patients, we believe that peripheral mechanisms of tolerance must play a critical role after the disappearance of chimerism. Detailed analysis of the lymphocyte subsets has revealed memory type cell predominance and an increased proportion of $CD4^+CD25^+CD127^-FOXP3^+$ regulatory T cells (T_{reg}) during the lymphopenic period [86]. Renal allograft biopsy of these patients also has demonstrated significantly higher FOXP3 mRNA levels than observed in normal kidneys without inflammatory responses [81]. Assays in 2 of 4 patients have been consistent with a suppressive tolerance mechanism at 6 months to 1 year, but in vitro assays performed at a later time have suggested deletion of donor-specific clones [86].

Full Donor Chimerism

Early trials in clinical applications were closely modeled on direct clinical evidence obtained from the unique setting of patients who underwent myeloablative HLA-matched BMT for hematologic disorders and years later developed renal failure requiring transplantation. When the kidney donor was the same individual who previously donated bone marrow, these kidney transplants succeeded without the need for immunosuppression. This demonstrated that stable full donor chimerism can confer tolerance to other tissues and organs transplanted from the same donor [87].

Full chimerism for induction of allograft tolerance has, until recently, been considered out of the realm of clinical applicability because of the risk of GVHD and the adverse effects of immunocompetence due to complete replacement of recipient myeloid and lymphoid lineages by HLA-mismatched donor cells. However, a novel protocol for HLA-mismatched

bone marrow transplantation was recently developed to achieve hematopoietic stem cell engraftment with a significantly lowered risk of GVHD. This protocol effectively deletes anti-host T cells attributable to GVHD through the administration of cyclophosphamide on days 3 and 4 after HSCT [88].

Investigators at Northwestern have modified this regimen by infusing novel tolerogenic "facilitating cells" which consist of CD8+/TCR- cells that include precursor plasmacytoid dendritic cells [89]. In animal models, these cells have been found to improve engraftment and to prevent GVHD, possibly through the induction of regulatory T cells [90, 91]. The full Northwestern protocol for induction of renal allograft tolerance consists of TBI (200 cGy on the day before transplant), fludarabine (30 mg/kg × 3 doses prior to transplant), cyclophosphamide (50 mg/kg on day -3), kidney transplantation on day 0, the administration of donor HSC combined with facilitating cells on day 1, and finally, another cyclophosphamide (50 mg/kg) treatment on day 3. Mycophenolate mofetil and tacrolimus are started on day 0 and slowly tapered off by one year (Table 23.1). A trial using this conditioning regimen has enrolled 15 recipients to date [55].

Nine of these 15 patients went on to develop full donor chimerism, and immunosuppression was successfully discontinued in 6 patients. Three patients developed transient chimerism, but this did not result in renal allograft tolerance; therefore, immunosuppression could not be discontinued. In the three other cases in which tolerance was not successful, weaning was halted with development of membranous nephropathy in one case, presumed rejection in one case, and loss of the renal allograft to thrombosis in the third case following what was believed to be an episode of sepsis due to an atypical viral infection that required stored autologous HSC transplantation.

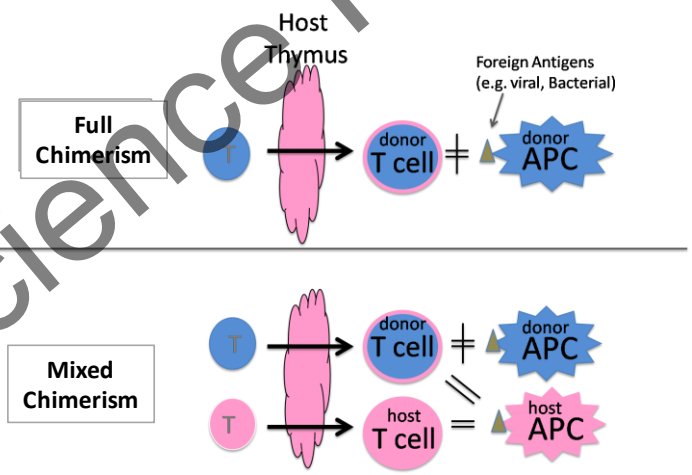

Figure 23.2. Antigen recognition in full donor chimerism and mixed chimerism. In both full and mixed chimerism, T cells are restricted to recipient MHC during development in the host thymus. However, there is no host APC available in full donor chimerism, which may result in immunoincompetence.

Despite these apparent successes, the approach used for induction of allograft tolerance with full donor chimerism has been controversial. First, the risk of GVHD cannot be justified in cancer-free patients for the purpose of inducing allograft tolerance. Although the risk of GVHD is significantly lower with the conditioning regimen utilized, it is not completely

eliminated [88]. Second, patients with full donor chimerism might be expected to suffer from immunoincompetence, since donor T cells that develop in the recipient thymus would be restricted to recipient type MHC as a result of positive selection. Therefore, donor type T cells would not recognize antigens effectively when presented by the donor type APCs, which would also be the only APCs available in full donor chimeras [92, 93] (Figure 23.2).

Conclusion

Tolerance induction is now a clinical reality in humans, at least for patients undergoing kidney transplantation. Although strategies to use HSC to induce tolerance differ in complexity and efficacy, outcomes are expected to improve as more patients are enrolled in clinical trials. Significant barriers remain for all-organ tolerogencity and further investigation is required, but the advent of routine tolerance induction after renal allograft transplantation may be closer at hand than currently projected.

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