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Deconstructing the germinal center, one cell at a time

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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.

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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 direct 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.

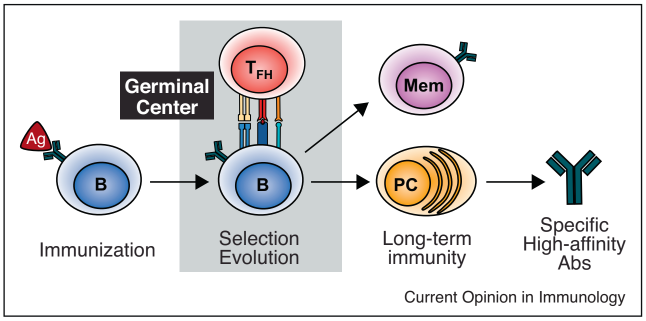
This selection process is highly regulated by complex molecular signals at multiple stages. Following immunization, antigen-specific B cell precursors are activated, binding antigen and moving to the outer follicular zones. Here, they present antigenic peptide on MHCII to specialized subsets of separately-activated follicular helper T (T_{FH}) cells to form GCs [3–6]. In these structures, 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 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

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).

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 *et al.* 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