Germinal center B cells govern their own fate via antibody feedback

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Efficient long-term protection from infection is mediated by high-affinity antibodies, which can be provoked by foreign structures that stimulate B cells and raise T cell help (Jacobson et al., 1974). The process is initiated by engaging the B cell receptor (BCR) of a few antigen-specific B cells from the vast repertoire created in the bone marrow by random variable region gene recombination. These activated B cells proliferate and within a few days differentiate into plasma cells producing low-avidity early protective antibody (MacLennan et al., 2003; Goodnow et al., 2010). As soon as the first specific antibody is produced, germinal centers (GCs) develop (Jacob et al., 1991a; Liu et al., 1991). In GCs, B cells undergo affinity maturation of their BCR genes over time and will differentiate into longer-lived plasma cells or emerge as memory lymphocytes. Affinity maturation of B cells is an example of Darwinian evolution, as it is comprised of repeated cycles (Kepler and Perelson, 1993) of reproduction (i.e., proliferation; Hanna, 1964) and variation of IgV region genes via hypermutation (Berek et al., 1991; Jacob et al., 1991b)
model of the GC reaction was developed that represents effects of soluble antibody with antibody concentration and affinity that is dependent on GC output. The model included masking of antigen by antibodies (using realistic on–off kinetics) and inhibition of uptake of antigen retained on follicular dendritic cells (FDCs), which impacts follicular T cell help (Meyer-Hermann et al., 2006). Both antibody feedback mechanisms, i.e., masking and retention, were made dependent on the affinity of antibodies produced by GC-derived plasma cells. With these parameters, the simulations revealed that antibody feedback accelerates affinity maturation (Fig. 1 b) and induces a timely end to the GC reaction (Fig. 1 c). To test these predictions, mice deficient in the secreted form of IgM (μs−/− mice; Ehrenstein et al., 1998) were immunized with immune complex (IC) to induce B cell activation and IC localization into B cell follicles. These mice developed GCs and, as predicted in silico, 4-hydroxy-nitrophenyl (NP)–specific IgG was of significantly lower affinity during the early stages of the GC response (Fig. 1 d). As μs−/− mice should still have antibody feedback through IgG, long-term development of GCs was followed in animals completely devoid of soluble antibody (IgHΔ1 mice; Waisman et al., 2007). As predicted in silico, GC responses were longer lived in the complete absence of soluble antibody (Fig. 1 e).

The outcome of these experiments thus motivated us to test in vivo whether there is an affinity-dependent equilibrium of

![Figure 1. Effects of antibody on affinity maturation.](image)

**RESULTS AND DISCUSSION**

To test the hypothesis that antibody feedback impacts the appearance of high-affinity B cell variants, a novel mathematical
antibody inside and outside GCs. Primed C57BL/6 mice (allotype IgMα) were immunized with ICs (IgMα-IC) composed of NP coupled to chicken gamma globulin (CGG) and a nonmutated IgMα antibody with low affinity to NP (clone Fab82, see Materials and methods). Exogenous IgMα-IC localized in the marginal zone and B cell follicles within hours of administration and by 24 h deposited on the FDC networks (Fig. 2 a). Concomitantly, endogenous antibody was displaced from the FDC network (IgMα in Fig. 2 [a and b]). 2 d after immunization, the time when the first NP-specific antibody–producing cells appear (Toellner et al., 1996), the injected antibody started to disappear and endogenous antibody reappeared on the FDC network (Fig. 2, a and b). 5 d after immunization, at the peak of the GC response, exogenous IgMα antibody on FDCs was completely replaced by endogenous antibody (Fig. 2 a, bottom right).

To determine whether antibody replacement is dependent on the interaction between antibody and antigen, we used additional IgMα monoclonal antibodies with affinities higher than clone Fab82. Two sets of IC with antibodies of lower intermediate (IntLow) and higher intermediate (IntHigh) affinities (Fig. 2 c) were created with NP-CGG and injected into primed IgMβ mice. In contrast to the low-affinity clone Fab82 (Fig. 2 a), both intermediate affinity antibodies were detectable in all mice in GCs 5 d after immunization (Fig. 2, d and e). Furthermore, significantly more antibodies were present in GCs of mice that had received the higher-affinity variant (IntHigh; Fig. 2, e [left] and f). Yet the total amount of NP-specific antibody on FDCs was similar whatever antibody had been injected (Fig. 2, d and e, right). These results demonstrate, for the first time, a dynamic process of antibody turnover within GC-localized IC and indicate that the replacement of antibody is affinity dependent.

To test whether antibody produced by plasma cells outside GCs can enter and affect B cell selection in established...
Figure 3. Effects of antibody injection on established GCs. (a) IgM (blue) injected 3 d after immunization with NP-CGG was analyzed 48 h later. IgD is brown, F, follicle; TZ, T zone. (b) Quantitation of IgM levels on FDC networks for IgM injected 3 or 6 d after NP-CGG immunization, analyzed 2 d later (dotted line: background staining level). (c) Apoptotic B cells as indicated by active form of caspase 3. Bars, 25µm. (d) Numbers of NP-binding GC B cells and NP-specific GC B cells staining for Annexin V 24 h after injection of NP-specific high-affinity antibody. (left) Representative FACS plot showing gating
effects of antibody feedback is the mechanism responsible for the effect on output, and affinity (Fig. 3i), supporting the conclusion that effects of antibody on B cell survival, GC volume, plasma cell straining antigen access and uptake by the GC B cells are both can be explained by competition for antigen using our new scheme.

...the affinity of endogenous antigen-specific IgG in substances amounts after higher-affinity endogenous antibodies had been produced (Fig. 3b, d8). Analyzing GC B cell differentiation revealed that when antibodies entered GCs, B cell selection was affected. Assessment of the active form of caspase-3 (Fig. 3c) and Annexin V staining (Fig. 3d) demonstrated increased apoptosis in NP-specific GC B cells. The amount of apoptosis correlated with the affinity of the injected antibody (Fig. 3e). Injection of the IntLow-affinity antibody at early stages had less effect than High-affinity antibody, and it had no effect in mature GCs, supporting the hypothesis that antibody entering GCs competes with GC B cells in an affinity-dependent way. Increased apoptosis substantially reduced GC volumes (Fig. 3a and f). Also, output from GCs was reduced with fewer plasmablasts present in the GC periphery (Fig. 3g). Surprisingly, administration of the antibody variants even affected the affinity of endogenous antigen-specific IgG in blood. High-affinity antibody drove the response quicker to higher-affinity IgG (Fig. 3h), but only when it was given at an early stage. Collectively, these observations demonstrate an increase in B cell selection stringency dependent on the affinity of antibody present inside GC.

We next asked whether these in vivo effects of antibody can be explained by competition for antigen using our new mathematical model. In silico experiments showed that constraining antigen access and uptake by the GC B cells are both necessary and sufficient to replicate the complex in vivo effects of antibody on B cell survival, GC volume, plasma cell output, and affinity (Fig. 3i), supporting the conclusion that antibody feedback is the mechanism responsible for the effects of antibody observed in vivo. Affinity-dependent variation in antigen uptake is an important factor because it determines the fitness of B cells to interact with T cells in silico (not depicted). To test in vivo whether competition from High-affinity antibody leads to reduced B cell–T cell interaction, we analyzed IgG1 heavy chain germline transcription, which is a good indicator of T-dependent B cell activation (Toellner et al., 1996). Indeed, within 6 h, injection of High-affinity antibody led to reduced production of IgG1 heavy chain germline transcripts (Fig. 3j). This confirms that antibody-dependent restriction of antigen access and uptake inhibits downstream T cell–B cell interaction, ultimately leading to death by neglect (Fig. 3j). Upstream processes such as expression of activation-induced cytidine deaminase (AID), essential for hypermutation, were not influenced (Fig. 3j).

Several mechanisms for the preferential selection of affinity-matured GC B cells have been proposed. T cells in GCs help the B cells that have been most efficient in taking up and presenting antigen (Meyer-Hermann et al., 2006; Allen et al., 2007; Victoria et al., 2010). Although BCR ligation has been shown to induce B cell activation and antigen presentation to T cells in an affinity-dependent way, this affinity dependence acts only over a relatively small affinity range (Fleire et al., 2006). As GC B cell affinity evolves, one would expect a mechanism that raises the selection pressure in line with the evolution of B cell clones (Tarlinton and Smith, 2000). During infections, large amounts of antigen are produced over a prolonged time. Therefore, direct competition of B cells for antigen or consumption of antigen is an unlikely mechanism to keep stringent selection pressure over a prolonged response. We show here that, dependent on its affinity, secreted antibody enters GCs and, over time, limits antigen access. As GC B cell affinity evolves, the selection pressure rises, gradually restricting access to and uptake of antigen. This may complement direct affinity-dependent B cell activation (Fleire et al., 2006) and lead to efficient directional selection of B cells over a large affinity range. Indeed, mice deficient in soluble IgM have delayed affinity maturation (Boes et al., 1998; Ehrenstein et al., 1998). This study focused on the analysis of effects of IgM antibodies on affinity maturation. Ig class switching may provide another layer of regulation, reducing antibody avidity once the response has advanced and providing a range of additional signals through the Ig heavy chain (Song et al., 1998; Hjelm et al., 2006).

One of the conundrums of GC biology has been whether responses in different GCs interact. Entry and exit of B cells from GCs have been observed, but these are typically naive B cells (Hauser et al., 2007; Schwickert et al., 2007), and different GCs usually show separate genealogies (Jacob and Kelsoe, 1992). The antibody-dependent selection mechanism demonstrated here makes inter-GC B cell migration dispensable,
as soluble antibody produces a systemic selection threshold. At some stage it can be expected that the restriction of antigen access is too strong to allow for B cell survival, ending the GC reaction. Indeed, in silico experiments predict a natural end to the GC reaction once antibody affinity is sufficiently high (Fig. 1, b and c), and mice without antibody feedback have prolonged GC responses (Fig. 1 d).

Artificially adding exogenous antibody of moderate avidity may be a way to manipulate vaccine responses. In silico modeling indicates that antibodies of low affinity added at the start of a reaction will accelerate the early stages of affinity maturation, and preliminary experiments using IgM in vivo show that this strategy may be effective.

Antibody feedback with a dynamic selection threshold accelerates optimization of Ig variable region genes during the development of an antibody response. Changes in selection pressure caused by consumption of limited resources or ecological changes induced by evolving species have been described as factors shaping evolution (Schoener, 2011). In the case of the GC, B cells produce their own selective environment, bootstrapping themselves by producing a mediator that tunes their own selection and provides adequate selection pressure throughout the entire reaction. This maximizes the speed of evolution of B cell clones systemically, which surely is an evolutionary adaptation to the enormous selection pressure caused by our continuous fight to adapt to new pathogens.

MATERIALS AND METHODS

Mice and immunizations. Specified pathogen–free C57BL/6j mice were primed with 50 μg alum–precipitated CGG mixed with 101 heat-killed Bordetella pertussis and challenged i.v. with 20 μg NP×4-CGG–soluble or complexed for 1 h with 180 μg NP-specific IgM×. NP-specific IgM× was injected i.v. at 90 μg/200 μl saline.

Unprimed soluble IgM-deficient µ−/− mice (Ehrenstein et al., 1998) and soluble IgG-deficient IgH−/− mice (Watzman et al., 2007) were immunized i.p. with NP-CGG complexed with low-affinity NP-specific antibody. All procedures on mice were covered by a project license approved by the UK Home Office.

NP-specific IgM× antibodies. Mice with targeted insertions for NP-specific V-regions of different affinities, QM (Cascalho et al., 1996), B1-8 (gift from M. Reth, Max Planck Institut, Freiburg, Germany; Sonoda et al., 1997), and B1-8high (M.C. Nussenzweig, The Rockefeller University, New York, NY; Shih et al., 2002), were immunized i.p. with NP-Ficoll 4 d before removing the spleens. Spleen cells were fused with NS0 plasmacytoma cells and selected in HAT (hypoxanthine-aminopterin-thymidine) medium. Supernatants from viable clones were screened for NP-specific antibody production and Ig class using ELISA plates coated with NP-JSA. Hybridomas were expanded and grown in hollow fiber bioreactors, and the antibody was purified by affinity chromatography using NP-Sepharose and dialyzed against PBS, pH 7.4. Antibody-binding kinetics were determined by plasmon surface resonance in a Biacore 3000 (GE Healthcare) using NP×4-JSA–coupled chips. 125 nM antibody in PBS, pH 7.4 (38°C), was flown at a flow rate of 5 μl/min for 30 min. This was followed by dissociation at 38°C with PBS. Low affinity (clone Fab82) was gift from F. Gaspar (University of Birmingham, Birmingham, England, UK).

Immunohistology. Spleen sections were prepared and double-stained as described previously (Marshall et al., 2011). The following additional antibodies were used: IgM× FITC (DS-1; BD), IgM× biotin (AF6-78; BD), rabbit anti–mouse active caspase 3 (C92-605; BD), and biotinylated peanut agglutinin (PNA; Vector Laboratories). FITC was detected with a secondary rabbit anti-FITC antiserum (Dako), followed by biotinylated swine anti-rabbit antiserum (Dako) and StreptABComplex/AP as described previously (Marshall et al., 2011). In the final step, color was developed using FastBlue and DAB (3,3′-diaminobenzidine; Sigma-Aldrich). A semiquantitative measurement of the appearance and disappearance of IgM×-IC in GCs was performed using ImageJ (National Institutes of Health). Spleen sections were stained for IgM× or IgM× using FastBlue plus DAB using DAB. To quantify FastBlue precipitate, blue and brown staining were separated using the color deconvolution plugin with inbuilt vectors for FastBlue and DAB. Regions of interest were drawn around areas representing ICs on the FDC network, and mean pixel intensity was determined. Median intensities of IgM× and IgM× staining were quantified from several spleen sections. The method was validated by comparing with staining intensities of parallel sections stained with FITC-labeled antibodies.

For fluorescence staining, biotinylated IgM× was incubated by Cy3-Streptavidin (Stratech). IgM× FITC and IgD Alexa Fluor 488 (ebioscience) were used. The slides were mounted in Prolong Gold antifade mounting medium (Invitrogen). Images were taken on a DM6000 fluorescent microscope (Leica).

ELISA. Serial dilutions of serum samples were analyzed by ELISA on NPe×-BSA–coupled microriter plates to detect NP-specific antibody. NPe×-BSA–coupled microriter plates were used to measure the high-affinity antibody fraction. Relative affinity was calculated by dividing the relative antibody concentration from NPe×-BSA–coupled plates by concentration derived from NP×-coupled plates.

Gene expression in GC cells. Splenic B cells of B1-8 mice (Sonoda et al., 1997) that are Igk deficient (Zou et al., 1993) and express eYFP (gift from J. Camañó, University of Birmingham; Srinivas et al., 2001) were prepared in RPMI1640 medium containing 5% FCS and 10 mM EDTA (Sigma-Aldrich) using CD43-labeled magnetic microbeads (Miltenyi Biotech). 105 B cells were injected i.v. into CGG–primed C57BL/6 mice. Hosts were immunized 24 h later with NP-CGG i.p. 4 d after challenge, 90 μg of High-affinity IgM× (clone 1.197) was injected i.v., and 6 h later, splenocytes were stained using Hoechst 33342, B220 PE-Cy5, Fas PE-Cy7, CD138 APC (BD), and NP-PE. GC B cells were sorted as B220high, eYFP+, IgMa+, Fas+, Annexin V+ as described previously (Marshall et al., 2011); other primers and probes were as described previously (Marshall et al., 2011); other primers and probes were from Applied Biosystems.

Flow cytometry. Splenocytes were stained with B220 FITC, Fas PE-Cy7, CD38 Pacific Blue (BD), and NP-PE. Cy5 Annexin V apoptosis detection kit (BD) was used for staining apoptotic and dead cells. Apoptotic GC B cells were gated as B220+, NP-binding or nonbinding, CD38+, Fas+, Annexin V+, 7-AAD− (Fig. 3 d).

Statistical analysis. All statistical analysis was performed using nonparametrical Wilcoxon Mann–Whitney U Test. Statistics throughout were performed by comparing data obtained from all independent experiments. P-values are indicated throughout with * for P < 0.05, ** for P < 0.01, and *** for P < 0.001.
Produced (or injected) antibodies with affinities orders of magnitude. We used (10 d), antibody affinity to the antigen in the different bins property, the amount of free antigen, by


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