Germinal Centers

Gabriel D. Victora[1] and Michel C. Nussenzweig[2,3]

[1]Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142; email: victora@wi.mit.edu

[2]Laboratory of Molecular Immunology, The Rockefeller University, New York, NY 10065

[3]Howard Hughes Medical Institute, The Rockefeller University, New York, NY 10065
email: nussen@rockefeller.edu

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Abstract

Germinal centers (GCs) were described more than 125 years ago as compartments within secondary lymphoid organs that contained mitotic cells. Since then, it has become clear that this structure is the site of B cell clonal expansion, somatic hypermutation, and affinity-based selection, the combination of which results in the production of high-affinity antibodies. Decades of anatomical and functional studies have led to an overall model of how the GC reaction and affinity-based selection operate. More recently, the introduction of intravital imaging into the GC field has opened the door to direct investigation of certain key dynamic features of this microanatomic structure, sparking renewed interest in the relationship between cell movement and affinity maturation. We review these and other recent advances in our understanding of GCs, focusing on cellular dynamics and on the mechanism of selection of high-affinity B cells.

Ig: immunoglobulin

SHM: somatic hypermutation

GC: germinal center

LZ: light zone

DZ: dark zone

FDC: follicular dendritic cell

INTRODUCTION

The immune system has the peculiar ability to respond to foreign substances (or antigens) by producing immunoglobulin (Ig) molecules that bind to antigens with extremely high affinity and remarkable specificity. The question of how a limited genome encodes for Igs that react specifically to a virtually limitless number of antigens puzzled immunologists for decades. The emergence of the theory of clonal selection and the discovery of VDJ recombination resolved critical parts of this problem. While VDJ recombination attributed the initial generation of Ig diversity to the combinatorial rearrangement of gene segments (1), clonal selection postulated that the subsequent expansion of B cell clones whose surface Ig bound to (or "recognized") specific antigens led to the production of serum antibodies (2, 3).

In parallel to these discoveries, work on serum preparations obtained from animals at different times after immunization showed that the affinity of the antibodies in serum increased dramatically with time (4, 5), in a phenomenon known as affinity maturation. Subsequent advances in the isolation of Ig molecules from single B cell clones showed that the high-affinity antibodies that emerged later in the immune response were not simply the products of VDJ rearrangement but were, in fact, highly somatically mutated versions of lower-affinity germline VDJ sequences (6–8). It is now clear that affinity maturation is the consequence of iterative rounds of Darwinian-like selection of high-affinity mutants generated by somatic hypermutation (SHM). The combination of SHM and affinity-based selection thus provides the fine-tuning of low-affinity germline VDJ rearrangements, greatly expanding the range of antigenic determinants to which Igs can bind with high affinity.

Affinity maturation takes place in structures known as germinal centers (GCs). GCs were first described by Walther Flemming in 1884 as distinct microanatomical regions of secondary lymphoid organs that contained dividing cells (reviewed in Reference 9). Although GCs were long believed to be the source of developing lymphocytes, immunization experiments showed that these structures developed only in response to antigen, and are in fact the sites of B cell clonal expansion during immune responses (9). Under experimental conditions, GCs initially form at around 6 days after a primary immunization, when foci of rapidly proliferating B cells begin to appear within the B cell follicles of lymph nodes and spleen. These foci increase rapidly in size and, within a few days of their formation, differentiate into the structure we know as the mature GC reaction (10).

Manual dissection of single cells from GCs made it clear that these structures are the site of SHM and affinity-based selection (11, 12). GC B cells express the enzyme activation-induced deaminase (AID) (13), which deaminates cytidine residues in the VDJ and switch regions of the Ig gene, leading to SHM and class switch recombination (14–16). The rate of mutation in the Ig variable (V-) regions during SHM in the GC is estimated to be as high as 10^3 per base pair per generation (10^6-fold the normal rate of somatic mutation) (7, 8). Because AID is targeted to ssDNA at sites where transcription is stalled (17–19), AID can also damage the genome at sites other than Ig loci, producing mutations in oncogenes and initiating chromosome translocations that lead to GC lymphomas (15).

From the very beginning, the GC was viewed primarily as an anatomical entity. GCs were clearly visible by conventional histology techniques, as was their division into "light" and "dark" zones (LZ and DZ, respectively) (9). Cells were found to proliferate extensively in the GC DZ, whereas antigen was found deposited on the follicular dendritic cell (FDC) network of the LZ (20, 21). Pulse-chase experiments suggested that B cells migrate between the two GC compartments and that DZ/LZ polarization reflects a progenitor-product relationship (22, 23).

The idea of the GC as a highly dynamic entity was proven correct when three groups, including our own, independently used multiphoton laser-scanning microscopy to visualize

the events taking place during the GC reaction (24–27). These studies provided a real-time picture of the GC reaction: B cells not only move constantly throughout the entire GC, but also engage in short, dynamic interactions with both T cells and antigen. Rather than an enclosed structure, the GC provides open access to "invading" naive B cells, and the peculiarity of the migration patterns of GC B cells cast doubt on the established models of how affinity-based selection takes place. In parallel, advances in our understanding of the helper T cells that both support and control the GC reaction have shed light on the important role these cells play in enhancing B cell affinity while controlling the emergence of humoral autoimmunity (28–31).

The aim of the present review is to summarize and discuss these and other recent developments in GC biology—with special emphasis on the cellular events leading to the selection of high-affinity B clones and affinity maturation—and to update the classical model of the GC proposed by Ian MacLennan (23) to accommodate these new findings. We focus mostly on in vivo experiments, in part because it is not yet possible to reproduce the GC reaction in vitro.

GERMINAL CENTER CELL TYPES

Under specific pathogen-free conditions, secondary lymphoid organs such as spleen and lymph nodes contain follicles primarily composed of naive B lymphocytes. Roughly one week after exposure to antigen, GCs develop in the center of these B cell areas, forming secondary follicles. Naive B cells are pushed aside by the developing GC, forming a compartment termed the B cell mantle (23). The most conspicuous anatomical feature of the GC is its division into two distinct compartments, a DZ, proximal to the T cell zone, and an LZ, proximal to the lymph-node capsule or the spleen marginal zone (**Figure 1*a***). The DZ consists almost entirely of B cells with a high nucleus-to-cytoplasm ratio, thus appearing "dark" by light microscopy (9). By contrast, B cells in the LZ are interspersed among a network of FDCs (**Figure 1*b***), which give this zone its "lighter" appearance (32). In addition to antigen-specific GC B cells and FDCs, the LZ also contains a substantial population of naive IgD^+ B cells, which are continually in transit through the GC (24, 33, 34). The LZ also contains T cells (**Figure 1*c***), most of which are of the $CD4^+$ lineage, but some of which are $CD8^+$ (35), as well as a small number of conventional dendritic cells (36, 37). Finally, tingible-body macrophages, a specialized group of phagocytes that engulf dying B cells that develop during GC selection, are found throughout the GC (**Figure 1*d***) (38).

Germinal Center B Cells

The great majority of cells in the GC are activated B cells. These differ from their naive counterparts in a number of important ways. GC B cells are larger than naive cells and display a highly polarized morphology, with evident leading edges and extended uropods (**Figure 1*c***) (24–26). Whereas naive B cells rarely divide, GC B cells are among the fastest dividing mammalian cells, with a cell-cycle time estimated at between 6 and 12 h (25, 26, 39, 40). GC B cells can be identified by their expression of high levels of Fas and *n*-glycolylneuraminic acid (the ligand of antibody GL-7), binding to peanut agglutinin, loss of surface IgD, and idiosyncratic changes in expression of CD38 (downregulation in mice and upregulation in humans), among other markers (41–46). GC cells also differ from resting B cells in their expression of the chemotactic G protein–coupled receptors Ebi2 and $S1P_2$. Ebi2 is highly expressed on naive but not on GC B cells, and its absence aids in the localization of GC B cells to the center of the follicle (47, 48). Ebi2 is a receptor for oxysterols, likely to be produced by stromal cells in the outer B cell follicle (49, 50). Unlike Ebi2, $S1P_2$ is upregulated in GC B cells and, by a yet unresolved mechanism, promotes the positioning of GC B cells in S1P-low regions in the center of the follicle (51).

A critical regulator of the GC B cell phenotype is the transcription factor Bcl-6 (52, 53). Bcl-6 acts primarily as a transcriptional

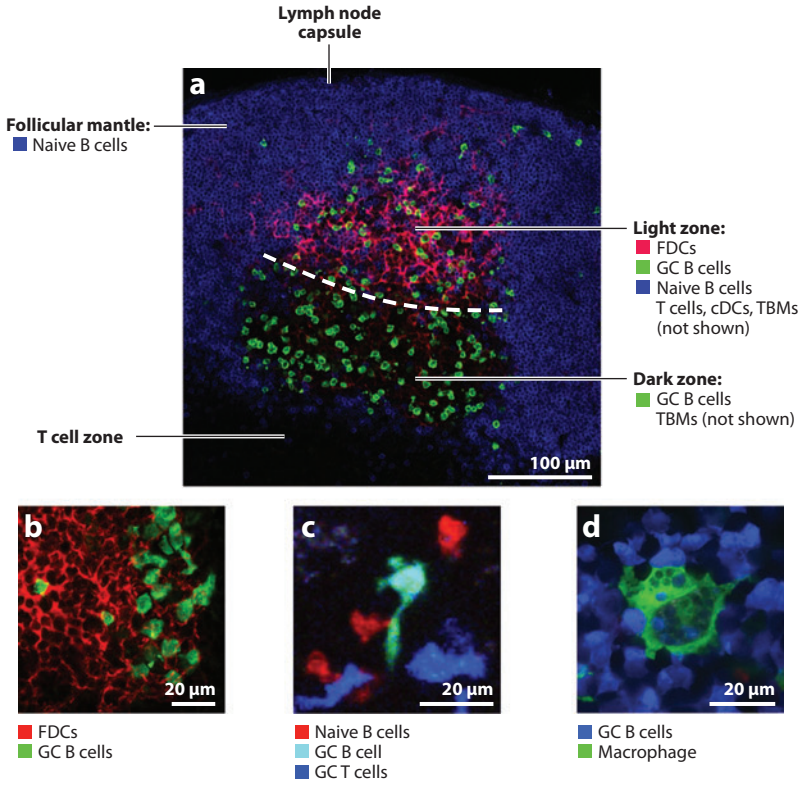

Figure 1

GC anatomy and cell types. (*a*) The GC is divided into light and dark zones. Frozen tissue sample stained with antibodies to CD35 (FDC network), IgD (mantle B cells), and GFP (which stained a fraction of GFP-expressing GC B cells). (*b*) Higher-magnification view of the FDC network. Frozen-tissue section stained with antibody to CD35 (*red*) and GFP (*green*) colors as in panel *a*. (*c*) Intravital multiphoton image showing the morphology of a GFP-expressing GC B cell, contrasted to dsRed-expressing naive B cells and CFP-expressing GC T cells. Note that the highly polarized morphology of GC B cells is fully retained only in live tissue. (*d*) Multiphoton image of a nonliving tissue explant showing a tingible body macrophage (from YFP expression, CD11c-YFP transgenic mouse) ingesting dying CFP-expressing GC B cells. Abbreviations: CFP, cyan fluorescent protein; FDC, follicular dendritic cell; GC, germinal center; GFP, green fluorescent protein; YFP, yellow fluorescent protein. Images by G.D.V. and Z. Shulman. Panel (*a*) reprinted with permission from Reference 31, © 2010 Elsevier.

repressor (53). In B cells, Bcl-6 is selectively upregulated during the GC stage (54, 55), and mice lacking Bcl-6 are incapable of forming GCs or producing high-affinity antibody (56, 57). Bcl-6 serves at least four important functions in generating the GC B cell phenotype. First, Bcl-6 silences the antiapoptotic molecule Bcl-2 in GC B cells, ensuring the maintenance of a proapoptotic state (58, 59). As we discuss below, this state is of fundamental importance for affinity-based selection and the prevention of autoimmunity that could arise as a result of SHM. Second, by repressing the expression of factors such as p53 and ATR, Bcl-6 contributes to a GC B cell's ability to tolerate DNA damage induced by rapid proliferation and AID activity (60, 61). Third, Bcl-6 plays an important role in preserving GC B cell identity by silencing the plasma cell master regulator Blimp-1, thus regulating exit from the GC to the plasma cell fate (62). Finally, Bcl-6 downregulates the expression of key mediators of both B cell receptor

(BCR) and CD40 signaling, possibly helping to fine-tune the responsiveness of GC B cells to selective signals (62, 63). The full range of genes and processes controlled by Bcl-6 is currently under investigation (63).

Phenotypic differences between light and dark zone B cells. A long-standing question is whether the anatomical polarization of the GC into LZ and DZ corresponds to phenotypic and/or functional polarization of the B cells in these compartments (23, 27). Two key observations indicated that these compartments may have distinct physiological functions. First, as discussed below, FDCs act as long-term reservoirs of intact antigen (20, 21, 64); second, the frequency of mitotic cells, as determined by histological observation, is greater in the DZ (9). Together, these findings suggested a model for the GC reaction in which the DZ is the site of B cell clonal expansion and antigen receptor diversification, whereas the LZ is the site of selection by antigen binding (23). B cells in the DZ were referred to as "centroblasts" because of their reported larger size and high-level proliferative activity. To accommodate the idea that cell division is segregated from selection, centroblasts were proposed to migrate to the LZ, after which they were thought to lose their blastic features and differentiate into the more quiescent and smaller "centrocyte" phenotype (23).

Although many aspects of the initial model are correct, experimental confirmation of these features was difficult owing to the rather complex microanatomy of the GC. Several attempts were made to distinguish centroblasts and centrocytes by flow cytometry (65–67). The marker most widely used for this purpose was CD77, a surface glycolipid originally described as expressed on a highly proliferative subset of human GC B cells, proposed to correspond to centroblasts (66). Despite the widespread use of CD77 as a marker of DZ cells, a number of studies failed to uncover functionally relevant differences between $CD77^+$ and $CD77^-$ GC cells (68–70).

Direct observation of GC B cells in intact mice (24, 26) or explanted lymphoid organs in culture (25) provided important information about the phenotype of LZ and DZ B cells. Surprisingly, LZ and DZ populations were indistinguishable in terms of size and morphology, and they showed similar dynamic behavior, moving at virtually equal speeds (24, 25). The only consistent difference between LZ and DZ B cell dynamics was the slightly more linear paths of B cells in the LZ (24, 25), possibly reflecting the higher density of CXCL13 in this zone (25). Intravital imaging also showed that a substantial number of naive B cells from the mantle region are continually transiting through the LZ (24). Thus, the small size attributed to centrocytes by histologists may have been due to the heterogeneity of the B cells found in this zone, rather than being an intrinsic property of LZ B cells. Together, these observations suggested that the differences between LZ and DZ B cells were less marked than implied by the terms centrocyte and centroblast, and that these terms can be misleading (27).

A breakthrough in the definition of LZ and DZ phenotypes came from work done by the Cyster laboratory (71). Using genetic and chemical approaches, Allen and colleagues showed that the chemokine receptor CXCR4 is responsible for retaining a subpopulation of GC B cells in the DZ (71). DNA synthesis was significantly enriched among CXCR4-high cells, suggesting that CXCR4 expression is a functionally relevant marker for DZ B cells (25, 71). A study profiling the gene expression of human GC cells purified on the basis of CXCR4 expression was the first to show distinct gene expression programs in putative DZ and LZ B cells that corresponded to cell division in the DZ and activation in the LZ (72). In the same study, CD77 expression correlated poorly with CXCR4 expression, further undermining the validity of CD77 as a centroblast marker (72). However, CXCR4 expression alone is insufficient to demarcate clearly distinct populations of GC B cells by flow cytometry, especially in mouse (71). Furthermore, CXCR4 could not be validated by histology as a marker for human

BCR: B cell receptor

DZ cells, possibly because the lower expression of this receptor in LZ cells is mostly due to internalization rather than to lower expression at the mRNA and protein levels (72).

These issues were resolved when in situ photoactivation was used to label LZ and DZ cell populations directly within intact GCs (31). As predicted by the initial intravital imaging studies (24), LZ and DZ cells were indistinguishable in terms of size or complexity, as determined by flow-cytometric forward- and side-scatter measurements. However, gene expression analysis on purified LZ and DZ populations showed that, despite extensive similarities, the two subpopulations differed in a number of important ways (31; also see below). Among these differences, we identified two surface markers—CD83 and CD86—that, when combined with CXCR4, can clearly distinguish between LZ and DZ cells by flow cytometry: LZ cells are $CXCR4^{lo}CD83^{hi}CD86^{hi}$, whereas DZ cells are $CXCR4^{hi}CD83^{lo}CD86^{lo}$ (31). Use of these cell-surface markers and gene signatures should allow for a more precise analysis of GC development, of the mechanisms that govern affinity selection, and, possibly, of the relationship between GC subpopulations and human B cell malignancies.

Functional polarization in the germinal center. Beyond their use as markers, upregulation of CD83 and CD86 also indicated that GC B cells in the LZ are in an activated state. This was confirmed by upregulation in the LZ of other activation-related genes, including CD69 and a number of "immediate early" genes (*Myc*, *Nfkbia*, *Junb*, *Egr1-3*, and *Batf*, among others) (31). Global analysis of genes induced in LZ B cells demonstrated the upregulation of the signatures of CD40 and BCR stimulation as well as of NF-κB and c-Myc engagement (31). These observations located the regulation of B cell activation to the LZ of the GC, supporting models in which positive selection of high-affinity mutants takes place in this compartment (23).

In contrast to selection, cell division was classically thought to be restricted to the DZ of the GC (23). This notion was based on direct histological observation of mitotic figures (9) and was supported by early H^3-thymidine incorporation experiments in which labeled cells appeared first in the DZ (22). This distinction was not entirely accepted, however, because among other reasons, proliferation antigen Ki67 can be readily detected in the LZ under certain conditions, especially in mouse (73, 74). In addition, cell division was occasionally seen to occur in the LZ by intravital microscopy (25), and short-term labeling with BrdU showed rapid uptake in the LZ as well as the DZ (25, 26). This apparent discrepancy was resolved by in situ photoactivation, which demonstrated that, although GC B cells enter the S phase in the LZ, cells in the G2/M phases of the cell cycle are nearly absent from this compartment (31). Consistent with this observation, G2/M phase cell-cycle genes were strongly enriched in the DZ when compared with the LZ. Thus, although entry into the cell cycle can be triggered in the LZ, B cells only rarely remain in the LZ through the completion of the cycle. A possible reason for this is that the signals that induce B cells to enter the S phase of the cell cycle also trigger their exit from the LZ, either toward the DZ or to a post-GC fate (25, 31, 40). The significance of the small number of cells that appear to complete the cell cycle in the LZ is unclear. However, these cells have been reported primarily in GCs formed in the presence of mitogen-containing adjuvants, which may account for the phenomenon (75).

In summary, data obtained by in situ photoactivation firmly establish that the GC is functionally polarized into a DZ in which B cells divide and a LZ in which B cells are activated and selected based on their affinity for antigen. In contrast, other classical ideas, such as the large size difference between centrocytes and centroblasts, were overturned. Finally, the definition of functionally relevant markers is likely to shed light on the extent to which phenomena such as selection-dependent apoptosis, commitment to the plasma cell fate, AID-dependent Ig diversification, and development of lymphoma are polarized between the LZ and DZ of the GC.

The Follicular Dendritic Cell Network

FDCs are radioresistant cells that form reticular networks in primary follicles and GCs (20, 32, 64, 76–78). In GCs, FDCs are concentrated in the LZ, where they constitute the primary anatomical marker for this zone in histology and intravital microscopy (24, 26, 27, 77, 79) (**Figure 1*a*,*b***). FDCs retain antigen in intact form on their surface for extended periods (21) and are thus believed to serve as an antigen reservoir during the GC reaction. According to this model, B cells with antigen-specific receptors are selected by antigen displayed on the FDC surface in the form of immune complexes (23). In addition to their role as an antigen depot, FDCs are thought to support GCs by secreting chemokines and cytokines that attract and sustain GC B cells.

FDCs retain antigen on their surface in the form of immune complex coated bodies, or iccosomes (80). Immune complex trapping relies primarily on complement receptors 1 and 2 (81). Although FDCs also express Fc receptor IIB (FcRIIB), this receptor is dispensable for antigen retention (81). Nevertheless, FcRIIB expression by FDCs impacts on GC B cell development and selection by reducing the effective number of Ig Fcs that can interact with the inhibitory FcR expressed by GC B cells (82). Even though FDC processes extend well into the DZ of the GC, immune complex trapping in vivo—as determined by staining with the antibody FDC-M2, which binds to complement component C4—is restricted to the LZ (77).

In addition to antigen display, FDCs are also thought to provide chemoattractant signals that help localize B cells to the GC. FDCs produce the ligand for CXCR5, CXCL13/BLC (32), which is the major chemoattractant for B cells and follicular T cells (83, 84) and may also play a role in LZ/DZ polarization (71). FDCs also express other molecules essential to GC maintenance, such as cytokines and integrin ligands. For example, loss of expression of ICAM-1 and VCAM-1 adhesion molecules by FDCs correlates with reduced GC size and affinity maturation (85). Finally, FDCs produce an array of cytokines, most notably IL-6 and BAFF, both of which may play a role in the GC reaction (76, 86–89).

Although the balance of the evidence suggests that FDCs play a major role in the GC, the idea that FDCs or FDC-bound antigen are absolutely required for GC development and affinity maturation has been challenged (reviewed in References 90 and 91). Affinity maturation is seen to occur to a variable extent in mice in which retention of antigen by FDCs is defective. Mice with a genetic deletion of the secreted form of IgM (92) or carrying a transgene encoding only the membrane form of anti-NP Ig (93) can form GCs, and show signs of antigen-dependent selection at higher antigen doses (50–100 μg), even in the absence of detectable immune complex deposition on FDCs. *Cr2*-deficient mice, which lack complement receptors in both FDCs and B cells, are also capable of forming GCs and of robust affinity maturation at higher antigen doses (50 μg), but not at lower antigen doses or in the absence of adjuvants (94, 95). In contrast to these models, mice lacking the lymphotoxin-α (LTα) gene completely lack FDCs and are incapable of forming GCs. Surprisingly, B cells in these mice show some degree of affinity maturation, even though repeated immunization with high doses of antigen (200 μg) is required (96). Together, these models suggest that, although retention of antigen by FDCs is essential to ensure affinity maturation under suboptimal conditions, this retention may be less important when antigen is widely available, especially within adjuvant depots.

Germinal Center T Cells

Only a small fraction of the cells in GCs are T cells; nevertheless, these cells are essential for GC maintenance and for affinity maturation. Athymic nude mice fail to develop GCs unless rescued by adoptively transferred thymocytes prior to immunization (97). Injection of antibodies that block the CD40-CD40L interaction, a major pathway by which T helper

cells deliver cognate help to B cells, is sufficient to dissolve an ongoing GC reaction (98). Likewise, humans with loss-of function mutations in either CD40 or CD40L fail to develop GCs; consequently, they have an excess of nonclass-switched, low-affinity serum antibody (99, 100). Patients with such mutations are highly susceptible to a multitude of bacterial and opportunistic infections, highlighting the importance of GCs to human health.

A specific population of GC T cells known as T follicular helper (Tfh) cells have been under intense investigation in recent years, and a number of excellent reviews are available on the subject (28, 30, 101, 102). Tfh cells were originally defined as $CD4^+$ T cells that express the chemokine receptor CXCR5, which is required for T cell entry into the B cell follicle (32, 84, 103). In addition to CXCR5, Tfh cells also express the CD28 family members ICOS and PD-1 (84, 104), the cytokine IL-21 (105), and, in humans, CD57 (106), among other molecules. Tfh cell development in mice is dependent on the expression of the transcription factor Bcl-6 (107–109). Intravital imaging studies have shown that access of T cells to GCs is dependent on Bcl-6 expression, as well as on the presence of the adaptor molecule SAP (110, 111). Although a set of markers that conclusively distinguish between Tfh and other activated T cells has not been defined (29), it is clear that Tfh cells play a fundamental role in the GC, as evidenced by studies showing that GCs develop abnormally when this population is altered (107, 109, 112–116).

Much less attention has been paid to other populations of GC-resident T cells, including $CD8^+$ T cells (106), Th17 cells (117), and $Foxp3^+$ regulatory T cells (118–120, 120a), all of which may play a role in positively or negatively regulating the GC reaction. The kinetics of appearance of the different GC-resident T cell populations and how this relates to GC progression are poorly defined and may provide insights into how the GC reaction is controlled. The role of Tfh cells in GC selection is discussed in depth in subsequent sections of this review.

Other Germinal Center Populations

In addition to T and B cells, GCs also contain tingible-body macrophages (TBMs) and a small population of conventional, bone marrow–derived dendritic cells (cDCs). TBMs engulf and eliminate apoptotic B cells that are frequent products of GC selection (**Figure 1*d***) (38). Defects in TBM function have been reported in patients with systemic lupus erythematosus (SLE) (121), and interfering with the ability of TBM to dispose of dying cells can lead to lupus-like disease (122, 123). Therefore, TBMs may contribute to limiting the emergence of autoreactivity in the GC.

The role of GC-resident cDCs is less well understood. These cells were first identified in human tonsil by immunohistochemistry (36), and $CD11c^+$ cells with cDC morphology have also been observed in mouse GCs by intravital microscopy using a CD11c-YFP transgenic strain (Reference 37 and our unpublished observations). However, the function of these cells in the GC has not yet been determined.

GERMINAL CENTER DYNAMICS

Intravital microscopy studies have clarified the events taking place before and during the GC reaction. Cells previously thought to slowly drift between the GC zones were instead found to be constantly moving along convoluted paths within the GC structure at a very rapid pace, pausing momentarily only to divide (24–26). Simultaneously, advances in chemotaxis research have clarified how this movement is regulated (47, 51, 71, 83, 124–126). Below, we discuss our current understanding of B cell dynamics in the GC, focusing primarily on migration between the GC LZs and DZs and on how this migration impacts affinity-based selection.

Interzonal Migration

Selection in a GC where antigen is localized in the LZ and cell proliferation in the DZ requires that GC B cells transit between the two zones. In a classic study, Hanna (22) pulse-labeled cells in vivo using radiolabeled nucleotides and

found that dividing cells appear first in the DZ and are detected in the LZ only several hours later. Centrocytes were thus thought to be the progeny of dividing centroblasts, suggesting a model in which the latter were constantly giving rise to the former. Upon the discovery of somatic mutation by Weigert (6, 7) and the observation that genetic variants arise in GCs (11, 12), it was proposed that the DZ functioned as a source of the genetic variants that migrated to the LZ to be selected by antigen displayed on FDCs (23).

However, a simple model in which movement of the centroblast progeny is unidirectional from DZ to LZ would likely fall short of explaining the remarkable efficiency of affinity maturation. First, the odds of achieving a dramatic increase in affinity by accumulation of random mutations are exceedingly low, even when considering the large number of mutants generated in the course of a GC reaction (127, 128). Second, studies that reconstructed the sequence of individual mutations leading to a high-affinity antibody showed that affinity increased in a stepwise fashion with each additional mutation, suggesting multiple rounds of selection (129, 130). Together, these observations led Perelson and colleagues to propose that B cells undergo iterative cycles of mutation and selection in the GC, in a process termed cyclic re-entry (127, 128). According to this model, a fraction of B cells positively selected in the LZ would return to the DZ for further rounds of proliferation, mutation, and selection in a cyclic fashion. This model accounts for both the efficiency of affinity maturation and its stepwise nature (127, 128).

Despite the attractiveness of the cyclic re-entry model, supporting evidence remained indirect until GCs were imaged by intravital microscopy (24–26). Three independent groups directly observed bidirectional migration between the two GC zones. However, the small number of events imaged and the limited observation time failed to reveal the net vector of migration from DZ to LZ predicted by cyclic re-entry (24–26). Moreover, it was argued that the low frequency of interzonal migration events was not sufficient to support a cyclic re-entry model, suggesting instead a model in which selection would occur in both LZ and DZ simultaneously (26, 131). Independent mathematical analyses of the three sets of intravital imaging data have questioned the validity of these objections (132) and suggested that more sophisticated analysis of the data may reveal a net flow of cells from the DZ to the LZ (133). This issue was resolved using in situ photoactivation to label GC B cells in the LZ or DZ directly (31). This new labeling system allowed direct observation over periods of many hours, circumventing the need to track individual cells traversing the LZ/DZ interface. The data uncovered a strong net vector of migration toward the LZ: Whereas up to 50% of DZ cells migrated from to the LZ in 4 h, less than 10% of LZ migrated in the opposite direction in the same period of time. Mathematical treatment of these data showed that this pattern corresponds to a fraction of roughly 30% of GC B cells re-entering the DZ after each round of LZ selection (31). In addition, this study showed that clonal expansion involves the return of B cells from the LZ to the DZ, providing further evidence of cyclic re-entry (31). A further implication of these findings is that, rather than differentiating linearly from "centroblasts" to "centrocytes" and then to plasma or memory cells, GC B cells are constantly shifting between what can be regarded as two closely related but functionally distinct states of a single population, which are associated with positioning in either LZ or DZ. True differentiation according to this model would be restricted to the positively selected B cells that receive selective cues to exit into the post-GC cell fates. Such a model is supported by the relatively small differences between LZ and DZ B cells with respect to size, dynamic behavior, and gene expression (24–27, 31).

Positional Cues that Regulate Positioning within the Germinal Center

Many of the cues that regulate the positioning of GC cells in the LZ and the DZ

have been elucidated. As noted previously, the chemokine receptor CXCR4 is essential for positioning GC B cells in the DZ, and interfering with this receptor either genetically or chemically is sufficient to disrupt LZ/DZ segregation (71). $CXCR4^{hi}$ GC cells are attracted by the chemokine CXCL12/SDF-1, which is expressed at higher levels in the DZ than in the LZ (71). Regulation of LZ positioning is somewhat less clear. CXCR5-expressing B cells move toward CXCL13, which is expressed by FDCs in the LZ (32, 71). However, the difference in expression of CXCR5 between LZ and DZ B cells is minimal (31). More importantly, although mice deficient in CXCR5/CXCL13 have abnormal follicle architecture (83, 124), GCs in these mice have readily distinguishable LZs and DZs (71). Thus, CXCR5 is not essential for GC LZ/DZ polarization. Other G protein–coupled receptors such as CCR6, Ebi2, and $S1P_3$ are also expressed more highly in LZ cells (31), and they may play a redundant role to CXCR5 in LZ positioning (133a).

Surprisingly, the GC reaction, and even affinity maturation, proceeds with little alteration in the absence of either CXCR4 or CXCR5 (51, 134, 135). These data suggest the possibility that the interplay of chemoattractants and their receptors in the GC may not be an essential requirement for the generation and selection of high-affinity B cell mutants. If this is indeed the case, it will be interesting to determine why such an elaborate pattern of migration has evolved and what the consequences of its disruption may be.

In summary, the overall pattern of B cell migration within the GC is becoming increasingly clear with the advent of intravital imaging and the clarification of the roles of key chemoattractant receptors. A number of questions are still open, however. Among these are the identity of the signals that trigger migration from the LZ to the DZ; the duration of the LZ/DZ cycle and each of its steps, and how this may vary as a consequence of affinity; the number of rounds of replication that a B cell will undergo in the DZ before migrating back to the LZ, and how this impacts DZ/LZ ratio; how cells exit the GC after selection and how this exit is controlled; how the signals that trigger selection are synchronized with those triggering migration; and finally, what the biological function is of GC compartmentalization.

GERMINAL CENTER SELECTION

The selective survival and expansion of rare GC B cell clones carrying somatic mutations account for the progressive increase in antibody affinity with time after immunization (5, 7, 8, 11). Although GC B cells do not secrete large quantities of antibody, they contribute to long-lived humoral responses by differentiating into plasma cells and memory B cells. Thus, GC selection involves survival, clonal expansion, and cell fate decisions.

Importantly, the default fate for a GC cell is to die by apoptosis (136). GC B cells express high levels of the death receptor Fas (41), lose expression of the antiapoptotic molecule Bcl-2 (137), and, when placed in culture, die within a few hours (136). Survival requires signals that induce expression of antiapoptotic proteins such as Mcl-1 (138) and cell proliferation. In the framework of a cyclic re-entry model, clonal expansion is intimately linked to return from the LZ to the DZ for additional cycles of proliferation and mutation. In addition, an unknown fraction of selected cells is triggered to leave the GC reaction and differentiate into antibody-secreting plasma cells or memory B cells (see below). Finally, in addition to positive selection, GC B cells carrying mutations that render them self-reactive may be "negatively" selected (139–141) by a process that appears to be defective in autoimmune diseases (142, 143).

Selection for Recycling and Clonal Expansion within the Germinal Center

Selective clonal expansion involves competition between B cells with different affinities: Whereas higher-affinity cells are selectively expanded, lower-affinity cells are eliminated by apoptosis, resulting in a GC in which B

cell clones have an increasingly higher average affinity for the immunizing antigen (23, 144). Once a B cell is in the GC, there is no known absolute threshold for selection; instead, this process operates on the basis of the relative affinity of competing clones. For example, in the absence of competition, B cells that express receptors with either high or low affinity for the hapten NP generate GCs with similar kinetics and that grow to similar sizes (145). However, low-affinity cells are incapable of forming GCs in the presence of higher-affinity competitors (145–147). Thus, lower-affinity cells are capable of sensing and responding to the presence of higher-affinity competition; that is, there must be some mode of information transfer, such that "losing" cells are triggered to stop proliferating or undergo apoptosis in the presence of "winning" cells.

Exchange of information along these lines is a common feature of many biological systems (148). For example, during development, vertebrate neurons are produced in excess and compete for limiting amounts of nerve growth factors released by their target cells, and losing neurons are induced to apoptose (148). This communication can take place in different ways, but a common theme seems to be the presence of a "limiting factor" that will allow only a certain proportion of competing cells to survive. In the case of the GC, communication between losing and winning cells was traditionally thought to occur by direct competition for a limiting amount of antigen on the surface of FDCs. According to this model, high-affinity cells would communicate their presence to lower-affinity cells either by consuming all of the available antigen—working essentially as an "antigen sink"—or, more likely, by "parking" on, and blocking access to, the antigen-rich sites on FDCs (136, 149, 150). In either case, the amount of antigen available for lower-affinity cells would be insufficient to trigger a BCR signal capable of rescuing these cells from apoptosis, leading to their elimination from the GC. Follicular T cells would play an accessory role, either providing survival signals to all cells or differentiation signals to cells selected by the antigen (151) (**Figure 2*a***). An alternative to the direct competition model is that the follicular T cells are the limiting factor in GC selection. According to this idea, the BCR captures and mediates the internalization and processing of differing amounts of antigen from FDCs in proportion to its affinity (152, 153), essentially mapping BCR affinity onto surface peptide-MHC (pMHC) density. The signals provided by direct BCR cross-linking by antigen would be sufficient to ensure the survival of all B cells over a wide range of signal intensities, but only those that display the highest levels of surface pMHC would receive help from a limiting number of GC-T cells. Thus, the T cell would provide help selectively to B cells with higher BCR affinity (**Figure 2*b***) (27, 101, 154, 155).

Both of these models highlight the distinction between survival and selection signals. Although signals from both the BCR and T cells are essential to maintain a GC reaction, in theory only one limiting factor is required to ensure competition and affinity maturation. For example, a B cell that loses expression of its BCR by hypermutation will die cell-autonomously from lack of a BCR-mediated survival signal, regardless of whether direct BCR stimulation by antigen is the limiting factor in GC selection (**Figure 2**). Distinguishing survival signals from limiting factors has been difficult because, irrespective of the model, both are dependent on the BCR.

Direct selection by antigen. The idea that B cells compete for signals induced by a limited amount of antigen deposited on the FDC surface originated with the very first studies of affinity maturation, in which the extent of the increase in antibody affinity was shown to be greater at lower antigen doses (5, 156, 157) (**Figure 2*a***). Attractive as this model may be, the mechanism by which it would operate within the dynamic GC context is not clear. In particular, it fails to account for the intercellular communication of relative affinity levels that would be required for effective competition. Whereas competition for BCR signals explains why higher-affinity B cells would

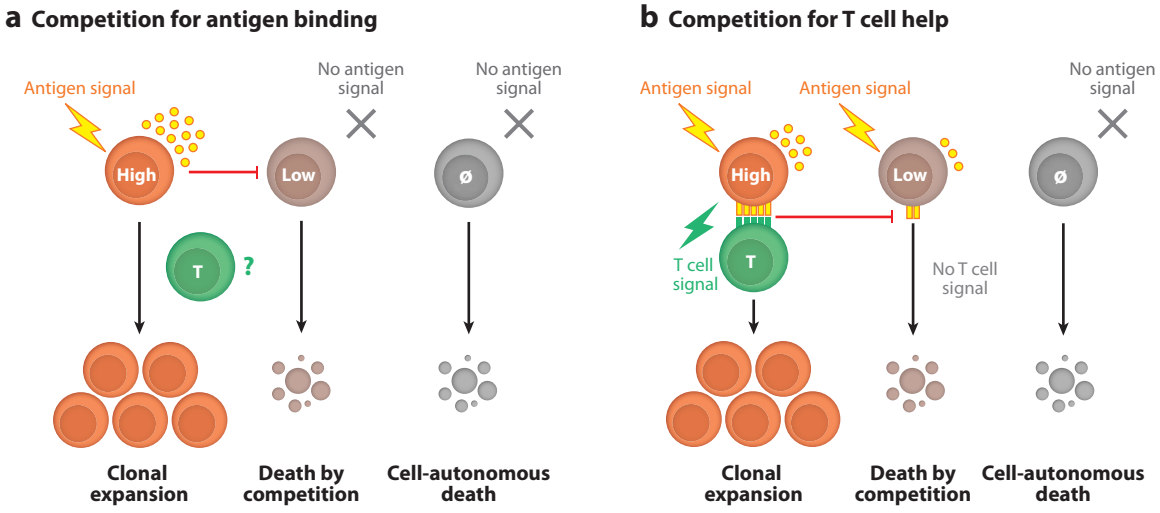

Figure 2

Models for germinal center (GC) competition. (*a*) Direct competition for antigen binding. Higher-affinity B cells (High) will take up all available antigen (*yellow circles*) or occupy all antigen-rich sites, preventing lower-affinity B cells (Low) from receiving any antigen signals through their B cell receptors (BCRs). This leads to expansion of higher-affinity clones secondary to BCR binding and death of lower-affinity clones from lack of a BCR signal (*red line*). In this model, antigen is the limiting factor, and the role of the T cell (T) is undefined. (*b*) Competition for T cell help. Both high- and low-affinity B cells bind antigen in an amount proportional to their BCR affinity. Both cells receive enough BCR signals to ensure survival, but higher-affinity B cells acquire more antigen and present more antigenic peptide on MHC-II (pMHC, *yellow rectangles*). Cells with higher pMHC compete favorably for a limiting number of helper T cells, leading to their expansion. Lower-affinity clones die from lack of T cell help owing to the sequestration of T cells by higher-affinity cells (*red line*). In this model, BCR signaling is essential for survival but not limiting, whereas T cells are the limiting factor driving selection. Note that, in both models, cells that have lost BCR expression or whose BCRs no longer bind antigen above a certain affinity threshold (Ø) die from lack of BCR-mediated survival signals. Exchange of information between higher- and lower-affinity B cells is represented by red lines. Although in theory only one limiting factor is required for selection, these two models are not necessarily mutually exclusive.

proliferate more vigorously than those with lower affinity, it does not provide any insight into how the latter are prevented from proliferating or induced to apoptose by the presence of the former, despite having sufficient intrinsic affinity to respond to antigen. The idea that higher-affinity cells would "park" on antigen-rich sites on FDCs in the LZ, thus blocking the access of low-affinity cells to these sites, is not supported by live imaging, as B cells in the GC fail to form prolonged, stable synapses with antigen deposited on FDCs (24–26). An additional theoretical consideration is that Ig somatic mutation in GC B cells is random and can create self-reactive cells (142, 143). Given that the GC contains self-antigens, autoreactive B cells would be positively selected if antigen binding were the sole determinant of selection.

The precise role of the BCR in GC selection has been difficult to define. Injection of an antigen bolus during the GC reaction leads to apoptosis and dissolution of the GC rather than to positive selection (31, 139–141). However, soluble antigen delivered in this manner may not assume the same physical characteristics as FDC-bound immune complexes and is therefore a poor model for the form of BCR stimulation that would lead to selection in the GC. In contrast, genetic experiments indicate that affinity maturation can occur, albeit to a lesser extent, in the absence of the complement receptor *Cr2*, serum antibody, and even FDCs, provided that a source of free antigen such as an alum depot is available (92–96). Thus, the exact form of the antigen that would trigger productive BCR signaling is unclear.

Genetic experiments in which BCR signaling is modified are also difficult to interpret, in part because BCR expression is essential for B cell survival (158) and because deletion or mutation of regulators of BCR signaling often abrogate GC formation. For example, mice lacking, or expressing hypomorphic mutants of, CD19, CD21, Igβ, PI3K p110δ, Dock8, and PLCγ2, among others, fail to develop, or develop stunted, GCs and T-dependent antibody responses (74, 159–166). Nevertheless, loss-of-function studies have been important in elucidating a role for BCR cross-linking in GC selection. For example, mice with hypomorphic mutations in CD19 or in the guanine exchange factor Dock8, in which GCs are reduced but not completely eliminated, show that BCR signals are important for optimal affinity maturation (74, 160, 167). Likewise, infection of CD19-deficient mice with vesicular stomatitis virus, a protocol that restores the appearance and maintenance of GCs, shows that affinity maturation is impaired in the absence of CD19 (168). Conversely, deletion of FcγRIIb, a negative inhibitor of BCR signaling, leads to increased T-dependent antibody titers, suggesting that, at a minimum, the FcγRIIb pathway regulates the differentiation of plasma cells, and possibly GC selection as well (82, 169). A clue regarding the mechanism by which the BCR may influence selection comes from experiments with mice deficient in CD45, a coreceptor that increases signaling through the BCR but does not affect its endocytic capacity. Although BCR signaling is altered in these mice, hypermutation and selection appear to be unaffected. Thus, selection can proceed under conditions of impaired BCR signaling, provided that antigen presentation remains unaltered (154, 170). In summary, although the BCR is essential for GC B cell survival and selection, the precise role of the BCR in the GC reaction, including a distinction between its dual role as a signaling and an endocytic receptor, remains to be determined.

Selection by T cell help. An alternative and possibly complementary idea to direct antigen stimulation through the BCR stipulates that GC-resident T cells are the limiting factor in affinity-based selection (**Figure 2*b***). There is abundant indirect support for this model, both theoretical and experimental, beginning with the discovery that antibody responses require T cell help (reviewed in Reference 171). In addition to experimental data, mathematical modeling of the GC reaction based on available multiphoton data and other theoretical considerations (132, 155) indicate that only competition for T cells would be capable of achieving the levels of affinity maturation encountered experimentally.

A role for T cells in regulating and maintaining the GC was uncovered experimentally by blocking the CD40-CD40L interaction with antagonistic antibodies to CD40L, which terminates the GC reaction (98). Secreted products of helper T cells, including IL-21, are also essential for normal GC development (172, 173). Consistent with these findings, GC size is closely correlated with the availability of follicular T cell help (115), and deregulation of Tfh cells leads to inappropriate GC B cell selection and humoral autoimmunity (112, 114, 116). One clue to how T cells select the high-affinity B cells was the observation that T cells are able to discriminate among B cells on the basis of the amount of pMHC displayed on the cell surface (174). T cells synapse preferentially with B cells with higher surface peptide density in vitro (174). This idea was corroborated by in vivo experiments showing that IL-4-producing T helper cells are more likely to form doublets with GC B cells bearing affinity-increasing mutations (175). Similarly, B cells deficient in the MHC-family molecule H2-O, a biochemical inhibitor of peptide loading onto MHC class II, outcompete H2-O-sufficient B cells in mixed knockout/wild-type GCs, even in conditions in which BCR affinity is equalized by forced expression of an NP-specific BCR (176). Finally, selection by T cells would be expected to proceed even in the absence of FDC-bound immune complexes, as long as another source of antigen is available, a prediction that has been confirmed experimentally (92–96). These and numerous other observations argue that helper

T cells play an essential role in GC selection and maintenance and that they may be a limiting factor regulating which B cells are allowed to progress in the GC.

Direct evidence for T cell–mediated selection was obtained by targeting T cell antigen to GC B cells in a BCR-independent manner (31). Ovalbumin (OVA) was targeted to GC B cells using an antibody to the surface lectin DEC205, which is highly expressed on activated B cells (31). By injecting mice with chimeric antibodies to DEC-205 fused to OVA (αDEC-OVA), Victora and colleagues increased the pMHC load on DEC-205-expressing cells while bypassing BCR stimulation (31). Administering this treatment to mice with ongoing GCs containing both DEC205-sufficient and -deficient B cells led to a dramatic increase in the proportion of the former and virtual elimination of the latter, even though BCR affinities were equivalent in the two populations. Thus, acutely skewing T cell help toward a subset of GC B cells in a BCR-independent manner leads to their robust selection, both for further proliferation and for export from the GC as plasmablasts (31). In a variation on this model, wild-type mice with ongoing GC reactions were injected with anti-DEC205-OVA. This approach generated a situation in which all GC B cells bore uniformly high surface pMHC levels, essentially blinding GC T cells to B cell affinity. Under these conditions, GC selection ceases to operate, and both serum affinity maturation and the selection of high-affinity mutants are abrogated, indicating that stronger BCR cross-linking alone is not sufficient to ensure B cell selection (31). Thus, recent experiments strongly support the view that T cells are the limiting factor in GC selection.

Although the data discussed above emphasize the limiting role of the T cell in affinity-based selection, they do not suggest that BCR stimulation is dispensable for the GC reaction. BCR signaling plays an essential role in ensuring GC persistence (159, 160, 170, 177, 178), and loss of BCR signaling by nonsense or structurally compromising mutations introduced during SHM is likely to induce cell-autonomous B cell death in the GC (**Figure 2**). The experiments discussed above also do not necessarily exclude the possibility of synergy between BCR signaling and T cell help. Synergistic enhancement of B cell survival by the BCR and CD40 pathways has been documented in vitro (136), though whether this happens in vivo, or what the molecular mechanisms for such synergy would be, is still not clear (179–181). Thus, even though GC T cells appear to be the limiting factor in affinity-based selection, they may work synergistically with signals emanating from the BCR, Fc receptors, and possibly other signaling pathways to enhance the efficiency of clonal selection and affinity maturation.

Open germinal centers. An intriguing feature of GC selection that has only recently become apparent is that mature GCs are open to invasion by B cells that were not present during the initiation of the GC reaction (24, 34). In addition to the naive B cells that frequent the LZ without participating in the GC reaction (24), B cells with higher affinity to the immunizing antigen can efficiently colonize GCs, ultimately replacing the lower-affinity clones that initiated the reaction (24). From the perspective of GC selection, this means that high-affinity clones may be able to spread from one GC to another, thus increasing the overall efficiency of affinity maturation. Although the presence of the same B cell clone in more than one GC has been reported to occur in human samples (182, 183), alternative explanations such as a common pre-GC or memory B cell precursor cannot be discarded, and as such, no concrete evidence of such "GC hopping" by high-affinity clones is currently available.

In addition to invasion by higher-affinity clones of the same specificity, ongoing GCs can also be replaced by a completely different antigen, provided that T cell help for the second reaction is available (33). This phenomenon, which is referred to as GC reutilization, suggests a disconnect between GC structure—as defined microanatomically—and the GC reaction—which refers to the

temporally restricted expansion and contraction of B and T cell clones specific for physically linked epitopes. It also provides a new perspective on GCs in sites prone to constant antigenic stimulation, such as Peyer's patches and tonsils. In these sites, it is likely that GCs are sequentially colonized by cells specific for different antigens. This has implications for our understanding of chronic GC reactions—such as those found in HIV and certain parasites with variable surface coats—and the process of epitope spreading that is characteristic of certain humoral autoimmune diseases such as SLE (33).

Selection for Export from the Germinal Center

In addition to cyclic re-entry, positively selected GC cells are exported from the GC as plasmablasts—the precursors of the plasma cells that will secrete antibody into serum and secretions (184)—or as memory B cells, which, upon re-exposure to antigen, will rapidly differentiate into plasma cells (185) or re-enter the GC reaction for further diversification (186, 187).

Selection into the plasma cell compartment. Commitment to the plasma cell fate begins while B cells are still in the GC. Blimp-1, the master regulator of plasma cell differentiation, is expressed by a small subset of GC cells before they are exported (126, 188, 189). Plasma cells differ in terms of affinity and hypermutation from GC and memory cells. Whereas the GC/memory compartments retain a fraction of cells with lower affinity for the NP hapten, the affinity of bone marrow plasma cells is almost uniformly high (190). These differences are accentuated in mice transgenic for the antiapoptotic molecule Bcl-2, in which plasma cell affinity remains high despite less stringent GC and memory cell selection, suggesting a qualitative difference between selection into the plasma cell versus the memory or recycling compartments (191). These observations have been confirmed and extended by Brink and colleagues, using an ingenious system in which mice bearing a transgenically encoded BCR specific for hen egg lysozyme (HEL) are challenged with HEL mutants that bind to this BCR with a range of affinities (192–194). Immunization with low-affinity HEL leads to the appearance of a characteristic somatic mutation (Y53D) in the transgenic BCR that increases its affinity by 80-fold. As in the NP system (190), post-GC plasma cells are heavily dominated by clones bearing the high-affinity mutation, even at a time point when this mutation is present in only a small fraction of GC cells (193, 194). Thus, unlike cyclic re-entry and memory cell differentiation, selection into the plasma cell compartment appears to favor cells with high absolute BCR affinity, rather than relying on intercellular competition.

As with cyclic re-entry, the relative importance of signals delivered through the BCR or by T cells to plasma cell differentiation has not been resolved. Although short-lived plasma cells can form very efficiently in T cell–independent responses (195, 196), a number of T cell–derived signals influence both the numbers and the affinity of the plasma cell pool in response to T-dependent antigens (197). In vitro studies have defined a clear molecular pathway by which ligation of CD40 can lead to plasma cell differentiation. This pathway involves CD40-mediated activation of the NF-κB pathway and induction of the transcription factor IRF4, leading to suppression of Bcl-6 and, ultimately, to expression of Blimp-1 and plasma cell differentiation (198–200). In agreement with these data, in vivo studies in which CD40 signaling is induced by either agonistic antibodies or genetic approaches consistently show that strong CD40 ligation increases the magnitude of the short-lived plasma cell response while curtailing the GC reaction (201–205).

Work from our laboratory has shown that increasing T cell help by targeting T cell antigen to B cells through DEC-205 leads to a dramatic increase in their recruitment into both pre- and post-GC plasma cell fates (31, 147). However, unlike direct stimulation of CD40,

DEC-205 targeting does not curtail the GC reaction, but instead potently drives B cells to recycle from the LZ to the DZ as well. Two reasons for this discrepancy may be that CD40 stimulation alone fails to mimic the whole range of signals exchanged between a follicular helper T cell and a B cell (206) and that other signals in addition to CD40 act in a dominant manner to drive a fraction of selected B cells back into the GC pathway. Alternatively, the decision to become a plasma cell upon CD40 ligation may be dose dependent, in which case the GC pathway is inhibited only by the supraphysiological levels of CD40 stimulation seen in CD40 transgenic or anti-CD40-treated mice. Evidence in favor of the latter is provided by data showing that transgenic T helper cells specific for a BCR-derived peptide direct GC B cells expressing this BCR preferentially to the plasma cell compartment, while preventing GC recycling and memory cell differentiation (207). Thus, supraphysiological T cell help triggers plasma cell differentiation even in the presence of the full complement of signals delivered by T cell help in vivo.

In addition to CD40, other T cell–derived signals can affect plasma cell differentiation in vivo. Mice lacking the surface receptor PD-1 or its ligands PD-L1 or PD-L2 have reduced numbers of plasma cells in both spleen and bone marrow, in a process that is B cell intrinsic. Though fewer in number, the resulting plasma cells are of higher affinity, suggesting more stringent selection of B cells by Tfh cells in the absence of this axis (208). Similarly, mice lacking IL-21 or the IL-21 receptor show decreased numbers of plasma cells, though these cells are of decreased rather than increased affinity (172). Interestingly, lack of PD-1 signaling leads to reduced production of IL-21 by follicular T cells (208), possibly providing a link between these two phenotypes.

Selection into the memory B cell compartment. Selection into the memory B cell compartment is the least understood of all the cell fate decisions made in the GC (197, 209). Memory cells can be generated from T-B cell interactions in a GC-independent manner (210, 211) as well as during T-independent B cell responses (212). Nonetheless, the majority of memory cells in wild-type mice responding to T-dependent antigens are likely to arise from the GC reaction, and the classic definition of memory B cell includes the presence of SHM in Ig genes (185).

Class-switched memory cells are indistinguishable from GC cells in terms of affinity and number of somatic mutations, although both are of lower affinity than plasma cells (190, 191). A recent review of the effects of a wide variety of genetic or chemical perturbations on GC and memory B cells shows that, overall, defects in the selection of GC cells are mirrored by defects in memory B cells, while often differing from effects in plasma cells (197). A possible interpretation of these observations is that, unlike plasma cells, memory B cells differentiate stochastically from among positively selected GC cells, and simply surviving apoptosis is sufficient for memory B cell differentiation. In support of this hypothesis, forced expression of Bcl-2 leads to an up to 20-fold increase in the number of IgG^+ GC/memory cells after immunization and to a marked decrease in the efficiency of selection of both memory and GC cells but not of plasma cells (213). Similarly, deletion of the proapoptotic receptor Fas leads to impaired selection in both GC and memory compartments (214). On the other hand, studies in mice deficient in the cytokine IL-21 or its receptor show an accumulation of memory cells concomitant with a reduction in GC cell numbers (172, 173). These memory cells are enriched in germline Ig sequences, suggesting that their precursors spent little if any time in the GC (172), which is in agreement with the notion that memory cells emerge early in the GC response (197). Thus, although memory B cells appear to be selected according to similar rules as recycling GC cells, how the decision is made to remain in the GC or exit as a memory cell remains poorly understood.

In summary, the available data on the differentiation of GC B cells along the memory or plasma cell fates suggest that, whereas very

high-affinity cells have an increased probability of being directed to the plasma cell fate, lower-affinity cells that pass positive selection can be directed into either the memory or recycling GC cell pools, in a decision that may be influenced by factors such as timing and the presence of IL-21. The recent development of strategies to trigger plasma cell differentiation from GC cells acutely (31) and to trace post-GC cells in vivo (186, 187) may help further our understanding of how these cells are selected within the GC.

Negative Selection in the Germinal Center

Because Ig SHM is random, there is a continual risk of producing autoreactive GC B cells. Therefore, GC reactions must include one or more checkpoints for removing the autoreactive B cells generated by SHM. These checkpoints are defective in patients that produce pathogenic autoantibodies (142, 143). Accordingly, high-affinity anti-DNA antibodies in human SLE patients are often heavily mutated (215, 216), and anti-dsDNA antibodies found in murine lupus models arise through SHM (217).

Data obtained from human subjects by antibody cloning also provide evidence for the emergence of self-reactivity by SHM. Although polyreactive and/or self-reactive BCRs are largely purged from the human naive B cell repertoire by checkpoints operating during B cell development (218), weakly self-reactive receptors are commonly found among memory B cells in healthy individuals (219) and are enriched in post-GC B cells in HIV-infected individuals (220). Artificial reversion of these antibodies to their germline configuration is sufficient to eliminate auto/polyreactivity, indicating that these properties emerge through hypermutation in the GC (219). Reversion to germline also eliminates the self-reactivity of most high-affinity autoantibodies from patients with SLE (216), indicating that both the low-affinity autoantibodies found in normal individuals and the pathogenic, high-affinity antibodies of SLE patients arise as by-products of hypermutation. These data, therefore, suggest the existence of a checkpoint within or after the GC that is capable of eliminating high-affinity self-reactive mutants from healthy individuals.

Direct evidence for deletion of autoreactive cells in GCs comes from experiments with B cells that express a transgenic antigen receptor with dual specificity for the hapten p-azophenylarsonate and for nuclear self-antigens. B cells with this specificity are actively eliminated in the GC, but they can be rescued by overexpression of Bcl-2 (221), implicating B cell apoptosis as an important mediator of GC-negative selection. The precise nature of the checkpoints that control the emergence of B cell autoreactivity in the GC are still debated. A variety of genetic defects can lead to GC deregulation and to the development of high-affinity autoantibodies (112, 114, 116, 217, 222–227). These checkpoints are thought to operate at both the B cell and T cell levels. B cell–intrinsic checkpoints include expression of the inhibitory Fc receptor FcγRIIb, the inhibitory BCR coreceptor CD22, and the downstream BCR signaling molecule lyn (82, 222–224, 226, 227). On the T cell side, humoral autoreactivity arises from defects in regulation of GC Tfh populations, either intrinsic to the Tfh cell (112, 225) or dependent on its interactions with regulatory CD4 or CD8 cells in the periphery (114, 116, 119, 120, 120a). The defect in the Fas/Fas ligand axis that leads to autoimmunity in the *lpr* and *gld* mutant mice (217, 228) or in humans to autoimmune lymphoproliferative syndrome (229, 230) appears to operate synergistically on both B and T cells (231, 232). A caveat of some of these genetic systems is that autoantibody-inducing mutations are present in either B or T cells throughout their entire development and not only during the GC reaction. Therefore, it is difficult to pinpoint these defects solely to the GC as opposed to the bone marrow or to the early stages of B cell activation by antigen or T cells. The use of conditional targeting approaches that delete genes specifically in the GC—as done in the case of Fas (231)—should help clarify this issue.

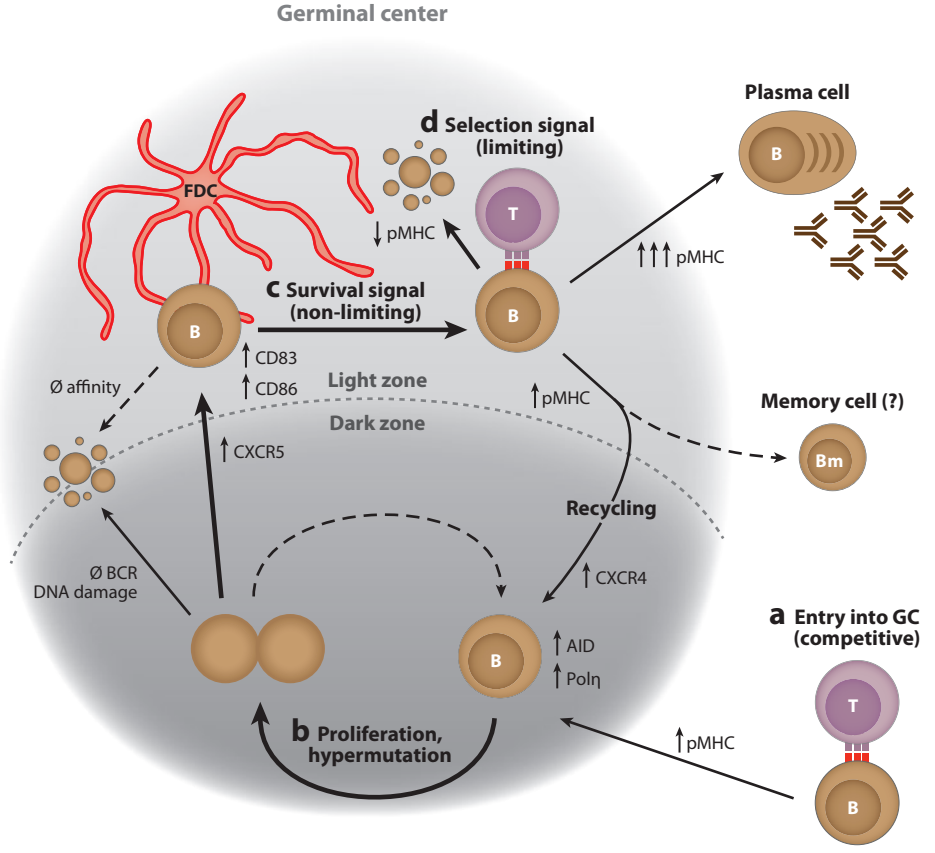

Figure 3

Proposed model for affinity-based selection in the GC. (*a*) B cells compete for a limiting number of T cells at the T:B border; only the cells with the highest affinity are allowed to enter the GC reaction. (*b*) GC cells in the dark zone (DZ) proliferate and turn on the somatic hypermutation machinery, which includes the enzymes AID and Polη. These cells are maintained in the DZ by high expression of chemokine receptor CXCR4. Cells that introduce mutations that impair BCR expression at this point (Ø BCR) die owing to the lack of a BCR signal. After one (or potentially more) cycles of division/mutation, surviving DZ B cells migrate toward the light zone (LZ), in a process that involves upregulation of chemokine receptor CXCR5. (*c*) B cells interact with antigen in immune complexes on FDCs. B cells with very low or no affinity for antigen (Ø affinity) may also die from lack of a BCR signal at this step. Antigen signals are not limiting at this point, and all B cells transiting to the LZ upregulate CD83 and CD86. BCR affinity determines the level of peptide-MHC (pMHC, *red rectangles*) on the B cell surface. (*d*) B cells present antigen to a limiting number of T helper cells. B cells with higher pMHC interact preferentially with T cells, preventing their interaction with lower-affinity B cells, in a competitive process. B cells that successfully interact with T helper cells can then follow three potential fates: recycling, with upregulation of CXCR4 and re-entry into the DZ for further proliferation/mutation; exit from the GC into the plasma cell fate (likely under conditions of high affinity/pMHC density); or exit into the memory B cell fate. The latter possibility has not yet been shown to be enhanced by interaction with T helper cells. In different points during this process, B cells that develop strongly autoreactive BCRs are eliminated by multiple checkpoints, which may be B cell intrinsic or T cell dependent (not shown). Abbreviations: AID, activation-induced cytidine deaminase; BCR, B cell receptor; DZ, dark zone; FDC, follicular dendritic cell; GC, germinal center; LZ, light zone. Arrow width represents the proportion of cells thought to follow a given path. Dashed arrows represent likely events for which the available data are inconclusive.

Regardless of mechanism, the diversity of the available mouse models of humoral autoimmunity argues for the existence of multiple checkpoints operating during or even after the GC reaction. It is also clear that these checkpoints are not entirely redundant, because genetically overriding a single T or B cell–intrinsic checkpoint is often sufficient to induce autoimmune disease. As is the case for positive selection, checkpoints for autoreactivity that are B cell intrinsic likely act in concert with checkpoints involving T cells and other cells so as to limit the generation of high-affinity autoantibodies by SHM.

Selection for Access to the Germinal Center

Classical studies using chimeric animals or simultaneous immunization with two different haptens suggested that GCs are initially colonized by a very small number of high-affinity precursors, maybe as few as one to three cells (10, 40, 233). By contrast, even cells with very low affinity for antigen are intrinsically capable of forming GCs under noncompetitive conditions (145–147). Likewise, many high-affinity somatically hypermutated antibodies to HIV cloned from patients have very low affinity for antigen when reverted to their germline configuration (220, 234). Thus, the absolute affinity threshold required for triggering the B cell GC program must be very low, indicating that a large number of naive B cells in a polyclonal repertoire have the cell-intrinsic potential to enter the GC reaction. By studying competition between B cells with high and low affinity for the hapten NP (145, 196), we have recently shown that entry into the GC is a competitive process and that presence of high-affinity competitors inhibits the proliferation and activation of lower-affinity B cells prior to GC coalescence (147). Intravital imaging and DEC205 targeting of T cell antigen (as described in Selection by T Cell Help, above) showed that, as in the GC, competition at the pre-GC stage is also mediated by a limiting number of helper T cells (147). Further investigation of affinity-based selection prior to the GC should lead to a greater understanding of the selection of B cell clones that found the GC reaction and may inform future vaccine design.

CONCLUSION

Nearly two decades have passed since the emergence of a model for the GC reaction that accounts for affinity maturation (23). On the basis of data that have become available in the interim, we propose an updated model of the GC in which competition for T cells is the driving force behind B cell selection and affinity maturation (**Figure 3**). According to the revised model, signals received by B cells through their BCRs are essential for survival, in a cell-intrinsic process that, though capable of distinguishing between ligands with differing affinities, is not sensitive to the presence of higher-affinity competitors. In contrast, engagement with a limiting number of T cells, before and during the GC reaction, is directly competitive and ensures the positive selection of only B cell clones with the highest affinity for antigen. This selection process is exquisitely synchronized with cyclic migration of B cells between the two GC compartments, proliferation taking place in the DZ and selection in the LZ. The years to come will most certainly generate a wealth of data that will further our understanding of the processes by which GCs ensure the robust generation of high-affinity antibodies while limiting the emergence of pathogenic self-antibodies and malignant transformation.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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