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What Are the Primary Limitations in B-Cell Affinity Maturation, and How Much Affinity Maturation Can We Drive with Vaccination?

A Role for Antibody Feedback

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We discuss the impact of antibody feedback on affinity maturation of B cells. Competition from epitope-specific antibodies produced earlier during the immune response leads to immune complex formation, which is essential for transport and deposition of antigen onto follicular dendritic cells (FDCs). It also reduces the concentration of free epitopes into the μM to nM range, which is essential for B-cell receptors (BCRs) to sense affinity-dependent changes in binding capacity. Antibody feedback may also induce epitope spreading, leading to a broader selection of epitopes recognized by newly emerging B-cell clones. This may be exploitable, providing ways to manipulate epitope usage induced by vaccination.

GREAT DEBATES

What are the most interesting topics likely to come up over dinner or drinks with your colleagues? Or, more importantly, what are the topics that *don't* come up because they are a little too controversial? In ***Immune Memory and Vaccines: Great Debates***, Editors Rafi Ahmed and Shane Crotty have put together a collection of articles on such questions, written by thought leaders in these fields, with the freedom to talk about the issues as they see fit. This short, innovative format aims to bring a fresh perspective by encouraging authors to be opinionated, focus on what is most interesting and current, and avoid restating introductory material covered in many other reviews.

The Editors posed 13 interesting questions critical for our understanding of vaccines and immune memory to a broad group of experts in the field. In each case, several different perspectives are provided. Note that while each author knew that there were additional scientists addressing the same question, they did not know who these authors were, which ensured the independence of the opinions and perspectives expressed in each article. Our hope is that readers enjoy these articles and that they trigger many more conversations on these important topics.

Editors: Shane Crotty and Rafi Ahmed
Additional Perspectives on Immune Memory and Vaccines: Great Debates available at www.cshperspectives.org

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Antibody-mediated protection from disease is a great success, and public vaccination programs have led to herd immunity against a large number of diseases. Still, we do not understand well how affinity maturation is regulated and how to translate this into good vaccine design. In recent discussions, it has been shown how to induce efficiently neutralizing antibody against antigens with temporal variation such as influenza or HIV. We need to find ways not only to target nonvariable epitopes that hide behind a screen of decoy epitopes that may vary over time (Midgley et al. 2011; Wrammert et al. 2011; Doria-Rose et al. 2014), but also to induce highest affinity antibodies that can efficiently neutralize their targets.

Affinity maturation of antibodies happens in germinal centers (GCs). They are the main source of affinity-matured memory B cells and plasma cells. Understanding the regulation of the evolution of B-cell clones within these structures is key to understanding how to manipulate affinity maturation of B-cell clones, their dominant specificities, and how we might design vaccines that induce high-affinity antibodies to specific antigens or epitopes. B cells in GCs expand and undergo immunoglobulin V(D)J gene hypermutation. These mutations produce variants with altered B-cell receptor (BCR) affinities that are positively selected or die as a result of neglect. This cycle typically is repeated many times. Reproduction, variation, and selection are the hallmarks of Darwinian evolution. Key to a directional evolution (in this case toward higher affinity) is a driver of directional selection. How selection is regulated in the GC has been subject to intense study over recent years. Signals from T follicular helper (T_{FH}) cells are the master regulators of GC responses, directing recirculation of GC B cells into the GC dark zone where proliferation and further hypermutation happen (Crotty 2011; Victora and Nussenzweig 2012). Signals from T_{FH} cells possibly also trigger differentiation of GC B cells into affinity-matured plasma cells or memory B cells. The efficacy of T_{FH} signaling to B cells is dependent on whether GC B cells are able to present sufficient antigenic peptide to the T cells. This has been shown in vitro (Batista and Neuberger 1998), and more recently in a series of studies in vivo (Victora et al. 2010; Victora and Nussenzweig 2012). The importance of T-cell-derived signals is also seen in T-cell-independent GCs that cannot be sustained for more than a few days (Garcia de Vinuesa et al. 2000), and was predicted in theoretical studies (Meyer-Hermann et al. 2006).

ROLE OF ANTIBODY FEEDBACK FOR THE INTERACTION OF B-CELL RECEPTOR WITH ANTIGEN

Key to bringing GC B cells into the position of presenting more antigen than competing B cells is the interaction between BCR and antigen. It is this trait that is the subject of directional evolution. The BCR makes contact with the antigen and delivers antigen into the endocytic pathway. It is also able to transduce signals into the B cell. While BCR signaling is important for the initiation of the endocytic event (Fleire et al. 2006), most GC B cells have "short-circuited" B-cell receptors and the signaling event itself is not involved in affinity-dependent selection in vivo (Khalil et al. 2012). Also, the reaction kinetics of unbinding of higher-affinity BCR from antigen are too slow or BCR signaling too fast to make BCR signaling a meaningful selection mechanism to differentiate between BCRs with different affinities (Foote and Eisen 1995). The faster BCR-binding reaction is limited by the diffusivity of the reactants to an on-rate of $10^6\ M^{-1}\ sec^{-1}$ (Raman et al. 1992), which quickly is reached when antibodies mature to higher affinity. Therefore, on-rate is not relevant for selection of higher-affinity BCR to antigen. Higher-affinity BCRs usually differ in the much slower unbinding reaction, or off-rate (Foote and Eisen 1995; Batista and Neuberger 1998). The off-rate is thought not to fall below $10^{-4}\ sec^{-1}$, because this translates into half-lives for BCR-antigen unbinding longer than the 8.5 min it takes for endocytosis to happen (Foote and Eisen 1995). As soon as it takes more time for the BCR to separate from the antigen than it takes to endocytose the antigen, it seems impossible that BCR interactions can differentiate further increases in affinity.

Therefore, the limit for affinity maturation is seen to have a ceiling of $K_a = 10^{10}$ (Foote and Eisen 1995). In vitro experiments using soluble lysozyme variants with different affinities to BCR showed that variations in B-cell-receptor antigen affinity led to differential antigen presentation and BCR affinity–dependent T-cell activation. These experiments also showed a ceiling beyond which B cells were not able to differentiate BCR affinity for antigen any more (Batista and Neuberger 1998; Guermonprez et al. 1998). Immobilizing antigen onto solid surfaces (as it would be on antigen-holding follicular dendritic cell [FDC]) leads to a larger affinity range (Batista and Neuberger 2000). All studies showing affinity differentiation by the BCR were done with μM to sub-nM concentrations of antigen (Batista and Neuberger 1998, 2000; Guermonprez et al. 1998). These low concentrations are necessary owing to the high affinity of all BCR–antigen interactions. Even for low-affinity BCRs, antigen concentrations above the μM range will lead to saturation of binding of the BCR.

Free antigen is not what B cells encounter in the GC; however, FDCs hold antigen in large tightly packed complexes of antigen bound by specific antibody. This is observed by the very prominent staining when antigen is detected using immunohistology, electron microscopy, or intravital microscopy (Chen et al. 1978; Kosco et al. 1986; Suzuki et al. 2009; Heesters et al. 2014). It was mentioned above that, to sense affinity differences, the concentrations of available antigen have to be in the range of the K_d of the BCRs to be tested (Batista and Neuberger 1998, 2000; Guermonprez et al. 1998). Antibodies present in immune complexes that are specific for the same epitopes as the ones tested by the GC B-cell's BCR are lowering the concentration of free epitopes. These antibodies initially are low-affinity nonmutated antibodies produced during the early extrafollicular plasmablast response or, later on, they may be derived from affinity-matured plasma cells from the GC response (Zhang et al. 2013). Competing antibodies derived from earlier stages of the ongoing response should limit concentration of free epitopes just adequately to allow interactions by higher affinity but not of lower affinity BCR variants (Zhang et al. 2016). This idea of epitope-specific antibody feedback leading to adequate regulation of GC B-cell selection was suggested in the first paper proposing affinity maturation in the GC (MacLennan and Gray 1986), but later forgotten.

WOULD ANTIBODY FEEDBACK INCREASE THE CEILING FOR AFFINITY MATURATION? MAYBE INDIRECTLY

Binding of BCR to specific epitopes is still dependent on the BCR reaction kinetics. The main difference under antibody feedback conditions is that binding of the BCR is slowed down because it now depends on the speed of the unbinding of the antibody covering the epitope.

If BCR binding is considerably delayed because of the presence of antibody feedback, this raises the question of whether the speed of endocytosis is the main factor for a ceiling of affinity-dependent BCR–antigen interactions. This question gains even more relevance as antibodies with $K_a > 10^{10}\ M^{-1}$ have been found, and dissociation rates of $K_{off} < 10^{-5}\ sec^{-1}$ are not impossible (Poulsen et al. 2011).

Is there actually more time available for the B cell to test for BCR binding? The theoretically maximum time available may be the time a GC B cell can survive in the absence of signals from T_{FH} cells. T-cell-independent GCs induced by the nonprocessable antigen nitrophenyl (NP)-Ficoll can last for 3 days until all GC B cells undergo apoptosis (Garcia de Vinuesa et al. 2000). The survival time for GC B cells in vitro that have received BCR stimulation but not T-dependent signals is ~1 day (Liu et al. 1989). Maybe there are processes before or after antigen uptake that give the B cell more time to test binding of BCR with immune complex. Intravital observations have shown that B cells dwell on FDC with antigen for up to 30 min (Suzuki et al. 2009). The same experiments showed that B cells rip off large bits of immune complex from FDC to take it with them. These experiments were also done in the absence of competing epitope-specific antibodies, so whether timings or amounts of antigen re-

trieved are different in the presence of antibody feedback is not known. The benefits of uptake of so much antigen are not clear. B cells seem to have intracellular compartments that preserve antigen (Thaunat et al. 2012). If these compartments contain antigen still bound by epitope-specific antibodies, they may allow longer periods for BCRs to compete with preexisting antibodies. This then would be compatible with dissociation rates $<10^{-5}$ sec^{-1}. The large kinetic delays associated with very low dissociation rates could lead to higher-affinity B cells being able to present sufficient peptide earlier than lower-affinity variants. Future in vivo and in vitro experiments should systematically test the role of competing antibodies for epitope-specific affinity maturation.

A ROLE OF ANTIBODY FEEDBACK FOR EPITOPE SPREADING?

It has been proposed that the enormous increases in serum avidity seen after immunization with complex antigens are more a result of clonal replacement and the generation of specificity variations rather than a result of increases of affinities of individual B-cell clones (Newman et al. 1992; Guermonprez et al. 1998). A further question is whether antibody feedback can be used as a tool to skew epitope usage, guiding responses toward easier accessible unbound epitopes (Brady 2005). Immune complexes have been used in human vaccination: The earliest active vaccines against diphtheria were complexes of diphtheria vaccine with antitoxin (Hajj Hussein et al. 2015). Passive immunization of mice with hyperimmune serum specific for a strain of *Actinobacillus pleuropneumoniae* followed by active immunization with strain variants leads to production of strain-specific unique antibodies (Stenbaek 1995). In simian immunodeficiency virus (SIV) infection models in macaques, passive immunization can skew specificities of developing antisera leading to earlier onset of production of neutralizing antibodies (Haigwood et al. 2004).

It is therefore important to study development of antibody affinities to complex antigens. Hapten-specific responses are useful and can be well analyzed as hapten-specific reagents are available, but it is more difficult to follow the behavior of individual clones. Even in hapten-induced responses, the range of B-cell clones taking part in the response changes over time. The NP-specific response in C57BL/6 mice initially is dominated by clones with canonical V(D)J recombinations (Jacob and Kelsoe 1992). Later, during the response, during recall, or when priming with carrier proteins was done, a broader repertoire of B-cell receptors can emerge, indicating activation of a broader range of B-cell clones specific for different epitopes (see below; Reth et al. 1978; McHeyzer-Williams et al. 1991; Nie et al. 1997).

A recent study by Kuraoka et al. (2016) analyzed responses to two complex antigens, *Bacillus anthracis*–protective antigen and influenza hemagglutinin. This provides insights into the parallel evolution of B-cell clones during the GC response. Analyzing such complex responses carries the caveat that the molecular specificities of individual B-cell clones are not immediately known. B cells may be coevolving in competition for the same antigen, but not necessarily for the same epitope. The study shows that GC B cells taken at specific times after immunization have a large variety of affinities. This is seen as absence of globally acting selection mechanisms such as antibody feedback (Zhang et al. 2013). However, the same study finds a consistent rise in median affinities of B-cell clones, which is compatible with a rise in general selection stringency. Variations in intraclonal affinities are expected to be substantial, as GC differentiation involves random mutation of antibody genes, which continuously produces lower-affinity variants that have to be weeded out over time. More importantly, without definition of the molecular specificities of individual B-cell clones it is possible that many of the different B-cell clones observed in the study bind different antigenic epitopes. Unless there is steric hindrance between neighboring epitope, antibody feedback should act on different epitopes independently, leading to different strengths in competition dependent on the affinity of antibodies that have developed specifically for a particular epitope. Therefore,

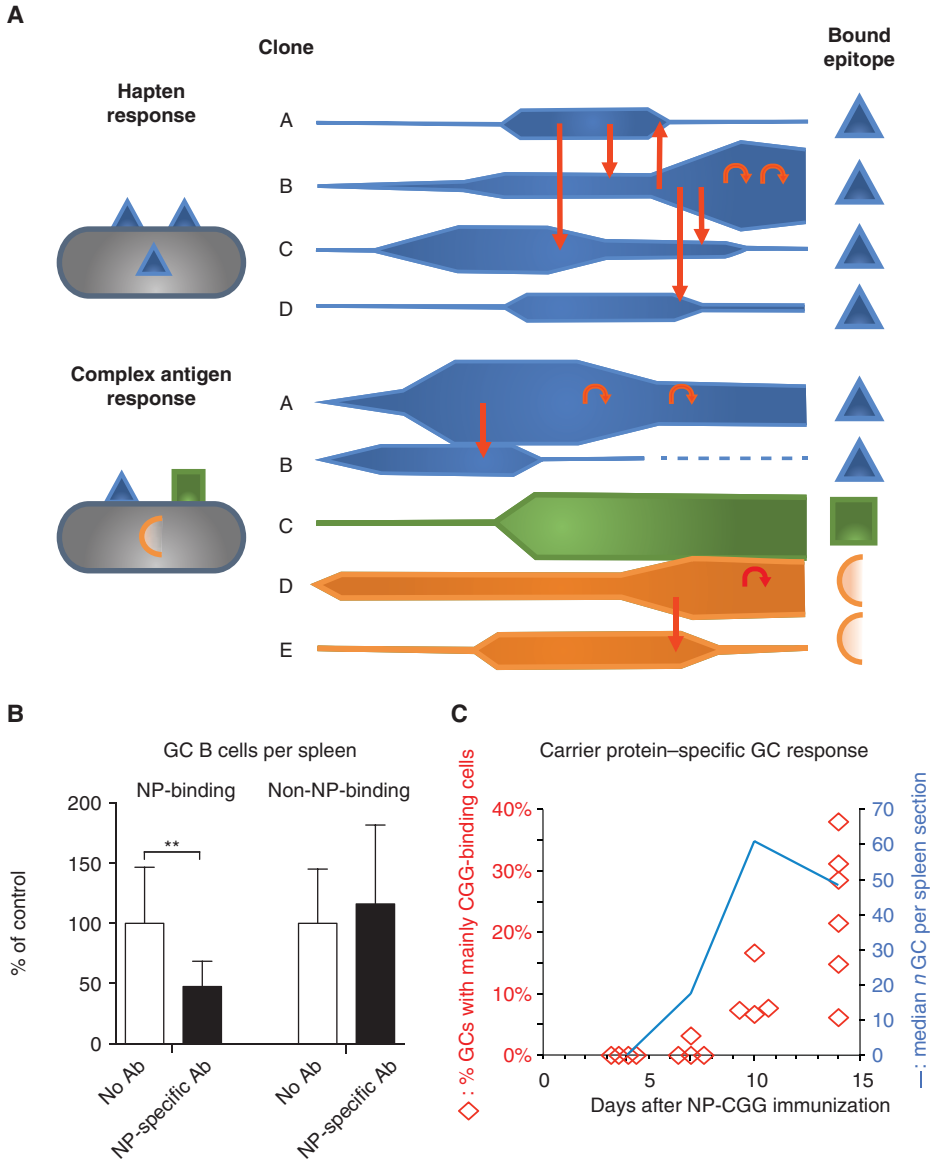

Figure 1. (*A*) Development of clones during a response to a single hapten antigen (*top*) or to a complex protein antigen (*bottom*). Antibodies produced by one clone may regulate the same clone or other clones of lower or similar affinity. Epitope specificities are symbolized by colors. Bar thickness represents clone size. Red arrows symbolize antibody feedback regulation through antibody produced by members of a clone. Affinity is not visually indicated. In the hapten response (*top*) clone A initially has higher affinity than clones C and D. Clone B at a later stage develops even higher affinity and starts to repress clones A, C, D, and finally itself once it reaches the limits of affinity maturation. In the polyclonal example (*bottom*) only clones specific for the same epitopes (symbolized by different colors) are capable of repressing each other. (*B*) Effect of injection of high-affinity anti-nitrophenyl (NP) immunoglobulin M (IgM) on numbers of NP-specific germinal center (GC) B cells and GC B cells not specific to NP. Data taken 24 h after injection. Experimental setup described in Zhang et al. (2013). (*C*) Frequency of carrier protein–specific GCs (diamonds) and number of GCs (line) during a primary splenic response to NP-chicken γ globulin (CGG). Experimental setup described in Toellner et al. (1996). Ab, Antibody.

one would expect large variations in affinity between different clones to different epitopes. Members of the same clone should bind the same epitope, and therefore have less variation of affinity. This fits with observations (Kuraoka et al. 2016).

One surprising result presented by Kuraoka et al. (2016) is that clones underrepresented in GCs at early stages can thrive later on. A recent study on complex antigens by Tas et al. (2016) shows similar clonal bursts that occur at various stages of the response. This may be explainable by antibody feedback. Only clones specific for the same epitope should regulate each other. However, owing to different V(D)J sequences used by different clones specific for an epitope, they may find themselves in different adaptive landscapes (Svensson and Calsbeek 2012). Different sequences require different key mutations that will lead to significant affinity enhancements at different stages of the evolutionary pathway of each clone. Emergence of higher-affinity mutations may lead to expansion of a clone, while plasma cells derived from this clone will suppress other lower-affinity clones specific for that same epitope. In the absence of significant additional affinity gains, antibodies derived from one clone may even suppress the expansion of that same clone (Fig. 1A). This may open options for clones with different specificities to thrive, access antigen, and recruit T-cell help. Indeed, we have seen similar phenomena when experimentally increasing antibody feedback to single epitopes on haptenated proteins. Injection of NP-specific antibody reduced the frequency of cells specific for NP, but left GC B cells with other specificities unaffected (Fig. 1B). At late stages of GC responses to NP-chicken γ globulin (CGG), increasing numbers of GC B cells were specific for other epitopes on the carrier protein (Fig. 1C). The easiest explanation for this is that affinity maturation of NP-specific antibody suppresses other clones specific for the NP epitope, allowing the expansion of slower maturing clones that are specific to other epitopes on the carrier protein.

In summary, future studies should consider not only the specificities and affinities of B cells, but also the presence of competing antibody and its affinity. While earlier studies showed that the affinity of BCR–antigen interactions can translate into affinity-dependent T-cell help, in the absence of competing antibody this had to be done at nM concentrations (Batista and Neuberger 1998; Guermonprez et al. 1998). Antibody feedback may restrict availability of antigen in real-life conditions. Experiments on antigen-induced B-cell activation should consider the presence of antibodies competing for specific epitopes. This requires availability of monoclonal antibodies with well-defined specificities and finely tuned affinity (Zhang et al. 2013). One should also bear in mind that variations in antibody class have additional effects on antibody function. Study of responses to complex antigens as models for naturally occurring antigens is certainly valuable; however, care has to be taken to define not only affinities but also epitope specificities of B-cell clones and antibody development (Newman et al. 1992). Understanding this better may well lead to new ways to use antibodies as a tool not only to increase affinity maturation, but also to guide B-cell responses toward epitope specificities wanted in specific contexts (Midgley et al. 2011; Wrammert et al. 2011; Doria-Rose et al. 2014).

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