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Germinal Centers

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Abstract

Germinal centers (GCs) are microanatomical sites of B cell clonal expansion and antibody affinity maturation. Therein, B cells undergo the Darwinian process of somatic diversification and affinity-driven selection of immunoglobulins that produces the high-affinity antibodies essential for effective humoral immunity. Here, we review recent developments in the field of GC biology, primarily as it pertains to GCs induced by infection or immunization. First, we summarize the phenotype and function of the different cell types that compose the GC, focusing on GC B cells. Then, we review the cellular and molecular bases of affinity-dependent selection within the GC and the export of memory and plasma cells. Finally, we present an overview of the emerging field of GC clonal dynamics, focusing on how GC and post-GC selection shapes the diversity of antibodies secreted into serum.

1. INTRODUCTION

A hallmark of the adaptive immune system is its ability to generate antibodies that bind with high affinity and specificity to a staggering variety of antigens of almost any molecular configuration (1). High-affinity antibodies protect from insults ranging from toxins to multicellular parasites but, when directed toward innocuous exogenous antigens or self-antigens, can also trigger allergies and autoimmune disease. How a limited genome can encode for such an enormous variety of high-affinity antibodies is one of the founding questions of immunology. Part of the answer can be found in V(D)J recombination and clonal selection theory, which propose that an extraordinary diversity of immunoglobulins is generated during B cell development, before antigen encounter (2, 3). However, pre-exposure diversification alone does not account for the observation that antibody affinity increases progressively, often by orders of magnitude, after antigen is introduced (4, 5), in a process termed affinity maturation (6). Early experiments revealed that affinity maturation involves a second round of diversification of immunoglobulin genes by somatic hypermutation (SHM) that is triggered by B cell exposure to antigen (7, 8). Competitive expansion of immunoglobulin mutants with improved antigen binding leads to progressive increases in affinity of immunoglobulin genes across the population; continuous differentiation of B cells into plasmablasts (PBs) and plasma cells (PCs) results in an increase in the affinity of serum antibodies over time (6). Nowhere is this selective process more evident than in the formation of broadly neutralizing antibodies to HIV, which often involves the acquisition and selection of hundreds of somatic mutations over several years of evolution (9, 10).

Affinity maturation takes place in germinal centers (GCs), microanatomical structures that form within the B cell follicles of secondary lymphoid organs upon exposure to antigen. These structures were first described in 1884 by Walther Flemming as foci of large lymphocytes that contained abundant mitotic figures, leading to the hypothesis that they might be involved in lymphocyte development (hence, germinal; reviewed in 11). Elegant experiments in which DNA from immunoglobulin genes was sequenced from single B cells extracted from GCs showed that GCs are the sites of SHM and affinity maturation rather than generative organs (12, 13).

In 1994, MacLennan penned a comprehensive article for the *Annual Review of Immunology* titled "Germinal Centers," summarizing what was known of the biology of the GC reaction (14). His review covered many of the key concepts that we rely on to this day, including the division of a GC into light and dark zones with different functions, the role of migration of B cells between these two zones, and putative mechanisms driving the positive selection of GC B cells with the highest-affinity receptors toward clonal expansion and PC differentiation. In 2007, three independent studies used intravital imaging to glimpse at the movement of GC B and T cells in real time (15–17), triggering a flurry of activity in the field of GC biology. We summarized these findings in 2012 in a second article for the *Annual Review of Immunology*, also titled "Germinal Centers" (18). In the ten years since, our understanding of GCs has progressed from the elucidation of GC B cell and T cell phenotypes to the detailed dissection of the cellular and molecular mechanisms of affinity-based selection. In this third installment of "Germinal Centers," we summarize recent developments focusing on GC B cells and affinity-based selection. We provide an overview of GC anatomy and cell populations before delving into the mechanisms that regulate affinity maturation. We end with a section on the emerging field of B cell clonal dynamics—the study of how individual B cell clones wax and wane in the course of the antibody response—which we expect will play an increasingly prominent role in our understanding of humoral immunity.

2. ANATOMY AND CELLULAR COMPOSITION OF GERMINAL CENTERS

In resting state, B cells are found in roughly spherical follicles located between the T cell zone and the capsule in lymph nodes or between the T cell zone and the marginal zone in the spleen. B cell follicles are held together by a specialized network of stromal cells known as follicular dendritic cells (FDCs) (19, 20). Cognate antigen exposure induces responding B cells to migrate to the border of the follicle and the T cell zone (T:B border), where T cell help allows them to expand into clones. A fraction of the B cells in these expanded clones home back to the center of the FDC network, seeding early GCs (21). In addition to their organizing role, FDCs serve as a depot for antigen that provides the substrate for affinity-dependent selection (19, 20).

Approximately 1 week after immunization, clusters of proliferating pre-GC B cells become polarized into two distinct zones. The light zone (LZ) is proximal to the lymph node capsule or splenic marginal zone within the FDC network and contains a mixture of GC B cells and $CD4^+$ T follicular helper (Tfh) cells. The dark zone (DZ), which is proximal to the T cell zone, is composed almost exclusively of GC B cells and a fine network of FDC-like DZ reticular cells (22). The higher density of stromal cells and lower density of cell nuclei in the LZ render the LZ lighter under classical histological stains (11).

The anatomy of the GC is closely related to its function. B cells in the DZ proliferate and undergo SHM, producing a population of cells that express closely related antibodies that differ in their ability to bind antigen; mutant GC B cells subsequently migrate to the LZ, where they test their newly mutated receptors on FDC-bound antigen and receive help from cognate Tfh cells, leading to positive selection of cells expressing higher-affinity antibodies (23). The segregation of proliferation/hypermutation and antigen-driven selection between DZ and LZ requires that GC B cells migrate dynamically between these two compartments, a framework known as cyclic reentry (24). Here, we discuss the phenotypes of the cells that constitute each of the GC zones. How zonal migration relates to affinity selection is addressed in Section 3.

2.1. Germinal Center B Cells

GC B cells differ from their naive precursors in important ways. Whereas naive B cells are quiescent, GC B cells are highly proliferative, with cell cycles that can be as short as 5–6 h (25). Accordingly, B cells in the GC are larger and more metabolically active than naive B cells (25–28). Despite such high metabolic demands, GC B cells appear to rely primarily on fatty acid oxidation rather than on glycolysis for energy production (27). Homing to the follicle center is promoted by changes in expression of several G protein–coupled receptors, including the downregulation of EBI2/GPR183, which normally attracts resting B cells toward the follicle edge (29, 30), and the upregulation of $S1P_2$ and, in humans, P2RY8, which helps constrain B cells to the FDC network (31, 32). Changes in expression of several surface markers are routinely used to distinguish GC from naive B cells by flow cytometry or histology. These include upregulation of the proapoptotic receptor Fas (33), the Eph-related tyrosine kinase ligand Ephrin-B1 (34, 35), *n*-glycolylneuraminic acid (recognized by the antibody GL-7) (36, 37), and Gal-β(1-3)-GalNAc, a ligand for peanut agglutinin (38); downregulation of surface IgD (39); and modified expression of the cyclic ADP ribose hydrolase CD38 (upregulated in human but downregulated in mouse) (40).

Key to affinity maturation is the abundant expression in GC B cells of the enzyme activation-induced cytidine deaminase (AID), the mutator enzyme that initiates SHM and is thus absolutely required for affinity maturation (41, 42). AID deaminates cytidine to uracil directly on DNA, creating U:G mismatches that upon repair lead to point mutations in variable regions of

immunoglobulin genes. AID-dependent mutations accumulate at a rate of 1 per 1,000 bp per cell generation, roughly one million times the baseline rate of mutation of the genome (43, 44). These mutations generate the sequence diversity upon which affinity-based selection will act. AID most efficiently targets the cytidine at position 3 within the WRCY motif (where W is A/T, R is A/G, and Y is C/T; better known by its reverse complement sequence RGYW). Mismatch repair with error-prone DNA polymerases such as Polη (*Polh*) can result in mutations other than the AID-dependent C/G-to-T/A transition scattered around the AID target (44, 45). This pattern leads to stereotypical intrinsic SHM patterns for each variable region (46, 47), which are often difficult to distinguish from mutations selected through affinity maturation. AID targeting to hotspots may constrain affinity maturation by creating a hierarchy of more and less accessible somatic mutations (46), as well as by preventing further SHM of highly mutated sequences of immunoglobulin genes in which AID hotspots are depleted (48). In addition to point mutations in the variable region of immunoglobulin genes, processing of AID-induced lesions can produce double-stranded DNA breaks in switch regions of immunoglobulin genes, triggering class-switch recombination to downstream isotypes IgG, IgE, and IgA. Although the GC was canonically thought to be the site of class-switch recombination, recent work suggests that switching occurs primarily before complete GC maturation in pre-GC B cells (49).

Bcl6 is the primary transcriptional regulator that is both necessary and sufficient to determine the GC B cell program (50, 51). Whereas *Bcl6* mRNA is expressed in naive B cells, protein expression requires B cell activation and T cell help (52–54). Bcl6 is a transcriptional suppressor with numerous targets that contribute to the GC B cell phenotype. For example, Bcl6 suppresses expression of the antiapoptotic gene *Bcl2* (55), leading to the high rate of apoptosis among GC B cells that underlies affinity-based selection (56). Other targets of Bcl6 are *Myc* (57–59) and *Prdm1* (encoding the transcription factor Blimp-1) (60), repression of which raises the threshold for positive selection and PC differentiation, respectively. Bcl6 also helps B cells tolerate the frequent DNA breaks that occur as a consequence of SHM by dampening expression of the DNA damage sensors such as p53, ATR, and CHEK1 (61–63). In addition to Bcl6, several other transcription factors and, notably, epigenetic regulators, including EZH2, TCF3/E2A, IRF4, and IRF8, play critical roles in generating and maintaining the GC phenotype. Their functions are reviewed in detail elsewhere (54, 64, 65).

2.2. Light Zone and Dark Zone B Cell Phenotypes

Given their unique functions, it is not surprising that the GC zones are populated by B cells in distinct phenotypic and transcriptional states. Classically, DZ B cells are described as large and mitotically active centroblasts, whereas the smaller, less proliferative LZ B cells are termed centrocytes (11, 14). Functional and in situ photoactivation experiments (23, 66) showed that DZ B cells express high levels of the chemokine receptor CXCR4, required for formation of an anatomical DZ, whereas LZ B cells are high in activation markers CD83 and CD86. Use of these markers has enabled identification of the key features of B cells in the two GC zones. For example, expression of late cell cycle genes (23, 67, 68) and completion of S phase and mitosis (69) are largely restricted to DZ B cells, as is expression of the SHM enzymes AID and Polη (67, 70). Meanwhile, activation-related gene signatures such as those of c-Myc and mTORC1, as well as signatures associated with B cell receptor (BCR) and CD40 ligation, are strongly enriched among LZ B cells (23, 26, 59, 67, 71, 72). Thus, the gene expression patterns of LZ and DZ B cells support the proposed roles of these zones in proliferation/SHM and antigen-driven selection, respectively.

Recent studies have identified the transcription factor Foxo1 as a key driver of the DZ phenotype (73, 74). Loss of Foxo1 expression in GC B cells produces GCs that lack an anatomical DZ,

in part due to absence of *Cxcr4* expression (74). Nevertheless, basic features of the DZ state, such as rapid proliferation and SHM, are largely maintained, as is the ability of GCs to foster affinity maturation (73–75). Thus, the prototypical DZ functions of mutation and proliferation do not absolutely require a physical DZ or a full-fledged DZ transcriptional program. Part of the role of Foxo1 appears to be to prevent the premature expression of the LZ program (74). Accordingly, deletion of *Cxcr4* in GC B cells, which also leads to their exclusive localization in the LZ (66, 76), does not result in upregulation of a LZ transcriptional program (77), possibly because of continued suppression of the LZ state by Foxo1 (74).

LZ and DZ B cells differ only subtly at the transcriptional level—much less, for example, than during clearer phenotypic transitions such as those from naive to GC B cell or from GC B cell to PB (23, 67). On the basis of this observation, we proposed that GC B cells might best be thought of as a single population oscillating through a cycle with two extremes, an LZ rich in activation signatures and a DZ characterized by expression of late cell cycle genes (67). A recent series of single-cell transcriptomic studies of mouse and human GC B cells provide additional support for this notion (68, 78–84). Whereas the number of subclusters identified varies widely according to the parameters used, these subclusters tend to represent different stages of the cell cycle (the signatures of which are difficult to deconvolute from those of the LZ-DZ cycle) or transitional states between more defined extremes in transcriptional space, without clear-cut distinctions between LZ and DZ B cells. A common feature is the presence of a small population of GC B cells with high expression of c-Myc and its related signatures (65, 68, 80, 82), likely corresponding to positively selected LZ B cells about to reenter the DZ (59). On the basis of these findings and given the key importance of Myc to positive selection (see Section 3.1), we propose a revised framework in which GC B cells cycle between three rather than two transcriptional poles (DZ, LZ, and Myc/positive selection), with the number of discrete steps between them defined by the depth of sequencing and the granularity of the downstream analysis (**Figure 1**).

Of note, despite the conservation of GC anatomy and LZ and DZ transcriptional programs across species (11, 67), disruption of LZ/DZ polarity by interference with the CXCL12/CXCR4 axis results in relatively mild phenotypes, such as slight decreases in GC magnitude and SHM burdens, with little to no impairment in affinity maturation (76, 77, 82).

2.3. Germinal Center T Helper Cells

GCs are strictly dependent on the presence of T helper cells, in that they fail to form in mice and humans deficient in $CD4^+$ T cells or in key components of T cell help (85, 86). Acute interruption of T cell help with blocking antibodies leads to dissolution of ongoing GCs, indicating that T cells are also required for GC maintenance (87, 88). In this section, we briefly review key features of the phenotype of these cells; their role in GC selection is discussed in greater detail in Section 3.

2.3.1. T follicular helper cells. GC-residing Tfh cells are distinguishable from naive or other effector T cells by expression of the chemokine receptor CXCR5, which promotes Tfh homing to the B cell follicle (89), and of the exhaustion marker PD-1 (90). GC residency is restricted largely to the Tfh cells expressing the highest levels of these markers; nevertheless, Tfh cells are less constrained to the GC than B cells are and exit and reenter GCs at a much higher frequency (91). In 2009, three papers simultaneously found that Bcl6, the master regulator of the GC B cell program, is also the primary transcription factor driving the Tfh phenotype, a finding that became key to the acceptance of Tfh cells as a bona fide T cell lineage (92–94). Since then, Tfh cell biology has developed into a field in its own right. Several reviews provide extensive insight into various aspects of Tfh cell biology, from their development to their role in disease (95–98).

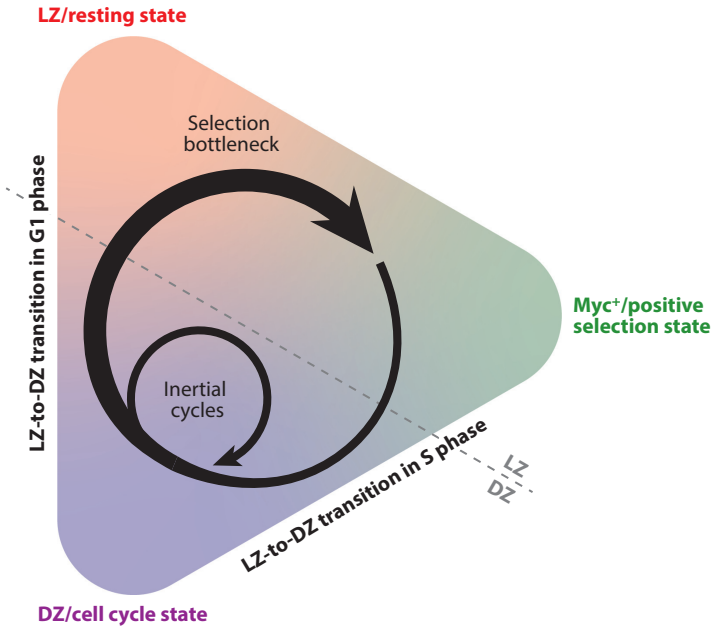

Figure 1

Proposed model for the transcriptional states of GC B cells transiting through the LZ/DZ cycle. GC B cells transition more or less smoothly between three transcriptional poles. Starting from the classic DZ transcriptional state (*purple*), characterized by high expression of late cell cycle genes, GC B cells move toward a preselection/counterselection state (*red*) in which cells physically located in the LZ express activation markers such as CD86 and CD83 but are otherwise resting in G1 phase. Following interactions with antigen and T follicular helper (Tfh) cells in the LZ (see Section 3.1 for further discussion), a small subset of LZ B cells enters the "positive selection" pole (*green*), characterized by activation of c-Myc and its downstream targets, entry into S phase, and physical and transcriptional transitions back into the DZ. Abbreviations: DZ, dark zone; GC, germinal center; LZ, light zone.

Tfh cells support the GC reaction through their expression of CD40L, a tumor necrosis factor (TNF) family member that engages CD40 on GC B cells, activating the NF-κB pathway and triggering B cell proliferation (see Section 3.1). Intravital imaging showed that Tfh cell help is delivered to GC B cells in the form of short but extensive cell–cell contacts (T–B entanglement) that take place repeatedly in the LZ and increase in duration upon positive selection (16, 99, 100), suggesting that B cells integrate the amount of help they receive from Tfh cells over time. A second key component of Tfh cell help is the cytokine IL-21, which promotes GC B cell proliferation and maintenance and the generation of long-lived PCs (101–103). Tfh cells can also acquire expression of several other cytokines (104). For example, Tfh-produced IL-4 synergizes with IL-21 to promote GC maintenance and PB differentiation (105–107), and allergen-primed Tfh cells can produce IL-13, promoting antigen-specific IgE production (108).

Tfh cells respond dynamically to their environment, especially to the amount of antigen presented by GC B cells. Tfh cell recognition of cognate antigen presented by GC B cells leads to Ca^{2+} flux and increased expression of IL-4 and IL-21 (99, 100). Because Tfh cells must remain limiting for efficient affinity selection, it was thought that these cells, unlike naive T cells, do not divide in response to antigen. However, it is now evident that Tfh cells divide in response to antigen to an extent proportional to the affinity of their T cell receptors, leading to increased representation of high-affinity Tfh cells over time (109). Conversely, in end-stage GCs, when

antigen is less abundant, Tfh cells can upregulate the T regulatory cell (Treg)–associated transcription factor Foxp3, which dampens their expression of CD40L and IL-21 and contributes to GC extinction (110). These observations suggest that the GC reaction is sustained by a feedback loop between B cells and Tfh cells, where both cell types respond to antigen availability and the affinity of their antigen receptors, ultimately determining GC maintenance and persistence (109).

The reliance on Bcl6 as a master regulator by both GC B cells and Tfh cells is remarkable from the evolutionary perspective (111). Bcl6 precedes the formation of GCs and affinity maturation evolutionarily (112), suggesting that this factor was co-opted by both T and B cells to allow GCs to emerge. Nevertheless, although intimately related, Tfh and GC B cells share little in terms of phenotype and functionality, and thus Bcl6 must act on at least partly divergent sets of downstream targets. This notion appears to be confirmed by Bcl6 chromatin immunoprecipitation studies in both cell types (111, 113). One shared feature, however, is the need for Bcl6 to repress expression of *Prdrm1* (encoding the transcription factor Blimp-1), which drives alternative fates in both cell types (plasmablasts and nonfollicular effectors in B and T cells, respectively) (60, 94). Suppression of possibly more evolutionarily ancient B and T cell fates may thus have been a first step in allowing GCs to evolve.

2.3.2. T follicular regulatory cells. A more recent addition is the subset of GC-residing Tregs that constitutively express Foxp3, referred to as T follicular regulatory (Tfr) cells (114–118). Tfr cells are thought to originate predominantly from thymic-derived Treg precursors, although Tfr cell differentiation from peripherally induced Treg precursors in specific settings has also been reported (119). Tregs differentiating into the Tfr cell phenotype upregulate Tfh cell–related transcriptional programs (including Bcl6 and Tfh cell markers CXCR5 and PD-1) to varying extents, thus occupying an intermediate position transcriptionally between Tfh cells and Tregs (114–116, 120, 121). Tfr cells have been proposed to regulate various aspects of the GC reaction, including GC formation, antigenic specificity, emergence of autoreactive or allergy-inducing B cell clones, and switching to various isotypes (114–116, 122–124); however, a consensus regarding which B cell phenotypes Tfr cells regulate, and by which mechanisms, has yet to emerge (117, 118). In addition to classic Tfr cells, other Treg-like populations in the GC have been reported. For example, human GCs contain $CD4^+$ T cells with regulatory properties that express IL-10 and CTLA-4, but not Foxp3, and may regulate class-switch recombination to IgE (125).

2.4. Other Cell Populations

Apart from B and T lymphocytes, GCs contain several other cell populations. The best understood of these are FDCs (19, 20). FDCs are stromal cells thought to derive from perivascular mural cell precursors in the spleen and marginal reticular cell precursors in the lymph node (126, 127) in a manner dependent on lymphotoxin-β and TNF-α (128). In steady state, FDCs are present diffusely in primary B cell follicles, where they maintain follicular architecture through secretion of the B cell chemoattractant CXCL13 (82, 129, 130). Upon immunization and GC formation, FDCs become compacted and enriched in the GC LZ (82, 127), where they serve the critical function of retaining the antigen that drives affinity-based selection of GC B cells (131). Antigen is held on FDCs in the form of immune complexes of antigen, antibody, and complement. FDCs express high levels of C3 receptors (CD21/CD35) and upregulate the immunoglobulin Fc receptor FcγRIIb upon immunization. Whereas the complement receptor appears to play a dominant role in antigen retention (132), FcγRIIb on FDCs contributes more subtly to the GC reaction, possibly by increasing the stringency of GC selection (133). As expected from their fundamental role in GC selection, acute ablation of FDCs leads to rapid termination of the GC reaction (130).

Recent developments include the findings that glycosylated antigens can also be loaded onto FDCs through the mannose-binding lectin pathway of complement, circumventing the need for preexisting antibody for efficient FDC targeting (134), and that FDCs can recycle surface-held antigen through endosomal compartments (135), potentially prolonging the life span of trapped antigen. Other recent work has described a second population of FDC-like reticular cells that secrete the CXCL12, providing the cellular basis for the attraction of $CXCR4^+$ GC B cells to the DZ (22, 82).

Other cell populations that reside within the GC environment have been less studied. Tingible body macrophages rapidly engulf and eliminate the enormous number of apoptotic B cells generated by immunoglobulin hypermutation and affinity-driven selection, and defects in this population may be associated with failure to clear apoptotic cells and humoral autoimmune disease (136, 137). Natural killer T cells can be recruited to GCs in response to lipid antigens and serve as a critical source of the cytokine IL-4 for GC initiation (138, 139). $CD8^+$ T cells are also present in the GC and may help clear virally infected B and Tfh cells (140).

3. CELLULAR AND MOLECULAR MECHANISMS OF GERMINAL CENTER SELECTION

GCs sort B cells into different fates—proliferation, death, export—according to the properties of their surface antigen receptors. This paradigm has fueled much of the work in GC biology, which focused on, for example, how B cells sense their immunoglobulin affinity and what cues trigger export of GC B cells into the memory B cell (MBC) or PB compartments. **Figure 2** summarizes key points in our current understanding of this selective process. In the following section we survey the concepts and experimental findings that provide the basis for this model.

3.1. Selection of High-Affinity B Cells Within the Germinal Center

The increase in the average affinity of GC B cells over time requires that B cells with higher affinity for antigen clonally expand (positive selection) and those with lower affinity proportionally contract. Although absolute affinity is likely to be important for some aspects of B cell selection and differentiation (88, 141–146), relative comparisons appear to play a dominant role in positive selection, as evidenced by observations that GCs grow to similar sizes when composed of high- or low-affinity B cells (141, 146) and that B cells of a given affinity are positively selected when competing against lower-affinity cells but counterselected when the affinity of the competition is higher (147, 148). How and to what extent GC B cells measure their affinity in relation to that of their peers is a longstanding debate in the GC field (see 18 for an in-depth discussion).

3.1.1. Death- and birth-limited modalities of selection. Classical studies of GC selection are centered on the notion that life–death decisions made by individual LZ B cells determine which clones are rescued from default apoptosis and allowed to reenter the DZ to proliferate, thus increasing in abundance over time (56). This has been referred to as death-limited selection (149) (**Figure 3*a***). This model implies that LZ B cells compete among themselves for access to one or more limiting factors in the LZ. Successful clones progress to DZ reentry, while failure leads to death by neglect (18). Substantial culling of LZ cells at the point of DZ reentry is supported by experiments tracking B cells as they undergo the LZ-to-DZ transition in vivo, which, when treated mathematically, showed that 70–90% of LZ B cells fail to reenter the DZ (23, 150). However, measurements of antibody affinity in caspase cleavage-reporter mice failed to detect enrichment for low-affinity B cells in the apoptotic LZ fraction, even though B cells expressing c-Myc, a marker of positive selection (59), were somewhat protected from death (151). Thus, whereas most

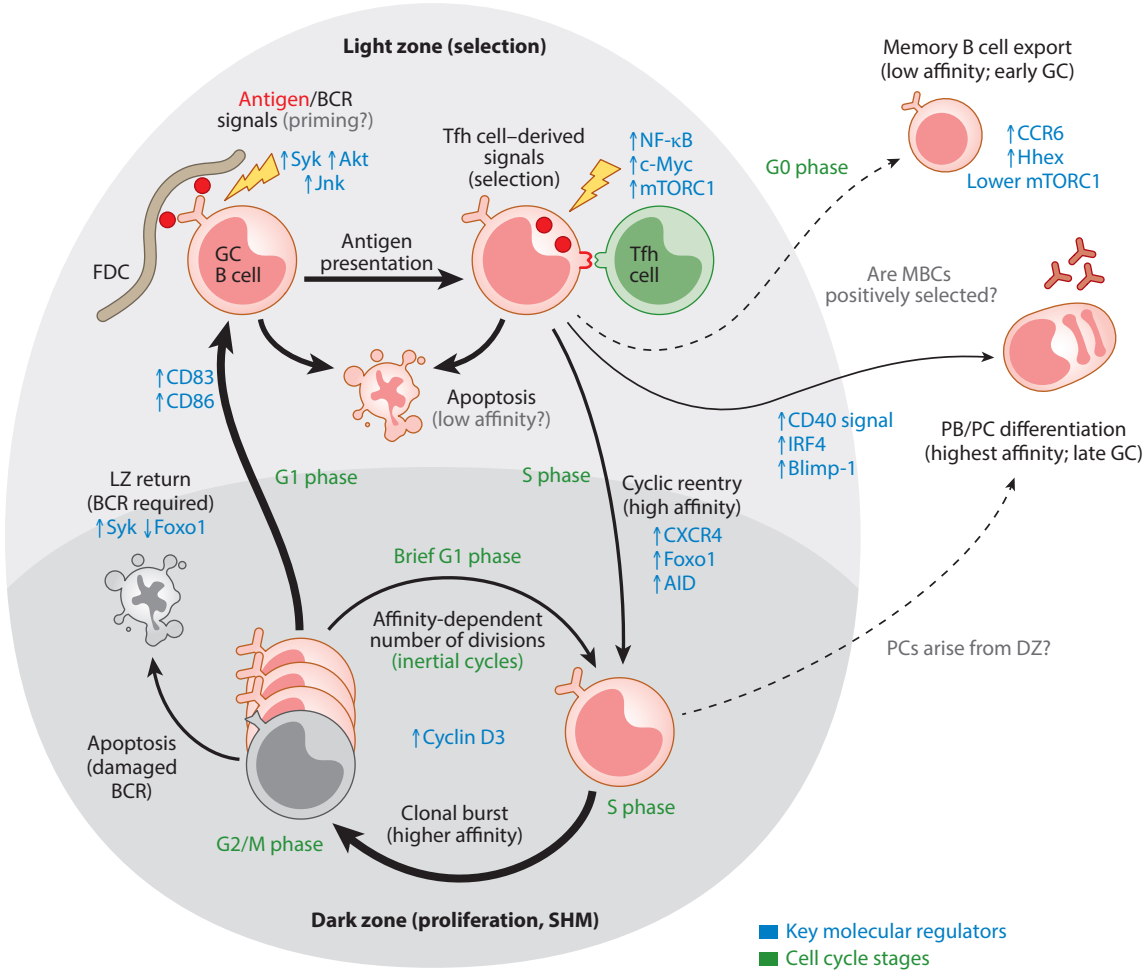

Figure 2

The selection cycle of GC B cells. Model depicting major events and decision points as GC B cells progress through the steps of affinity-based selection. Arrow thickness is roughly proportional to the frequency of each fate. Dashed arrows represent putative transitions. Key molecular regulators are in blue font, and cell cycle stages associated with each transition are in green font. Abbreviations: BCR, B cell receptor; DZ, dark zone; FDC, follicular dendritic cell; GC, germinal center; LZ, light zone; MBC, memory B cell; PB, plasmablast; PC, plasma cell; SHM, somatic hypermutation; Tfh, T follicular helper cell.

LZ B cells die before they can reenter the DZ, the preferential culling of low-affinity cells required for death-limited models has yet to be demonstrated experimentally.

Our work has identified an additional form of selection (birth-limited; 149) (**Figure 3*b***), in which the relative abundance of a clonal variant in the GC is determined not by its ability to survive and reenter the DZ but rather by its capacity for sustained proliferation after the LZ-to-DZ transition, set by the strength of the selective signals it received in the LZ (23, 25, 26, 68, 69, 71, 150). On average, GC B cells enter the cell cycle twice in the DZ for every cycle started in the LZ, but this ratio can be increased severalfold by artificial delivery of strong positive selection signals (25, 69). Clonal dynamics studies (discussed further in Section 4) indicate that efficient affinity maturation requires clonal bursts, events in which the descendants of a single B cell take over a

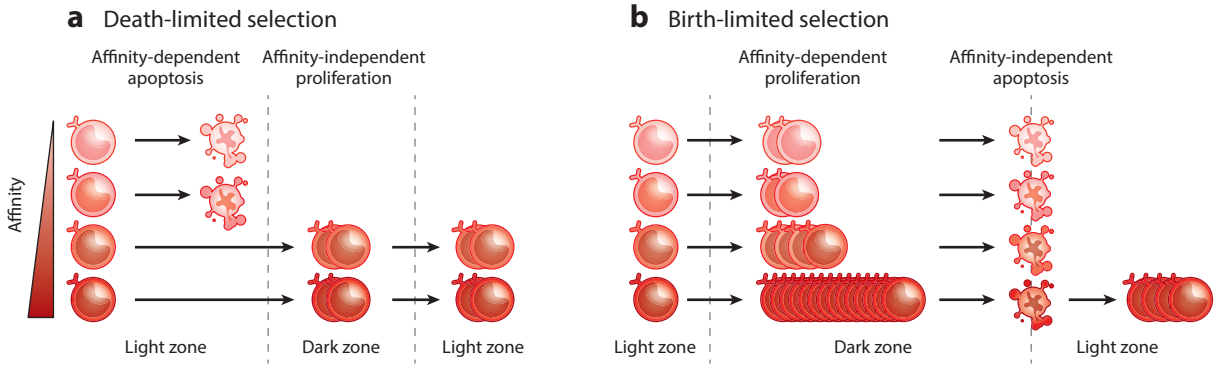

Figure 3

Death- and birth-limited models of positive selection in the GC. (*a*) In purely death-limited selection, only higher-affinity B cells are allowed to reenter the DZ, after which they divide a fixed number of times. Selection derives from the elimination of low-affinity B cells by apoptosis in the light zone. (*b*) In a purely birth-limited framework, B cells across the affinity spectrum are allowed to reenter the DZ, where they undergo a number of cell cycles proportional to their affinity. GC cell numbers are maintained steady by continuous (but affinity-independent) culling of cells by apoptosis. Abbreviations: DZ, dark zone; GC, germinal center.

large fraction of a GC in a matter of days, supporting birth-limited selection as a prominent driver of positive selection also under physiological conditions (152, 153). These models are not mutually exclusive, in that selective apoptosis of lower-affinity B cells may follow, and thus complement, birth-limited selection. In practice, therefore, death- and birth-limited models of selection may operate in parallel or in alternation to achieve efficient affinity maturation. Alternation between periods in which selection is predominantly death- or birth-limited could explain why selection of dominant clones occurs to different extents in different GCs (152). Below, we discuss cellular and molecular aspects of positive selection as they apply to each of these frameworks.

3.1.2. Cues triggering positive selection in the germinal center. GC B cells are positively selected for clonal expansion on the basis of the affinity of their cell surface receptors for antigen. The BCR is both a signaling and an endocytic receptor and thus can function directly as a signal transducer by binding to antigen on FDCs or indirectly by capturing and internalizing antigen in proportion to its affinity. In the latter mode, presentation of captured antigen to Tfh cells allows them to discriminate between B cells with different affinities for antigen (154). Early studies showed that, whereas ligation both of the BCR and of CD40 was capable of prolonging the survival of GC B cells in culture (56), strong BCR cross-linking alone results in GC B cell death in vivo (155, 156), setting the stage for a decades-long debate over the relative contribution of BCR and T cell–dependent signals to positive selection.

A role for Tfh cells in positive selection was established by experiments providing a small number of GC B cells with antigen in a BCR-independent manner via the surface receptor DEC-205. In those experiments, BCR-independent antigen delivery produced a burst of B cell proliferation (23, 26, 69, 70, 151, 152). Conversely, when antigen delivery via DEC-205 was used to equalize the amount of peptide–MHC among GC B cells, selection of high-affinity B cells ceased (23), implying that signals from the BCR alone are insufficient to sort B cells on the basis of affinity. Further support comes from a series of intricate experiments in which egg lysozyme–specific B cells are pulsed ex vivo with lysozyme coupled to ovalbumin (OVA) and then adoptively transferred into OVA-immunized mice. Pulsed B cells remain in GCs for extended periods in the absence of further BCR stimulation, as long as help from cognate Tfh cells is present (157). Delivery of additional

OVA to these B cells via DEC-205 is sufficient to trigger their positive selection, again in the absence of any BCR ligands (158). An attractive feature of T cell–driven selection is the notion that Tfh cells can engage in positive feedback loops in which signals delivered by Tfh cells, including CD40L in mice and the neurotransmitter dopamine in humans, induce B cells to upregulate surface and soluble mediators that attract further help from Tfh cells (100, 159, 160). Such loops could explain how relatively modest gains in BCR affinity can elicit disproportionately large clonal bursts (152, 153).

Genetic evidence for a role of Tfh cells in positive selection is less clear. For example, B cells deficient in herpes virus entry mediator (HVEM), a negative regulator of a B cell's ability to receive help, are at a proliferative advantage in GCs (161). By contrast, B cells with lower expression of MHC-II, due to either lack of the MHC chaperone H2-O (162) or haploinsufficiency for class-II MHC (163), are not counterselected over time, whereas both defective genotypes are clearly at a disadvantage in pre-GC competition. A drawback of the latter experiments is that, whereas MHC molecules are measurably limiting at the pre-GC stage, it is not clear whether this is also the case in the GC, where the amount of antigen presented by B cells is typically too low to be determined experimentally.

Our understanding of the role of BCR signaling in GC selection is less developed. Recent studies have shown that, despite the strong attenuation of BCR downstream signaling by suppressors such as PTEN (164, 165), GC B cells can use their receptors to sense antigen (166–169). However, the role of BCR signals in discriminating between B cells with relatively higher or lower affinity is still poorly defined. As noted above, and in contrast to DEC-205 delivery of T cell antigen, provision of BCR-binding antigen to GC B cells acutely in the absence of added T cell help results in their deletion (23, 155, 156, 170). Nevertheless, concomitant BCR activation increases positive selection driven by suboptimal doses of T cell antigen delivered through DEC-205 (158), suggesting possible synergy between BCR and Tfh cell–derived signals. GC B cells appear to be positively selected on the basis of not only their affinity but also their isotype, with IgG-expressing cells showing a selective advantage over their IgM-expressing counterparts (171). Because deletion of the IgG_1 cytoplasmic tail did not impair selection of IgG^+ GC B cells in this model, it is not clear whether isotype-driven selection operates at the level of BCR signaling or through other mechanisms such as differential antigen retrieval for presentation to Tfh cells.

A setting in which BCR signaling is clearly playing a role is in the elimination of B cells whose surface immunoglobulins have been truncated or otherwise damaged by SHM. Sequencing of the immunoglobulin genes of B cells undergoing apoptosis in the GC showed that cells with truncated or structurally compromised immunoglobulins undergo apoptosis almost exclusively in the DZ (151, 172). Because SHM is also thought to occur predominantly in the DZ (23, 67), these findings imply that B cells must sense the presence of a functional BCR before they are allowed to transition from the DZ to the LZ (151, 172). In support of this notion, the point of the GC cycle at which B cells are most sensitive to BCR stimulation is during G2/M phase, which occurs predominantly in the DZ (164). Likewise, BCR signaling via Syk and Akt is required to eject Foxo1 from the nucleus, enabling DZ B cells to access the LZ (167, 173).

3.1.3. Molecular control of positive selection. The key molecular event in positive selection is the induction of transcription factor c-Myc. c-Myc and its targets are expressed predominantly by LZ B cells with higher affinity for antigen (23, 59, 72), and expression of c-Myc and its downstream signatures is induced in GC B cells by interaction with Tfh cells (26, 71). Although c-Myc is expressed in only a small fraction (~5%) of GC B cells, inhibition of its activity is sufficient to shut down a GC (59, 72), implying that most or all GC B cells must pass through a c-Myc-dependent state in their cycle around the GC. Genetic titration of c-Myc directly controls the size of the

proliferative burst undertaken by GC B cells upon positive selection, providing a link between c-Myc induction and positive selection (71). The signaling pathways required to induce c-Myc in GC B cells are still being defined. c-Myc is a target of NF-κB (174, 175), the c-Rel subunit of which is required for GC maintenance (176). In vivo, BCR signaling on its own is insufficient to drive nuclear translocation of NF-κB and c-Myc expression in GC B cells, whereas CD40 ligation is (166, 167). Nevertheless, studies in which GC B cells are stimulated jointly via the BCR and CD40 pathways show synergistic induction of c-Myc (166, 167), suggesting that BCR ligation may sensitize GC B cells to respond productively to selective signals delivered by Tfh cells.

Signals delivered in the LZ have to induce selected B cells to undergo several rounds of proliferation after moving to the DZ, where they are no longer in contact with antigen or Tfh cells. In naive B cells activated in vitro, continuous c-Myc expression is required to sustain a proliferative burst (177). In the GC, however, B cells lose expression of c-Myc as they migrate away from the selective signals delivered in the LZ. Most DZ proliferation thus occurs by "inertia," in the absence of c-Myc (59, 68, 72). Recent studies have uncovered several requirements for inertial cell cycling. Strong Tfh cell help accelerates cell cycling in the DZ by increasing the rate of DNA synthesis during S phase (25). Activation of c-Myc and mTORC1 in the LZ increases cell biomass and anabolic capacity, allowing B cells to survive sustained proliferation in the absence of further growth signals (26, 71). The transcription factor AP4 helps prolong the expression of c-Myc-related transcriptional programs in the DZ (101). Finally, cyclin D3 boosts the expression of E2F targets exclusively in the DZ, providing the first specific regulator of DZ cell cycling (68, 178). Coordinated induction of these growth and proliferative pathways is required for efficient positive selection, creating interesting cell size versus proliferation dynamics. For example, both preventing cell growth by blocking mTORC1 (26) and accelerating inertial proliferation by a gain-of-function mutation in cyclin D3 (68) result in GC B cells that are smaller than normal because they cycle beyond their growth capacity. Conversely, GC B cells lacking cyclin D3 grow at a normal rate but fail to undergo inertial cycles, resulting in larger-than-normal size (68).

In conclusion, although direct demonstration of an association between Tfh cell help and positive selection in unmanipulated GCs is still lacking, experimental manipulations of antigen presentation by GC B cells show that Tfh cell help is at least sufficient to trigger B cell clonal expansion and positive selection. Whereas the precise role of affinity-dependent BCR signals in selective survival and proliferation of higher-affinity GC B cells remains to be determined, it is likely that a background of auxiliary signals transmitted by the BCR plays a role in allowing GC B cells to respond effectively to Tfh cell help.

3.2. Selection for Export from the Germinal Center

To secrete antibody into serum and mucosal surfaces, B cells must first be exported from the GC into the PC compartment. This can happen either directly, as GC B cells continuously differentiate into PBs and later PCs, or indirectly, when B cells that exited the GC as MBCs later differentiate into PB and PCs upon secondary antigen encounter. Despite much recent progress, the cues that direct GC B cells to differentiate along either route are still incompletely understood.

3.2.1. Selection into the plasmablast fate. High affinity for antigen favors differentiation of GC B cells into proliferative PBs, some of which subsequently enter the quiescent PC compartment. High-affinity monoclonal B cells differentiate readily into PBs and PCs (141, 143), and when lower-affinity hapten-specific or monoclonal B cells are assayed, those that become PBs and PCs tend to be enriched for affinity-enhancing mutations at time points where these mutations are still rare within the GC (144, 179). How high affinity is translated into PB differentiation is an area of

active investigation (180). As with positive selection, Tfh cell help is thought to play a prominent role in driving cells toward the PB fate. In vivo, delivery of T cell antigen to B cells via DEC-205 triggers a marked expansion of the DEC-205$^+$ PB population as well as greatly increased serum antibody levels (23), showing that Tfh cell help has at least the potential to trigger PB differentiation from GC B cells. Mechanistically, ex vivo stimulation of GC B cells with anti-CD40 leads to nuclear translocation of the NF-κB subunit p65/RelA (167), which is specifically required for PB differentiation from GC precursors (176). Conversely, loss of a single copy of *Cd40* is sufficient to inhibit PB export from GCs, whereas GC representation of haploinsufficient cells is not affected (181). By contrast, in mice immunized with sheep red blood cells, acute blockade of CD40L or depletion of T cells has only a mild if any effect on the proportion GC B cells expressing Blimp-1, the key transcriptional driver of the PB/PC identity (88, 182). Whereas these findings suggest that pathways other than CD40L-CD40 may promote PB/PC differentiation, at least in response to particulate antigens, questions remain about the extent to which B cells expressing relatively low levels of Blimp-1 are indeed committed to leaving the GC as PBs (182).

Other work supports a role for BCR signaling as a driver of PB export from the GC. Compelling data come from studies of the IgE response, which show that IgE-bearing B cells are extremely short-lived within GCs, likely because signaling via the IgE receptor itself promotes rapid differentiation toward a PB fate (183–185). Likewise, B cells made to switch toward IgG by cre-mediated recombination (in the absence of AID and therefore also of SHM and affinity maturation) showed that IgG_1 BCRs become enriched among bone marrow PCs but not in the GC (186). Barring potential differences in the ability of different isotypes to retrieve antigen from FDCs (171), these studies strongly suggest that BCR signaling directly affects PB differentiation.

3.2.2. Selection into the memory B cell fate. Relatively less is known about the selection of GC B cells into the MBC compartment. Recent studies have identified putative MBC precursors (pre-MBCs) in the GC, defined variously as CCR6$^+$ (187), Ephrin-B1$^+$S1pr2$^-$CD38$^+$ (34), and CD38^{int}Bcl6^{low}Ephrin-B1$^+$ (188); as high expressors of a fluorescent reporter suggestive of exit from the cell cycle into G0 phase (189); or as transcriptionally resembling spiked-in MBCs by single-cell mRNA sequencing (79). Pre-MBCs are found almost exclusively in the LZ, have transcriptional features that overlap with those of true MBCs, and display functional features suggestive of memory, including the ability to survive longer and respond better to stimulation in vitro (34, 187, 189). The transcription factors HHEX and TLE3 are required for full differentiation of MBCs from GC precursors (79). Additional complexity comes from studies showing that mature MBCs can be segregated into different subsets with different differentiation potentials upon recall (190–193) or derived from more or less proliferative precursors (194). Although it is not clear that all of these subsets are generated in the GC (as opposed to the extrafollicular reaction), it may be that the choice to exit the GC as an MBC is further regulated to allow for export of different MBC subsets.

Mechanistically, MBC differentiation appears to require cessation of proliferation, downregulation of Bcl6, and upregulation of antiapoptotic factors such as Bcl2 (34, 187, 189, 195; reviewed in detail in 65). Thus, the MBC fate choice differs fundamentally from DZ reentry and PB differentiation pathways in that it involves the long-term survival of B cells that are not positively selected to proliferate. What types of signals trigger pre-MBCs to survive but not proliferate is an open question. Studies show that recently exported MBCs generally display lower affinity for antigen than do contemporary GC B cells (187, 196–198; 189 is a notable exception), suggesting that the signal for MBC export is relatively weaker than signals promoting DZ reentry and PB/PC differentiation. Accordingly, strong Tfh cell help appears to inhibit MBC differentiation, partly by inducing downregulation of the transcription factor Bach2 and upregulation of mTORC1, both

of which counter the MBC fate choice (26, 188, 196). Whether MBCs are selected stochastically from among GC B cells not positively selected by T cell help or whether signals exist that specifically instruct MBC differentiation is not yet clear. The range of signaling pathways that could trigger MBC development from GC precursors is reviewed in detail in Reference 65.

3.2.3. General models for post–germinal center fate decision. Integrating classic and recent studies, Kurosaki and colleagues (199) proposed a conceptual framework in which affinity is the primary determinant of GC B cell fate decisions (**Figure 4*a***). B cells with no or low affinity for antigen die by neglect. Of those that do not die, B cells with the lowest affinity exit the GC as MBCs; those with intermediate affinity reenter the DZ for further proliferation; and those with the highest affinity are exported to the PB and PC compartments (199). A twist to this model (**Figure 4*b***) comes from the finding that, when observed well after the contraction of the GC response, long-lived MBCs are derived predominantly from pre-GC and early GC stages, whereas long-lived PCs tended to arise from the later phase of GC (200), an observation confirmed by multiple subsequent studies (79, 145, 196). Because average B cell affinity increases with time spent in the GC (201), the contribution of temporal changes in B and T cell phenotype is difficult to disentangle from that of increasing immunoglobulin affinity, and further work is required to separate these two factors. Be that as it may, the finding that high-affinity pre-MBCs are readily found in later-stage GCs (187) and the routine cloning of antibodies with very high affinity and often extraordinarily high SHM burdens from the human MBC pool (202) indicate that any temporal and affinity-dependent restrictions to MBC generation are unlikely to be absolute.

3.3. Negative Selection

SHM has the potential to generate self-reactive immunoglobulins and humoral autoimmunity (203), leading to the notion that GCs must include one or more strategies to eliminate or counterselect autoreactive B cells. Two main classes of checkpoints have been proposed. First, negative selection proper entails elimination by apoptosis of GC B cells that acquire self-reactivity through SHM. In theory, self-reactive B cells would be exposed to much higher concentrations of antigen than when responding to foreign antigen, triggering apoptosis by activation-induced cell death (155, 156). Such a mechanism is restricted to self-antigens that are abundant in the GC, and, indeed, a study using hen egg lysozyme mutants as pseudo-autoantigens showed that negative selection operates to a measurable extent only when antigen is expressed in the GC at high levels but not when it is expressed distally in the liver or kidneys (204). On the other hand, monoclonal antibodies cloned from apoptotic LZ B cells in a polyclonal system were not noticeably enriched for self-reactivity when compared with nonapoptotic cells, and prevention of apoptosis by a *Bcl2* transgene did not increase self-reactivity among GC B cells (205). These findings argue either that negative selection is not a frequent enough phenomenon to be readily observable in polyclonal settings or that self-reactive cells are dealt with by mechanisms other than apoptosis.

A second proposed checkpoint, termed clonal redemption, posits that dual self-/foreign-binding B cells can be rescued by mutation of their immunoglobulin genes away from self-reactivity. Clonal redemption has been documented by experiments in both mice and humans, in response to model antigens and blood group polysaccharides, respectively (206, 207). A detailed analysis of the mutational trajectory leading one B cell clone away from self-binding and toward foreign binding showed that rapid selection for mutations that decrease affinity for the self-antigen is followed by slower improvement in affinity to its foreign counterpart (208). A remarkable case of clonal redemption comes from the study of human public B cell clones that acquired the ability to bind to multiple strains of *Plasmodium falciparum* by insertion of the coding sequence for the

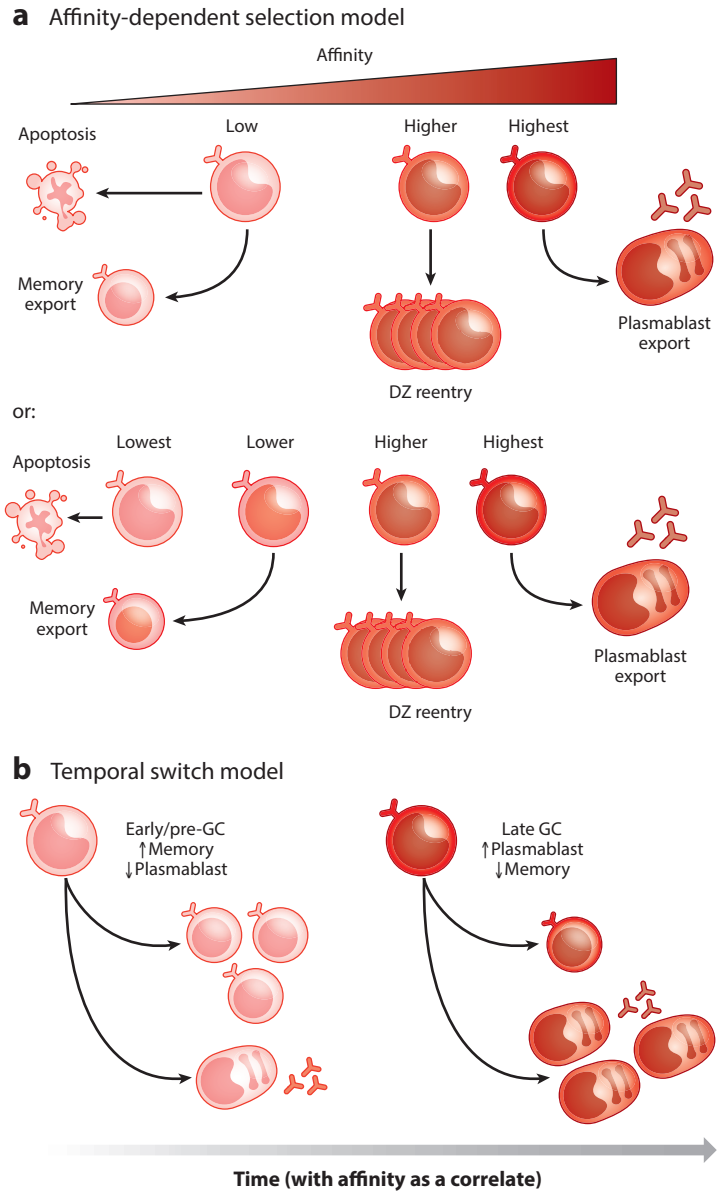

Figure 4

Models for post-GC B cell fate choice. (*a*) According to the affinity-dependent model, cells with lower affinity (either absolute or relative to their neighbors) that do not die by neglect differentiate into MBCs. Among B cells with higher affinity that are positively selected, those with the highest affinity are exported as PBs, whereas those with relatively lower affinity reenter the DZ. It is not clear whether MBCs arise stochastically from the pool of B cells that failed to be positively selected (*top*) or whether they are actively instructed to differentiate by weak positive selection signals (*bottom*). (*b*) In the temporal switch model, early GCs tend to favor the export of MBCs, whereas PBs arise preferentially later in the course of the GC reaction. Because GC B cell affinity increases progressively with time, the temporal switch model is not incompatible with affinity-dependent fate decisions. Abbreviations: DZ, dark zone; GC, germinal center; MBC, memory B cell; PB, plasmablast.

collagen-binding protein LAIR-1 into different sites within the *IGH* gene, which confers binding to the broadly expressed RIFIN antigens of *P. falciparum* (209, 210). Sequence analysis of LAIR-1 insertions, themselves subject to SHM, showed that these hybrid antibodies almost invariably accumulated mutations that led to loss of collagen binding, even when overall SHM was low, suggesting strong pressure for loss of self-reactivity. Mechanistically, clonal redemption requires positive selection of cells that fail to bind an antigen. Although counterintuitive, this phenomenon may potentially be explained by competition between self-antigens and foreign antigens for access to the BCR: B cells that lose self-reactivity would in theory be able to bind more foreign antigen, which, unlike self-antigen, contains the foreign peptides that Tfh cells require to drive B cell selection. Clonal redemption may therefore provide indirect evidence for a Tfh cell–centered model of GC positive selection.

Also remarkably, a recent study of patients with cryoglobulinemic vasculitis detected the frequent presence in pathogenic autoreactive B cell clones of typical lymphoma driver mutations that unleash BCR signaling or promote GC B cell proliferation (211). These findings suggest that corruption of the normal B cell proliferation programs promotes, or may even be required for, the emergence of at least some humoral autoimmune diseases in humans, possibly by allowing B cells to bypass checkpoints that normally prevent GC entry, excessive SHM, or PB differentiation by autoreactive B cells.

4. CLONAL DYNAMICS OF THE GERMINAL CENTER REACTION

The unique ability of GCs to evolve the affinity of antibodies in real time has justifiably led to a strong focus on using reductionist models to dissect affinity-based selection mechanistically. As described above, most of our understanding of GC selection derives from studies of monoclonal immunoglobulin knock-in mice and of functionally monoclonal responses to haptens, where a single epitope, usually associated with a highly dominant V-gene, is assayed. However, as made clear by studies of neutralizing antibodies to viruses such as HIV, influenza virus, and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), antibody effectiveness is determined not only by affinity but also by the fine epitope specificity of the pool of antibodies that emerges from the B cell selective process (212–214). Likewise, lack of clonal diversity in the antibody pool facilitates viral escape from the humoral immunity by mutation (215–217). These observations have fueled a growing interest in the field of B cell clonal dynamics, defined as the study of how the rules of selection described in previous sections play out in polyclonal settings and how they shape the ultimate diversity (and therefore potency and escapability) of the antibody pool.

Understanding GC clonal dynamics involves a shift in focus from intraclonal competition (i.e., that between SHM variants of the same clone, for which the definition of success is well defined as increased affinity for a single epitope) to interclonal competition (i.e., that between different clones with different antigen or fine epitope specificities, which may have different strategies for being positively selected within the GC environment). According to our current understanding, individual early GCs are composed of tens to hundreds of distinct clones [defined as groups of B cells with distinct V(D)J rearrangements] (152), and this diversity is lost only slowly with time (152, 201). Thus, interclonal rather than intraclonal competition is the driving force behind most of GC selection and affinity maturation. **Figure 5** presents a tentative model describing various known bottlenecks to clonal diversity across the B cell response as defined in mice. We briefly review the literature supporting this model in the sections below.

4.1. Clonal Selection for Access to the Germinal Center

Even though clonal diversity in early GCs is high (152), clones that seed the GC represent only a subset of all naive B cells with intrinsic potential to enter GCs in response to a given antigen.

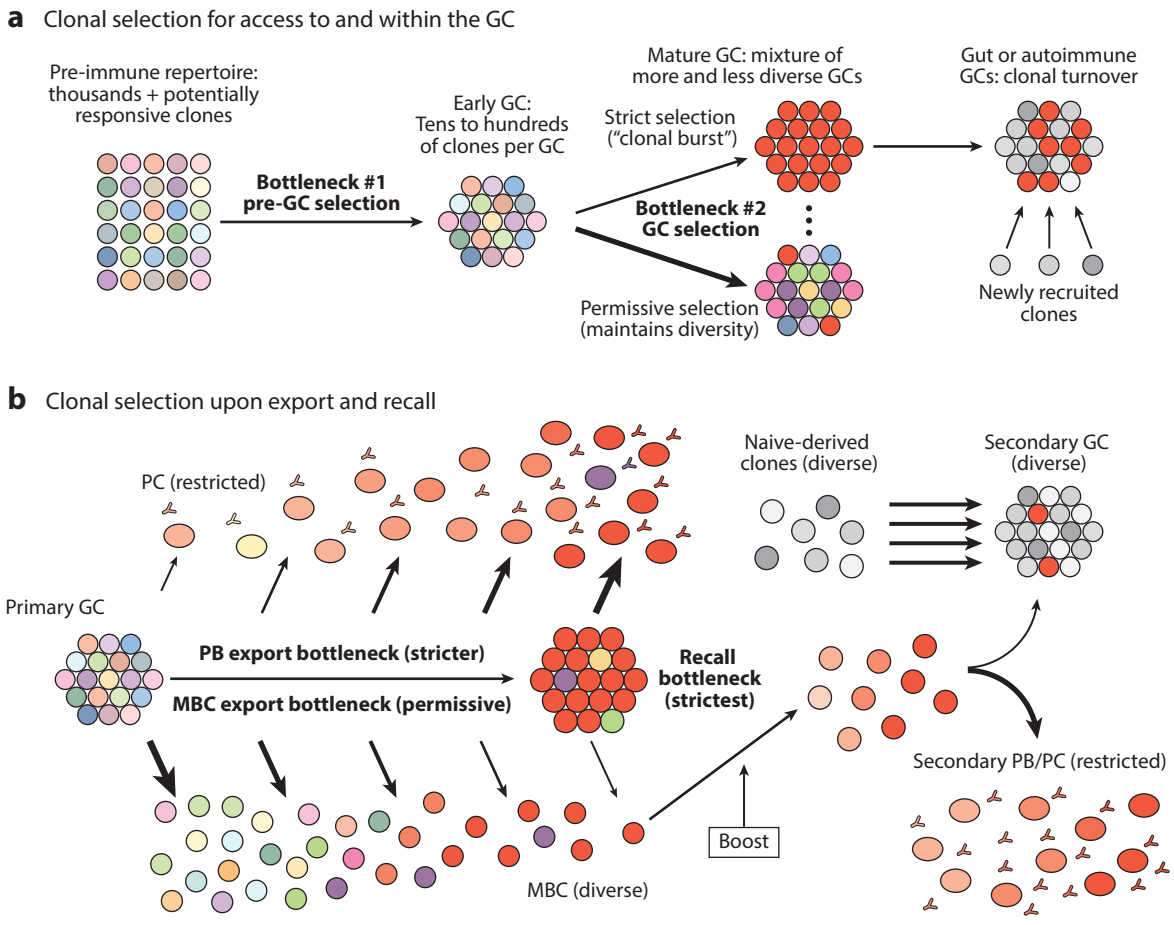

Figure 5

Overview of the clonal dynamics of the GC response. Proposed model depicting the effects of sequential bottlenecks on the clonal diversity of B cells (*a*) prior to and within the GC and (*b*) upon GC export and recall, based primarily on mouse models (e.g., 145, 152, 153, 197, 198, 200, 201, 232, 233). Different clones [distinct V(D)J rearrangements] are represented by different colors, with darker hues corresponding to higher affinities within the same clone; clones not derived from the initial response are in grayscale. Arrow thickness is roughly proportional to the frequency of each fate. Abbreviations: GC, germinal center; MBC, memory B cell; PB, plasmablast; PC, plasma cell. Panel *a* adapted from Reference 226.

Evidence for this comes from the finding that very-low-affinity B cells that do not enter GCs in polyclonal settings are able to do so efficiently when competition from other clones is eliminated (141, 148, 218). Before entering GCs, B cells that acquire the immunizing antigen must present this antigen to T helper cells at the T:B border, which deliver signals that allow B cells to progress toward the GC path (21, 219). Competition between pre-GC B cells for T cell help limits access to the GC at this stage (148, 162, 163, 220). By relaxing this bottleneck, increases in the availability of T cell help (e.g., by transfer of monoclonal T cells) can improve recruitment of lower-affinity B cells to GCs (221). At the extreme, when T cell help is hyperabundant and competition from other clones is completely eliminated, even B cells with theoretically no specificity for the immunizing antigen are allowed GC access (183, 222). Nevertheless, different model antigens recruit different numbers of B cell clones into GCs, following a logic that cannot be explained solely by the

availability of T cell help. For example, immunization with OVA generates GCs with substantially fewer clones than immunization with 4-hydroxy-3-nitrophenylacetyl (NP)-OVA—even though these antigens share the same T cell epitopes—likely as a result of the higher availability of naive precursors capable of binding NP (152). Similarly, naive B cells with subthreshold affinity can be recruited to GCs when precursor frequency is increased, suggesting that even very-low-affinity B cells will access GCs if given enough chances to do so (223, 224). The clonal composition of early GCs is therefore likely to be a function of both the availability of T cell help and the frequency and quality of the naive B cell precursor pool.

An unexplained but widespread observation is that, despite this early affinity-dependent bottleneck, most B cells that enter GCs are not readily identifiable as antigen specific, either by flow cytometry with fluorescent antigen probes or by monoclonal antibody assays (152, 194, 201). B cells with BCRs specific for antigens other than the one used for immunization are largely excluded from GC participation, even when they represent the large majority of B cells (152). Therefore, non-antigen-binding B cells in GCs are likely specific for the immunizing antigen but fail to be detected by standard methods. Possible explanations include the threshold affinity for in vivo recruitment being lower than what we can readily detect experimentally (197, 213) or antigen being available to B cells in vivo in a form different (dark antigen) from that used in typical binding assays (201). The fraction of GC B cells that bind antigen appears to be amenable to experimental manipulation, however. For example, a change in antigen that allows tighter binding to alum adjuvant increased the proportion of antigen-binding cells in the GC (225). An often overlooked consequence of poor antigen binding by GC B cells is that the common practice of identifying the B cells participating in a GC reaction by flow cytometry by first gating on antigen binders will almost invariably exclude a major fraction of the cells involved in this response (197).

4.2. Clonal Diversity Within the Germinal Center

The high clonal diversity of early GCs is expected to progressively narrow as GC selection expands fitter B cell clones at the expense of their less-fit competitors. However, two studies published almost simultaneously (152, 201) highlighted that GC selection, when viewed at the clonal level, does not appear to be as stringent as prescribed by these models, and substantial diversity of often low-affinity clones is maintained throughout the lifetime of immunization-induced GCs. Single-GC analysis using in situ photoactivation and multicolor fate-mapping showed that radical loss of diversity occurs in only the small fraction of GCs in which a single B cell, typically carrying affinity-enhancing somatic mutations, undergoes a jackpot-style clonal burst that is large enough to eliminate most of its intra- and interclonal competitors (152). This observation led us and others to propose that GC selection is fine-tuned in its stringency so that factors such as stochastic noise in selection and the inherent stochasticity of SHM allow for strong selection of high-affinity variants to occur in some cases, and tolerance toward lower-affinity B cells allows most GCs to maintain a varying degree of clonal diversity that may be important for antibody-mediated protection (149, 226, 227). Recent work suggests that the strictness of GC selection may be tunable depending on the antigenic setting. For example, selection in mesenteric lymph node and Peyer's patch GCs of germ-free mice is much more likely to generate GCs dominated by a single clone than is any other setting we have examined so far (153), with GCs converging on stereotyped clonotypes of unknown specificity present in almost every mouse (47, 153).

Important in this framework is the notion that individual GCs most likely evolve in isolation from one another, with little if any B cell exchange between adjacent structures. Coarse-grained evidence for such isolation comes from classic studies showing that neighboring GCs can have entirely distinct clonal compositions (228). This notion is in agreement with more recent studies

(152, 223) and is also supported by mechanistic understanding of the positioning cues that restrict GC B cells to their structure of origin (229, 230). As with geographical islands in Darwinian evolution, restriction to individual GCs of B cells that acquire large fitness advantages may be required to prevent these lineages from taking over the entire GC response, and could thus play a role in maintaining overall clonal diversity.

Whereas exchange of GC B cells between GCs is uncommon, invasion of GCs by naive-derived B cell clones has been documented. Naive-derived B cells can colonize ongoing GCs under two circumstances: when they express BCRs with higher affinity for antigen than those within the GC at the time of invasion or when they are specific for a different antigen for which independent T cell help exists (15, 231). The consequences of invasion are most easily observed in chronic GCs that arise in response to the gut microbiota or in autoimmune disease models. In the gut, fate-mapping has shown that most GC B cells are rapidly replaced, with a calculated half-life of approximately 2 weeks for mesenteric lymph nodes (153). Likewise, in a mouse model in which a minor population of genetically engineered ribonucleoprotein-specific B cells is used to trigger GC formation, GCs initiated by these founder cells are gradually replaced by nontransgenic B cells specific for other autoantigens, leading to epitope spreading (232). Thus, in chronic GC settings, there is a distinction between the GC reaction—i.e., one or more clones that enter the GC, affinity mature, and then exit—and the GC structure, which is present for the long term, housing different GC reactions as B cell clones constantly turn over.

4.3. Clonal Selection at Export and Recall

The rules governing export of clones form the GC (Section 3.2) can affect not only the affinity but also the clonal diversity of the PB and MBC compartments. Both affinity-dependent and temporal switch models of selection (**Figure 4**) suggest that MBCs should be more clonally diverse than PBs, because higher-affinity B cell clones are likely to be both rarer and longer-lived in the GC reaction. This notion is largely supported by fate-mapping studies, which place the diversity of MBCs in the hundreds of clones or higher (145, 197), compatible with the total diversity of GC B cells (152). Conversely, large-scale single-cell sequencing studies of mice have shown that PB compartments resulting from influenza infection are less diverse than contemporaneous GCs are and tend to derive from clones that are also more expanded within GCs (233). The more diverse MBC population is therefore the one most likely to contain the usually nonimmunodominant clones that cross-react with different viral variants (such as those specific for the influenza hemagglutinin stem), a prediction that has been confirmed experimentally (198, 234, 235).

In mouse models, the strictest bottleneck to clonal diversity operates at the time of recall of MBCs to the secondary PB response. Of the hundreds of MBC clones generated by priming, only a handful of dominant clones are recalled as secondary PBs, usually through repeated recruitment of multiple members of a few higher-affinity dominant clones (145). Sequencing and serum-level studies suggest similar focusing also occurs in humans, at least in the setting of influenza immunization (216, 236, 237). Notably, whereas most secondary PBs derive from MBCs, secondary GCs in the mouse prime–boost setting are composed predominantly of naive-derived B cells with only a minor MBC-derived component, leading to early secondary GCs that are just as diverse and poorly mutated as early primary ones (145, 197). Substantial participation of naive-derived B cells in secondary GCs in humans boosted with influenza vaccine has also been reported (238). The MBC-derived fraction in this setting is highly variable between subjects and generally higher than in mice, which may reflect either the variable but likely extensive histories of influenza antigen exposure in humans or more profound biological differences in MBC post-reactivation fates between humans and mice. Although our picture of the clonal dynamics of repeated antigenic

exposure is still a work in progress, understanding these dynamics may help us predict the outcomes of sequential immunization regimens, including those common in influenza vaccination settings and those proposed as vaccination protocols to induce broadly neutralizing antibodies to HIV.

5. OUTLOOK

In the decade since our last article in the *Annual Review of Immunology*, our understanding of GC biology has gained much in detail. Careful dissection of the cellular and molecular details of affinity-driven selection—relevant to basic B cell biology but also to lymphomagenesis—has paved the way for an increasing awareness of how GCs operate in polyclonal settings, with clear implications for vaccinology and autoimmunity. Despite such progress, multiple fundamental questions remain open, and the GC field is thriving. For one, the central debate on how B cells measure their affinity, both in absolute terms and in relation to that of their neighbors, is yet to be settled, with distinct and often incompatible results emerging from different experimental systems. In parallel, the recalcitrance of the HIV and influenza problems, compounded by the emergence of coronavirus disease 2019 (COVID-19), has led to widespread interest in the role SHM and GC selection have to play in immune protection. Moving forward, one can expect that recent technical advances will play a dominant role in shaping the ways in which we address these questions. Critically, for a reaction in which cells behave largely autonomously, the recent explosion of high-throughput single-cell transcriptome and immunoglobulin sequencing techniques promises us access to the clonal and phenotypic details of thousands of individual cells. The development of protocols to access immunization-induced GCs in higher primates foreshadows a shift in studies of GC biology to the human setting, with important gains in terms of relevance to human disease but also with commensurate challenges given the lack of tractable experimental systems and the complexity of antigenic exposure histories. We look forward to what the next ten years have in store.

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