Germinal centers; programmed for affinity maturation and antibody diversification

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Summary

The seminal discovery by Eisen that antibodies undergo improvements in antigen-binding affinity over the course of an immune response led to a long running search for the underlying mechanism. Germinal centers in lymphoid organs are now recognized to be critically involved in this phenomenon, known as antibody affinity maturation. As well as improving in affinity for a specific epitope, some antibody responses maintain or even increase their breadth of antigen-recognition over time. This has led to another intense line of research aimed at understanding how broadly neutralizing anti-pathogen responses are generated. Recent work indicates that germinal centers also play an important role in the diversification process. We discuss current understanding of how germinal centers are programmed to support both affinity maturation and antibody diversification.

Highlights

- **GCs can support B cells with a range of affinities for the inducing antigen**
- **GC selection involves a LZ bottleneck and differential expansion in the DZ**
- **Transition between DZ and LZ states is guided by a timed gene expression program**
- **Redemption of self-reactive B cells through SHM contributes to antibody diversity**

Introduction

Germinal centers (GCs) are sites where B cells undergo antigen-driven somatic hypermutation (SHM) of their immunoglobulin (Ig) variable (V) regions and selection for improved antigen binding [1-4]. GCs form over a period of several days following antigen exposure, and individual lymphoid organs can contain a few or dozens of these isolated B cell clusters. GCs become organized into two main zones, a T zone proximal dark zone (DZ) and a

distal light zone (LZ). The DZ is the most active site of cell division. DZ GC B cells (also known as centroblasts) express high amounts of activation-induced cytidine deaminase (AID), driving SHM. The LZ contains LZ GC B cells (also called centrocytes) that have mostly left the cell cycle, as well as T follicular helper (Tfh) cells and a dense network of follicular dendritic cells (FDCs) that display surface bound antigens. The LZ is the principle site of antigen-mediated and T cell-mediated selection of GC B cells. During the process of SHM and selection, many B cells undergo cell death within the GC and their corpses are cleared by tingible body macrophages. GC B cells move continually within the GC, traveling to the DZ in response to the CXCR4 ligand, CXCL12, that is made by DZ stromal cells and being directed to the LZ by the CXCR5 ligand, CXCL13 [5,6]. GC B cells undergo repeated rounds of mutation and selection, through a process of cyclic reentry from the LZ to the cycling DZ state [3]. The output of the GC includes memory B cells and long-lived antibody producing plasma cells (PCs). Here we review the growing evidence that GC responses against complex antigens are not geared solely to generating the most highly focused, highest affinity responses, but that they encompass a permissiveness that allows the generation of diverse responses with a range of antigen binding affinities.

Experimental versus real world antibody affinity maturation

Subsequent to the classical work of Eisen and Siskend that was performed in rabbits [7], the study of antibody affinity maturation has predominantly been performed in mice and has relied largely on two experimental systems; the tracking of endogenous B cells following hapten-protein conjugate immunizations (most commonly NP), and the adoptive transfers of B cells from immunoglobulin (Ig) heavy chain knockin mice such as those specific for hen egg lysozyme (HEL). These approaches have shown that affinity maturation can occur via a process of stringent competition that leads to the best variants rapidly replacing their neighbors. However, in both experimental systems, all cells recognize the same epitope on the antigen (intraclonal competition) and relatively large affinity increases (1-2 log) are conferred via just single amino acid substitutions within the variable region. While this may provide a reasonable model for how “easy” affinity maturation events occur, the extent to which these responses reflect what is going on during affinity maturation against more complex antigens that have multiple epitopes, and against which many clones may compete, appears more limited.

The development over the last decade of relatively high throughput single B cell antibody cloning technologies has led to an array of insightful studies characterizing the molecular details and maturation pathways of medically and scientifically important antibodies from both humans and mice, with a strong focus towards understanding how antibodies with broadly neutralizing potential function. In line with the mouse studies, affinity maturation of some “real” antibodies occurs through simple changes in the CDRs of the IgHV region causing cells to rapidly gain neutralizing potential [8]. Often, however, the developmental pathways of neutralizing antibodies are much more complex, with cells needing to pass through multiple low or moderate affinity intermediate steps en route. One such example is the influenza specific antibody CR6261, the first identified member of a family of broadly neutralizing human antibodies that utilize IgHV1-69 and recognize a conserved region on the stem of the virus’

haemagglutinin glycoprotein [9]. Despite earlier studies indicating that activated B cells compete with one another based upon the affinity of their BCRs even at the time of GC entry [10,11], the unmutated founder of this antibody was determined to have an extremely low affinity (below detection in soluble form with the assay used). To acquire its full activity, CR6261 needed to accumulate a minimum of seven specific mutations in two regions of its IgHV gene. The stem region of the HA glycoprotein that CR6261 recognized is known to be a conformationally challenging site for antibodies to access when in its native form, likely contributing to why multiple mutations are needed to achieve full binding potency [12]. This theme of complex pathways to the generation of high affinity antibodies is reinforced in the large body of work that has been performed to decipher the somatic mutation history of broadly neutralizing HIV-1 gp120 antibodies [13]. In many cases, these antibodies need to accrue very large numbers of somatic mutations before eventually binding difficult to access conserved regions of this glycoprotein. These findings do not directly contradict simple models of affinity-based discrimination in the GC, however they do infer that GCs should be relatively permissive to low and moderate affinity antibodies so as to not severely restrict the pool of somatic variants from which more complex antibodies can develop. There is also strong evidence that the generation of diversity in GCs contributes to immunity by providing pools of B cells from which recall or maturation towards similar but different antigens can occur following reinfection or in response to viral escape mutants [8,13].

Diversity generation and retention in germinal centers

Two recent studies from Tas et al. and Kuraoka et al. have begun to reconcile observations made through human antibody cloning technologies with how affinity maturation might occur *in situ* in GCs by tracking endogenous responses to complex protein antigens [14,15]. In both studies, GCs were found to be much more permissive to retaining cells expressing low and moderate affinity receptors than had previously been appreciated. In order to facilitate a large scale analysis of GC derived antibodies, Kuraoka et al. developed a novel feeder cell based *ex vivo* culture system to expand and differentiate single GC B cells, such that they could screen the antibody products derived from them. When they then compared the relative antigen binding characteristics of the antibodies from 8 days and 16 days post immunization with recombinant proteins derived from influenza A virus and *Bacillus anthracis*, they found clear evidence that the mean antibody affinity improved with time as might be expected. Importantly, however, this did not occur at the expense of retaining low or moderate affinity cells, and it did not cause a narrowing of clonal diversity as defined by usage of different V_H gene segments. Furthermore, order of magnitude affinity differences were observed even when only clonally related cells within the same branch of a phylogenetic tree were considered, indicating significant permissiveness in terms of affinity even when competition was for the same epitope. It will be interesting in the future to determine how long such variants can remain in GCs alongside each other.

Little clonal exchange occurs between GCs during acute responses. This led Tas et al. to interrogate the nature of clonal dynamics and relationships at play within separate GCs from single lymph nodes [14]. To achieve this, they

applied a range of creative “brainbow”, photo-conversion, and micro-dissection techniques that allowed them to both visualize lineage relationships via expression of color combinations and to isolate single cells for antibody cloning based characterization. This current study revealed that the seeding of primary GCs is a highly oligoclonal affair (50-200 clones per GC) and that the threshold affinity requirement is very low. The visual nature of the analysis in the tamoxifen inducible “brainbow” system made it possible for the authors to examine multiple GCs from within a single lymph node. Once established, the fate of individual GCs varied greatly, with some foci becoming dominated by single clones while others remained highly diverse throughout the study period. When homogenization did occur, the kinetics could be fast, with some GCs becoming >50% clonally dominated by 9 days post-tamoxifen treatment and >80% by day 11. Remarkably, homogenization occurred even within GCs from Peyer’s patches and mesenteric lymph nodes where the responses are to gut-associated organisms. When immunization driven “clonal bursts” did occur, the expected increases in affinity over the ancestoral seeding cells were observed, suggesting that the outgrowths probably contribute to affinity maturation. However, affinity alone was not sufficient to cause clonal bursts because identical clones that carried the same affinity conferring mutations were also found in non-bursting GCs from the same lymph nodes. Furthermore, selection caused by high affinity antibody-antigen interactions also was not necessary because some clonal bursts involved lower affinity cells. Importantly, the authors note that affinity maturation may not require clonal bursts, since affinity enhancement within clonally related populations was also observed in GCs that were not bursting. In the future, it will be important to try to understand why clonal bursting occurs in some GCs and not others, what causes it, to what extent it contributes to affinity maturation and whether any relationship exists between bursting and the differentiation of memory or plasma cells. In addition, whether bursting involves a period of clonal expansion without additional somatic hypermutation is an appealing idea to consider as this might provide a means for increasing a population prior to risking changing the specificity further. The observation that multiple paths branch for a single common ancestor cell in some bursts is at least consistent with this possibility.

Reconciling clonal diversity with pathways of selection

So how then do current models of antibody affinity maturation fit with the observations from antibody cloning and GC clonal dynamics studies? At their most simple, popular models of selection in GCs involve B cells competing for T cell help based upon the amounts of antigen that they are able to gather through their BCRs [2,16]. Those cells that express higher affinity receptors capture more [17], allowing them to present greater amounts of peptide complexed with MHC class II and thereby favoring their making productive interactions with a limiting number of Tfh cells (i.e. competition is for access to T cell help). The outcome is that the ‘helped’ B cells are selected to continue participating in the GC (cyclic reentry), while the low affinity cells receive inadequate help and undergo cell death. Engagement of T cell ICOS by B cell ICOSL during brief encounters within the GC contributes to a positive feedback loop that augments CD40L expression and provision of T cell help [18]. Experimentally, the provision of more T cell help to small subsets of GC B cells causes them to quickly dominate responses [16]. In addition, the findings during anti-NP responses that the LZ-DZ bottle neck is tight [16], and that W33L affinity

conferring mutations were enriched 20-fold in populations of NP-reactive LZ cells that express c-Myc and are thought to have just undergone selection [19], suggests that selection may be stringent at this stage. However, two recent papers from Gitlin and coworkers revealed that affinity improvements do more for GC B cells than simply enhance their chances of cyclic reentry [20,21]. By making use of a doxycycline controllable cell division marker system, the authors identified that the quality and/or quantity of T cell help received by a GC B cell during selection directly influences their subsequent proliferation rates, the rate at which they accrue somatic mutations, and the number of divisions that they will go through during their next DZ cycle. While the average number of divisions that a cell would pass through was calculated as being 3, anywhere between 1 and 6 cycles was deemed possible. Therefore, those GC B cells that are better able to bind antigen might not only have an increased likelihood of survival and cyclic reentry but will also clonally expand to a greater degree, further diversifying their Ig genes (Fig. 1). This mechanism may help explain the cause of clonal bursts observed by Tas et al. [14], although the number of cell cycles required to reach a “bursting” stage is presumably considerably higher than will be achieved via a single selection event. It has not yet been determined what the relative contributions of each of these mechanisms might be in conferring affinity increases during non-perturbed complex responses. Simple mindedly, pathways that preferentially expand high affinity cells without their directly repressing lower affinity cells might seem better suited when diversity generation is needed.

Multiple factors are likely to contribute to clonal diversity and permissiveness during GC responses. Stochastic elements have been suggested as one factor [3,14], and another may be that the binding properties of antibodies affect how peptides are presented [22]. Mutations may occur that cause the antibody to protect an epitope from proteolysis more effectively and thereby increase the amount of MHC-peptide that is presented without increasing binding strength for the antigen. The physical separation of individual GCs most likely contributes to the overall diversity of the response by supporting ‘islands’ of clonal evolution [14]. The finding that B cell confinement to GCs is dependent on sphingosine-1-phosphate (S1P) mediated signaling via S1PR2 and Gα13 opens up avenues for testing how disruptions in confinement affects the diversity of the response [23]. While GCs may initially operate as separate entities, once they give rise to plasma cells antibody can travel via circulation to reach other GCs. The ability of soluble antibody to influence ongoing selection events has been demonstrated in the context of anti-hapten responses [24]. A study that modeled events associated with the GC response against an antigen that is present in multiple variant forms, such as HIV-1, provided evidence that antibody feedback could help in broadening the antigen-binding range of the responding B cells [25]. However, it is not yet known how many PCs can be generated by a single GC and thus how strongly the products of one GC might impact the ongoing response in others. Moreover, secreted antibody could influence an ongoing response in multiple ways, including by steric interference by binding to the site recognized by the clonally related B cells, negative feedback through FcγRII crosslinking [26], and increasing antigen accessibility by facilitating more efficient deposition of remaining (or newly generated in the case of replicating agents) antigen on the FDC network [27]. In the case of rapidly evolving chronically infecting viruses, such as HIV-1, antibody can also have quite rapid effects on the ongoing GC response by selecting for altered forms of the antigen. Elegant work tracing the anti-gp120 response

evolving in a single HIV-1 infected individual showed that somatically mutated antibody generated against one surface of the glycoprotein can select for viral variants that influence the selection of B cell clones recognizing another surface of gp120 [28,29].

A germinal center program to promote antibody affinity maturation

GC B cells are in a unique state of activation that is highly adapted for antibody affinity maturation. The movement of cells between the DZ and the LZ is associated with the exiting of cell cycle, a reduction in expression of SHM promoting genes and increases in expression of activation-associated markers [16,30]. Surprisingly, GC B cells transition between DZ and LZ states at almost normal rates when all cells are physically restricted to the LZ as a result of *Cxcr4* genetic deficiency, leading to the proposal that GC B cell behavior is controlled at least in part by an intrinsic cellular program that involves a “timer” like component [6]. The transition between DZ and LZ states correlates with (and presumably determines) cell movement between the respective areas, but is not caused by it. Given the findings that proliferation rates and DZ residence times are determined by the nature of T cell interaction [20], it seems that the “depth” of induction of the DZ program is one way by which certain clones are preferentially expanded and/or diversified. However, new findings suggest that DZ behavior may also facilitate effective affinity maturation by helping ensure that subsequent selection events are efficient.

Photo-conversion based *in vivo* cell tracking experiments have taught us that the dynamics with which cells transition between states and locations are very rapid; up to 50% of GC B cells move from the DZ to the LZ every 4-6 hours [16]. Consequently, the period between when a cell acquires a new mutation and when it tests its new post-SHM BCR is probably very short. To help deal with the challenge of screening new Ig variants so soon after their generation, the GC B cell program appears to involve a stage where pre-existing MHC class II complexes are specifically and rapidly targeted for proteolytic degradation [31]. Then, upon returning to the LZ, cells reverse this process and stabilize these proteins. This corresponds with a period of increased MHCII synthesis suggesting that LZ cells may favor antigen presentation by increasing their generation of new complexes and sheltering them from degradation. Mechanistically, fluctuations in MHCII levels are controlled by ubiquitination of the beta chain by the E3 ligase March1. Increases in Cd83, a LZ associated antagonist of March1 [32], contribute to reduced ubiquitination at that stage but other Cd83 independent mechanisms of March1 regulation also contribute to DZ associated fluctuations [31,33]. These changes are analogous to those undergone by dendritic cells during their maturation [34]. In theory, by reducing the chances of peptide-MHCII carry-over following SHM, such a pathway could aid diversity generation and maintenance in the GC by allowing new somatic variants to be screened during each LZ selection stage for their ability to still bind antigen (as opposed to screening for an increase in antigen binding). Interestingly, populations of DZ cells with the lowest surface MHCII levels were enriched for cells that have recently undergone multiple cell divisions but were currently in G1 or early S phase, raising the possibility that they may represent a stage at which old peptide-MHCII complexes are purged late in the DZ cycle in advance of the next selection event (GC B cells exit cell cycle prior to returning to the LZ). Consistent with this

idea, the frequency of DZ cells in active cell cycle (S, G2, M) was found to correlate with levels of the transcription factor AP4, whose abundance is expected to decrease with time post-selection [35]. However, as with many questions in GC B cell biology, better fate-mapping approaches are needed to directly prove or disprove this point.

The molecular pathways controlling the functions of DZ cells and the timing of LZ transitioning are not well understood, however two important players in the regulation of these processes have recently been identified. The first of these, FoxO1, is a transcription factor that also plays important roles during B cell development. In GC B cells, nuclear (active) FoxO1 is only found in DZ cells and a small subset of c-myc+ LZ cells that presumably are undergoing selection [36,37] (Fig. 2). Curiously, FoxO1 is responsible for driving much (but not all) of the DZ transcriptional signature but is not needed for cells to behave like DZ cells in terms of AID induction or proliferation, indicating that other selection-induced pathways must act in synergy. Results from these mice indicate that the normal coordination of LZ and DZ processes is critical for effective selection since the functional consequence of FoxO1 deficiency was a defect in affinity maturation. The other recently identified player is AP4, a downstream target of c-myc [35]. In contrast to FoxO1-deficient mice, animals lacking AP4 in B cells generate normal looking DZ subsets but their GCs are smaller (despite normal Tfh cell frequencies) and the number of cell divisions that each DZ cell passes through is seemingly reduced. As such, this transcription factor is probably a regulator of the cell intrinsic “timer” [6]. While induced by c-myc, the study provided strong evidence that AP4 levels and/or maintenance can be driven via IL-21 signaling, with IL-21 being a Tfh-derived cytokine that is essential for GC maintenance [38], suggesting that the amount of exposure to this cytokine may be one way by which T cells determine the number of cycles a DZ cells will pass through [20]. It is interesting to note that B cell specific AP4 knockout mice showed significant defects in their ability to form neutralizing antibodies when analyzed almost three months after LCMV infection [35]. This was in spite of apparently normal affinity maturation against NP-CGG. Like some of the human pathogen examples listed above, neutralizing antigens on LCMV virions are thought to be inherently challenging targets [39]. These experiments are complicated to interpret given the generally reduced GC sizes and mutation accrual in the AP4-deficient mice, however they are consistent with the notion that some of the DZ components reviewed here may help drive clonal diversification and more complex affinity maturation events.

A striking feature of GC B cells is their probing, dendritic morphology and dynamic, searching behavior [2]. They appear to continually move over the antigen-laden FDC network without showing evidence for extensive pausing. Despite apparently intimate contact between GC B cells and FDCs, the process of antigen uptake by GC B cells has so far not been visualized. This contrasts to the situation for naïve B cells [40]. One explanation for the difficulty in visualizing this process might be the lower BCR levels on GC versus naïve B cells [41]. An important recent discovery provides another explanation by using *ex vivo* high resolution microscopy to reveal that the BCR in GC B cells does not coalesce into a central supramolecular activation complex (cSMAC) but instead forms multiple peripheral (p)SMACS [42]. Elegant force measurement experiments showed that GC B cells produced

stronger tugging forces on the BCR and GC B cells extracted antigen with better affinity discrimination than naïve B cells, consistent with the importance of MHC peptide presentation as a key determinant of selection [16].

The extent of BCR signaling occurring in GC B cells is under active investigation. *Ex vivo* staining of fixed GC B cells suggested BCR signaling may be minimal except in the G2 phase of the cell cycle [43], whereas exposure of GC B cells to antigen in lipid bilayers revealed strong activation of BCR signaling pathways with the exception of PKCδ and NFκB activation [42]. FoxO1 is negatively regulated by PI3K and AKT signaling [41,44]. However, it is not known what receptors are acting on PI3K and AKT to inhibit FoxO1 in LZ cells. BCR signaling seems likely to be involved [42] and Tfh-derived signals (CD40L, IL21, IL4) can also activate AKT [41]. GC B cell S1PR2 negatively regulates AKT via Gα13 [23,45]. In accord with this pathway contributing to FoxO1 regulation, mice lacking Gα13 [illegible] with a LZ phenotype in mucosal GCs ([46] and Jagan Muppidi and J.G.C. unpublished). The S1PR2 ligand, S1P, is thought to be more abundant at the perimeter than in the interior of the GC [47,48]. It seems possible that encounter with S1P at the LZ GC perimeter might be one factor that helps switch off AKT, turn on FoxO1 and favor transition to a DZ state. In humans, GC B cells express a second Gα13 coupled GPCR, P2RY8, that may also contribute to control of AKT and thus possibly FoxO1 and cyclic reentry [45,49]. Unequal PI3K/AKT/mTOR signaling, possibly as a result of asymmetric partitioning of AKT during cell division, may also contribute to cell fate decision-making in the GC [50] Recent studies have identified roles for additional promoter- and enhancer-binding factors and microRNAs in GC B cells including canonical and non-canonical NFkB family members, Bach2, E2A (TCF3), Id3, YY1, EZH2 and miR155 [51-59]. This work includes initial insights into how regulation of cell metabolism can be anticipated to be a crucial factor influencing GC B cell competitiveness and fate. Studies in Burkitt lymphoma, a lymphoma thought to be of GC DZ cell origin, suggest that E2A (TCF3) may contribute to establishing or maintaining features of the DZ state in a manner controlled by ID3 [60].

Auto-reactivity; negative selection, retention and conversion of auto-reactive clones

Autoreactivity is a prominent feature of the pre-immune B cell repertoire and many recirculating B cells are IgM^{lo}, reflective of self-antigen engagement and entry into an anergic state [61]. It has long been questioned why cells of this type would be kept in the peripheral B cell repertoire rather than being eliminated. The thought that autoreactive B cells might somatically mutate away from self-reactivity was raised in early theoretical work [62] and experimental support for this possibility has now emerged in multiple studies [61,63-66]. In recent work, the Goodnow group used a model system to reveal the remarkable antigen-recognition gymnastics that can be achieved through SHM to ‘redeem’ autoreactive B cells and make them foreign-antigen selective. Using repeated immunization with a foreign antigen that differed from self only in being multimerized on a cell surface, anergic B cells became GC B cells and accrued mutations that reduced affinity for the soluble autoantigen, allowing the cells to preferentially bind and take up the multivalent foreign antigen [61]. This process seems unlikely to be a

rare phenomenon associated with responses to simple protein antigens. The human VH4-34 element confers autoreactivity against poly-N-acetyllactosamine carbohydrates (I/i antigen) on RBCs and is carried by about 5% of B cells. In accord with their autoreactivity, these cells have an anergic phenotype. An analysis of foreign antigen-reactive VH4-34 containing antibodies revealed that they carried mutations away from self-reactivity; using an immunization approach in humans, it appeared that during mutation away from self-reactivity the B cells could suffer a reduction in reactivity to the foreign antigen [65].

A large body of work has revealed that the majority of unmutated germline ancestors of bnAbs against HIV-1 are autoreactive [67-69]. Why these antibodies often begin as autoreactive has been speculated to be because the difficult to access conserved sites on HIV-1 gp120 require features (such as a hydrophobic patch) that typify polyreactive antibodies [70]. A novel and seemingly extreme example of this process is the acquisition by some type of recombination event of an entire Ig-fold from the collagen receptor, LAIR1, in malaria antigen-binding antibodies [66]. In these antibodies, the LAIR1 Ig domain has undergone SHM that causes loss of collagen (self) binding. In the simplest case, mutations that improve foreign specificity may decrease self-recognition in a process of specificity-selection [63]. However, the studies of Goodnow and colleagues highlight how more complex processes can occur that seemingly decrease affinity for both the self- and foreign antigen as measured by a typical binding assay, yet allow selection [61]. This type of mutation and selection program might be 'read out' as retaining low affinity cells in the GC when, in fact, the lower affinity cells are binding the foreign antigen more avidly *in vivo* due to less distraction by self antigen, and thereby transmitting more effective selection signals (Fig. 3). This might involve more uptake of the foreign antigen and receipt of more effective T cell help. Alternatively, relief of continual BCR engagement by a ubiquitous autoantigen may be necessary for upregulation of surface BCR levels and transmission of survival or other maintenance signals [41,61]. In this scenario, one purpose for the separation of GC cells into LZs and DZs may be to provide cells with a respite from BCR engagement by foreign (LZ-restricted) antigen; in the case of a ubiquitous self antigen or polyreactivity with many self antigens, this respite would not occur leading to negative selection [61]. This process might be one reason why cells trapped in the LZ due to CXCR4-deficiency were outcompeted over time despite showing a relatively intact amount of SHM [6]. Mutation away from self-reactivity to achieve an improvement in foreign antigen binding may contribute to antibody diversification within GCs and may be one reason for the apparent persistence of low affinity B cells in GCs.

Conclusions

Studies discussed in this review provide evidence that GCs often accommodate multiple B cell clones for long periods of time rather than being rapidly dominated by single 'winner' clones. Some of the factors that allow GCs to support affinity-based clonal evolution while also accommodating clonal diversity are coming to light, but much remains to be understood. This includes a need to define more comprehensively how higher affinity cells are selected, including a better understanding of the BCR and T cell inputs involved, how the concurrent presence of variant forms of the antigen and innate stimuli impact the process, and how multiple inputs are integrated and

read-out by the B cell across time. Understanding the extent to which gain and loss of self-antigen recognition shapes the evolving B cell response will also continue to be enlightening. We can anticipate that continued use of multi-color and photoconvertible reporter systems, and antibody cloning, as well as single cell tracking, and single cell sequencing will be among the approaches that help advance our knowledge of this critical phase of the humoral immune response.

Acknowledgements

We thank J. Muppidi, S. Mintz and D. Radtke for critical reading of the manuscript. O. Bannard is a Sir Henry Dale Fellow (Wellcome Trust/Royal Society). J.G. Cyster is an Investigator of the Howard Hughes Medical Institute. This work was supported in part by Wellcome Trust grant 105654/Z/14/Z and by NIH grant AI45073.

Figure Legends

Fig 1. Affinity maturation through programmed clonal expansion and discriminatory selection. High affinity GC B cells gather greater amounts of antigen through their BCRs than their lower affinity neighbors, thereby enabling them to present more peptide to T cells. The provision of more help may drive GC B cells to proliferate faster and more times while in the DZ state (i). LZ selection may in some cases be discriminatory of strength of antigen binding (ii, upper) but in other cases may be permissive to any amount of binding (ii, lower). To what extent T cell help influences affinity maturation via preferential clonal expansion (quality of help) versus discriminatory selection during the LZ-DZ bottleneck (chances of receiving help) is not clear. The contribution of BCR signaling to cell fate decision-making in the GC is not resolved. Certain factors such as antibody feedback, stochastic noise and epitope directed processing might increase the permissiveness of a discriminatory checkpoint.

Fig. 2. Molecular pathways contributing to the regulation of the GC DZ program. Selected LZ GC B cells (LZ*) transiently co-express c-myc and mir-155, enabling cyclic reentry. The switching off of PI3K signaling pathways during the LZ-DZ transition de-represses the activity of transcription factor FoxO1 which contributes to the re-induction of the DZ associated gene expression programs (e.g. Cxcr4). Different quantities or qualities of T cell help may drive GC B cells to complete a number of divisions in the DZ that is proportional to their BCR affinities, thereby preferentially expanding and diversifying the “better” clones. Levels of AP4 are influenced by T cell derived cytokines and contribute to determining the cell division number. While in the DZ state, MHCII turnover is increased in DZ cells as a result of increases in March1 activity, while CD83 increases in LZ cells antagonizes the function of this ubiquitin ligase and helps stabilize peptide-MHCII complexes for improved selection efficiency. Other important factors known to contribute to controlling GC B cell behavior are listed.

Fig. 3. A possible pathway for the "redemption" of self-reactive B cells in GCs. Anergic self-reactive B cells may be efficiently recruited into GCs when their BCRs cross-react with foreign T-dependent antigens, thereby providing them with the opportunity to mutate away from binding self. "Redeemed" variants may be selected for continued participation in GCs even when the somatic mutations that decrease reactivity to self also reduce BCR affinity towards the immunizing antigen. Reductions in self-reactivity might improve a cells ability to capture and present foreign antigens due to them decreasing receptor occupancy (i.e. decreasing the amount of distraction by self), thereby conferring cells with a competitive advantage. The apparent permissiveness of GCs provides "redeemed" but low affinity B cells with the opportunity to further affinity mature towards the immunizing antigen during subsequent rounds of SHM and selection.

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