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Affinity-based selection and the germinal center response

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Summary: Interactions between B-cell antigen receptors (BCRs) and their ligands have a complexity and variability that is unparalleled within known biology. Each developing B cell undergoes gene rearrangements to generate a BCR encoded by a unique pair of immunoglobulin (*Ig*) variable region genes, which serves to make the antigen-binding capabilities of primary BCRs incredibly diverse. Further diversification of the BCR repertoire takes place when antigen-activated B cells enter the germinal center (GC) response and undergo somatic hypermutation (SHM) of their *Ig* variable region genes. To develop optimal antibody responses against foreign antigens, the key B-cell survival and differentiation decisions made in the GC are based primarily on the affinity of the BCR (and therefore subsequent antibodies) for foreign antigen. However, the secondary diversification of BCRs by SHM also carries the risk of generating new self-reactive specificities and thus autoantibody production. Herein, we review the role of antigen affinity/avidity in controlling pivotal events both leading up to and during the GC response. The emergence of self-reactivity during the GC response is also examined, with particular focus on the threat posed by cross-reactive GC B cells that bind both self and foreign antigen.

Keywords: *germinal center, B cells, antigen affinity, selection, differentiation, autoimmunity*

Introduction

The B-cell antigen receptor (BCR), comprised of membrane immunoglobulin (Ig) heavy and light chain molecules, is the trigger for both initial and ongoing B-cell activation and the driving force behind humoral immunity. The BCR repertoire of each individual has the capacity to recognize an incredibly diverse range of molecular structures, a feature that allows humoral immune responses to be raised against virtually any foreign pathogen or other antigen that breaches the body's outer defenses. The same Ig molecules that initially define the BCR eventually become the effector molecules of humoral immunity, being secreted as soluble antibodies following the differentiation of antigen-activated B cells into plasma cells. As predicted by Burnet's clonal selection theory (1), this process results in the production of antibodies that specifically bind to structures carrying foreign antigens and selectively target them for elimination.

The fundamental mechanism underlying the diversity and versatility of ligand-binding by BCRs is *Ig* gene V(D)J recombination. This process occurs independently during the development of every B cell and culminates in the generation of clonally defined variable region coding exons in both the heavy and light chain *Ig* genes. The two variable region domains encoded by these rearranged exons combine to form the initial antigen binding site of the BCR, a process that has been estimated to result in a B-cell repertoire of over 10^9 distinct binding specificities (2). As a result of this diversity, the nature of the ligands that can be bound by the BCR is virtually unlimited. This property is unique to the BCR as even the closely related T-cell antigen receptor (TCR), which also carries variable regions encoded by the products of V(D)J recombination, is constrained by the requirement to recognize peptides presented in the context of class I or II major histocompatibility complex (MHC). Thus, Ig molecules have been indentified that bind to virtually every class of biological molecule (proteins, carbohydrates, nucleic acids, and lipids) as well as synthetic chemicals and other molecules that have no natural biological occurrence (3).

In addition to the diversity of structures they are capable of binding to, Ig molecules can also bind to antigen ligands with affinities that span a range of over six orders of magnitude. Biologically relevant interactions of BCRs with antigen have been identified that have affinity constants (K_a) as low as $3–5 \times 10^4/M$ (4, 5) whereas antigen-binding affinities as high as $10^{11}/M$ have also been recorded (6). The wide affinity range of Ig molecules expressed by different B-cell clones can be explained in part by the inherent diversity of the primary BCR repertoire caused by V(D)J recombination. However, it is now well accepted that the secondary diversification of *Ig* variable region genes by somatic hypermutation (SHM) has an enormous impact on shaping the BCR repertoire, particularly in generating the specificities with the highest affinities for antigen.

Both SHM and the selection of those rare B cells that acquire increased affinity for foreign antigen occur in a specialized physiological structure within secondary lymphoid tissues called the germinal center (GC). With the support of antigen-specific T follicular helper (Tfh) cells, GC B cells proliferate extremely rapidly (dividing as many as four times every 24 h) and undergo SHM of their *Ig* variable regions genes with each division. New BCRs carrying amino acid changes introduced by SHM are expressed by GC B cells and surveyed against foreign antigen localized within the GC. B cells with an improved ability to bind foreign antigen preferentially survive and ultimately produce the high-affinity antibodies that sustain long-term immunity. B cells that acquire reactivity with self-antigens are an inevitable but undesirable product of the GC response and may form the basis for an autoantibody response unless their survival or differentiation can be controlled.

The mechanisms by which GC responses select for B cells with high-affinity for foreign antigen but against low affinity and potentially self-reactive B cells have been the subject of intense research over the last 20 years. The dynamic and complex nature of the GC response, coupled with the absence of appropriate *in vitro* models, have conspired to keep many of the secrets of the GC hidden from view. However, recent technological and experimental advances have started to shed new light on how the GC operates and how antigen affinity directs the fate of each GC B cell via their rapidly evolving BCRs. Before examining these new advances, however, it is worth revisiting the evolution of our current understanding of SHM, the GC response, and the significance of antigen affinity.

Evolution of the concepts of SHM and GC function

These days, the concept that *Ig* gene SHM and affinity-based selection of antigen-activated B cells takes place within the GC is immunological dogma. However, this model of humoral immunity only really gained general acceptance around 20 years ago when the link between the GC, SHM, and affinity maturation was firmly established (7, 8). The concept that a SHM-like mechanism was responsible for antibody diversification was proposed during the 1950s (9). At this point of time, the nature of the *Ig* genes remains unknown and the generation of antibody diversity by SHM of one or a few antibody genes was an attractive one. Importantly, however, this process was proposed to diversify the repertoire before antigen activation. Advances in protein chemistry in the 1950s and 1960s elucidated the primary structure of the Ig polypeptide chains. Amino acid sequencing of Ig light chains revealed that only the amino-terminal half of the protein chain varied between individual molecules, leading Sidney Brenner and César Milstein to postulate in 1966 that the SHM is enzymatically directed to the relevant parts of the *Ig* gene (10), quite incredibly predicting the existence of AID nearly 40 years prior to its discovery! The rapid development of molecular biology in the 1970s and 1980s resulted in the characterization of the *Ig* genes and the finding that the primary mechanism underlying variable region heterogeneity was apparently not SHM, but V(D)J recombination (11). It soon became apparent, however, that SHM did in fact take place and complemented V(D)J recombination in contributing to Ig variable region diversification (12). Significantly, SHM was found to

operate independently of V(D)J recombination, acting not during early B-cell development, but following encounter with foreign antigen and recruitment into an immune response (13).

GCs were first identified in the late 19th century and named according to the original concept that they were responsible for the production of new lymphocytes. Experiments performed in the 1920s and 1930s established that GCs formed in secondary lymphoid tissues in 'reaction' to the entry of foreign antigens and were in fact part of the immune response (14). During the 1960s and 1970s some of the key features of the GC were identified including the complement-dependent localization of antigen on the follicular dendritic cells (FDCs) present within GCs, the dependence of GCs on T cells, and predominance of B cells within GCs (14). The recognition during the 1980s that SHM occurred only in antigen-activated B cells strongly implicated the GC as the site of *de novo* SHM (13, 15), a fact that was subsequently confirmed in 1991 through molecular analysis of micro-dissected GC B cells (7). The contemporaneous demonstration that affinity-based selection also takes place in the GC (8) set in place the basic model of GC function that is still generally accepted today.

Antigen affinity versus avidity in the control of B-cell responses

Before discussing the role of antigen affinity in the GC response, it is worth reflecting on what is meant by antigen affinity both in its strictest sense and in its common usage. The affinity of an Ig molecule (BCR or secreted antibody) for a particular antigen is defined in terms of the interaction of a single pair of Ig heavy and light chain variable region domains (e.g. monovalent Fab fragment) with its binding site (epitope) on the antigen. The affinity of a monomeric binding interaction can be quantified by its affinity constant K_a (units/M), which in turn is a function of the 'on' and 'off' rates of the binding interaction. In practice, all BCR and antibody molecules carry two antigen binding sites, meaning that antigens that either naturally carry multiple epitopes or are arranged in a multimeric or repeating structure can be engaged by both of the binding sites of an Ig molecule. In the case of BCR molecules, the situation is even more complex, with multivalent antigens having the potential to recruit multiple divalent receptors into microclusters on the B-cell membrane (16) and thus establish higher order binding interactions with active BCR signaling complexes. Because multivalent interactions increase the strength of binding between Ig and antigen, they cannot be described in terms of a basic affinity measurement. Instead, multivalent interactions between Ig and antigen are most accurately described in terms of the avidity of binding.

Although avidity is clearly a function of the basic affinity of the interaction, it is influenced by a number of other factors including the density and spacing of epitopes on the antigen and the flexibility and density of the Ig molecules involved. Avidity is typically not quantified in absolute terms, rather in relative terms or semi-quantitatively. Thus, the interaction of an Ig molecule with multivalent antigen will be of higher avidity than its interaction with a paucivalent one, the relative difference potentially expressed in terms of the amount of input Ig or antigen required to achieve a certain level of binding or responsiveness.

It is not uncommon for the interactions between BCR/antibody and antigen to be described in terms of binding affinity, be it in terms of the actual K_a or as a 'high' or 'low' affinity interaction. In most physiological scenarios, however, it is the avidity of the interaction that is likely to be more significant. A good example of this is the role of BCR:antigen affinity/avidity in the recruitment of naive B cells into an immune response. The complexity of the primary repertoire is such that only a very small fraction [typically <0.01% (17)] is recruited into the response against any particular foreign epitope. Although this naturally includes the cells carrying BCRs with the highest affinity for the antigen, it is apparent that the overall avidity of the interaction is the primary determinant of recruitment. For example, immunization of mice with protein carriers [e.g. keyhole limpet hemocyanin (KLH), and chicken γ-globulin (CGG)] that have been densely modified with chemical haptens [e.g. (4-hydroxy-3-nitrophenyl)acetyl (NP)] activates and recruits B cells with affinities (K_as) for the hapten epitope as low as $3–5 \times 10^4/M$ (4, 5). In contrast, a modified version of the protein antigen hen egg lysozyme (HEL) that binds to a transgenic BCR with approximately 10-fold greater affinity ($2.3 \times 10^5/M$) fails to activate or recruit B cells expressing this BCR when the antigen is arrayed on the surface of a sheep red blood cell (SRBC) (T. C., R. B., unpublished data). Consistent with these observations, thermodynamic measurements suggest that a $K_a > 10^6/M$ is require to obtain detectable triggering of a BCR by a paucivalent antigen, but this affinity threshold is reduced significantly by cross-linking the antigen and thus increasing the avidity of the interaction (18).

The concept that epitope density as well as intrinsic affinity plays a major role in determining the strength of BCR:antigen binding is an important one in considering how these interactions guide B-cell responses *in vivo*. Avidity, which has been

referred to as 'effective affinity', is in nearly all scenarios likely to be the key determinant of how a B cell responds. However, in any given scenario such as GC response to a specific antigen, responding B cells are not likely to experience differences in epitope density. Thus, it is fair to postulate that differential responsiveness to antigen between B cells in the same environment is determined primarily by their intrinsic affinity. For the remainder of the article, avidity is discussed primarily when comparing independent responses to different antigens. However, whereas acknowledging avidity to be the more absolute determinant of B-cell responsiveness, relative affinity for antigen is discussed as the primary determinant of differential responsiveness of B cells responding simultaneously to the same antigen.

An *in vivo* system for analyzing affinity/avidity-based B-cell selection

Assessing the role of antigen affinity/avidity in the control of B-cell responses carries two major challenges. First, the naive B cells that respond to antigen typically interact with it over a range of affinities that are difficult to measure. Even when the response is at its peak, responding B cells typically comprise only a small fraction (1–5%) of total lymphocytes, making it difficult to distinguish B cells that are specific for particular epitopes and almost impossible to assess their affinities. Second, the dynamic nature of the response, particularly once SHM commences in the GC, means that it is extremely hard to track changes in BCR specificity as the response progresses.

One approach employed to circumvent these problems has been to develop systems whereby the responses of B cells expressing a transgenically defined BCR are tracked *in vivo*. To this end, our laboratory developed the SW_{HEL} strain of mice, so-called because these mice produce anti-HEL B cells with the ability to undergo Ig class switching as well as SHM in response to antigen (19, 20). Responses to T-dependent antigen, including GC responses, are elicited by transferring small numbers of SW_{HEL} B cells into CD45 congenic recipient mice and challenging them with HEL conjugated to SRBCs. If the reader is interested, further details on the experimental procedures and analyses used to track the *in vivo* responses of SW_{HEL} B cells can be found elsewhere (21, 22).

The SW_{HEL} model provides the opportunity to track the response of B cells that bind antigen with a homogeneous and defined affinity. However, the BCR of B cells produced in SW_{HEL} mice [specificity of HyHEL10 mAb (23)] binds to HEL with an extremely high affinity ($2 \times 10^{10}/M$) that would normally only be seen after extensive SHM and affinity maturation. To provide a system that more accurately reproduces the antigen-binding affinities one would expect to find in the primary B-cell repertoire, we generated a panel of recombinant HEL proteins carrying one or more point mutations that altered the amino acid side chains involved in interactions with HyHEL10 (24). Mutation of one, two, or three HyHEL10 contact residues produced the HEL^{1X}, HEL^{2X}, and HEL^{3X} proteins, respectively. These recombinant HEL proteins bind HyHEL10 with progressively decreasing affinity and, when conjugated to SRBCs, elicit strong GC responses from SW_{HEL} B cells (24, 25). As well as providing a more physiological range of binding affinities to study primary B-cell responses, this panel of antigens can be used in conjunction with the SW_{HEL} model to directly assess the effects of primary BCR affinity on *in vivo* B-cell responses. Moreover, knowledge of the initial binding affinity and variable region coding sequences of the BCR, combined with the ability to track both SHM events and changes in antigen affinity over the course of a GC response (25), means that the SW_{HEL} model can also provide significant insights into the regulation of the GC response by antigen affinity.

Affinity and avidity in early B-cell differentiation and GC recruitment

The recruitment of a naive B cell into a T-dependent response requires a BCR:antigen interaction of sufficient avidity. This involves not only activation BCR-dependent signaling pathways but also BCR-mediated internalization and processing of foreign antigen into peptide fragments for presentation on the B-cell surface in conjunction with class II MHC molecules. Proliferation of antigen-activated B cells requires signals provided to them by antigen-specific $CD4^+$ T helper (Th) cells that recognize cell surface class II MHC-peptide complexes. T-cell help is primarily delivered via CD40 ligand as well as mitogenic and immunomodulatory cytokines, such as interleukin-4 (IL-4), IL-5, IL-13, and IL-21. The delivery of both 'signal 1' (BCR stimulation) and 'signal 2' (cognate T-cell help) to B cells by virtue of the interaction of foreign antigen with the BCR is critical not only for initiating B-cell responses but also for sustaining these B cells as they proliferate, differentiate, and eventually participate in the GC reaction (26, 27).

The initial phase of TD B-cell responses is marked by the movement of responding B cells through secondary lymphoid tissues, guided by the combined activities of the chemotactic receptors CCR7, CXCR5, and EBI2 (28–30). Around 3–4 days after their initial encounter with antigen, proliferating B cell blasts undergo synchronous differentiation either into either GC B cells or extrafollicular plasmablasts (31–33). Early

memory B cells are also generated at this point (32–35), but cease proliferating and take no further part in the response.

Although B cells entering the GC response are destined to undergo SHM and affinity-based selection, those that undergo plasmablast differentiation typically are not subject to SHM and contribute directly to the early antibody response based on their original antigen specificity. SHM of cells that enter the extrafollicular response can occur, however, particularly in responses driven by endogenous self-antigens (36–38). The fundamental mechanism that determines whether individual B-cell blasts undergo GC versus plasmablast differentiation remains unknown. As they occur in spatially distinct portions of secondary lymphoid tissues, differential migration of responding B cells may make an important contribution, as suggested by the differential requirement for EBI2 expression in GC and extrafollicular plasmablast responses (39, 40). Nevertheless, affinity/avidity for antigen does play a significant role in determining the relative contributions of particular specificities to each compartment.

Comparison of responses of SW_{HEL} B cells to SRBCs conjugated to our panel of recombinant HEL proteins revealed that the contribution of responding B cells to the plasmablast compartment increased according to their relative affinity for foreign antigen (24). This effect was not nearly as pronounced for the GC response, meaning that lower affinity B cells are able to make a more significant contribution to the GC response as opposed to the plasmablast response. Similar results have been reported using other Ig transgenic systems (41). Importantly, alteration of the density of HEL proteins on conjugated SRBCs showed that avidity as opposed to affinity *per se* is the critical determinant of the relative contribution of responding B cells to the GC and plasmablast responses (24). Consistent with this, B cells with relatively low affinity for the hapten epitope NP can contribute equally to both GC and extrafollicular plasmablast responses when challenged with a protein antigen carrying NP groups at high density (4). A detailed analysis of the effect of affinity on the plasmablast versus GC responses indicated that lowering antigen affinity did not reduce the number of cells committed to plasmablast differentiation, but impaired their capacity to proliferate and survive (32). Thus, compared with plasmablasts, early GC B cells are significantly less sensitive to BCR affinity-dependent signals for their ongoing proliferation and survival.

Despite the ability of relatively low affinity B cells to enter the GC response, competition from higher affinity B cells has been found to reduce their contribution to the GC. This is evident in populations of B cells with a range of affinities for HEL (24) as well as the hapten NP (5, 42). As discussed elsewhere, the ability of a B cell to interact with antigen has the potential to modulate BCR-dependent activation signals (signal 1), but also the extent to which antigen presentation, and therefore T-cell help (signal 2), occurs. In theory, therefore, the reduced ability of low affinity B cells to compete with a high-affinity B cells could be due to impaired access to one or both of these signals. In practice, independent analysis of these two BCR-dependent signals during an *in vivo* response is extremely difficult. Nevertheless, Schwickert and colleagues (43) recently employed a series of elegant experimental approaches to demonstrate that the innate superiority of higher affinity B cells to acquire and present antigen, and therefore access T-cell help, plays a major role in their preferential entry into the GC reaction.

Affinity and avidity in GC positive selection

The major role of the GC is to preferentially select B-cell clones that bind to foreign antigen with increased affinity. Although it would seem obvious that BCR affinity is fundamental to this process, in light of our previous discussion, it is worth considering whether antigen avidity has any distinct role to play.

A significant factor in this respect is the nature of the foreign antigen that localizes to the GC and drives affinity/avidity-based selection. This is widely thought to be the antibody:antigen immune complexes that localize on the surface of FDCs within the light zone of the GC (44–46) (*Fig. 1*). Data indicating that GCs can be initiated and progress in the absence of immune complexes (47) suggest that alternative antigen sources within the GC, possibly not even associated with FDCs, may also be important. Although this is a topic of some controversy (46, 48, 49), for the purpose of this review we assume that GC responses are driven primarily by FDC-bound immune complexes, with the caveat that alternative forms of antigen may involved.

In considering the role of avidity, the arrangement of foreign antigen in the GC may significantly alter its cross-linking ability compared with earlier extra-GC stages of the response. Although B cells entering the GC may in this case recognize antigen with different avidity compared with earlier in the response, antigen within the GC is likely to have a relatively consistent epitope density, meaning that avidity would essentially be directly related to affinity. It seems reasonable, therefore, to discuss the selection of GC B cells in relation to their relative affinity for GC-localized antigen.

The most widely accepted model for the process of positive selection in the GC is as follows (*Fig. 1*): (i) Responding,

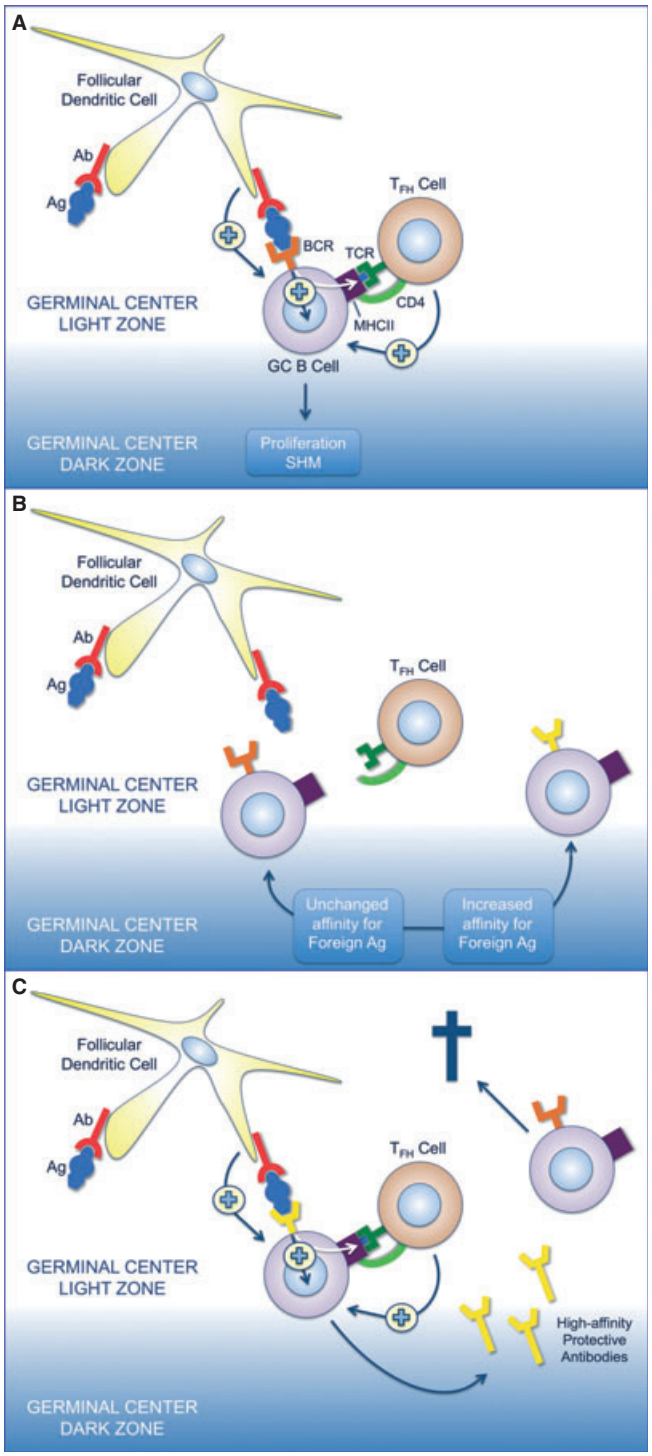

***Fig. 1.* Schematic depiction of the key events during affinity-based selection of somatically hypermutating B cells in the germinal center response**. See text for details.

mostly non-dividing, B cells in the light zone (LZ) of the GC (centrocytes) bind antigen presented in the form of immune complexes on FDCs (*Fig. 1A*); (ii) Antigen is internalized and presented to antigen-specific T follicular helper cells (Tfh; 50) localized predominantly in the LZ (*Fig. 1A*); (iii) B cells that receive sufficient antigen-dependent stimulus and Tfh-mediated help begin cell division and migrate to the dark zone (DZ) (*Fig. 1A*); (iv) DZ B cells (centroblasts) downregulate their BCR, divide and undergo SHM of their *Ig* variable region genes (*Fig. 1A*); (v) After one or more rounds of cell division and SHM, DZ B cells migrate back to the LZ and express their 'new' somatically mutated BCR (*Fig. 1B*); and (vi) LZ B cells that have improved their affinity for antigen via SHM preferentially cycle through this process and thus are positively selected at the expense of lower affinity specificities (*Fig. 1C*). Although some of the earliest attempts to visualize this dynamic process by two-photon intravital microscopy raised doubts as to whether GC B cells did mostly cycle between the LZ and DZ in this fashion (51, 52), a recent study incorporating flow cytometric and gene array analysis of LZ and DZ populations has indicated that this general scheme appears to be accurate in describing some if not all GC responses (53).

A central component of this model of the GC response is that GC B cells compete with each other for access to antigen based on their relative affinity of their BCRs. This results in GC B cells with the highest relative affinity for antigen preferentially receiving key survival and proliferative signals, resulting in their selective propagation or 'positive selection'. It is apparent that relative rather than absolute affinity for antigen is the key to this process. Thus, low-affinity B cells survive and proliferate unhindered in early GCs and only disappear later when higher affinity B cells develop (25). Lower affinity GC B cells are not immediately destined to die once a higher affinity clone emerges. Low- and high-affinity B cells can coexist within the GC response for many days (25), presumably because high-affinity B cells must be expanded over several proliferative cycles before they are present at sufficient frequencies to effectively exclude lower affinity B cells from accessing antigen. As there is no intrinsic difference in the rate of SHM that takes place in low- and high-affinity GC B cells (25, 54), the emergence of a high affinity clone in the GC does not ensure that it will come to dominate the response. Thus, before a single high affinity clone has the opportunity to dominate a GC response, independently generated high affinity clones can emerge and potentially be co-selected with or outcompete the previously generated high affinity specificity.

On the basis of detailed *in vitro* binding studies, Batista and Neuberger postulated that there is an upper threshold to the BCR affinity for foreign antigen beyond, which any further increases in binding affinity will not result in significantly improved access to antigen and thus will provide no selective advantage to GC B cells (18). This value (10^{10}/M) agrees with

a previous theoretical value proposed by Foote and Eisen (55) and seems to fit well with data from *in vivo* systems. First, it is rare that monoclonal antibodies are found with an affinity for their immunogen that greatly exceeds this value (6, 18). Second, the differential responses of SW_{HEL} B cells to high and low affinity HEL variants support this concept. Thus, although SW_{HEL} GC B cells accumulate predictable and affinity-increasing somatic mutations in response to HEL proteins they recognize with low initial affinity (HEL^{2X}, HEL^{3X}), their response to high affinity HEL^{WT} is marked by scattered mutations that are often silent or make conservative amino acid substitutions. In other words, there does indeed appear to be a ceiling to affinity maturation and this ceiling is at or below the affinity with which the native SW_{HEL} BCR binds to HEL^{WT} ($2 \times 10^{10}/M$).

Because the BCR is so fundamental to GC selection, it is worth noting that GC B cells have surface BCR densities that are on average 10-fold lower than those on naive B cells (32). Analysis of early B-cell differentiation has revealed that down-regulation of surface BCR levels coincides with the differentiation of proliferating follicular B-cell blasts into GC B cells, around days 3–4 of a model T-dependent response (32). This is true of both switched (IgG^+) and unswitched (IgM^+) B cells, both of which contribute to the initial seeding of the GC (32). Although the reason for this variation in BCR density remains unclear, it seems likely that high BCR levels are expressed prior to GC formation so that B cells with a wide range of antigen affinities can be recruited into the response. On the other hand, reduction of surface BCR levels upon GC B-cell differentiation may occur to facilitate more stringent affinity-based selection of somatically mutated variants. Consistent with this idea, the IgD class of BCR that is expressed specifically on naive B cells appears to exist primarily to facilitate high level BCR expression and promote early B-cell responses (56–58), but is not expressed by GC B cells (59).

What then is the key role of affinity in driving positive selection in the GC? Those B cells that bind preferentially to antigen in the GC are likely to receive stronger BCR signals (signal 1), but also to present more antigen to Tfh cells and thus receive more help (signal 2). Within the GC, however, the situation is likely to be more complex. In particular, although other sources of antigen may also be important (47), the localization of antigen in immune complexes on FDCs brings with it the possibility of GC B cells receiving multiple stimulatory signals that depend on their interaction with antigen, but that are delivered via receptors distinct from the BCR (*Fig. 1*). These include signals delivered from complement fragments deposited on the immune complexes (via B-cell CD21/35) and from synapses formed between FDCs and B cells by complementary pairs of intercellular adhesion molecule pairs such as ICAM-1/LFA-1 and VCAM-1/VLA-1 (60). These signals, which act as another type of signal 2, are likely to make an important contribution to the selection of high-affinity B cells in the GC (61).

The relative roles of BCR signals, antigen-associated co-signals, and Tfh help in driving positive selection of high-affinity GC B cells remain to be determined. Recent data showing that delivery of increased Tfh help to GC B cells increases their proliferation and survival (53) is consistent with the preferential delivery of these signals playing a role in positive selection. Although this may be true in the recruitment of cells into the GC (43), it remains unclear that this occurs under normal selective conditions within the GC and certainly does not exclude a prominent role for either BCR or antigen-associated signals. It is entirely possible that a combination of all three inputs provides an integrated stimulus that preferentially sustains high-affinity GC B cells. It appears, however, that a BCR stimulus alone is not sufficient for positive selection, as exogenously delivered antigen rapidly kills rather than serves to propagate GC B cells (62–64). Whatever the precise stimuli involved are, a recent study indicates that it is preferential survival rather than proliferation of high-affinity GC B cells that is likely to be primary basis for positive selection (65).

Affinity in the differentiation of GC B cells into plasma and memory cells

Although the positive selection of high-affinity B cells in the GC reaction is fundamental to achieving long-term immunity, this is only actually achieved when high-affinity GC B cells differentiate into either long-lived plasma cells or memory B cells (33). The precise signals that trigger the progression of GC B cells down either of these differentiation pathways remain to be determined. However, affinity for antigen appears to play an important role, particularly in the case of plasma cell differentiation.

Activated B cells are capable of differentiating into plasma cells in the complete absence of BCR signals (66) suggesting that plasma cell differentiation may be a stochastic process that progresses independently of antigen affinity. However, the long-lived plasma cells that reside in the bone marrow are known to have heavily somatically mutated variable region genes and to produce high-affinity antibody specificities (67, 68), suggesting that affinity-based selection does play a prominent role in their production. One way of reconciling these observations is to propose that stochastic differentiation of GC B cells into plasma cells occurs over the course of the response. In this way, mature GCs containing only high

affinity specificities would generate the final wave of plasma cells that would by default occupy the bone marrow plasma cell niche. However, simultaneous comparison of the GC and bone marrow plasma cell compartments during a T-dependent response has revealed that high affinity mutations were more frequent among the plasma cells (67, 68), suggesting that high-affinity GC B cells are preferentially selected to undergo plasma cell differentiation. Further evidence for this was obtained in experiments using SW_{HEL} B cells, which revealed that post-GC plasma cells are almost exclusively derived from high-affinity GC B cells, even when these cells made up <25% of all GC B cells (25).

As with positive selection in the GC, the specific mechanism by which antigen affinity drives the plasma cell differentiation of high-affinity GC B cells is difficult to determine. Enhanced BCR signaling is one candidate, as this has been shown to trigger degradation of the Bcl6 transcription factor (69), which can in turn de-repress the plasma cell transcription factor Blimp-1 (70). However, the enhanced provision of T-cell help to GC B cells drives a burst of plasma cell production (53), suggesting that this input may be important in driving plasma cell differentiation as well as positive selection. Indeed the fact that high-affinity GC B cells preferentially undergo both positive selection and plasma cell differentiation raises the possibility that these two processes may indeed be mechanistically linked.

In contrast to the bone marrow plasma cell compartment, post-GC memory B cells are not as greatly enriched for high-affinity B cells (67). Rather, it seems that the progression from GC B cell to memory B-cell is more stochastic, the overall affinity of the memory B-cell compartment increasing more or less in line with the GC response (68). Consistently, unswitched memory B cells typically have lower levels of SHM and lower affinity for the immunogen than IgG^+ memory B cells (17). As opposed to terminally differentiated plasma cells, it may be advantageous to maintain relatively low-affinity memory B cells to provide the flexibility to respond to variant pathogens that have mutated the initial target antigen (17) and thus ameliorate the effects of original antigenic sin (71).

Affinity for self-antigen and regulation of the GC response

Previously in this article, we discussed the importance of B cells receiving both a signal 1 (BCR signal) and signal(s) 2 (T-cell help and/or other non-BCR stimuli) to initiate and perpetuate B-cell responses. The concept of complementary signals being required for lymphocyte activation is in essence the hypothesis proposed over 40 years ago by Bretscher and Cohn (72) to explain how the immune system achieves self-nonself discrimination. A critical requirement of this model is that signal 2 is uniquely associated with foreign and not self-antigen. Assuming the T-cell repertoire is self-tolerant (73), this appears at first glance to be an effective way of preventing the T-dependent activation of self-reactive B cells. Thus, B cells that bind self-antigen would receive signal 1, but would not receive signal 2 due to the absence of self-reactive T-cell help. However, if a self-reactive B cell binds to a cross-reactive epitope on a foreign antigen, there is in theory no barrier to them presenting peptides from the foreign antigen to activated Th cells responding to this same antigen. For this reason in particular, there is a need for the immune system to silence or delete self-reactive B cells generated in the primary repertoire (B-cell self-tolerance; 74). Indeed, it appears that as many as 85% of initial BCR specificities are removed from the primary repertoire during the development of immature bone marrow B cells into mature, long-lived, peripheral B cells (75, 76).

The enforcement of both B and T lymphocyte self-tolerance would appear to provide an effective barrier against the activation of self-reactive B cells present in the primary repertoire. However, the fact that B cells undergo a secondary diversification of their antigen receptors by SHM presents a separate challenge for the immune system to maintain self-tolerance. The implications for self-tolerance of the discovery of SHM were not lost on researchers at the time (15). Thus, as with the primary diversification of the BCR repertoire by V(D)J recombination, it was appreciated that the SHM of Ig variable regions in the GC not only had the potential to generate crucial new specificities with (high) affinity for foreign antigen, but also potentially dangerous ones that bound strongly to self-antigens. Indeed, the threat posed by self-reactive B cells generated by SHM seems particularly alarming as they by definition would emerge in the midst of an active immune response. The potential for SHM to precipitate autoimmunity was confirmed by the identification of pathogenic autoantibodies that were both heavily hypermutated and had undergone apparent antigen-based selection (77–79). Given the fact that autoantibodies could be produced from GC B cell precursors, it was reasonable to ask whether any mechanism existed to prevent self-reactive B cells generated in the GC from producing an autoantibody response.

In considering how self-reactive GC B cells could be controlled, the first case that can be mentioned briefly is that of GC B cells that acquire self-reactive BCRs, but at the same time lose affinity for the original stimulatory foreign antigen. Under these circumstances, the fate of the cell is presumably

the same as all GC B cells that acquire reduced antigen affinity after SHM that is they are deprived of the stimulatory signals associated with binding to the foreign antigen and soon die (*Fig. 1C*). Exposure of these cells to the target self-antigen would not be expected to rescue these cells as the BCR stimulus in this case would not be associated either with FDC-associated co-signals or Tfh help. This hypothesis is supported by the fact that isolated cross-linking of the BCR on GC B cells in the absence of co-signals leads to rapid cell death (62–64).

A more challenging scenario for the maintenance of self-tolerance arises when somatic mutations acquired by GC B cells not only bestows them with a self-reactive BCR but also increases their affinity for foreign antigen. Herein, the danger of generating self-reactivity within an active immune response becomes apparent, as a conflict arises between positive selection pressure from the ongoing response to foreign antigen and any negative selection pressures that may ensue from the acquisition of self-reactivity. The generation of cross-reactive specificities during an immune response is not merely a theoretical possibility: around 3.5% of monoclonal antibodies generated against viral antigens have been shown to have cross-reactivity with various self-antigen targets (80), and the production of autoantibodies that cross-react with microbial antigens are a well-characterized consequence of many infectious diseases (81–86). In light of these observations, the key question that arises is whether any mechanism exists to override the positive selection of high-affinity GC B cells under circumstances where they have acquired a self-reactive BCR.

On the basis of current knowledge of affinity-based selection events in the GC, we wish to propose a model for how the immune system deals with self-reactive GC B cells that maintain strong reactivity with foreign antigen. In addition to adhering to current concepts in GC selection, the model seeks to explain how self-tolerance can in general be maintained, but be susceptible to subversion relatively frequently by GC B cells encoding cross-reactive autoantibodies. This model is summarized in *Fig. 2* and explained in detail below.

Assuming they have no self-reactivity in the first instance, B cells that bind foreign antigen with sufficient affinity/avidity are expanded in the follicle and then recruited into GCs (*Fig. 2A*). GC B cells that acquire somatic mutations improve their affinity for foreign antigen, but do not result in self-reactivity, interact unimpeded with the foreign antigen (*Fig. 2A*). As has been discussed, under these circumstances the high-affinity GC B cells preferentially receive signals from FDC-associated foreign antigen and Tfh cells, are positively selected, and differentiate into plasma cells producing high-affinity antibodies directed against the original foreign antigen (*Fig. 2A*).

If a GC B-cell acquires self-reactivity at the same time as improved affinity for foreign antigen, we propose that two basic scenarios are possible. If the target self-antigen in question is expressed ubiquitously (or just in the GC microenvironment) and is recognized with sufficient affinity by the revised BCR, the GC B cell may be diverted from the stores of foreign antigen and instead preferentially interact with self-antigen (*Fig. 2B*). In the same way that high- and low- affinity GC B cells compete for antigen, we propose that the sources of foreign and self-antigen in the GC 'compete' to interact with the cross-reactive GC B cell and that the dominant interaction will depend to some extent on the relative affinity of the BCR for the two antigen sources. It has been argued, however, that self-antigens are likely to be present at higher concentrations in the GC than the limiting amounts of foreign antigen presented on GCs (60), which would swing the balance in favor of the B cell binding to self-antigen. In any event, we propose that GC B cells that preferentially interact with self-antigen in the GC will undergo cell death due to their inability to access the co-signals associated with FDC-presented foreign antigen or Tfh cells (*Fig. 2B*).

The second potential scenario upon acquisition of a cross-reactive BCR by a GC B cell is where expression of the target self-antigen is either extremely low or absent from the GC, such as for a tissue-specific protein. In this case, we propose that there is no immediate barrier preventing the self-reactive GC B cells from undergoing positive selection based on their cross-reactivity with foreign antigen (*Fig. 2C*). The provision to these cells of signals from FDC-associated foreign antigen and Tfh cells would also result in their differentiation into plasma cells and the production of high-affinity antibodies directed against the original foreign antigen. However, once released into extracellular fluids, these same antibodies would also be free to access and bind to the distal self-antigen target and potentially contribute to organ-specific autoimmune disease (*Fig. 2C*).

The model we have proposed describes a situation that could potentially arise out of any GC response. Importantly, it does not require any overt compromise of the normal mechanisms of self-tolerance. In particular, it does not require any contribution from self-reactive T cells as it is based on the continued provision of help to cross-reactive GC B cells by the anti-foreign Tfh cells that initiated the GC response. Rather, we suggest that the potential inability of the immune system to control autoantibody production from anti-foreign GC B cells that cross-react with tissue-specific self-antigens may represent a 'hole' in the body's self-tolerance defenses. Evidence that this may be the case can be seen from the many instances

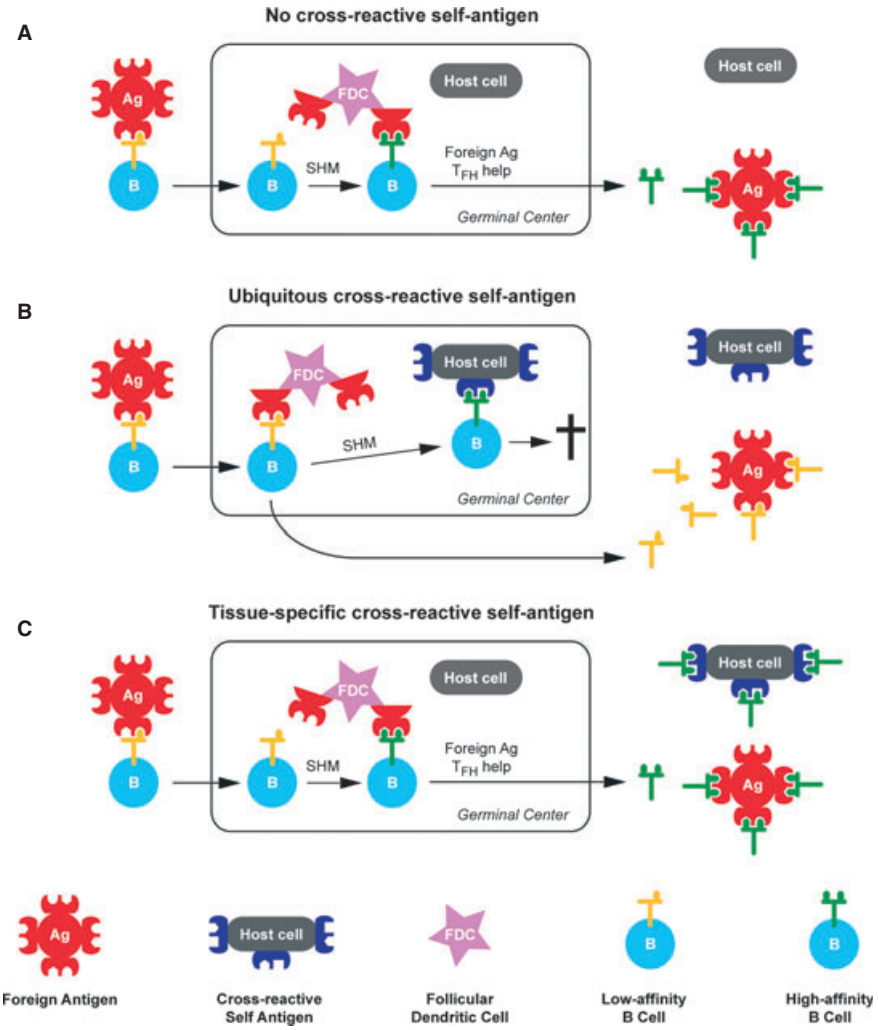

***Fig. 2.* Proposed model for the removal of self-reactive B cells produced in the germinal center response depending localization of self-antigen expression**. See text for details.

of postinfectious autoantibodies that recognize tissue-specific self-antigens as well as microbial epitopes. Examples of these include Guillain–Barré syndrome, where antibodies against *Campylobacter jejuni* lipooligosaccharides cross-react with peripheral nerve gangliosides (82), and rheumatic carditis, where antibodies against *Streptococcus pyogenes* M protein cross-react with cardiac myosin (83).

For reasons addressed earlier in this article, it has been extremely difficult to answer questions regarding the selection events occurring in the GC. This is even more challenging in regard to self-reactive specificities potentially undergoing negative selection as these may exist only transiently in the GC and would be virtually impossible to identify. Current experiments using the SW_{HEL} B-cell system are aimed at circumventing these problems and testing our proposed model of how self-reactive B cells are controlled in the GC. This includes testing if the existence of tissue-specific, cross-reactive autoantibodies such as those associated with postinfectious autoimmunity, can be explained by an inability to control self-reactive GC B cells when the target self-antigen is not expressed in the GC microenvironment.

Concluding remarks

Although all B cells are in theory created equal, each clone can progress down a myriad of different response pathways depending on the interactions of the cell's unique BCR with the antigenic universe. Progression of B cells into GCs, and their fate once inside, are determined primarily by the affinity of the BCR for foreign antigen and the way in which SHM molds this affinity over the course of the response. Although the inherent dynamism and complexity of the GC have conspired to shroud many of the details of its inner workings, new experimental systems and technologies are providing

fresh insights these processes and how antigen affinity guides them. Acquisition of affinity for self-antigen is a constant threat in the GC responses and, depending on the balance between affinity of GC B cells for foreign and self-antigen and the localization of self-antigen expression, has the potential to result in autoantibody production. Nevertheless, the benefits of the GC response in providing high-affinity antibodies for long-term immunity presumably outweigh the autoimmune risk, as the GC has clearly evolved to become the sophisticated apex of the adaptive immune response.

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