BBS821 proposal evaluation (example)

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Abstract

The induction of humoral responses involves regulated interactions between B cells, antigens, CD4 T_H cells, and dendritic cells. These interactions are critical for appropriate cytokine production, isotype switching, affinity maturation, and the formation of long-lived plasma cells. Improper regulation of the signaling molecules involved in these interactions can lead to defects in the immune system.

X-linked Lymphoproliferative syndrome (XLP) is an often fatal immunodeficiency. Mutations in the gene encoding the protein SAP were shown to be the cause of this disease. SAP is an SH2-containing intracellular adaptor protein that interacts with members of the SLAM family receptors including SLAM, 2B4, CD84, NTBA (Ly108), Ly9 (CD229), and CRACC. Deficiency in SAP leads to a decreased production of Th2 type cytokines, a lack of NKT cells, and abrogation of T-cell dependent humoral immune responses, including a lack of T-dependent immunoglobulin isotype switching. This defect was shown to be an intrinsic CD4 T cell defect.

CD4 T cells express SLAM, NTB-A, Ly9, CD84, and a small subset expresses CRACC. CD4 T cells capable of providing crucial help for isotype switching were found to express high levels of CD84 and Ly9 on their cell surface. CD84 is a homophilic receptor that is expressed on T cells, B cells, dendritic cells, macrophages, and a few other cells types. Expression of CD84 is increased upon TCR stimulation and becomes tyrosine phosphorylated, a requirement for the recruitment of SAP. CD84 is higher on memory B-cells, than naïve B cells, and $CD84^{hi}$ B cells contain a higher proportion of cells expressing switched Ig isotypes. Considering that CD84 is a self ligand, it is possible that T-cell:B-cell interactions through CD84 are responsible for isotype switching.

Therefore, ***it is hypothesized that SAP dependent CD84 signaling is required in CD4 T cells to mediate B cell isotype switching.*** To address this hypothesis it will be determined if CD84 signaling is essential for B cell isotype switching, and if CD84 signaling through SAP in CD4 T cells is required for B cell isotype switching and the regulation of expression of important T cell induced cell contact ligands such as CD40L and ICOS.

Background

Immune responses must be tightly regulated to allow a potent response against foreign pathogens while preventing the development of autoimmune disease. The induction of humoral responses involves regulated interactions between B cells, antigens, CD4 T_H cells, and dendritic cells. The humoral immune response is the branch of adaptive immunity that is mediated by secreted antibodies produced by B lymphocytes. Naïve B lymphocytes can be activated to secrete antibodies in either a T cell independent or a T cell dependent manner[1]. T dependent antibody responses result from the interactions between antigen-specific B cells and activated antigen specific T helper (Th) cells in germinal centers. These interactions are critical for appropriate cytokine production, isotype switching, affinity maturation, and the formation of long-lived plasma cells[1]. These effector functions are required for successful clearance of both intracellular and extracellular pathogens and often involve the precise regulation of different signaling molecules.

Immune responses can be mediated through a diverse set of surface receptors which activate intracellular signaling cascades. Improper regulation of the signaling molecules involved can lead to defects in the immune system, resulting in autoimmunity or immunodeficiency. For example, mutations in the SH2D1A gene, encoding the protein SAP, were determined to cause the immunodeficiency termed XLP (X-linked Lymphoproliferative disease)[2] affecting about 1 in 500,000–1,000,000 Caucasian males.[3] Up to 60% of XLP patients will develop fulminant infectious mononucleosis with the majority of them dying within 1-2 months following infection[3]. Different SH2D1A mutations were found to alter the stability and or function of SLAM-associated protein (SAP), a small SH2 domain containing protein. SAP is predominantly expressed in T cells, NK cells, and NKT cells and has been shown to associate with SLAM family receptors including SLAM, 2B4, CD84, NTBA (Ly108), Ly9 (CD229), and CRACC through tyrosine based motifs in their cytoplasmic domains[2]. SAP functions as an important intracellular adaptor protein in immune signaling. Deficiency in SAP leads to a decreased production of Th2 type cytokines, a lack of NKT cells, and abrogation of T-cell dependent humoral immune responses, including a lack of T-dependent immunoglobulin isotype switching[4]. This defect is intrinsic to the CD4 T helper cells, as

SAP-/- B cells could still undergo isotype switching through the help of WT CD4 T helper cells[5]. As mentioned, SAP deficiency in CD4 T cells causes a decrease in Th2 type cytokine production, and also results in the aberrant expression of both ICOS and CD40L. ICOS is a member of the CD28/CTLA-4 family and is expressed by activated T cells. ICOS interacts with its ligand, B7RP-1, which is constitutively expressed on B cells and mediates germinal center formation and immunoglobulin class switch recombination[6]. CD40L is also expressed on activated T cells and engages its ligand, CD40, on B cells. Mutations in either CD40L or CD40 have been shown to cause hyper immunoglobulin M syndrome, which is characterized by defective germinal center formation, high levels of IgM, and low serum levels of IgG, IgA, and IgE[7]. Considering the importance of these two surface molecules in T dependent antibody responses, and that SAP regulates T cell mediated humoral responses independent of cytokine production[8], it is likely that their aberrant expression in SAP-/- CD4 T cells is the cause of the observed humoral defects. The aberrant expression of these molecules most likely results from signaling defects through one of the SLAM family receptors expressed on these cells.

CD4 T cells express SLAM, NTB-A, Ly9, CD84, and a small subset expresses CRACC. SLAM-/- and Ly9-/- mice both exhibit normal isotype switching, indicating that the lack of isotype switching in SAP-/- mice is not due to defective SAP signaling through these two receptors[9 10]. CRACC is only expressed on a small subset of CD4 T cells and its interaction with SAP is controversial, implying that CRACC may also not be responsible for mediating isotype switching in a SAP-dependent manner[2]. NTB-A has been implicated in innate immune functions, and the administration of NTB-A Fc-fusion proteins in-vivo had no affect on IgG1 isotype switching and moderate affects on IgG2a and IgG3 isotype switching in response to a T dependent antigen[11]. Considering SAP-deficient mice have severe defects in the production of all IgG isotypes it is possible that NTB-A is not the mediator of B-cell isotype switching. CD4 T cells capable of providing crucial help for isotype switching were found to express high levels of CD84 and Ly9 on their cell surface[12]. CD84 is a homophilic receptor that is expressed on T cells, B cells, dendritic cells, macrophages, and a few other cells types. Expression of CD84 is increased upon TCR stimulation and becomes tyrosine phosphorylated, a requirement for

the recruitment of SAP[13]. CD84 is higher on memory B-cells, than naïve B cells, and $CD84^{hi}$ B cells contain a higher proportion of cells expressing switched Ig isotypes[14]. Considering that CD84 is a self ligand, it is possible that T-cell: B-cell interactions through CD84 are responsible for isotype switching.

Specific Aim

The primary aim of this proposal is to determine if SAP dependent CD84 signaling in CD4+ T cells is required for B cell isotype switching.

Sub Aim I: Determine if CD84 is essential for B cell isotype switching.

Experimental Design

To determine if the expression of CD84 on Th cells is required for B cell isotype switching, OT-II CD4 T cells lacking CD84 along with SW_{HEL} B cells will be adoptively transferred into a recipient mouse. OT-II cells are class II restricted CD4 T cells that recognize an OVA epitope, and the SW_{HEL} B cells specifically recognize hen egg lysozyme (HEL) and are capable of class switch recombination. The recipient will then be challenged with covalently conjugated HEL-OVA. After a period of 4 days, defects in antibody forming cells will be analyzed.

OT-II CD4 T cells lacking CD84 will be used to determine if CD84 is required for B cell isotype switching. Since there is currently no CD84 knock out mouse, Nucleofector™ technology (Amaxa, Cologne, Germany) will be used to deliver a shRNA to knock down CD84 in CD4 T cells. Amaxa nucleofection is a non-retroviral-mediated gene delivery system based on an improved electroporation method. This method will be used because it has been shown to efficiently transfect primary mouse CD4 T cells. First, a population of pure CD4 OT-II cells will be isolated for nucleofection. The modified RNA-interference plasmid pSuper-H-$2K^k$ will be used. This plasmid contains a multiple cloning site to clone in the gene of interest followed by an internal ribosomal entry site and a gene for the truncated mouse H-$2K^k$ class I molecule[15]. It will be used because it allows for the gene of interest along with the H-$2K^k$ gene to be translated in one bicistronic message. This will allow for selection and enrichment of transfected cells through magnetic H-$2K^k$ selection and since this is a truncated surface molecule, it should not interfere with any cellular functions. After selection for transfected T cells, FACs can

be used to verify CD84 knock down. Since CD84 is a surface molecule, if the truncated H-2K^k does seem to interfere with cellular functions, it is also possible to select for transfected T cells by positively depleting any CD84 positive T cell.

SW$_{HEL}$ B cells[16] (CD45.2+), will be adoptively transferred into (CD45.1+) congenic recipients along with equal numbers of CD84 depleted OT-II cells, WT OT-II cells transfected with a control shRNA, or OT-II cells transfected with a shRNA to SAP. Hosts will then be challenged with covalently conjugated HEL-OVA intravenously along with LPS. This system will allow for recognition of the antigen by both B and T cells, allowing for T cell dependent B cell help. It also eliminates problems encountered with antigens, such as influenza, that can generate class-switched T cell independent antibodies which could skew interpretations of the results.

Since it has been shown that as early as 4 days after adoptive transfer of SAP -/- OT-II cells using the same system as described above[5], defects in antibody forming cells (AFCs) can be observed, analysis of AFCs will be done on day 4. On day 4, splenic AFCs will be analyzed using ELISPOT and HRP-conjugated Abs specific for mouse IgM, IgG1, IgG2a, IgG2b, and IgG3 to determine different anti-HEL antibody responses. This will be used to determine if class switch recombination has occurred.

Possible Results and Interpretations

If CD84 is required on Th cells for B cell isotype switching, then the results from the host receiving the SAP deficient OT-II cells and the host receiving the CD84 deficient OT-II cells should be similar. The host receiving the WT OT-II cells is the positive control and should show antibody forming cells for IgM and all of the IgG subtypes. The host receiving the SAP deficient OT-II cells is the negative control, as it has been published that in the system described, SAP deficient T cells can not mediate class switch recombination. The results for this host should indicate that the IgM levels are similar between the WT, however, they should show a significant decrease in IgG subtypes. If CD84 is required for isotype switching, it would be expected that like the host receiving the SAP deficient OT-II cells, IgM levels would be comparable to WT, and there would also be a decrease in all IgG subtypes.

Alternatively, the host receiving the CD84 deficient OT-II cells may have IgM levels comparable to WT, and have levels of the IgG subtypes that lie somewhere in between the positive control (WT OT-II cells) and the negative control (SAP deficient OT-II cells) without being significantly different than either one. This could be due to an incomplete knockdown of CD84, or loss of the shRNA in vivo, thus the T cells are beginning to regain expression of CD84. It will be important to analyze the OT-II cells for expression of CD84 during the 4 day period to ensure that the protein is actually depleted throughout the course of the experiment. It is unlikely that this ambiguous result would be due to the use of LPS as an adjuvant because it should have the same affect on the host receiving the SAP deficient OT-II cells. If the results for this negative control are significantly different than the WT control, the possibility of the adjuvant skewing the results should be ruled out.

It is also possible that the host receiving the CD84 deficient OT-II cells may have IgM levels comparable to WT and show a significant decrease in IgG subtypes but not be directly involved in mediating B cell isotype switching in a SAP dependent manner. The knockdown could be affecting signaling through other molecules known to interact with CD84, T cell activation, or something else upstream of isotype switching. This issue will be addressed in Sub Aim II.

Sub Aim II: Determine if CD84 signaling through SAP in CD4 T cells is required for B cell isotype switching.

Experimental Design

CD84 knockdown in OT-II cells, as described in Sub Aim I, could be affecting signaling through other molecules known to interact with CD84 and any results obtained are also not specific for SAP dependent signaling through CD84. This sub aim will determine if CD84 signaling through SAP in CD4 T cells is required for B cell isotype switching.

CD84 can also bind EAT-2, a SH2-domain containing protein that is 45% homologous to SAP, and any defects observed in the absence of CD84 could be due to signaling through SAP or EAT-2[13]. To determine if SAP mediated signaling through CD84 can regulate B cell isotype switching, OT II cells will be isolated and retrovirally

transduced to over-express CD84_Y262F, Y298F. This is the CD84 receptor with each of the two tyrosine residues critical for SAP binding mutated to phenylalanine. It has been shown that these mutations block tyrosine phosphorylation and subsequent SAP binding and have no affect EAT-2 interaction[13]. The retrovirus will contain the gene of interest and also an IRES and then the GFP gene so that efficiency of retroviral transduction can be determined.

Using a similar system as described in Sub Aim I, SW^{HEL} B cells (CD45.2+), will be adoptively transferred into (CD45.1+) congenic recipients along with equal numbers of OT-II cells retrovirally transduced to over-express the CD84 mutant receptor, or WT OT-II cells retrovirally transduced to only express GFP. Hosts will then be challenged with covalently conjugated HEL-OVA intravenously along with LPS. On day 4, splenic AFCs will be analyzed to determine if class switch recombination has occurred.

Over-expression of the CD84 mutant receptor should act as a dominant negative receptor, and prevent SAP dependent signaling through the endogenous CD84 receptor, without affecting EAT-2 signaling. The phosphorylation status of endogenous CD84 will be used to ensure that signaling through the endogenous CD84 receptor when the CD84 mutant is being over-expressed is impaired. This will be done by activating OT-II cells either over expressing the mutant CD84 or GFP *in vitro* and doing western blot analysis using antibodies specific for phosphorylated CD84 Y262 and Y298. The mutant CD84 should be present in levels greatly exceeding endogenous CD84 and prevent homophilic interactions between the endogenous CD84 and CD84 present on other cells, thus inhibiting its phosphorylation which should be evident in western blot analysis.

It will also be possible to separate the retrovirally transduced OT-II cells over-expressing the mutant CD84 into different populations based on GFP positive expression. It would be assumed that the more GFP expression, the more mutant CD84 is being expressed as well. Then, the same experiment can be repeated except a 'gradient' of mutant CD84 expressing OT-II cells can be used to determine if there is a threshold of SAP dependent CD84 signaling required to be blocked to inhibit isotype switching.

Possible Results and Interpretations

If SAP dependent signaling through CD84 is required for B cell isotype switching, the dominant negative CD84 should block isotype switching in this system. The WT OT-II cells retrovirally transduced only to express GFP serve as the positive control for the experiment and it is expected that analysis of anti-HEL specific IgM and IgG subtypes will indicate that class switch recombination has occurred in response to the HEL-OVA challenge. In comparison to the WT control, it would be expected that IgM levels would be comparable and that levels of IgG subtypes would be greatly decreased in the CD84 mutant OT-II cells. Ideally, the levels of IgG subtypes would decrease as the level of mutant CD84 expression in OT-II cells increased. It is also possible that the levels of IgG subtypes would be comparable to the WT control at the lowest expression of mutant CD84 and then greatly decrease once the mutant CD84 reached a certain level of expression with no change in any increase of expression. This would indicate that either all endogenous CD84 signaling had been blocked, or there is a certain threshold of CD84 signaling through SAP that is required for isotype switching.

Alternatively, no difference between the IgG subtypes from hosts that received either WT or CD84 mutant OT-II cells may be observed, indicating that SAP dependent signaling through CD84 is not required for isotype switching, the CD84 mutant is not acting as a dominant negative receptor, or that there is still endogenous CD84 signaling occurring. If no difference is observed, it would be possible to use the system set up in Sub Aim I, where CD84 was depleted from OT-II cells, and perform a rescue experiment. The CD84 depleted OT-II cells can either be retrovirally transduced with WT CD84 or CD84_Y262F, Y298F. Both genes would have to have silent mutations so that they would not be targeted by the shRNA against CD84 in those cells. Then, the same experiment could be done using these OT-II cells and adoptively transferring them into recipient mice with SW$_{HEL}$ B cells, challenging them with HEL-OVA, and analyzing AFCs 4 days after the challenge to determine if isotype switching occurred. This experimental setup would rule out the possibility that the mutant CD84 was not acting as a dominant negative and bypass any issues with endogenous CD84 signaling.

Sub Aim III: Investigate if CD84 signaling in CD4 T cells modulates the surface expression of CD40L and/or ICOS.

Experimental Design

It was previously shown that humoral immune responses could be rescued in SAP-/- mice without the restoration of Th2 cytokine defects[7]. This implies that humoral immunity and Th cytokine defects in SAP-/- mice may involve distinct signaling pathways. Expression of CD40L and ICOS on CD4 T cells is required for T-dependent isotype switching[17 18]. Both of these surface molecules are aberrantly expressed in SAP-/- T cells and could cause the defect in T cell mediated B cell isotype switching. To investigate if signaling through CD84 affects the surface expression of CD40L and/or ICOS, WT OT II cells and OT II cells in which CD84 has been knocked out using Amaxa Nucleofector™ technology, as described in Sub Aim I, will be stimulated in vitro with peptide pulsed APCs.

WT OT-II cells will be isolated through magnetic negative selection. Either CD84 or SAP will be knocked down in this cell type through nucleofection, as described earlier. Separately, the WT, CD84 deficient, or SAP deficient OT-II cells will be stimulated *in vitro* using OVA-peptide pulsed APCs. Both expression and kinetics of ICOS and CD40L will be evaluated by flow cytometry every 6hrs for a period of 48hrs.

Possible Results and Interpretations

The expression pattern of both ICOS and CD40L in CD84 deficient OT-II cells will be compared to the WT and SAP deficient OT-II cells. Previously, it was shown that SAP-/- CD4 T cells show delayed and impaired expression of ICOS and elevated expression and kinetics of CD40L expression when activated[8]. The SAP deficient OT-II cells therefore serve as a control for aberrant ICOS and CD40L expression. The WT OT-II cells should show normal upregulation and kinetics of both ICOS and CD40L expression. If CD84 signaling is required for proper regulation of expression of either of these two molecules, the resulting data will be comparable to the SAP deficient OT-II cells. If the results for ICOS and CD40L expression are similar between the SAP deficient and CD84 deficient OT-II cells, it will help to elucidate the mechanism of how CD84 contributes to B cell isotype switching.

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