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CLONAL AND CELLULAR DYNAMICS IN GERMINAL CENTERS

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Abstract

Germinal centers (GCs) are the site of antibody affinity maturation, a process that involves complex clonal and cellular dynamics. Selection of B cells bearing higher-affinity immunoglobulins proceeds via a stereotyped pattern where B cells migrate cyclically between the GC's two anatomical compartments. This process occurs in a timeframe that is well suited to analysis by intravital microscopy, and much has been learned in recent years by use of these techniques. On a longer time scale, the diversity of B cell clones and variants within individual GCs is also thought to change as affinity maturation progresses; however, our understanding of clonal dynamics in individual GCs is limited. We discuss recent progress in the elucidation of clonal and cellular dynamics patterns.

Introduction

The high-affinity antibodies typical of a secondary immune response emerge from their lower-affinity precursors through a process of random mutation and targeted selection known as affinity maturation. This process takes place in specialized structures—germinal centers (GCs)—that form within the B cell follicles of secondary lymphoid organs upon infection or immunization [1-5]. While in these structures, B cells undergo somatic hypermutation (SHM) of their immunoglobulin (Ig) genes, triggered by the enzyme activation-induced cytidine deaminase (AID)[6]. A minority of B cells with affinity-enhancing mutations are then selected based on the increased ability of their antigen-binding B cell receptors (BCRs) to retrieve antigen from the surface of follicular dendritic cells (FDCs) and present it to a limiting number of GC-resident T follicular helper (Tfh) cells [4,7].

GCs are divided into two anatomically distinct compartments—a dark zone (DZ) and a light zone (LZ). A major feature of the GC reaction is the close association between affinity-based selection and B cell migration between these compartments: upon positive selection in the LZ, GC B cells transit to the DZ, where they proliferate and mutate their Ig genes, subsequently returning to the LZ to test their mutated Igs against antigen retained on FDCs.

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In recent years, the emergence of multiphoton microscopy has dramatically increased our ability to observe this migratory process in real time, providing invaluable insight into the mechanics of GC selection [7-11]. These and other studies have been reviewed extensively elsewhere [3,4,12]. In the present review, we discuss specific points regarding the interplay of clonal and cellular dynamics in the GC that in our view remain incompletely understood.

Clonality in the early GC

Before the LZ and DZ form, and thus before *intraclonal* GC selection can begin, GCs must develop by expansion of precursors selected from within a large pool of naïve B cells that compete *interclonally* (Fig. 1). Early studies of GC clonality using allelically marked mixtures of B cells or immunization with two distinct antigens estimated that B cells within mature GCs are the progeny of as few as 1-3 precursor clones [13,14]. Because cells in mature GCs have presumably gone through several cycles of purifying selection, these early studies were in fact reporting on the number of surviving clones rather than of founder clones [15]. Later studies showed that clonal diversity in early GCs can be substantially higher than in mature GCs, suggesting that GCs may initially grow by accretion of many B cell clones that are subsequently filtered by selection to yield the 1-3 clones of mature GCs [16]. Studies in which Ig gene rearrangements were amplified from single cells picked from individual human GCs also support a more complex pattern of GC clonality [15]. Access of B cell clones to the early GC is controlled by a balance between a low B cell-intrinsic activation threshold [17-20] and interclonal competition for T cell signals that regulate B cell entry into the GC [20], possibly by triggering the downregulation of the G-coupled receptor Ebi2 [21,22]. For example, B cells with very low affinity for nitrophenol haptens, which are largely excluded from GCs when transferred into wild-type mice, form normal GCs when in the absence of competition from other B cell clones [18-20]. Interclonal competition is also likely to constrict the breadth of antibody specificities that are allowed entry into the GC. Knowledge of how to manipulate this early selective step may therefore improve our ability to generate antibody responses to non-immunodominant epitopes.

As the GC reaction proceeds, B cell selection shifts from interclonal competition to a system increasingly dominated by competition among variants of a single clone generated by SHM [16]. This progressive “monoclonalization” is limited by the segregation of individual GCs from the B cell perspective, which allows several different clonal trees to evolve simultaneously in different GCs. A further contributing factor may be the invasion of ongoing GCs by newly activated B cells with a competitive advantage in antigen binding or access to T cells [9,23]. The need for competitive advantage limits invasion to special circumstances, such as when T-cell help specific for the incoming B cells is available [23]. Thus, GC invasion and reutilization may be confined to sites prone to constant antigenic stimulation, such as mucosal associated lymphoid tissue. Accordingly, following oral immunization, circulating B cells repeatedly re-enter antigen-experienced GCs, as revealed by the clonal relationship between GC B cells derived from multiple Peyer’s Patches [24].

Dynamics of selection in the mature GC

Within mature GCs, selection and cell division are polarized towards LZ and DZ, respectively, although the extent of cell division in the LZ may vary slightly depending on the model employed [7,8,25,26]. Polarization of GCs into these two compartments relies on gradients of the chemokines CXCL13 and CXCL12, whose receptors—CXCR5 and CXCR4—are upregulated in LZ and DZ B cells, respectively [7,27]. Early attempts at mathematical modeling of the GC reaction concluded that efficient affinity maturation could only be attained if cells were allowed to cycle functionally between periods of proliferation and selection, and, by extension, to cycle physically between DZ and LZ [28,29]. These predictions led to a model for affinity maturation known as *cyclic re-entry*, which would be confirmed experimentally only after the introduction of intravital microscopy to the field a decade later [7-10].

The close association between interzonal migration and affinity maturation has raised interest in the mechanisms that trigger the transition of GC B cells from one zone to another (Fig. 2). Because LZ to DZ migration is equivalent to positive selection [7], most of the emphasis has been placed on the cellular and molecular mechanisms driving this transition. It was predicted mathematically [30,31] that LZ to DZ migration is restricted by the ability of a GC B cell to recruit help from a limiting number of GC-resident Tfh cells, which is in turn determined by the ability of the B cell to retrieve and present antigen deposited on FDCs. To directly test this hypothesis, we developed a system in which the density of cognate peptide on a small subset of GC B cells expressing the surface lectin DEC205 could be artificially increased by injection of the T cell antigen fused to a monoclonal antibody to this protein. DEC205-expressing B cells thus targeted followed the predicted steps of selection, migrating in mass from LZ to DZ and then proliferating vigorously within the GC or differentiating into plasmablasts [7]. Importantly, the larger fraction of untargeted (DEC205^{-/-}) GC B cells almost entirely disappeared from GCs 48-72h following treatment, leading to the conclusion that Tfh are the limiting factor in GC selection [7].

In such a model, the primary function of the BCR would be that of an endocytic receptor, and the role of signaling downstream of the BCR would be limited to tasks other than executing the selection of higher affinity clones and variants. Indeed, when cognate peptide levels are equalized among all GC B cells by anti-DEC205 targeting, affinity maturation ceases to operate despite the variation in BCR affinity among GC B cells [7]. The notion that BCR signaling plays a limited role in selection is further supported by work by Shlomchik and colleagues showing that phosphorylation of proximal components of the BCR signaling pathway is surprisingly absent from IgM⁺ GC B cells (with the notable exception of a brief spike during the G2/M transition of the cell cycle) [32]. This absence is potentially due to high activity of SH2-containing phosphatases, which is supported by the finding of residual BCR-proximal phosphorylation in mice bearing a natural low-expressing variant of the FcγRIIb promoter [33]. These findings suggest that affinity-based selection—which presumably corresponds to the G1-S transition of the cell cycle—can proceed in the absence of overt BCR signaling. Whether this lack of downstream activity is also the case for isotypes other than IgM is at present not clear. Whereas little work has been done on IgG signaling in GCs, a series of recent studies suggest that the IgE BCR may have unique

signaling properties in GC cells [34-36] [Reference to C. Allen's review in this series]. Thus, although affinity maturation requires affinity-dependent retrieval of antigen from FDC and presentation to Tfh, signaling downstream of the BCR appears to play a secondary role in defining which B cells will be positively selected, and may be more important in guiding post-GC differentiation or in the culling of autoreactive clones [37-40].

Much less is known about how GC B cells are driven to return from DZ to LZ. Forcing interactions between GC B and T cells by targeting antigen to DEC205 induces a protracted period of residency of GC B cells in the DZ (24-48 hours, compared to the ~4 hour half-life under non-manipulated conditions). Mathematical modeling indicates that B cells must undergo at least 5-6 cell divisions while remaining in the DZ if the DZ/LZ B cell ratios obtained in experiment are to be achieved [41]. This suggests that the strength of the signal provided to B cells by Tfh cells determines the number of divisions (and the length of time) that a B cell will subsequently undergo (spend) in the DZ. Because the phenotype of LZ cells is dominated by a strong signature of NF- κ B activation [7,25], we hypothesized that, once a B cell exits this proliferative phase, it would be free to migrate back to the LZ, where it would upregulate the LZ signature genes as a result of contact with activating signals from antigen and T cells [25]. However, subsequent work by Cyster and colleagues using mice with CXCR4-deficient B cells showed that physical location in the LZ alone is insufficient to drive the LZ phenotype [26]. This suggests that B cells have an intrinsic "timer" that determines the switch in phenotype from DZ to LZ, either by directly triggering the LZ gene expression program, or by rendering B cells susceptible to activating signals present in the LZ. A model therefore emerges in which Tfh cells in the LZ extrinsically trigger selected B cells to migrate to the DZ. B cells then enter a proliferative state, in which they divide one or more times. Release from this state, return to the LZ, and acquisition of a LZ phenotype (or of the ability to respond to activation signals in the LZ) are temporally controlled in a B cell-intrinsic manner.

A common theme across these studies is that they place positive selection by Tfh cells in control of both LZ to DZ and DZ to LZ migration. Whereas the former occurs by triggering B cells to enter cell cycle and migrate to the DZ, the latter is achieved by setting the B cell DZ timer in accordance with the strength of the T-B interaction (Fig. 2). The notion of a GC reaction controlled by access to T cell help has important functional implications. A system in which selection is dependent on two consecutive steps—affinity-dependent retrieval of antigen from FDCs followed by cognate engagement of helper T cells—is likely to be more noisy (i.e., not all selected cells are higher-affinity, and vice-versa) than one in which B cells are selected based on direct binding to antigen. This may explain why selection proceeds at a slower rate than expected [30], or why heterogeneity of affinities in serum increases rather than decreases as a function of time [42]. While theoretically distracting the system from achieving the highest affinity in the shortest amount of time, such built-in noise is likely to be advantageous in most real-life scenarios, where a diversity of antibodies is required [43].

Less is known about what triggers the differentiation of GC B cells into post-GC plasma cells and memory B cells. We have reviewed the evidence for the role of Tfh cells and other signals in a previous article [4].

Tfh cell dynamics

Whereas the dynamics of B cells in the GC are known to some detail, those of Tfh cells have only begun to be elucidated [44]. After an initial period of interaction with B cells at the follicle border [45-47], CD4 T cells invade the B cell zone, initially dispersing throughout the follicle and only gradually accumulating within GCs [45,48]. Entry into the follicle and GC is dependent, among other factors, on expression of CXCR5, SAP, and Bcl6 in the Tfh cells themselves, and of ICOSL on naïve follicular B cells [49-52]. However, not all T cells expressing CXCR5 are located inside GCs, and neither do all T cells physically within GCs express CXCR5 [48]. Together with data showing that CXCR5 deficiency only partially impairs the formation of GCs and T cell homing to these structures [53], the lack of specificity of CXCR5 as a marker suggests that additional cues must exist that control Tfh residency in the GC.

In contrast to B cells, GC Tfh cells are highly polyclonal within a given GC, and the same clones can be found across many different GCs at approximately equal ratios [48,54-56]. Equal distribution among different GCs can be explained both by the absence of an overt founder effect and by continuous exchange of Tfh cells between mature GCs [48]. Furthermore, newly activated T helper cell clones can invade ongoing GCs and provide help to cognate B cells within these GCs [48]. At present, one can only speculate on why GCs should be open to free exchange of Tfh cells while B cell exchange is limited. As noted above, being self-contained from the perspective of B cells allows GCs to evolve different responses in parallel, preventing a single B cell clone from dominating the GC response. On the other hand, free exchange of Tfh cells between GCs and engagement of newly activated Tfh clones may ensure that B cell evolution is supported by a broad assortment of T cells, which may contribute to the robustness of the system, for instance in face of frequently mutating T cell epitopes (Fig. 3).

Clinical Implications

Most vaccines currently licensed for human use are thought to work by eliciting neutralizing antibodies [57]. Recently, the ability to induce broadly neutralizing antibodies (bnAbs) against HIV and influenza by vaccination has emerged as an important and potentially achievable goal [58,59], highlighting the importance of understanding the GC reaction in detail. In this context, a crucial question is how breadth and neutralization can evolve within a reaction that selects variants solely on the basis of affinity/avidity. A recently published study of the co-evolution of virus and bnAbs within a single patient and from the very onset of infection provides some insight in this sense [60]. BnAbs in this patient appeared to have evolved during a period when many variants of the virus were present simultaneously. One possible explanation for this finding is that, in such a scenario, GC B cells with greater breadth would also have higher avidity because of their ability to bind multiple viral variants on the surface of FDCs, which could lead to their preferential selection over B cells with similar or even higher affinity but more narrow breadth. A second important observation is that the most potent bnAbs against HIV carry an inordinately large number of somatic mutations (up to 30% of amino acids), which appear to be functionally required for broad neutralization [61-63]. Such high mutational load is almost an order of magnitude higher

than what is currently attainable, for example, by influenza vaccination [64]. How such antibodies evolve during infection and whether they can be induced by vaccination are open questions in the GC field.

Conclusion

Recent years have witnessed a leap in our understanding of GC dynamics, driven to a large extent by the advent of intravital microscopy. Nevertheless, important gaps remain in our knowledge of how B cells are selected to enter, thrive in, and exit the GC. Filling in these gaps will undoubtedly contribute to future efforts to design more effective vaccines against challenging pathogens.

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Highlights

- Germinal center clonal diversity is kept low by multiple bottlenecks
- Germinal center selection is inextricably linked to cellular dynamics
- T cell help regulates multiple aspects of germinal center B cell dynamics
- Better understanding of germinal center dynamics will aid vaccine development

Figure 1.

Potential model for clonal dynamics during germinal center formation. GCs are seeded by a small fraction of the large repertoire of naïve B cells potentially responsive to the immunizing antigen by pre-GC competition for T cell help (Bottleneck #1), generating GCs composed of a limited number of clones. These GCs are then further purged from less competitive clones by a second round of competition for T cell help in the mature GC (Bottleneck #2), yielding the 1-3 clones observed in experiment.

Figure 2.

Interplay of dynamics and selection in the germinal center reaction. B cells activated by antigen migrate to the T zone–follicle (TB) border, where they compete for a limiting number of helper T cells. Selected B cells migrate to the center of the follicle, where they proliferate extensively, giving origin to mature GCs. In these structures, GC B cells cycle between periods of proliferation and AID-driven mutation in the dark zone (DZ) and antigen-driven selection in the light zone (LZ). LZ-DZ cycling, selection for cyclic re-entry, and exit from the GC as memory (B_{MEM}) or plasma cells (PC) are likely to be controlled by interaction with a limiting number of T follicular helper (T_{FH}) cells. Changes in expression of selected G-protein coupled receptors required for the various steps in B cell migration are indicated in red. Cell phenotypes and cell-cycle stages are indicated in blue.

Figure 3.

Tfh dynamics in germinal centers. From the B cell perspective, GCs are thought to be “islands” dominated by one or a few clones that differ between neighboring GCs. In contrast, Tfh cells freely exchange between GCs, and Tfh clones are distributed in approximately equal proportions among GCs from the same LN. Additionally, newly activated T cells (light green) can join and contribute to ongoing GC reactions.