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Deconstructing the germinal center, one cell at a time

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Abstract

Successful vaccination relies on driving the immune response towards high specificity, affinity and longevity. Germinal centers facilitate the evolution of antigen-specific B cells by iterative rounds of diversification, selection, and differentiation to memory and plasma cells. Experimental evidence points to B cell receptor affinity and amount of antigen presented to follicular helper T cells as main drivers of clonal evolution. Concurrent studies suggest that modifiers of cognate contact, temporal mechanisms, and stochastic factors can also shape diversity and influence differentiation to memory and plasma cells, but molecular pathways driving these selection decisions are unresolved. Due to rapid cycles of transcriptional change in the germinal center, single-cell resolution is imperative to dissect mechanisms dictating the mature antigen-specific repertoire. Future studies linking high-resolution analysis of this diverse evolving population with cellular outcome are needed to fully understand the complex mechanisms of selection driving antigen-specific humoral immunity.

Introduction

Vaccination remains an important public health tool to prevent infection and the spread of disease. By driving the evolution of antigen-specific B cell populations, vaccines elicit robust antibody-mediated immunity while bypassing infection. Affinity maturation through clonal selection in germinal centers (GCs) allows evolution of the B cell repertoire to generate antibodies against virtually any foreign antigen [1] (Figure 1). Though antigen affinity is a major driving force for selection, patterns of molecular signals drive B cells through this process, ensuring the production of not only antibody-producing plasma cells but also memory B cells that can respond and re-diversify to secondary challenge [2]. Understanding the regulation of this process *in vivo* is paramount to formulating novel vaccines to produce efficient and diverse immune responses.

This selection process is highly regulated by complex molecular signals at multiple stages. Following immunization, antigen-specific B cell precursors are activated, binding antigen and moving to the outer follicular zones. Here, they present antigenic peptide on MHCII to

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specialized subsets of separately-activated follicular helper T (T_{FH}) cells to form GCs [3–6]. In this structure, B cells undergo cycles of Darwinian evolution through repeated rounds of expansion, diversification, and selection by limiting numbers of cognate T_{FH} cells to form a both a diverse and highly-specific repertoire in both the memory and plasma cell compartments.

Central to understanding these concurrent processes of diversification, affinity maturation, and exit are spatial, temporal, and transcriptional dynamics in the GC. Robust model antigen systems and recent advances in genetic and imaging approaches currently allow access to this complex and ever-changing population of GC B cells. In this review, we will outline literature informing our present understanding of GC physical structure over time as it relates to transcriptional programs as well as the cellular and molecular mechanisms that regulate them in the primary and secondary response. Finally, we will discuss future directions of the field, with an eye on uncovering dynamics of evolutionary development by using the power of single-cell resolution.

Spatiotemporal control of GC B cell programs

The physical organization of the GC is reflective of and intimately tied to spatiotemporal function. Originally observed in histological sections of secondary lymphoid tissue, GC B cells were described to reside in two compartments that would be known as the “light zone” and “dark zone” (LZ and DZ, respectively) [7]. The LZ contains B cells that bind antigen trapped on the follicular dendritic cell network and interact with GC-associated T_{FH} cells. The DZ contains large numbers of proliferating cells undergoing rapid division and somatic hypermutation. Early pulse-chase experiments using ^{3}H-thymidine and BrdU [8,9] implied movement between the two zones that was later suggested to be controlled by CXCR4- and CXCR5-mediated chemotaxis [10]. In a series of seminal studies using two-photon microscopy, the real-time dynamics of cellular movement during early GC events [11] and dynamic cycling between the LZ and DZ [12–14] were directly visualized for the first time.

In more recent studies, Victora and colleagues utilized a fluorescent photoactivatable reporter to label DZ and LZ GC B cells *in situ* to provide direct confirmation of the connection between GC localization, cellular phenotype, and gene expression [15]. They found that DZ B cells were characterized by increased expression of CXCR4 protein and mRNA, along with upregulation of distinct patterns of expression for cell cycle and somatic hypermutation machinery. In contrast, CD86 mRNA and protein were upregulated in LZ cells, and these cells also displayed increased transcription of gene programs essential for antigen presentation [15]. Together, these studies revealed that distinct GC processes were dependent upon cell location within the GC structure, and that GC B cells undergo spatiotemporal cycling through progressive stages of transcription over the course of clonal evolution.

Characterization of DZ and LZ transcriptional programs informed further studies to dissect regulators of GC B cell movement and location-specific processes. The cell cycle regulator c-Myc, previously found to have low or completely absent activity in bulk GC B cells, was revisited and shown to be transiently expressed in selected subsets of GC B cells in the LZ

[16,17]. The short-lived expression of Myc in the LZ was suggested to initiate downstream regulators, such as AP4, to mediate multiple rounds of division and somatic hypermutation in the DZ [18]. Additionally, high expression of the transcription factor FOXO1 in DZ B cells appeared to control DZ formation, organization, and gene expression [19,20], likely working in concert with Bcl6, an important regulator of GC B cell function [21–23]. Furthermore, the presence of FOXO1+ cells in the LZ suggested an initiation program for DZ entry established during cognate selection [20].

Paired analysis of repertoire, transcription, and protein

While visualizing the spatiotemporal organization and movement of GC B cells and T_{FH} cells in real time has illuminated essential information regarding the cellular dynamics of GC clonal selection, many of these studies relied heavily on adoptive transfer systems of transgenic Ag-specific T and B cells. It remains important to consider the complex evolutionary dynamics within an intact polyclonal system, where there is a much broader range of binding affinities competing for selection. Additionally, while genetic experiments are informative for mechanistic analysis of GC processes, there is no one definitive marker for GC B cells, and many surface markers and transcription factors active in the GC also play a large role in its formation, making results from these experiments complicated to interpret. Finally, single-cell resolution is necessary to disentangle the complex, heterogeneous population of cells in the GC. Analyzing repertoire, mRNA, and protein levels from the same cell allow integrated analysis of antigen-specific affinity, molecular programming, and cellular phenotype to make conclusions about the mechanisms of GC selection and differentiation.

Utilizing this approach, Victora and colleagues used multicolor labeling to illustrate clonal selection and dominance within GCs of an intact polyclonal system [24]. To visualize clonal composition of individual GCs in real time, B cell clones were irreversibly labeled with a heritable color label after GC formation, and the GC was allowed to progress. Clonal dominance was indicated by the heterogeneity of colors visualized *in situ*. Microdissection and repertoire analysis of single cells within individual GCs linked clonal dominance with affinity maturation. Remarkably, lower-affinity B cells were able to maintain fitness even within GCs that contained higher-affinity competition, providing evidence that mechanisms independent of BCR affinity are important in GC clonal composition [24].

In recent studies, we have integrated surface phenotype and transcription with BCR repertoire analysis of single cells to detail how antigen-specific GC B cells progress through stages of selection [25]. Transcription of 96 genes for single cells was obtained using high-order quantitative PCR and paired with immunoglobulin repertoire to link gene expression with clonal selection. Multidimensional clustering using the machine learning algorithm, t-distributed stochastic neighbor embedding (t-SNE), was utilized to visualize patterns of similar gene expression within the heterogeneous GC population. Four clusters of similar gene expression emerged, with a clear pattern of progression defined most clearly by expression of *Cd83* and *Polh*, two genes whose differential mRNA and protein expression had been previously established as part of the hallmark LZ and DZ programs, respectively, in bulk RNA-seq [15] (Figure 2). Expression of *Polh*, which encodes the DNA polymerase

that introduces somatic hypermutations in the DZ [26], was highly expressed in the DZ program, while *Cd83* expression characterized cognate interactions between T_{FH} cells and GC B cells. Transitions between these four stages were correlated with genes important for cognate contact (Stage 1 to 2), DZ transition (Stage 2 to 3), proliferation and diversification (Stage 3 to 4), and LZ re-entry (Stage 4 to 1) [25].

T_{FH} cells as molecular controllers of clonal evolution

To drive clonal evolution and initiate progression through the GC, effective selection pressure is required. Two-photon imaging studies first illustrated contact between GC B cells and T_{FH} cells in real time *in vivo*, lending appreciation for a possible role of T_{FH} cells to provide selection pressure [12–14]. To further investigate the effect of this interaction on GC selection, Nussenzweig and colleagues manipulated peptide presentation levels on MHCII independently of BCR crosslinking via utilization of the endocytic receptor DEC205. The increased expansion of GC B cells that presented more antigen supported the model that T_{FH} cells serve as the primary limiting selection pressure for GC B cells [15]. Indeed, further study using this system revealed that cell division and hypermutation by GC B cells was proportional to the amount of antigen presented to cognate T_{FH} cells [27], and the cells presenting the highest levels of pMHCII to T_{FH} cells had sustained increases in intracellular free calcium and IL-4 and IL-21 expression [28], suggesting an active mode of selection. Evidence linking increased antigen presentation on MHCII to rapid cell cycle progression in the DZ [29] provided a mechanism for clonal evolution. These results support a model in which T_{FH} cell help in the LZ determines the timing of proliferation and diversification in the DZ to preferentially shape the GC repertoire [30]. In addition to controlling re-entry into the DZ, antigen affinity may also regulate GC exit by inducing memory or PC transcriptional regulators. Kurosaki's group correlated *Bach2* expression with antigen affinity to show induction of the post-GC memory program in lower-affinity, less mutated cells, which would preserve a wide range of repertoire diversity in the memory compartment [31].

Despite much evidence pointing to peptide presentation as a primary mode of selection, concurrent mechanisms also influence GC clonal evolution. Even though individual GCs may become dominated by a single clone, high-affinity GC clones can be found evolving alongside low-affinity clones, even within the same GC [24]. Heterogeneous populations of T_{FH} cells may select multiple antigen-specific B cell clones binding different epitopes, and could contribute to the maintenance of clonal variety [32,33]. Sets of costimulatory molecules expressed on GC T_{FH} cells and GC B cells can modify the signaling consequences of cognate contact and direct post-GC fate [32]. In a complete knockout of PD-1, a costimulatory molecule highly expressed on T_{FH} cells, decreased *Il4* and *Il21* expression by T_{FH} cells was accompanied by lowered long-lived antigen-specific PCs following immunization [34]. Furthermore, ICOS-ICOSL interactions between T_{FH} cells and GC B cells were found to enhance cell-cell contact and initiate a feed-forward signal of positive selection events to enhance antigen-specific affinity selection in a competitive adoptive transfer system [35].

Differential selection may also rely on the timing of the immune response. Shlomchik and colleagues proposed that low-affinity, antigen-specific B cells with few mutations differentiate into memory B cells early on, and are followed by differentiation of high-affinity plasma cells later in the response [36]. While studying the humoral response to a Th2-type infection, Craft's group attributed this switch to modulation of T_{FH} cell phenotype over the course of infection, suggesting T_{FH} cell-mediated temporal control by differential cytokine secretion [37]. These ongoing studies continue to emphasize the importance of T_{FH} cells in shaping GC B cell populations and outcomes.

Negative selection in the GC

Because of the prevalence of DNA damage driving the rapid mutational processes in the GC, stringent negative selection mechanisms must be in place to prevent autoreactivity [38] and cancer [39]. Follicular regulatory T (Tfr) cells have emerged as negative regulators that dampen the GC response [40–42] and may play an integral role in preventing aberrant pathological outcomes; however, their mechanism of action is largely unresolved. Like T_{FH} cells, they express the co-receptors PD-1 [43] and CTLA-4 [44,45], which modify the potency of their activity and may serve as modulators of negative selection. Furthermore, *in vitro* co-culture experiments have suggested that T_{FR}-mediated suppression of B cell activation relies on the presence of T_{FH} cells and may epigenetically restrict access to genes important to GC B cell differentiation, notably *Aicda*, *Myc*, and *Pou2af1* [46]. T_{FR} cells can be specific for both foreign and self-antigen [47]. Nevertheless, it is unclear how antigen-specificity and TCR affinity contribute to T_{FR} cell differentiation, as they do for T_{FH} cells [48], and how cognate contact might contribute to T_{FR} cell function.

Another requirement for GC negative regulation may be to preserve diversity of BCR specificity across many antigen epitopes. Plasma cell differentiation itself within the GC may serve as a negative feedback loop. Our studies have shown that PCs, enabled by expression of MHCII, costimulatory molecules, and antigen presentation machinery, can negatively regulate the T_{FH} cell program in an antigen-specific manner [49]. Another study by Toellner and colleagues provided evidence that antibody secreted by differentiated PCs can bind antigen in the GC, limiting access to antigen and driving BCR evolution by providing an advantage to specificities that have not already been produced [50].

Memory and the secondary response

The ability to produce memory B cells that can rapidly differentiate into memory-response PCs (mPCs) and re-diversify in memory-response GCs (mGCs) upon antigenic rechallenge is a hallmark trait of the GC [2,25]. Experimental evidence from several studies points to heterogeneity of memory cells with varying capacities for mPC versus mGC differentiation. Antibody class is suggested to be a major divergent trait induced in the primary response because it has been correlated to divergent differentiation pathways upon re-challenge. In a murine malaria model, Pepper and colleagues found that IgM+ memory B cells (MBCs) preferentially expanded and generated PCs upon secondary infection [51], while Jenkins' group revealed that phycoerythrin (PE) immunization with complete Freund's adjuvant (CFA) induced domination of the secondary response by class-switched MBCs [52].

Reynaud, Weill, and colleagues suggested that both IgM+ and class-switched cells participated in the secondary response in different ways. Immunization with sheep red blood cells induced antigen-specific IgG1+ MBCs that preferentially differentiate to mPC, while antigen-specific IgM+ MBCs induced secondary GCs [53]. Alternatively, a virus-like particle (VLP) prime-boost immunization strategy by Bachmann's group drove a robust IgG + and IgM+ mPC response with negligible mGCs forming [54]. Finally, Shlomchik and colleagues found that CD80 and PD-L2 could define MBC heterogeneity in a class-independent manner. After immunization with NP-CGG in alum, CD80+ PD-L2+ MBCs seeded mGCs, and CD80− PD-L1− MBCs became mPCs regardless of antibody class [55]. These seemingly conflicting results may indicate that memory-response programming may be dependent on immunization or infection conditions.

An additional important characteristic of memory B cells is longevity, which is crucial for lasting antigen-specific immunity. The enzyme activation-induced cytidine deaminase (AID), which is responsible for both class-switch recombination (CSR) and somatic hypermutation (SHM) [56], has been implicated as a key driver in MBC longevity. Jenkins and colleagues originally suggested class-switch recombination as the key factor for persistence, as PE-specific IgM+ MBCs survived longer than class-switched MBCs [52]. However, in a more recent study, Nussenszweig's group used a complex genetic model to disentangle AID-driven mechanisms of CSR and SHM to conclude that levels of SHM were a better indicator of MBC longevity, independent of class [57]. Single-cell resolution is still needed to dissect divergent programming of populations arising post-GC to understand mechanisms of antigen-specific regulation. Regardless, antigen experience, independent of class, is most likely a strong component of memory B cell programming [58], which itself diverges in a class-specific manner [59].

Conclusions and future questions

While much information has been uncovered from bulk analysis, single-cell approaches are needed to uncover the ever-changing clonal and molecular dynamics of heterogeneous populations within the GC. The studies already performed will inform future work using techniques such as single-cell RNA-seq to detail more completely the molecular mechanisms of GC cyclic progression. Pairing these analyses with information on clonal selection and evolution within the GC, as well as its products memory B cells and PCs, will enlighten our understanding of these changing dynamics. Finally, studies pairing GC B cell evolution with active programs of T cell-mediated selection will help to design novel vaccine formulations that induce efficient immune responses against antigen.

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Highlights

- Germinal centers (GCs) facilitate antigen-specific evolution of B cell repertoire.
- Follicular helper T cells regulate GC evolution, memory, and plasma cell formation.
- Complex molecular signals drive rapid transcriptional changes in GC B cells.
- Single-cell analysis details regulation of the diverse, evolving GC population.

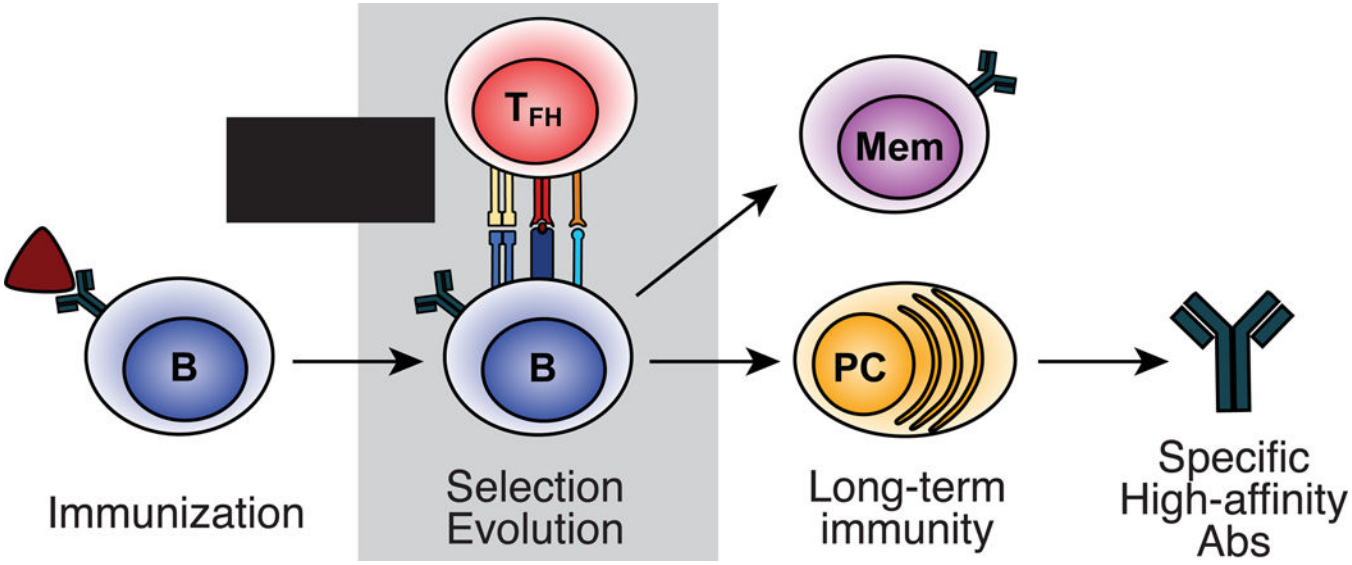

Figure 1. Immunization-driven antigen-specific immunity

Immunization with protein antigen primes naïve antigen-specific B cells and T cells separately. Activated B cells uptake bound antigen, processing and presenting antigenic peptide on MHCII to T_{FH} cells and a germinal center is formed. The population of germinal center (GC) B cells undergoes evolution toward higher antigenic affinity and specificity, marked by continual antigenic binding, processing, and presentation to cognate **T_{FH} cells**, which deliver selection signals resulting in further diversification or exit to join the memory compartment (Mem) or differentiate to plasma cells (PC), which secrete specific, high-affinity antibodies (Abs).

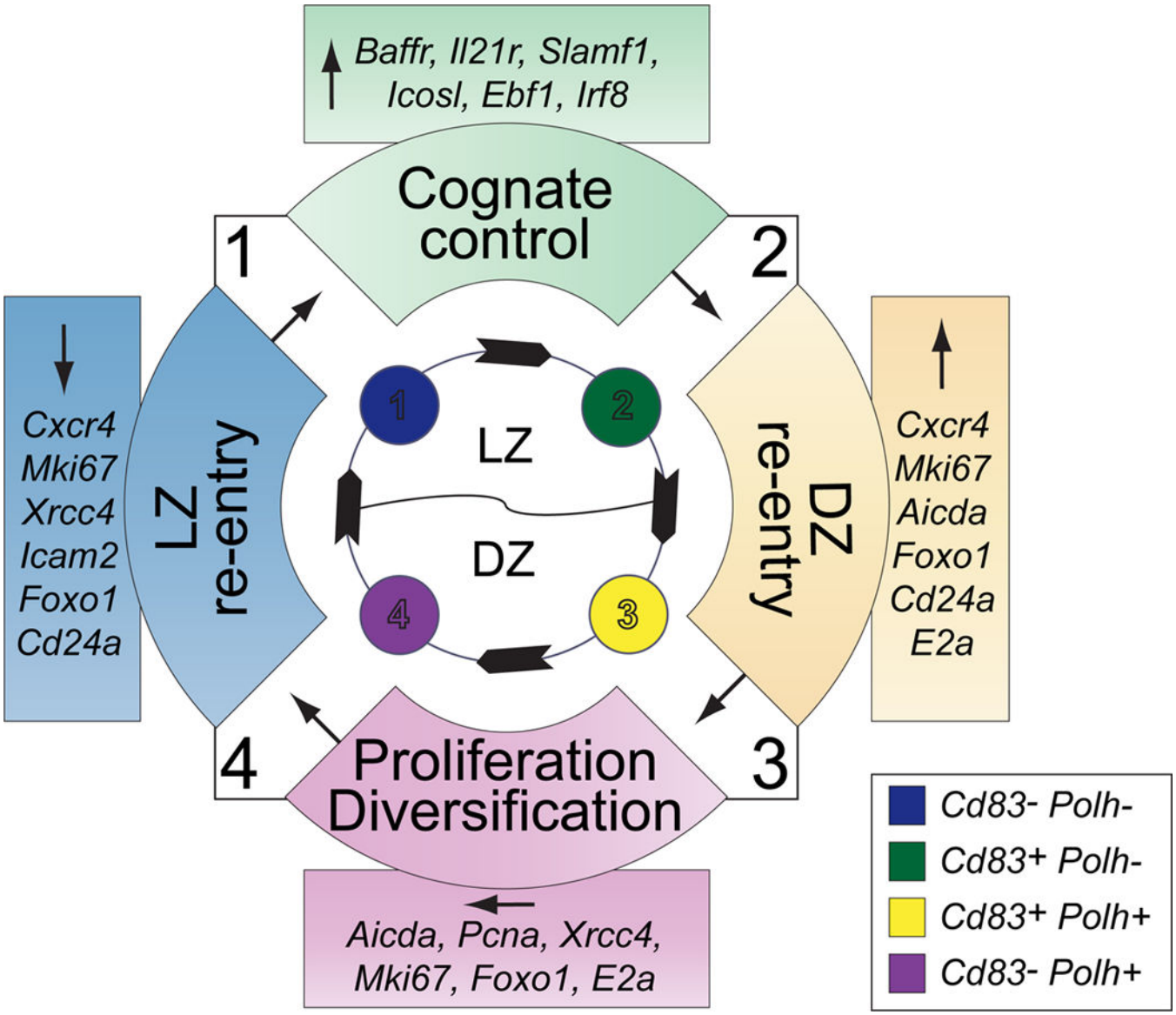

Figure 2. Stage-specific regulation of genetic programs in the GC

Gene expression of single cells in the GC assorted into four distinct patterns of gene expression **(Stages 1–4)**. Gene clusters were determined according to patterns of similar expression using the machine learning algorithm t-distributed stochastic neighbor embedding (t-SNE), and order of cyclic progression computationally inferred by the trajectory detection algorithm Wanderlust. Genes in colored boxes change significantly between the stages marked in the colored semicircles in the direction indicated by the arrow. **Stages 1–2** represent cognate control in the light zone (LZ), where germinal center (GC) B cells pick up antigen trapped on the follicular dendritic cell network, processing and presenting them to limiting numbers of cognate follicular helper T cells (Tfh), which select B cells based on levels of cognate antigenic peptide. Between **Stages 2–3**, selected B cells travel to the dark zone (DZ), upregulating genes associated with somatic hypermutation and proliferation, as well as the chemokine receptor *Cxcr4*, which enables spatial migration.

From **Stages 3–4**, GC B cells diversify the B cell receptor and proliferate, maintaining high levels of *Aicda* and *Mki67*, among others. Finally, from **Stages 4–1**, GC B cells re-enter the LZ, downregulating genes necessary for affinity maturation and prepare to re-express the new B cell receptor to pick up and present antigen once again.