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Germinal center selection and the development of memory B and plasma cells

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Summary: A hallmark of adaptive immune responses is the generation of long-lived protection after primary exposure to a pathogen. In humoral responses, this protection stems from a combination of sustained antibody titers and long-lived memory B cells (MBCs), with the former deriving from long-lived plasma cells (PCs). Both types of cell are thought to primarily derive from the germinal center (GC), a unique structure that forms during the immune response to many types of antigenic stimuli. GCs are seeded by antigen-specific B and T cells that were previously activated in the early stages of the response. The GC does not directly or immediately generate effector function; rather, it is a site of intense B-cell proliferation and cell death. GC B cells undergo both somatic hypermutation and isotype switch, and a Darwinian process very efficiently selects B cells with higher fitness for survival and expansion. GC B cells adopt a unique activation and transcriptional state, and the cells become poised to differentiate to either MBCs or PCs. Despite this general understanding of the events in the GC, the mechanisms that control both affinity selection as well as differentiation have not been well worked out. In this review, we address what is known about what determines whether GC B cells become MBCs or PCs. This is discussed in the broader context of the origins of both cell types, whether from the GC or potentially other sources. We present a model encompassing recent data from several laboratories including our own that suggests that the GC undergoes a temporal switch that alters the nature of its output from MBCs to PC as the response progresses. We will discuss B-cell receptor signaling in the GC as it relates to potential mechanisms for affinity-based selection during the reaction.

Keywords: *B cells, cell surface molecules, cell activation, cell differentiation, memory, signal transduction*

Introduction

B-cell immune responses, though demonstrating flexibility based on the context of the immunogen and the host, have a stereotypical set of phases (1). Shortly after BCR engagement, partially activated B cells in murine spleen migrate to the T zone–B zone border (2, 3). During the ensuing few days, B cells proliferate there and, in the case of T-dependent antigens, undergo productive interactions with T cells (1, 4, 5). Some of these cells migrate to the T zone-red pulp border, marginal sinus bridging channels, and into the red pulp (6). They continue to proliferate and differentiate there, creating

clusters of proliferating B cell and plasma blasts, some of which undergo isotype switching but are generally of low affinity (7, 8). Individually, these cells are short-lived, though depending upon the stimulus, this extrafollicular (EF) response can persist for a few days up to several weeks, or in the case of persistent self-antigens, the lifetime of an animal (9, 10).

Concurrently, some T cells and B cells continue to interact at the T-B border of the spleen or the interfollicular region of the lymph node (LN), where cells of both types begin to express the transcriptional repressor Bcl-6 (B-cell lymphoma-6) (4, 11). Some of these cells, evidently by altering expression of G-protein-coupled receptors for small molecule chemoattractants and chemokines, migrate into the follicle where they continue to proliferate, forming a nascent germinal center (GC).

GCs are specialized microenvironments within secondary lymphoid tissues in which B cells undergo extensive rounds of proliferation, somatic hypermutation, and antigen-affinity driven selection (1, 12, 13). B cells participating in this reaction that gain affinity for the cognate antigen preferentially expand compared with less avid siblings. Eventually the GC is oligoclonally populated with the progeny of more fit cells (14). During the reaction, via mechanisms that remain unclear, some cells undergo differentiation to either plasma cells (PCs) or memory B cells (MBCs), each of which is long-lived and can serve to protect the host against re-exposure or to help clear persistent primary infections.

Given the dynamism and plasticity of the GC, its intricate microanatomy, as well as the multiple potential fates of GC B cells, it is no surprise that despite much recent progress there are still many unsolved mysteries. In this review, we focus on three related issues that remain controversial. First, though dogma holds that both memory and long-lived PCs (LLPCs) derive exclusively from the GC, we reexamine the experimental support for this and the biological relevance of possible exceptions to this notion. Second, what are the mechanisms that drive selection of higher affinity cells in the GC? And finally, how is the differentiation of GC B cells into either memory or LLPCs controlled?

What are the sources of ~~memory cells~~ and PCs?

It has become well-accepted that the GC is the source of both MBCs and LLPC. How do we actually know this? In fact, it is almost certain that the GC is a source of such cells and the real question may be is it the only source? To address this question first requires a definition of a MBC.

Although over the years MBCs have been characterized as isotype switched or mutated cells, or as expressing certain markers such as CD27 in ~~the~~ human, a more general definition is preferred to avoid biasing results towards one or another type of cells. We define a 'memory B cell' as a member of a clone that has responded to antigen by proliferation and remains in the animal in a resting state and at expanded frequency long after the initial stimulus. Their survival is independent of persisting T-cell help and continuous contact to cognate antigen (15–17). MBCs have a lower threshold for antigenic stimulation and can enter cell cycle more rapidly compared with naive B cells (18–20). MBCs differ from their naive precursors also in their dependence on B-lymphocyte stimulator (BLyS) (also called BAFF) as a survival factor (21) and in their reactivation requirements (19, 22–24).

MBCs can derive from the GC

The idea that ~~memory cells~~ derived from the GC was first demonstrated by ~~Thorbecke~~ et al (25), who used peanut agglutinin (PNA) to purify GC B cells by 'panning' and then demonstrated that most of the secondary response in recipient animals derived from the PNA^{pos} cells. However, even in this experiment, the PNA^{neg} cells were a source of some degree of memory response, as carefully noted by the authors. It seems likely that this experiment has never been repeated with more sophisticated cell purification technology. The remaining support for the linkage of the GC with memory cells comes from experiments in which GCs are blocked or inhibited with antibodies (e.g. anti-CD40L) or genetically, with a correlative loss of MBCs (26). Limitations in most of these studies include that the blockage was not specific for GCs; for example, CD40L is required for early T-B interactions prior to the GC as well as optimal T-cell priming (27, 28). Surgical removal of GC B cells (or precise lineage marking, see below), a prerequisite for absolute proof of this idea, has not been achieved. The second type of evidence is even more correlative: that both GC B cells and ~~memory cells~~ tend to have mutations and tend to have switched isotypes (29). Again, this supports but does not prove the linkage, and in particular does not exclude an extra-GC origin for IgM MBCs that have been long known to exist (30–33) as well as un-mutated MBCs, for which there is also considerable evidence (34, 35).

Evidence for extra-GC memory

It may come as a surprise to most modern readers that as early as the 1970s and 1980s, multiple groups provided direct evidence that T-independent type 2 (TI-2) antigens, presumably

in the absence of GCs, could promote development of MBC (36, 37). They also demonstrated that lack of a secondary response by these B cells was mediated by persistent antigen-specific immunoglobulin (Ig), as responses were revealed upon transfer of the B cells to antibody-negative environments (38). These data were more recently confirmed by Obukhanych and Nussenzweig (39), who also measured the half-life of these cells, which was not different from that of naive B cells.

Adding to this story are data from Alugupalli et al. (40) that [1] msii stimulates B1b cells to generate protective TI memory. Similarly, mice with B cells lacking Bcl6 do not make GCs but do make a form of unmutated, long-lasting memory in response to hapten immunization (41). Hence, it does appear that when GCs are bypassed, B-cell memory can form (41, 42). However, in many respects, this memory is of a different quality than that formed in a GC reaction. In general, TI memory populations harbor fewer isotype switched cells, which have less somatic mutation and affinity maturation. Importantly, these cells do not live indefinitely, as it seems at least many GC-derived MBCs do (43, 44). Further, there may be differences in cell surface phenotype (39), though this bears further investigation.

TI responses seem to represent a relatively clear instance in which GC-independent memory can form. One caveat is that under strong stimulation and with a high frequency of antigen-specific cells, there can be transient TI GCs (45); the biological significance of these GCs is unclear. Nonetheless, the above results raise the question of whether GC-independent memory can form even in a TD response. MBCs have been observed at early time points (days 7–10), prior to the peak of the GC response, suggesting a possible extra-GC origin (41, 42, 46, 47) (Weisel and Shlomchik, manuscript in preparation). In pioneering work, Inamine et al. (47) inhibited GC formation early in the response using anti-inducible costimulator (ICOS) and then measured the numbers of memory phenotype cells at later time points. Surprisingly, the numbers of such cells were little affected, though the affinity and mutation content of the resultant memory population was reduced. These data suggested that memory could form in the absence of GC B cells, though it was possible that cells formed and expanded to fill the compartment before antibody-mediated inhibition of the GC response was complete. The fact that the number of memory cells remained the same suggests that memory cells of higher affinity and/or that formed later in the response would normally replace cohorts of cells formed earlier. This subject also needs to be explored more fully.

Implications

The above considerations have several implications. First, it would be ideal to have a method to indelibly mark cells that had been in the GC or to mark cells that were activated and not in the GC. Jacob et al (48) were perhaps the first to recognize this need and devised a system based on expression of Cre via the truncated I-E^{d} promoter, which had shown specificity for the GC. This system was revealing in many respects, but did experience some lack of specificity of cell marking, based on immunohistology, as well as some inefficiency. Hence, it was not suitable for making hard conclusions as to the GC or extra-GC origin of cells at later time points, though in general the data of Jacob's group provided good evidence that GC B cells are precursors of MBCs. A cell marking experiment was also undertaken by Dogan et al., using an AID-Cre mouse (31). Unfortunately, as class-switch and hence AID expression occurs in many contexts outside the GC, such as in early EF reactions and at the T-B border (2, 49–51), this method—while surely marking many cells in the GC—is not specific for the GC. Thus, though the concept is a good one, better systems are yet needed for marking as well as inducibly deleting genes in GC B cells.

Second, these considerations suggest that within a single response there could be multiple sources of MBCs and that these could differ in qualities. Data from Takahashi et al. (34) revealed that the IgG1^{+} memory compartment of mice immunized with NP-CG in alum contained substantial numbers of unmutated B cells, an observation confirmed by several other groups (31, 32, 54, Weisel and Shlomchik, manuscript in preparation). These cells could have emigrated from early GCs, whose B cells have few or no mutations (52), or derived [2] from an extra-GC source. It is also possible that extra-GC-derived memory cells are mostly IgM and less frequently IgG.

IgM and IgG memory cells have long been recognized in humans and mice. In mice, depending upon the immunization and system, IgM memory cells may even predominate over IgG, in keeping with the large fraction of GC B cells still expressing IgM even late in the response (53). Emerging data suggest that IgM memory cells are more prone to regenerate GCs, while IgG memory cells tend to generate AFC upon restimulation. These data led to the proposal that IgM and IgG mark distinct subsets of memory cells (31). Our group has defined subsets of memory cells based on phenotypic markers independent of isotype, including CD21/23, CD73, CD80 and PD-L2 (CD283) (35, 54). Although the functional significance of these subsets of memory cells remains unclear, these subsets do differ in mutational content as well as dependence on

BLyS for survival. The relationship between subsets defined by such surface markers versus by Ig isotype has also yet to be resolved.

Given that subsets of MBCs differ in numbers of mutations and requirements for survival factors, it could be that the memory compartment evolves in terms of function and affinity with time after the initial exposure to antigen. Supporting this idea, we found that the fraction of B cells with mutations in the mutated $CD80^+$ subset increased over time, suggesting selective survival (54). Notably, it seems that the number of MBCs overall declines from a peak at 8 weeks post-immunization with NP-CGG to about 1/3 this number at 16 weeks (43). Thus, at a minimum, 2/3 of 'early' MBCs are not destined to be very long-lived. Intriguingly, Pape et al. (32) recently suggested that IgG but not IgM memory cells are relatively short-lived, a finding that seems at odds with some other data including that on recall IgG responses. Further investigation of this area is clearly needed, but it can already be concluded that not all MBCs are mutated or isotype switched nor are all mutated cells long-lived memory cells.

These studies in mice suggest some caution in the interpretation of memory studies in humans and the conventional understanding of phenotypic designations for memory cells. Although subsets of MBCs exist in humans (55), including cells that lack CD27 (56), most workers simply refer to $CD27^+$ cells in human as memory cells. It seems more likely that this protein marks a cell that has been activated, without respect to its actual longevity. Since more than 2/3 of murine memory phenotype cells do not last 8 weeks, it could be that many or even most $CD27^+$ cells in a person at any given time are relatively recently generated memory cells and not long-lived. This view could explain why human MBCs appear more 'activated' by phenotype of gene expression analysis compared with murine B cells induced by a defined antigen (57–61). In humans, exposed constantly to a more stimulatory environment than laboratory mice, there is a higher fraction of such cells (i.e. as many as 20% of human peripheral blood B cells are $CD27^+$) (62, 63). This would also be consistent with a short half-life for most $CD27^+$ cells, a notion that is supported by *in vivo* heavy water labeling studies (55, 64).

Origins of LLPCs

The notion that LLPCs derive from the GC is well established. Antibody titers from GC-independent responses wane relatively quickly, while LLPCs may last a lifetime (15, 65). Blocking GCs with anti-CD40L results in a dramatic loss of LLPCs and standing antibody titers (27, 66, 67). Care is needed in interpreting phenotypes of animals that lack certain molecules, such as CD40 or ICOS/ICOSL, and in which GCs and LLPCs do not form [these mutant mice are summarized in Good-Jacobson and Shlomchik (26)]; such mutations block many steps in the B-cell and T-cell immune response and cannot be thought of as specific for the GC. However, a number of mutations—many recently characterized—lead to defects in GC maturation or progression and are also associated with loss of LLPCs. These mutations include defects in CR1/2, IL21R on B cells, PD-1 and PD-L1/2 (68–70), and CD80 (Good et al., ~~unpublished data~~). As the early stages of the immune response are all relatively normal in these mutants but LLPCs are still lacking, they more convincingly link the normal GC reaction with LLPC generation. Again, gene marking studies or GC-specific inducible deletion would more firmly establish the GC as the exclusive site for LLPC development.

While GCs are a major source for LLPCs, controversy has arisen recently over whether EF non-GC responses can under some circumstances generate LLPCs. Early studies using model TI antigens, such as NP-Ficoll, had not found evidence for substantial LLPC generation (6, 39, 71). The protective efficacy of some carbohydrate-based vaccines, which presumably elicit only TI response without GCs, has suggested that they may elicit LLPCs. Taillardet et al (72) have investigated this further in animal models, reaching the conclusion that LLPCs are indeed generated independent of the GC in response to a *Streptococcus pneumoniae* capsular polysaccharide vaccine. CpG DNA was a critical and potent adjuvant for this effect; though not directly demonstrated, such an immunization would be expected to generate a very vigorous EF plasmablast (PB) response—in spleen (73), with some AFCs migrating to BM—and/or possibly even directly in BM. Although the response was indeed detectable for many months, it remains possible that this was due to persistent antigen stimulating new rounds of PB development, much as persistent auto-antigens lead to lifelong generation of short-lived PBs in lupus-prone mice. The turnover of AFCs was not directly examined in these studies, and the authors did conclude that the amount of antibody secreted per AFC generated by the TI antigen was much less than that elicited by a TD form (72); this would be consistent with PBs being the AFCs in the former case and LLPC being the AFCs in the latter.

Short-lived AFC generation

Short-lived AFCs definitely derive from EF responses and can be visualized forming and dividing there as PBs (9, 71, 74).

They can clearly form in TI responses but can be augmented in the presence of T cells, which interact with B-cell blasts and PBs at the EF site. Some (2) but not all (75) TD responses to inert antigens have an EF PB phase that precedes the GC reaction in onset. Although acute EF PBs generally have little mutation in their V regions, they readily undergo class switch (2, 49, 51, 76, 77), which can occur independent of T cells. As expected from the expression of AID that would be required for class switch (49), EF PB responses do undergo V region mutation, provided the response is persistent, as it would be in response to self-antigens or possibly persistent pathogens. It could also be that to initiate somatic mutation, the B-cell stimulus might need to include both BCR and TLR ligation (74, 78); however, this requires further investigation. In any case, properties conventionally associated with GC-derived cells can certainly be observed in EF AFC responses, again emphasizing the need for more direct experiments to establish precursor-product relationships *in vivo*. It is no longer reasonable to infer that a cell must have derived from a GC simply based on the presence of mutations or isotype switch.

Upon maturation, GCs probably begin to produce a wave of GC-derived AFCs that seed the spleen in addition to the BM. These may be of intermediate or variable duration. Several publications (67, 79, 80) including some from our laboratory (~~71~~, Weisel and Shlomchik, manuscript in preparation), have consistently noted a biphasic profile in the numbers of splenic AFCs, which would be consistent with a GC origin of a second wave of AFCs. Nonetheless, AFC numbers monotonically decline in the spleen post-immunization with NP-CGG, whereas they stabilize in the BM, possibly indicating that even GC-derived AFC are not necessarily long-lived, particularly if they remain in the spleen (69, 80).

How does selection in the GC work?

Although some long-lived cells likely derive from outside the GC, iterative mutation with selection of high affinity mutants mainly occurs in the GC in many contexts (81). How does this process actually work? This can be analyzed at several levels: (i) migration and cell interaction patterns that underlie selection; (ii) consequences of selection—reduced proliferation, increased cell death, or differentiation; and (iii) signals that differentially affect the fate of higher affinity cells. Migration patterns have been extensively reviewed elsewhere (82). Suffice it to say that there is not yet agreement or compelling data on a uniform model for how cells migrate in the GC (83–86), and there is no specific location that has been proven for either selection, mutation, or division, despite the commonly repeated notion that proliferation occurs in the DZ and selection in the LZ. We focus below on the other aspects.

Proliferation versus survival in driving selection

A major open question has been whether differential survival, proliferation, or both would drive selection. GC B cells both die and proliferate rapidly, making either process a good subject for selective modulation. Selection on mutants in the GC had been computer-modeled by a number of groups, most of which had assumed that higher affinity cells would proliferate faster rather than die more slowly (87–90). However, the literature has been mixed in terms of whether workers have assumed more fit cells would proliferate more or die less (91). Prior work had shown that overexpression of bcl-2 family members in B cells could lead to exaggerated GC responses with less evidence of selection (92, 93); however, such studies only show the effects of artificial expression not whether protection from death, via bcl-2 family member expression or otherwise, is the actual mechanism of selection.

Our laboratory assessed the fate of low and high affinity GC B cells using transgenic mice with fixed affinities. We examined whether when interclonal competition was markedly reduced in mice expressing a uniformly higher or lower affinity BCR, there were intrinsic differences in GC B-cell proliferation or survival (94). Indeed, low affinity cells had almost three times as many GC B cells undergoing cell death compared with medium affinity cells. Interestingly, the low affinity cells if anything were proliferating faster than the medium affinity cells, possibly because GCs in this strain remained small and did not fill up the available niche (95). Hence, in the absence of robust competition, low affinity GC B cells have an inherent propensity to die. Shih et al. (96) extended these findings to a competitive situation, demonstrating a very strong effect of high affinity cells in suppressing the clonal expansion of low affinity ones. This may involve additional or separate mechanisms that have yet to be elucidated.

How does the B cell 'know' it is higher affinity?

Apart from the consequences for the lower affinity B cell in a GC is the question of how affinity is sensed by the B cell. For selection to operate, the BCR affinity must play a central role, with a different biological outcome for a B cell harboring a higher versus lower affinity. In principle, differences in BCR signaling based on affinity or differences in the ability to elicit T-cell help signals based on ability to capture and present

antigen to T cells could each result in affinity-based selection; these two are not mutually exclusive.

Liu and Maclennan (97) showed that GC B cells could be rescued from death most efficiently by a combination of BCR and CD40 signals, suggesting that both pathways might be important, albeit in an *in vitro* culture. Much emphasis has been placed on differential BCR signaling based on affinity (98, 99); again, these studies have been *in vitro* using naive B cells. B-cell lymphomas have also been shown to respond differentially to BCR signals based on their affinity (100). The effects of affinity on BCR signaling and function could depend not only on the starting cell (GC B cell vs. naive vs. lymphoma cell line) but also on the antigen recognition system used. Some investigations of BCR recognition of antigen have used a highly idealized system involving B cells recognizing antigen on planar membranes (101, 102). Although it is sometimes stated that *in vivo* antigen recognition only or usually occurs on surfaces (103–105), this is actually difficult to prove even if one can set up a system in which it appears that such recognition is taking place *in vitro* (106) or *in vivo* (107). As a counterpoint, several investigators have convincingly demonstrated that soluble antigens rapidly find essentially all antigen-specific B cells in follicles and in the marginal zone (50, 108, 109). Moreover, whether B cells encounter intact pathogens or just fragments or soluble proteins shed from them is not clear, and probably both do occur. Thus, balance is needed in discussion of how B cells in GCs recognize antigen, and while the planar membrane system is elegant and informative, it likely does not reflect a number of *in vivo* settings.

The role of T cells in selection

In addition to direct affinity-based effects on B cells via BCR signaling, T-cell signals have been implicated in GC survival, if not precisely affinity-based selection. As noted, interrupting CD40 signals results in GC decay (67). IL21, another key signal coming from T-follicular helper cells, is also required to sustain GC reactions beyond a certain point (70). Based on the failure to observe B-cell arrest on FDCs and the transient nature of T-B interactions in the GC, Cyster et al (82) speculated that T-cell help might be a limiting factor in B-cell selection. Recent fascinating studies by Schwickert et al. (110) on the pre-GC and early GC response demonstrate that providing B cells with the ability to present more antigen results in increased proliferation and GC seeding. This suggests that at least in part extra T-cell help can compensate for lower affinity. Surprisingly, these studies also showed that competition does not reduce the ability of a cell to acquire and present antigen *in vivo*; thus, how high affinity cells markedly suppress coexisting low affinity cells in the GC remains a mystery. This same group, in separate studies, concluded that in mature GCs at steady state, DZ B cells migrate to the LZ with a half-time of ~2 h (86). They suggested such migration was a requirement for iterative selection. However, they also obtained another intriguing and puzzling result which is not easily reconciled: when they provided strong exogenous T-cell help to ongoing GC B cells, all B cells migrated to the DZ, where they stayed for over 48 h. While this was thought to reflect the response of the GC B cells to T-cell help and was interpreted as evidence that T cells are normally the limiting factor in GC selection, it is unclear why these DZ B cells did not move to the LZ with a half-time of 2 h, as would have occurred in steady state. Hence, this experiment shows that GC B cells can indeed respond in a dramatic way to delivered T-cell help, but it does not formally prove that this is how selection occurs in the steady state. For as yet unclear reasons, in analogous experiments, Heiser et al. (111) paradoxically found that upon presenting excess T-cell help to GC B cells, the GC response was inhibited, raising further questions about how T-cell help influences GC B-cell fate. As some T-follicular helper cells have recently been shown to also have a T-regulatory cell phenotype (112–114), the situation could be more complex than previously appreciated. Nonetheless, naive B cells *in vitro* were shown by Batista and Neuberger (100, 115) to differentially present antigen to T cells over an almost 10,000-fold range of kinetic off-rates, providing a basis for T cell-mediated selection. Thus, while recent work has provided some new insights and systems into selection, there is need for further studies *in vivo* and *in vitro* using GC B cells rather than naive cells.

We have been studying spontaneous and inducible BCR signaling in direct *ex vivo* GC B cells and recently found that such cells are greatly impaired in their engage canonical BCR signaling pathways (A. Khalil, J.C. Cambier, and M.J. Shlomchik, manuscript in preparation). One explanation is that the relative unresponsiveness of the BCR to signals is a means to raise the affinity threshold, thus promoting affinity-based selection. Another is that T cells are in fact the primary selecting force and that BCR signaling is not required. In this context, biochemical studies have shown that at the level of individual BCRs, there is an exclusive fate in promoting downstream canonical signaling versus directing antigen for degradation and presentation (116, 117). Thus, it is possible that GC B cells are specialized for presenting antigen to T cells to the exclusion of classical BCR signal transduction.

How is the differentiation fate of a GC B cell determined?

Apart from multiple rounds of selection that occur while clonal progeny retain a GC B-cell phenotype, differentiation to long-lived memory and AFCs must also occur. Considering the number of long-lived cells generated in a typical response compared with the peak number of GC cells, such events must be relatively rare. Thus, it is no surprise that the mechanisms that control the fate of GC B cells have not yet been elucidated.

Differentiation of GC B cells into either MBCs or LLPCs could be controlled in a number of ways: (i) extrinsic signals from other cells, including cytokines and contact-dependent signals; (ii) affinity-based instructive signals via the BCR; (iii) temporal differences that could reflect the accumulation of signals over multiple divisions or over time; or (iv) stochastic effects that are either random or reflect the details of local signals and positions of GC B cells at critical times ('right place, right time' model). This latter model is particularly difficult to test and would be considered a default explanation in the absence of a more specific one; it will not be further discussed here.

Instructive signals that could determine GC B-cell differentiation

There have been a number of publications suggesting that specific signals to B cells promote one or another fate (51, 118–121). In many cases, the cells subjected to various stimuli were studied *in vitro* and in some cases were naive B cells rather than GC cells (122, 123); as GC B cells differ greatly in gene expression from naïve or even other types of activated B cells (57, 124), the same signals could generate very different outcomes. In one study using human GC B cells, CD40 signals derived only from activated T cells could induce a MBC phenotype in culture (125). Analogous studies in mice support the notion that GC B cells have unique responses to CD40 ligation, including adoption of a memory phenotype (126). Cytokines could also instruct fate. It has been proposed that IL-10 promotes GC B cells to become PCs (127), whereas IL-24 has the opposite effect (128). On the other hand, IL-2, IL-10, and CD40L were observed to drive murine GC B cells to a memory phenotype, a process partially inhibited by IL-4 (129), which is itself a cytokine made by at least some T-follicular helper cells (130).

GCs formed with IL-21R-deficient B cells generate few LLPCs and a somewhat larger number of MBCs. This observation led to the suggestion that IL-21 could control centrocyte differentiation and by extension PC generation, though other explanations were also put forth (70). This idea is concordant with the PC-inducing effects of IL-21 on human naive B cells *in vitro* (131). We have found the PD-1 signaling to T-follicular helpher cells, via B cell-derived PD-L1/2, promotes GC B-cell maintenance and LLPC formation; we proposed this was due to induction of IL-21 secretion by T-follicular helper cells, but the possibility exists that reverse signaling via PD-L1/2 on B cells could play a role (69). Hamel et al. (132)made similar observations in an anti-proteoglycan model of arthritis.

The potential role of affinity in directing GC B-cell fate

Another idea that has gained credence is that affinity of BCR interactions is the mechanism by which PCs are selected. Originally, the Brink group (120) reported that higher affinity correlated with a larger AFC response in the early phase of the B-cell immune response and concluded that affinity was an instructive signal. This was followed by a similar report on the GC to PC transition (121). To their credit, this group persisted in a more detailed study of this phenomenon and eventually reached the conclusion in a third paper that affinity was promoting overall cell expansion rather than directing PC differentiation (118). They found that when B cells were higher affinity, there were more PCs because there was an overall greatly expanded responding population and that this expanded population underwent PC differentiation in a similar fashion as less-expanded lower affinity responders. Thus, at least for the early response, affinity controls expansion but is not an instructive differentiation signal for PCs. It seems very likely that the same caveat holds true for the GC experiments, and thus, the same explanation is likely to hold for the apparent increase in PCs in the higher affinity case. This is a very important point, as it seems to us that the field has not universally recognized this caveat.

A temporal switch in GC output?

Another possible explanation for how MBC versus LLPC generation is controlled in the GC is via a developmental switch in GC output over time. We would propose that the early GC reaction is largely dedicated to the production of MBCs, whereas the late GC reaction preferentially generates LLPCs. This model is depicted in Fig. 1. Although the cellular composition of an established GC (d8–21) seems to be stable in terms of quantitative relationships (133), there is much circumstantial evidence to support this hypothesis. There are a number of mutants in which early failure of the GC correlates with a lack of LLPCs with minimal impact on MBC

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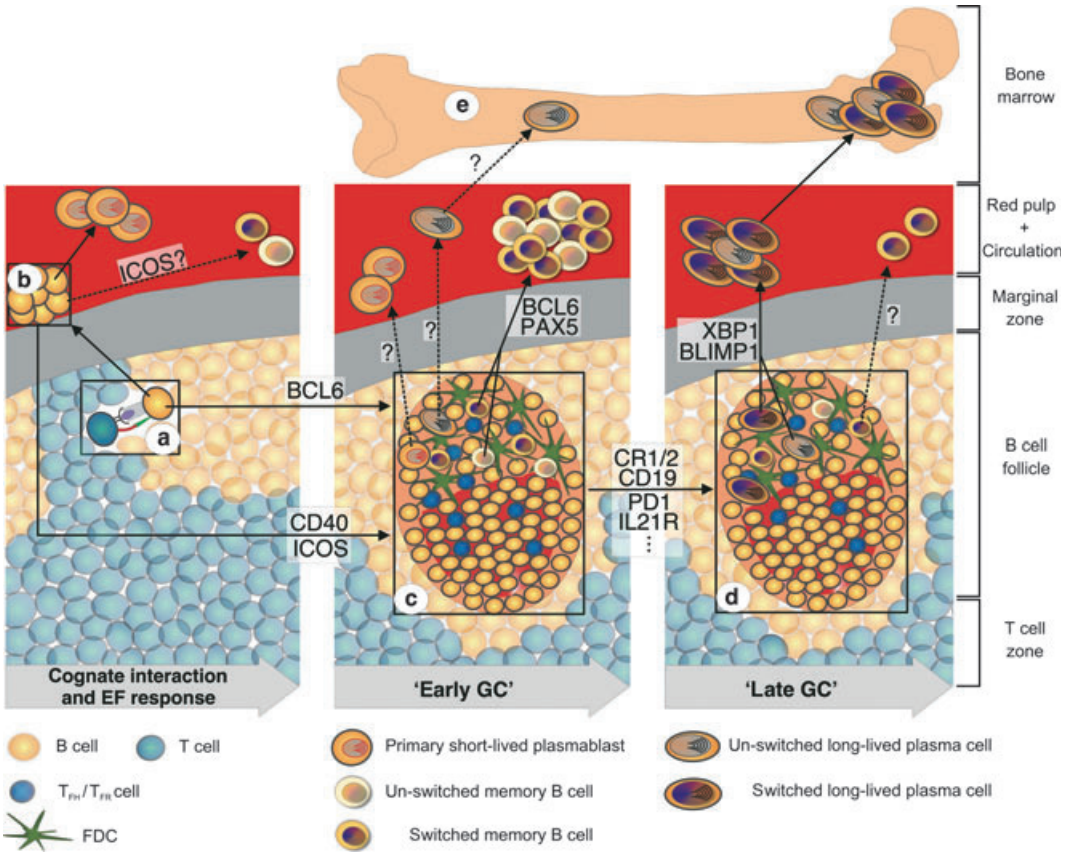

Fig. 1. Stages of a T-cell-dependent immune response. After antigen encounter B cells migrate to the T-B border to interact with cognate T cells (A). Successful B-T interaction leads to an early extrafollicular response (EF) (B), which is characterized by strong proliferation and generation of short-lived plasmablasts at the marginal sinus and in the red pulp. This serves as the source of an initial wave of mainly germline-encoded, unswitched antibodies (left panel). Alternatively, B cells that upregulate Bcl6 re-enter B-cell follicles and establish germinal center (GC) reactions (C). 'Early' germinal center reactions mainly give raise to memory B cells of either switched or un-switched isotype (middle panel). Various proteins, including CR1/2, CD19, PD-1, IL21R, have been identified playing a role in sustaining the GC reaction. With time the output of the GC reaction switches from initially mainly memory B cells to predominantly ~~long-lived plasma cells (PCs)~~ at later time points (D, right panel). Immunoglobulin genes of PCs generated during 'late' GC reaction are almost exclusively somatically mutated and isotype-switched. These ~~long-lived~~ PCs preferentially migrate into the bone marrow (E) where they survive for long periods, secreting large amounts of antibody.

development. These include deficiencies in or blocking of: CD21/35, IL21R, PD-1 signals, ICOS (47), CD80 (Good-Jacobson et al., unpublished data), and CD19 (134, reviewed in 26). Consistent with this hypothesis, MBCs have fewer V region mutations than LLPCs and tend to be of lower affinity (135). Also consistent with this proposal is the early appearance of memory phenotype B cells, whereas LLPCs accumulate later and continues to increase in number until 4–5 weeks or more post-immunization with NP-CGG in alum (67, 69). Perhaps the best existing evidence in favor of this explanation comes from Takahashi et al. (34), who showed that at various time points MBCs harbored fewer V region mutations than did concurrent GC B cells, suggesting that at late time points the GC was not contributing to the MBC compartment and implying that perhaps the GC was instead devoted to LLPC generation. They did not analyze PCs however.

Despite the attractiveness of this hypothesis in explaining a number of disparate observations, it has not been directly tested. To do would require labeling of proliferating GC B cells under differing circumstances (e.g. at given time points or in mice with key mutations) to establish precursor-product relationships. Our results from such experiments strongly support this hypothesis (Weisel and Shlomchik, ~~unpublished data~~).

Even if it turns out that the GC shifts its output over time, this would only be a partial answer to this longstanding problem in B-cell biology. It raises the question of how and why the GC does this. Are certain signals accumulated? Is this change B-cell intrinsic, or does it reflect a different composition or function of T-follicular helper cell in the GC? Nonetheless, if it can be proved that the GC does switch its output upon maturation, this will be an important insight that could

ultimately lead to a detailed mechanistic explanation of this elusive issue.

Concluding remarks

The origin of many MBCs and perhaps all LLPCs is the GC reaction. Selection for higher affinity mutants is quite efficient in the GC, a process that affects the quality of both the MBC and LLPC compartments. Yet, despite great new insights into the migration of cells in GCs as well as the effects of T-cell help on GC B cells *in vivo*, exactly how selection is mediated has not been solved. Similarly, the signals that control differentiation fate towards these cellular outcomes remain unclear. We have proposed the hypothesis that the GC tends to switch its output over time, with the early GC making mainly memory cells and the late GC mainly LLPCs. Whether this is a stochastic or strictly time-dependent event or whether it reflects the time-dependent availability of key instructive signals remains to be determined. On the other hand, where and why MBC are formed outside of the GC is poorly understood. It seems clear that additional immunization contexts should be studied to understand the full breadth of the long-lived progeny of antigen-activated B cells that can be generated. Recent studies have revealed that the resultant MBC and PC compartments are more complex than originally thought. In the case of MBCs, subsets exists that differ in cell surface marked expression, dependence on BLyS for survival, somatic hypermutation content, isotype switch, and gene expression. This heterogeneity could be a consequence of contributions from both GC-dependent and -independent sources; it could also reflect time-dependent differential output from the GC. Most importantly, the functional roles of these subsets of MBCs in protection of the host along with the LLPC compartments remain to be determined. This is an exciting avenue for future research, with implications for pathogen resistance, vaccine design, and even autoimmune disease.

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During the copy-editing of your paper, the following queries arose. Please respond to these by marking up your proofs with the necessary changes/additions. Please write your answers on the query sheet if there is insufficient space on the page proofs. Please write clearly and follow the conventions shown on the attached corrections sheet. If returning the proof by fax do not write too close to the paper's edge. Please remember that illegible mark-ups may delay publication.

Many thanks for your assistance.

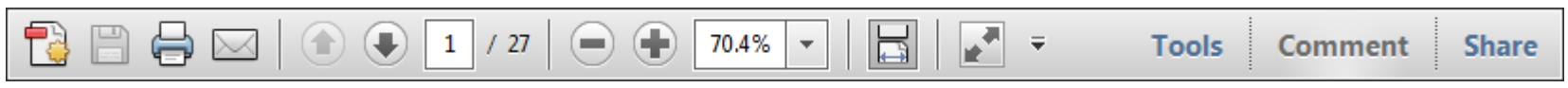
Query reference	Query	Remarks
1	**AUTHOR: Please expand B. hermsii.**	
2	**AUTHOR: To facilitate sequential numbering, reference numbers have been reordered. Please check.**	
3	**AUTHOR: Please note that "manuscript submitted" has been changed to "unpublished data" here and in subsequent occurrences. Please provide all author names with initials for all unpublished data.**	
4	**AUTHOR: References [9.] and [78.] are identical. Hence, reference [78.] is deleted and rest of the references is renumbered. Please check.**	
5	**AUTHOR: References [39.] and [73.] are identical. Hence, reference [73.] is deleted and rest of the references is renumbered. Please check.**	
6	**AUTHOR: References [41.] and [49.] are identical. Hence, reference [49.] is deleted and rest of the references is renumbered. Please check.**	
7	**AUTHOR: References [42.] and [48.] are identical. Hence, reference [48.] is deleted and rest of the references is renumbered. Please check.**	
8	**AUTHOR: References [52.] and [115.] are identical. Hence, reference [115.] is deleted and rest of the references is renumbered. Please check.**	
9	**AUTHOR: Please check the amendments made in the reference [63].**	
10	**AUTHOR: References [69.], [84.] and [117.] are identical. Hence, reference [84.] and [117] are deleted and rest of the references is renumbered. Please check.**	
11	**AUTHOR: References [71.] and [141.] are identical. Hence, reference [141.] is deleted and rest of the references is renumbered. Please check.**	
12	**AUTHOR: References [72.] and [139.] are identical. Hence, reference [139.] is deleted and rest of the references is renumbered. Please check.**	

13	**AUTHOR: References [85.] and [86.] are identical. Hence, reference [86.] is deleted and rest of the references is renumbered. Please check.**	

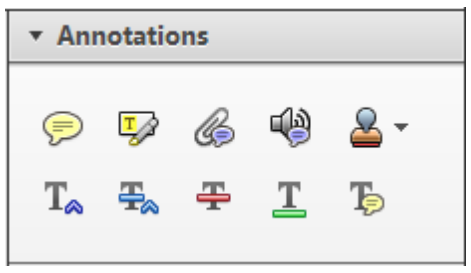
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 8.0 or above). (Note that this document uses screenshots from Adobe Reader X)
The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

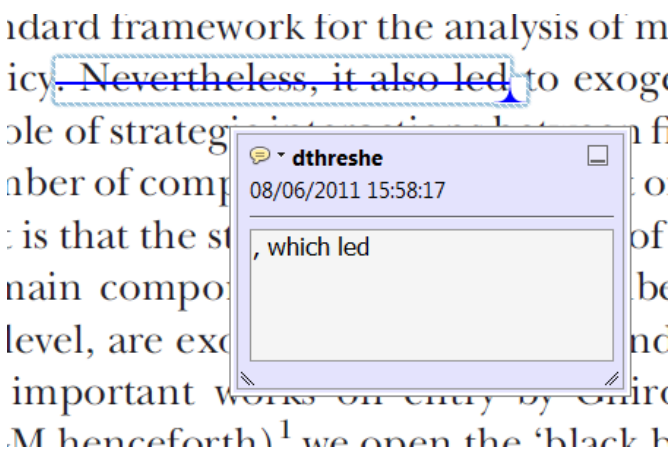


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

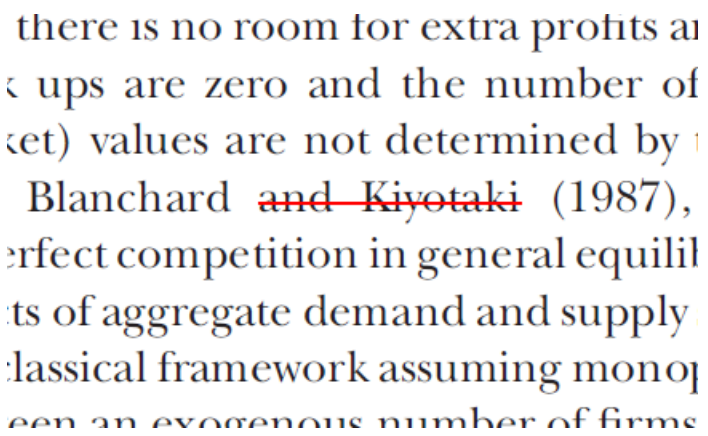


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

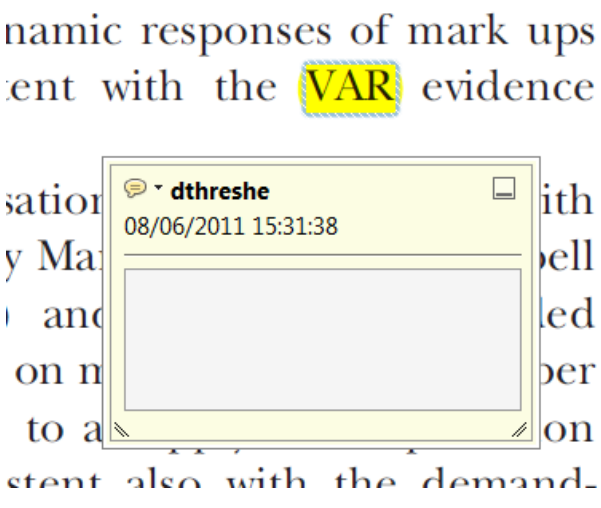


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

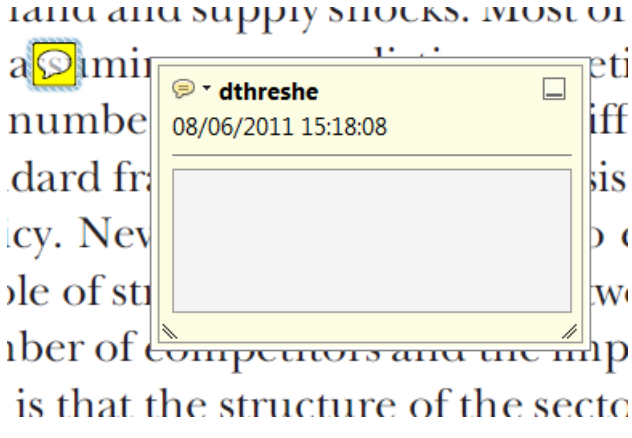


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



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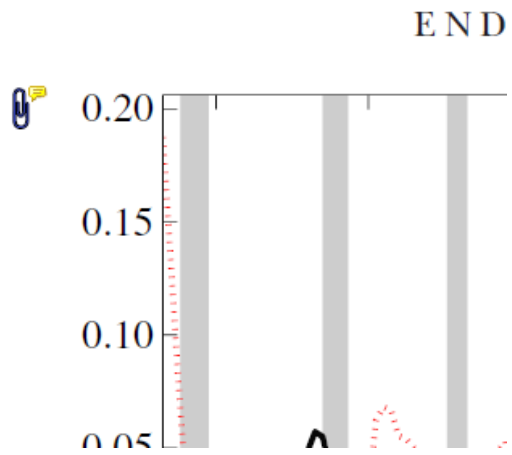
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

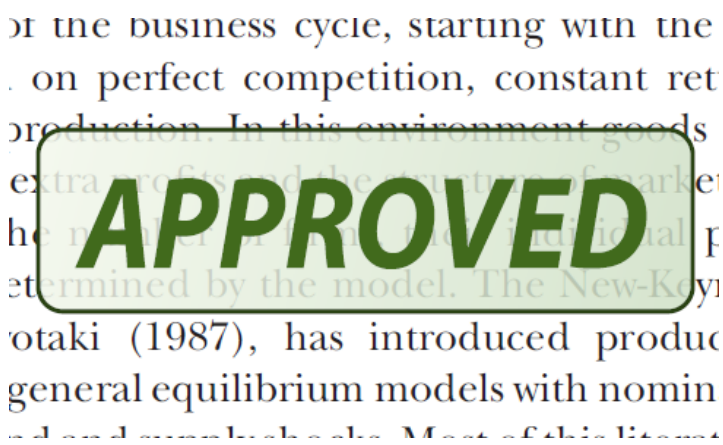


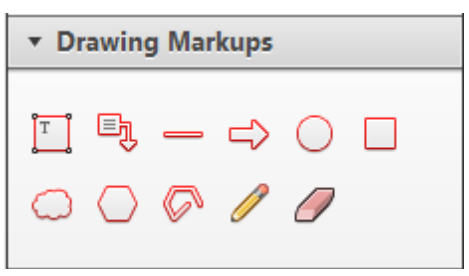
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

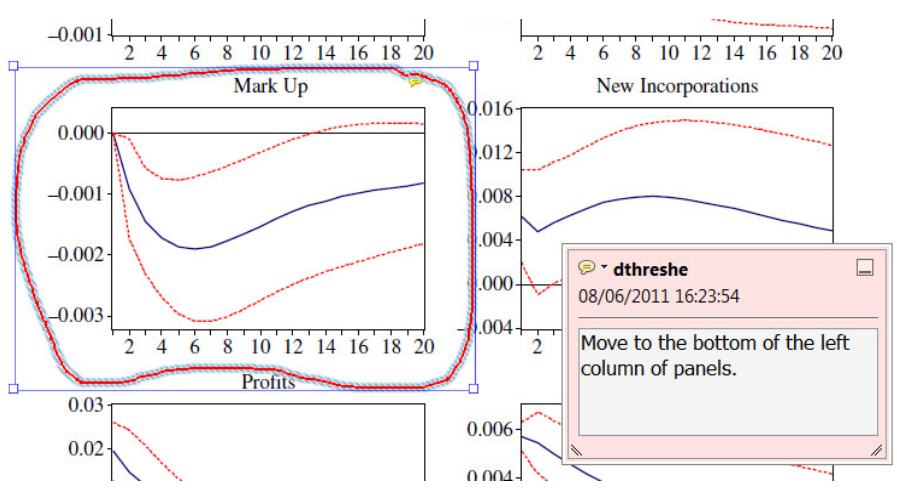
- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).





7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..



How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: