Textbook Germinal Centers?

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Models for the development and function of germinal centers (GCs) have been so widely discussed in the original literature that they now appear in immunology textbooks. Unfortunately, many of the tenets of these models have not yet been subjected to adequate experimental scrutiny. Indeed, recent studies have called several of their principal assumptions into question. In addition, the term germinal center has been applied to a diverse assortment of focal processes of B cell proliferation and differentiation. This variability might be explained by alterations in the progression of a single textbook GC process. Alternatively, distinct developmental pathways may create unique classes of GCs with specialized functions. The Journal of Immunology, 2004, 172: 3369–3375.

Nearly twenty years ago, the work of two factions of immunologists studying Ag-driven B cell responses using either serological and histological, or molecular genetic and hybridoma techniques coalesced (1–8). Collectively, their findings implicated germinal centers (GCs)3 as the principal sites in which the many steps of memory B cell development occur. Subsequent seminal studies by Kelsoe and colleagues (9) and Berek et al. (10) demonstrated that B cell Ag receptor (BCR) hypermutation and selection take place in GCs. These revelations spawned models explaining the maturation of the B cell response in GCs leading to the generation of memory (2, 3, 11).

Such models have been so widely publicized that a version is presented in most immunology textbooks. Unfortunately, many of the central tenets and assumptions of these models remain to be adequately tested. Moreover, the results of recent studies have painted a much fuzzier picture of the role of GCs in the maturation of the memory B cell response than is portrayed by these models. In addition, although components indispensable for the GC response clearly exist, recent studies have also revealed that the forces that drive B cells to form these structures and down the memory pathway are more robust and diverse than previously thought.

It is important to acknowledge that insightful investigations of the GC response have been conducted in a variety of animal models. However, due to space considerations and because much of the published data have been derived from the analysis of GCs in mice (mainly spleen) and humans (mainly tonsil), I will restrict detailed discussion to these two species.

Textbook models of the GC reaction

According to these models, a requisite step preceding GC formation involves CD4 T cells activated by a protein Ag presented to them by APCs such as interdigitating dendritic cells (DCs) in the T cell zones of peripheral lymphoid organs (Fig. 1). Follicular B cells whose BCRs have engaged the Ag migrate to and undergo cognate costimulatory interactions with these T cells. The B cells then become primary Ab-forming cells (AFCs) or precursors destined for the GC. The factors that determine which of these choices is taken, and whether a single primary B cell clone is even capable of differentiating down both of these paths are subjects of controversy (12). Several GC precursor B cells then migrate back into a follicle, acquire a unique cell surface phenotype (e.g., surface IgD− peanut agglutinin +) and rapidly proliferate within the stromal environment created by follicular DCs (FDCs). The phenotypic and functional diversity, and developmental interrelationship of follicular stromal elements including FDCs are poorly understood. Therefore, I will operationally refer to FDCs present within follicles lacking GCs as primary FDCs and those associated with the GC reaction as secondary FDCs. After this phase of brisk proliferation, which creates the GC dark zone, is underway, Ab V gene hypermutation is induced, resulting in the generation of diverse mutant progeny of the founding clones. Although inroads have been made recently on its mechanism, hypermutation remains a mysterious process, particularly with respect to its requirements for induction and regulation (13). The rapidly dividing B cells, which express little surface (s)BCR and are termed centroblasts, then exit cell cycle, re-express sBCR, and migrate to a sector of the GC rich in secondary FDCs and also containing CD4 T cells, called the light zone. GC B cells in the light zone are termed centrocytes, and are programmed to die unless survival signals are provided by accessory cells.

FDCs can retain Ag on their surfaces in the form of immune complexes (ICs) due to expression of C and Fc receptors (14, 15). If their BCRs engage Ag on secondary FDCs, the centro-
cytes obtain survival signals, and extract and internalize the Ag. Competition for FDC-bound Ag is intense, and only those centrocytes expressing mutant BCRs that have acquired high affinity for the Ag survive. Others die by apoptosis, and are engulfed and degraded by macrophages. Surviving centrocytes subsequently either re-enter the centroblast pool, or process and present the Ag to GC T cells, resulting in induction of Ab H chain class switching, and differentiation to either pre-AFCs or memory B cells that exit the GC.

Because hypermutation can generate or enhance the autoreactivity of BCRs, Klinman and coworkers (16) originally proposed that memory B cell precursors are subjected to a peripheral tolerance checkpoint. Support for this idea has come from several experimental angles (16–19). This putative facet of GC function has been incorporated into textbook models by assuming that autoantigens are readily available in the GC, and that the avid binding of autoantigen to the BCR expressed by a GC B cell precludes the productive interaction of this cell with either secondary FDCs or GC T cells, leading to death by apoptosis (17, 20).

Undoubtedly, the textbook principle that FDCs and the Ag on their surfaces play a critical role in driving the GC response as well as in the phenotypic selection of BCR mutants was motivated by early work demonstrating that visually impressive amounts of Ag can be localized and retained on the FDC reticulum for extended periods (21, 22). However, past observations that GC formation may precede evident IC trapping by FDCs in certain situations cast the first doubt on this idea (1, 23). This suspicion has been reinforced by recent studies on mice deficient in the ability to produce secreted Ab (and thus ICs) demonstrating that GC formation and at least the first stages of BCR hypermutation and affinity-based selection can occur in the absence of detectable levels of Ag on primary and secondary FDCs (24). Conversely, we found that the stringency of affinity maturation in mice with dramatically enhanced deposition of ICs on primary and secondary FDCs due to an FcR deficiency was not relaxed (25), as would be expected from textbook models. Affinity maturation takes place both during and well after the GC response has waned (26). Moreover, studies pioneered...
by Chaplin and colleagues (27) have shown that hypermutation and affinity maturation occur, albeit with reduced efficiency, in mice incapable of normal FDC development due to a deficiency in lymphotoxin (LT)α. A subsequent investigation revealed that LTβ-deficient mice, phenotypically similar to LTα-deficient mice, can mount lymph node GC responses associated with affinity maturation, but these GCs lack secondary FDCs and IC deposits (28). Such data point out that for an essential role of FDCs and their bound Ag in GC formation and the affinity maturation process is currently lacking (29).

However, GC responses in FDC-deficient LTα and LTβ knockout mice are not observed or sustained, respectively (27, 28). We and others have also found that GCs of normal size can be induced in mice deficient in B cell activating factor belonging to the TNF family, but these GCs contain poorly developed secondary FDC reticula and are short lived (30, 31). Collectively, these findings suggest that, although FDC-bound Ag is not required for the induction and early stages of the GC response, FDCs themselves may be necessary to maintain the response. Indeed, in vitro studies have shown that FDCs can function as potent accessory cells for promoting B cell clustering, proliferation, survival, and differentiation (32–34).

FDCs and their bound Ag have also been proposed to play a central role in maintenance of the memory B cell pool, in homoeostatic regulation of antipathogen serum Ab levels, and in induction of anamnestic B cell responses (32, 35–37). A role for persistent Ag in the survival of memory B cells has been rendered unlikely by an elegant experiment conducted by Rajewsky and colleagues (38). After memory cells acquired new BCRs in vivo due to induced gene targeting, they continued to persist, despite apparent absence of an exogenous ligand for these BCRs. In contrast, there is experimental support for the idea that FDCs and their bound Ag facilitate the activation and differentiation of memory B cells (15, 32, 36, 37, 39). Whether secondary FDCs and the Ag they trap function in the putative GC peripheral tolerance checkpoint remains to be tested.

The role of T cells and cognate T cell-B cell interactions

Another component of textbook GC dogma is that GCs form only in response to T cell-dependent (TD) Ags. Past histological studies of several T cell-independent (TI) responses did not reveal Ag-induced GCs (40, 41). Also, immunization of mice deficient in factors critical for cognate T cell-B cell interactions with TD Ags have resulted in either no or drastically impaired GC responses (mutations that reduce or ablate the GC response are tabulated in Ref. 42). Early questions regarding the validity of this TD rule were motivated by the work of Kabat and colleagues (43) on the immune response of mice to the TI Ag α(1–6) dextran, in which efficient GC formation was observed, but subsequent studies indicated that this GC response required T cells (44).

Nonetheless, recent investigations by our laboratory have shown that a high dose of the TI Ag (4-hydroxy-3-nitrophenyl)-acetyl-Ficoll can induce follicular GCs in mice that completely lack T cells (45). B cells expressing a transgenic anti-(4-hydroxy-3-nitrophenyl)acetyl BCR can also give rise to GCs in athymic mice after immunization with this TI Ag (46). Importantly, however, these responses are sporadic and short lived, and the BCRs expressed in these TI GCs contain no or only a few somatic mutations (45, 47), suggesting that at least low levels of T cell help are important for the efficiency of induction and progression of the response. This notion was first suggested by Cerny and coworker (48), who showed that only small numbers of CD4 T cells are required for a TD GC response.

A newly published study (49) has revealed that BCR hypermutation and affinity-based selection take place in GCs induced by TD Ag immunization of mice containing only γδ T cells. Thus, GC progression and several of the processes associated with the intermediate stages of the response can occur with the provision of only noncognate forms of T cell help. Nonetheless, the GC response in these mice was quantitatively reduced, as was serum IgG production and degree of priming for a secondary response. Apparently, conventional T cell help is important for certain steps in GC B cell differentiation.

Early support for this idea came from a study demonstrating that, in normal mice, GC T cells were of the αβ type and expressed TCR structures consistent with specificity for the inducing TD Ag (50). In addition, when reagents that block CD40 ligand and B7.2 function were administered to mice after GCs were induced by a TD Ag, the results were GC dissolution or inhibition of memory development, and perturbation of hypermutation, respectively, consistent with the idea that cognate T cell help is constitutively required for progression of the GC response (51, 52).

Finally, recent studies have revealed that GC T cells express CXCR5, required for lymphocyte homing to follicles, and that CXCR5+CD4 T cells are efficient B helper cells (53, 54). In total, current data are inconsistent with the textbook idea that cognate T cell-B cell interactions are critical throughout the GC response, but they do seem necessary for promotion of development of B memory cells, and may be crucial for the generation of TD Ag-induced GC-derived AFCs.

Elucidation of the role of T cell-B cell interactions in the GC response will likely be assisted by continued studies of the characteristics and capabilities of the T cells that reside in this microenvironment. Kelsoe and colleagues (55, 56) have published the surprising results that mouse GC T cells are members of a unique subpopulation of the αβ CD4 compartment that undergoes hypermutation of its Vβ genes as well as Ag-driven selection. Such behavior was not envisioned by textbook models and, if borne out, will mandate their substantial revision.

Subcompartmentalization of GCs and GC function

FACS purification and analysis of subpopulations of human tonsillar GC B cells previously localized histologically provided initial support for the textbook prediction that different steps in GC B cell development take place in distinct subregions of the GC (57). However, examination of acutely induced mouse splenic GCs has generally revealed far less striking substructure than is typical of human tonsillar GCs. The issue of whether textbook light and dark zones formed in such mouse GCs was carefully addressed by Berek, Kosco-Vilbois, and colleagues (58). These kinetic studies confirmed that mouse GCs develop two zones, one rich and one poor in secondary FDCs (defined as expressing high levels of the FDC-M1, FDC-M2, and CD23 markers; the FDC-M2 marker has been recently shown to be a processed form of C4 (59)) and CD4 T cells. However, these experiments also revealed that nonproliferating B cells (centrocytes?) were only transiently found in the secondary FDC-rich zone, at a time when BCR selection events were known to already have taken place. In addition, throughout the response, nearly all GC B cells appeared to express sBCR.
In corroboration of these studies, we found that only secondary FDCs express high levels of the IgG FcR FcγRIIB in the mouse, and that such FDCs are restricted to a sector of splenic GCs in which most GC T cells are also seen (15, 60). In addition, we observed that the majority of murine GC B cells are in cycle throughout the response, and display a uniform histological phenotype (30, 60). Whether BCR hypermutation and selection take place in different locales in GCs remains to be rigorously addressed. However, in the mouse, it seems as if BCR selective processes can act on a rather phenotypically homogeneous population of actively proliferating GC B cells.

**Ectopic GC-like structures**

Perhaps the strongest blow to the concept of a textbook GC response has been the discovery of GC-like structures outside of B cell follicles and even secondary lymphoid organs. Analysis of B cell locale and activity at sites of inflammation in humans with autoimmune diseases provided the first indication that GC-like structures could form in ectopic sites (61). In general, these structures bear many, but not all of the characteristics of follicular GCs. Studies of ectopic GCs present in inflamed synovia of arthritis patients by Berek and colleague (62) have shown that BCR hypermutation and selection, as well as clonal expansion and AFC differentiation, can take place in these microenvironments. If a peripheral tolerance checkpoint does not operate in ectopic GCs, perhaps due to their unusual locale preventing the recruitment of necessary cellular components, these structures may promote (62), rather than prevent, the proliferation and differentiation of autoreactive B cell clones created by hypermutation.

**The evolutionary perspective**

The ability to form GCs appears to be restricted to warm-blooded animals, despite the fact that all vertebrates examined are capable of V gene hypermutation (66). In addition, T cell-B cell interactions leading to augmented AFC responses and Ab class switching seem to evolutionarily predate GCs and the development of secondary lymphoid organs with segregated substructure (67, 68). Moreover, the capacity of developing B cells to coalesce, creating focal regions of clonal expansion and BCR diversification outside of secondary lymphoid tissue, is an evolutionarily ancient property: such processes are used by a variety of vertebrates to generate primary Ab repertoires in gut-associated lymphoid tissue (69). Indeed, many of the molecular and cellular mechanisms characteristic of the GC response seem to

![Diagram of GC response](http://www.jimmunol.org/DownloadedFrom)

**FIGURE 2.** Stepwise progression of a textbook GC response. The development and functioning of a textbook GC in a series of discrete phases is illustrated. The initiation of each step is indicated by a numbered red octagon (see The plasticity of GC responses for details). All other illustrations are as described in Fig. 1. Events between steps one and three are assumed to be driven by soluble Ag, whereas in the terminal stages of the response, events are suggested to be driven by Ag in the form of ICs.
have been in place well before the appearance of GCs in evolution, and these mechanisms appear to have originally evolved to create diverse primary, not secondary BCR repertoires.

From this perspective, modern follicular GCs can be viewed as accessories that may have evolved to increase the resistance to pathogens of the more metabolically active warm-blooded animals. Nahm et al. (68) have argued that acquisition of the ability to maintain high body temperature translated into faster pathogen replication, antigenic variation, and dissemination during infection. The evolutionary answer to this fitness challenge may have been the ability to gather pathogen-activated B cells into a nurturing microenvironment where clonal expansion and AFC development were accelerated due to juxtaposition of responding B and accessory cells, and where the generation of more effective BCRs through hypermutation and selection took place simultaneously with pathogen activity. Subsequently, the need to maintain self tolerance in the face of such Ag receptor instability may have resulted in the evolution of a GC peripheral tolerance checkpoint.

Apparently, multiple, perhaps redundant, pathways are involved in the formation and maintenance of focal regions of B cell proliferation and differentiation in higher vertebrates. Some of these could have arisen rather early in evolution, and been maintained in higher organisms due to their utility in certain contexts. Others may have been modified over evolutionary time to better suit the character of the particular host and its pathogens. Whatever the case, the term germinal center has now been applied to a diverse assortment of such processes observed both inside and outside of B cell follicles.

The plasticity of GC responses: stepwise development or distinct but related processes?

Within the framework of textbook models, this variety would best be explained by assuming that the development of GCs is a multistep process that can terminate at any one of several stages, depending on variables like dose, type and persistence of Ag, level of T cell help, associated inflammation, and stromal environment (Fig. 2). The earliest stages would involve the aggregation and initial proliferation of activated B cells (phase 1). B cells stimulated by either TD or certain TI Ags could reach this point. Next, development of the secondary FDC reticulum and the induction of BCR hypermutation and phenotypic selection processes would occur (phase 2). Only GCs formed by B cells activated by Ag and at least some form of T cell help would be capable of proceeding to this stage. Subsequent steps would result in the recruitment of T cells and the segregation of these T cells and FDCs into two zones (phase 3). Some of these events might fail to occur in ectopic GC-like structures due to deficiencies in the nonlymphoid microenvironment. Finally, selected B cells would either re-enter the proliferating and hypermutating pool of cells, or interact with T cells and be induced to commit to AFC or memory lineages (phase 4). Only during robust and sustained αβ TD B cell immune responses in follicles (e.g., high-dose protein Ag in inflammation-inducing adjuvant
or rapidly replicating pathogens) would these latter stages occur efficiently.

Alternatively, the concept that GC responses follow a textbook linear pathway could be dismissed. In this light, the form and function of this microenvironment could be viewed as far more malleable than previously imagined. For example, the utility of induction of GCs by certain TI Ags might be to promote expansion of B cell clones expressing V regions selected over evolutionary time to recognize common multivalent structures on the surface of such pathogens, resulting in a more vigorous AFC response (Fig. 3A). Because hypermutation does not take place efficiently in TI GCs (45, 47), the specificity of the selected V regions would not be altered, and such a process would not pose a threat to self tolerance. The presence of T cells and perhaps secondary FDCs would not be required for TI GCs to perform this function. Particular subsets of mature B cells might preferentially nucleate TI GCs, contributing to their unique characteristics (70). B cell receipt of inflammatory stimuli associated with pathogen infection might be required to make this response efficient, perhaps explaining the sporadic nature of TI GC responses induced by purified TI Ags in T cell-deficient mice (45).

GCs induced throughout the latter stages of TD responses (i.e., after the Ag/pathogen is cleared) or during periods of subclinical infection could facilitate the stringent positive selection of high affinity, and negative selection of autoreactive memory B cells from a large pool created during the acute phase of the response (Fig. 3B). This might take place largely through interaction of these cells with the FDC network (and, perhaps, its retained Ag) and not require (and, in fact, could be inhibited by) high levels of T cell help or the formation of light and dark zones specialized to promote B cell proliferation and hypermutation, and differentiation, respectively. Immunization with low doses of purified protein Ags might mimic the situation that develops after pathogen clearing or low-level infection, and thus predominantly induce this class of GC. Its utility would be in creating small, but highly specific memory B cell clones to single foreign epitopes, allowing the memory compartment to simultaneously possess effective immunity to a wide range of Ags/pathogens using a minimal number of B cells. This would also maximize sensitivity to the pathogen, while minimizing the risk of induction of cross-reactive autoimmunity during subsequent anamnestic AFC responses (18).

Only during acute, high-dose infection by inflammation-inducing pathogens might the formation of GCs akin to those that inspired textbook models (Fig. 1) be required. This could represent a situation in which innate and TI B cell responses had proven ineffective yet had primed the TD response (71). The main function of this type of GC would be to generate numerous class-switched AFCs that would clear the infection due to their production of moderately specific and relatively long-lived serum Ab. Such GCs would also give rise to a sizable population of moderately specific memory cells, which could migrate to other tissue sites and produce rapid local AFC responses as needed to neutralize the pathogen body-wide during periods of reinfection. The generation of light and dark zones would facilitate the rapid production of these two effector populations, by spatially segregating clonal proliferation, hypermutation and perhaps selection, and accessory cell interaction and induction of differentiation.

Concluding remarks

Significant hurdles remain to deciphering the many remaining mysteries surrounding the development and function of GCs. Current textbook models appear inadequate to explain the robust and variable nature of the GC reaction. This inherent plasticity should serve as a sobering influence to investigators in the field, because interventions that target factors involved in the GC response may usually result in altered progression, or transformation of the classes of this response, rather than its elimination. Future advances will require new tools and techniques that allow studies of the GC response to transcend the descriptive and focus on the mechanistic. These needs are particularly pressing in the mouse system, because the development of reagents, and purification and culture conditions needed to characterize the cellular and molecular players that participate in different stages or classes of the GC response has not kept pace with the ability to alter this process through reversed genetics approaches.

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