Immune Complex-Mediated Enhancement of Secondary Antibody Responses

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Immune Complex-Mediated Enhancement of Secondary Antibody Responses

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Immunologic memory is a hallmark of the vertebrate immune system. The first antigenic exposure leads to a slow and modest immune response, whereas repeated exposure, even many years later, leads to a rapid and exaggerated response that is two to three orders of magnitude greater than the primary. In the case of humoral immunity, the increased efficacy of recall responses is due to the production of amplified levels of Ag-specific Ab, as well as the accelerated kinetics of their production. Current thinking suggests that this is due to selective activation of long-lived, Ag-specific memory B cells. A downside of restricting secondary responses solely to memory cells is that the repertoire of the memory B cell pool remains static while pathogens continue to evolve. In this study, we propose that during secondary responses, naive Ag-specific B cells participate alongside memory cells. We show that immune complexes formed in vivo between the Ag and pre-existing Abs from the primary response activate these naive B cells, inducing them to respond with accelerated kinetics and increased magnitude. Thus, the continued recruitment of new B cell clones after each antigenic exposure enables the immune system to stay abreast of rapidly changing pathogens. The Journal of Immunology, 2010, 184: 6293–6298.

Unlike primary Ab responses, which occur after an extended lag and are too late to prevent infection, secondary responses occur rapidly after Ag encounter. The current understanding of Ab responses is that Abs formed during the secondary response come from memory B cells that differentiate into secondary plasma cells (1, 2). These cells are activated during the primary response and continue to persist in the host for a lifetime; their expressed Igs have been altered through somatic hypermutation and isotype switching, but still bear the Ag specificity of their progenitors (3, 4). If this were the case, the repertoire of Abs responding to a particular Ag would remain static. The cells activated upon primary exposure would be the source of all subsequent Ab, meaning that Abs seen years later would be virtually identical to those seen early in memory. This would be disadvantageous to the host, especially because the pathogens continue to evolve. Interestingly, Ab repertoires of responding B cells change with time and repeated Ag exposure (5). The Abs responding following a third or fourth Ag exposure are not identical to those responding to a primary or secondary exposure (5). The Abs responding following a third or fourth Ag exposure are not identical to those responding to a primary or secondary exposure (5). The Abs responding following a third or fourth Ag exposure are not identical to those responding to a primary or secondary exposure (5).

Recently, our laboratory has shown that a large portion of the secondary Ab response comes from non-germinal center-derived B cells (11). These cells were Ag-specific, and their expressed IgV regions lacked somatic hypermutation. Whereas a small component of B cell memory comes from extra germinal center B cells (12), these cells are not numerous enough to account for the observed population. One possible explanation for this population is that some or all of these non-germinal center-derived cells are naive B cells that have been activated only upon secondary Ag exposure. If this were true (i.e., that a large portion of secondary Ab responses come from naive cells), it would explain how and why Ab repertoires change upon each subsequent Ag exposure.

During the primary response, naive B cell differentiation and Ab production occur several days after Ag encounter. In contrast, following secondary antigenic exposure, B cells expand with a shortened lag phase and produce larger quantities of Abs. The difference between the primary and secondary exposures is the presence of memory B cells and pre-existing Ag-specific Abs. These Abs can form immune complexes (ICs) with the incoming Ag, and it is known that ICs can induce the production of higher Ab titters than Ag alone (13). One possible mechanism of IC-mediated enhancement is the activation of complement cascade. ICs, particularly those containing the Ab isotypes IgG2a and IgG3, are able to activate the classical complement pathway (14). Because the complement receptor CD21 is part of the B cell coreceptor complex, this could lead to enhanced B cell activation. In addition, ICs are able to bind to a variety of cell types, particularly dendritic cells through FcγRs. For dendritic cells, engagement of FcγRs leads to cell activation, which results in enhanced Ag presentation and increased expression of costimulatory molecules (15). These effects lead to more efficient B and T cell activation.

In this study, we propose that the shortened lag phase and exaggerated Ab production characteristic of secondary responses is due to IC-mediated enhancement of naive and memory Ag-specific B cells. We show that ICs are able to activate naive B cells with accelerated kinetics, that naive B cells are activated early during secondary responses, and that this activation is dependent on activating FcγR engagement.

Materials and Methods

Mice and immunizations

Animals were housed in an American Association of Laboratory Animal Care-accredited facility under specific pathogen-free conditions at the Emory University Vaccine Research Center. We purchased FcγR-/- mice...
ICs stimulate secondary responses through FcγR binding, not complement activation

Having confirmed the potent stimulating capabilities of ICs, we next sought to determine the extent to which ICs participate in the generation of secondary Ab responses, because ICs form naturally in vivo following secondary immunization. To determine the role of ICs in the secondary response, we targeted two of the main activating pathways used by ICs—FcγR binding and complement activation.

ICs exert their potent stimulating effect on a variety of cell types by binding to FcγRs. To determine whether this action plays a role in the generation of secondary Ab responses, we blocked ICs from binding to FcγRs by using CD32-Ig, a recombinant soluble FcγR dimer that binds the Fc regions of ICs with higher avidity than do cell-surface FcγRs (21, 22). We immunized cohorts of B6 mice i.p. with 50 μg NPCG/alum, allowed them to develop to the memory phase (> day 30), and administered CD32-Ig 2 d before a secondary immunization with 20 μg NPCG i.v. Four days after immunization, we harvested the spleens and quantitated them for NP-specific plasma cells using ELISPOT assay. Mice that received CD32-Ig prior to immunization had significantly reduced IgG (p = 0.035) and IgM (p = 0.034) B cell responses compared with mice that received secondary NPCG immunization without the CD32-Ig treatment (Fig. 2A). Thus, blocking the FcγR binding of ICs in vivo resulted in severely diminished secondary immune responses, suggesting that ICs play an essential role in the development of secondary Ab responses through their interactions with FcγRs.

Next, we examined the role of the complement pathway during secondary Ab responses by depleting complement using cobra venom factor (CVF). CVF is a C3b analog that induces uncontrolled activation of the complement cascade, resulting in temporary depletion of all complement proteins (23). We gave NPCG-immune C57B/6 mice three injections of 5 μg CVF prior to administering a secondary immunization of 20 μg NPCG i.v. We bled the mice before and 1 d after CVF treatment and examined the level of complement depletion by ELISA for C3. Mice that received CVF treatments had significantly lower levels of the complement protein C3 (one order of magnitude lower; p < 0.0001) as compared with both their pre-CVF levels and with mice that had not received CVF, confirming successful complement depletion (Fig. 2B). Four days after secondary immunization, we harvested and examined splenocytes for NP-specific Ab-secreting cells by ELISPOT (Fig. 2C). Mice that received CVF prior to secondary immunization had statistically insignificant reductions of both IgG- (p = 0.59) and IgM-producing (p = 0.70) cells.

Results
ICs enhance immune responses in naive animals

Previous reports have identified enhanced immune responses after immunization either with ICs or with specific Ab administered passively prior to immunization (19, 20). To revisit these results, we immunized naive mice with ICs formed from NPCG and a monoclonal anti-NP Ab. Four days after immunization, we examined their sera for anti-CGG Ab levels (Fig. 1A) and splenocytes for the development of NP-specific Ab-secreting cells by ELISPOT (Fig. 1B). Mice receiving ICs had anti-CGG levels of 5416 ± 1484 ng/ml; this was significantly greater (p = 0.0043) than that observed in mice immunized with NPCG/alum (226 ± 52 ng/ml). Similarly, IC-immunized naive mice exhibited significantly greater (p = 0.0006) numbers of NP-specific Ab-forming cells than did those immunized with NPCG/alum. From these data, we conclude that ICs are able to stimulate Ag-inexperienced cells in such a way as to produce a more rapid and efficient immune response than Ag alone.

Adoptive transfers

Single cell spleen suspensions of TcR transgenic OT-II mice (on C57BL/6 background) were RBC-lysed, washed, and resuspended in PBS. Purified TCRβ5.1.52 CD4 T cells (1–2 × 10^6) were administered to Ly5.1 congenic mice in the tail vein 1 d prior to immunization.

ELISA

Serum NP or CGG Ab titers were measured by modification of an ELISA assay. Ninety-six–well microplates were coated with a solution of 20 μg/ml NP-BSA (Biosource Technologies, Navato, CA) or 20 μg/ml CGG (Sigma-Aldrich) in PBS overnight at 4°C. Plates were blocked for 1 h at 37°C using a solution of PBS containing 4% nonfat dry milk (Bio-Rad, Hercules, CA). Serum samples were applied and allowed to react at room temperature for 1.5 h. Anti-mouse Abs with specificity for IgG or IgM isotypes coupled to HRP (Southern Biotech, Birmingham, AL) were applied and allowed to incubate for 1.5 h at room temperature. Plates were developed with tetramethylbenzidine substrate and read at 450 nm absorbance. Values were compared with known dilutions of IgG or IgM to calculate Ab concentrations. An ELISA for complement component C3 was performed as directed using a mouse C3 ELISA kit (Immunology Consultants Laboratory, Newberg, OR).

ELISPOT assay

ELISPOT assays were performed as described (18) with the exception that 96-well nitrocellulose plates (Millipore, Billerica, MA) were coated overnight with 20 μg/ml NP-BSA in 100 μl PBS. Spots were visualized using an ELISPOT reader (Cellulara Technologies, Cleveland, OH) and counted manually.

Statistics

Unpaired, two-tailed Student t test was used to generate all statistical values except where otherwise indicated. For statistical designations: *p* < 0.05; **p** < 0.01; ***p** < 0.001.

Promisc from Taconic Farms (Albany, NY) and C3−/− mice from The Jackson Laboratory (Bar Harbor, ME). All other studies were performed using C57BL/6 mice (Charles River Laboratories, Wilmington, MA). The hapten NP coupled to chicken γ globulin (CGG; Biosearch Technologies, Navato, CA) (NPCG) was used for these studies. Primary immune responses were induced by i.p. injections of 50 μg alum-precipitated NPCG, CGG, or OVA with 250 ng pertussis toxin (List Biological Laboratories, Campbell, CA). Secondary responses were induced by tail-vein injection of 20 μg soluble NPCG. In experiments in which ICs were used for immunization, these complexes were made in vitro by incubating 25 μg NPCG with 50 μg NP-specific mAb (pEVCHy1) at 37°C for 2 h and were administered i.p. For IC blockade experiments, 10 μg each of plasmids encoding the human CD32A-Ig R and H isoform DNA was mixed and given i.v. in 1.6 ml sterile PBS 2 d prior to secondary immunization (16). Human CD32A-Ig is capable of binding mouse IgG subtypes. For depletion of complemen, mice received 5 μg Cobra Venom Factor ( Sigma-Aldrich, St. Louis, MO) i.p. at 28, 24, and 4 h prior to secondary immunization as described (17). Emory University Institutional Animal Care and Use Committee approved all studies.

Previous reports have identified enhanced immune responses after immunization either with ICs or with specific Ab administered passively prior to immunization (19, 20). To revisit these results, we immunized naive mice with ICs formed from NPCG and an anti-NP Ab in vitro (IC) or NPCG precipitated in alum (Alum). Four days after immunization, sera and spleens were harvested for ELISA and ELISPOT assays, respectively. A, The levels of serum anti-CGG Ab in the two groups are shown. Error bars denote mean ± SEM. B, The number of NP-specific Ab-secreting plasma cells per 10^6 splenocytes is plotted. Error bars denote mean ± SEM. Data shown are representative results from two independent experiments (n = 5–6). **p** < 0.01; ***p** < 0.001.
The CVF treatment decreased the level of C3 but did not completely eliminate it (Fig. 2B). To confirm these results in the complete absence of C3, we examined secondary responses in C3-deficient mice (C3−/−). We immunized C3−/− and control wild type mice with 50 μg NPCG/alum and >30 d later gave them a secondary challenge of 20 μg NPCG i.v. Four days later, we harvested splenocytes and tested for NP-specific Ab secreting cells by ELISPOT (Fig. 2D). C3−/− mice exhibited no deficiency in mounting a secondary Ab response; they mounted comparable IgG and IgM Ab-secreting cells per 10^6 splenocytes compared with wild type mice, producing fewer Ag-specific IgG secreting cells in response to repeat antigenic exposure.

Mice that lack the signaling chain of activating FcγRs have diminished secondary Ab responses

FcγRs bind IgG in many forms, including as ICs. Once IgG has bound to these receptors, a signal is sent to the cell through an attached signaling chain. Some FcγRs cause cell activation when IgG ICs are bound. These receptors contain the γ-signaling chain (FcRγ) and are found primarily on APCs (24). To determine whether the secondary Ab response was a product of signaling through an activating FcγR, we examined secondary responses in mice lacking FcRγ−/−. The prediction is that these mice would fail to mount a secondary response. We immunized cohorts of FcRγ−/− and wild type FcRγ+/+ mice with 50 μg NPCG in alum and allowed them to develop immunity (>30 d). We bled these mice and examined their sera for Abs by ELISA to determine whether γ-chain deficient animals could mount a successful primary response. We found that FcRγ−/− mice mount similar IgG (p = 0.10) responses compared with FcRγ+/+ animals (Fig. 3A).

We next immunized these mice with 20 μg soluble NPCG. Four days later, mice were sacrificed and examined for secondary B cell responses by measuring NP-specific Ab-secreting splenocytes by ELISPOT (Fig. 3B). As anticipated, FcRγ−/− had a significantly diminished secondary B cell response (p = 0.01) compared with control wild type FcRγ+/+ mice, producing fewer Ag-specific IgG secreting cells in response to repeat antigenic exposure.

To better understand the kinetics of secondary responses in FcRγ−/− mice, we collected serum at various time-points following secondary immunization and measured Ag-specific IgG Abs by ELISA (Fig. 3C). Control FcRγ+/+ wild type mice developed normal IgG secondary responses, with a rapid increase in Ab levels peaking 8 d after secondary immunization. However, IgG responses in FcRγ−/− mice developed much more slowly, with a modest increase in Ab levels not appearing until day 15 after secondary immunization. Overall, FcRγ−/− mice achieved significantly lower IgG titers. These data show that signaling through the FcR γ-chain, initiated by IC binding, is critical for the rapidity and enhanced magnitude of secondary Ab responses.

ICs stimulate naive CD4 T cell activation

One possible way that ICs could enhance B cell responses is by augmenting CD4 T cell help via increased APC-mediated pHMCHII presentation to CD4 T cells. To determine whether ICs could generate rapid activation and expansion of naive CD4 T cells, we transferred CFSE-labeled Ly5.2+ OVA-specific TCR transgenic OT-II CD4 T cells into congenic Ly5.1 C57BL/6 mice. We then immunized these recipients i.v. with immune-complexed OVA, OVA alone, or PBS. Four days later, we sacrificed the recipient mice and examined the donor Ly5.2+ CD4 T cells for activation and proliferation (Fig. 4A). Activation, as measured by upregulation of
The number of donor CD4+ Ly5.2+ T cells per spleen is diminished with control wild type FcRγ−/− mice were immunized with 50 μg NPCG in alum. Thirty days after immunization, sera were collected and analyzed for Ab levels by ELISA. Results were compared with control wild type FcRγ−/− mice receiving identical treatment. Levels of NP-specific IgG are plotted in nanograms per milliliter, with time relative to secondary immunization denoted on the y-axis. For all experiments error bars denote mean SEM. Data shown are representative of two independent experiments (n = 3–5).

As expected, blocking FcRγR binding with CD32-Ig. prevented naive NP-specific B cell participation in the response (Fig. 5B). As a result, we conclude that naive B cells can be enticed in to secondary responses via IC-mediated activation.

Memory CD4 T cells alone are not sufficient to enhance the Ab response through naive cell activation

In the above experiment (Fig. 5B), we showed that CGG-immune mice exhibited enhanced primary responses to NP upon immunization with NPCG. We attribute this enhancement to the presence of CGG-specific Abs. However, CGG-primed T cells are also present and capable of helping newly activated B cells. To observe the effect of CGG-primed T cells alone on the Ab response, we isolated 5 × 10^6 CD4+ T cells from CGG-immune B6 mice and adoptively transferred them into individual naive B6 mice. One day following transfer, we immunized these mice with 20 μg NPCG i.v., and 4 d after immunization we examined splenocytes by ELISPOT for NP-specific Ab producing cells (Fig. 6). Compared with mice receiving naive T cells prior to immunization, the mice exhibited enhanced primary responses to NP upon immunization with NPCG. As anticipated, no more NP-specific B cells than a normal primary immunization were generated in this cohort. This finding shows that the presence of ICs, generated with CGG-specific Abs, is essential for the recruitment of naive B cells in the secondary response. Unlike a normal secondary response, more of the responding NP-specific cells expressed IgM rather than IgG. Because all NP-specific cells must be naive and will have not yet undergone germinal center reactions, this is unsurprising. To confirm that this NP-specific naive B cell activation requires ICs, we blocked FcRγR binding with CD32-Ig.

A B C

FIGURE 3. Mice that lack the signaling chain of activating FcγRs have diminished secondary Ab responses. A, FcRγ−/− mice were immunized with 50 μg NPCG in alum. Thirty days after immunization, sera were collected and analyzed for Ab levels by ELISA. Results were compared with control wild type FcRγ−/− mice receiving identical treatment. Levels of NP-specific IgG are plotted in nanograms per milliliter. B, NPCG-immune mice were given a secondary immunization with 20 μg NPCG i.v. Four days after immunization, splenocytes were harvested and examined ex vivo by ELISPOT. The number of NP-specific IgG-secreting cells per 10^6 splenocytes is plotted. C, Serum was collected at various time points before and after secondary immunization and analyzed for NP-specific Ab levels by ELISA. Levels of NP-specific IgG are plotted in nanograms per milliliter, with time relative to secondary immunization denoted on the x-axis. For all experiments error bars denote mean ± SEM. Data are representative of two independent experiments (n = 4–6). *p < 0.05.

FIGURE 4. ICs stimulate naive CD4 T cell activation. CFSE-labeled, naive Ly5.2+ OT-II TcR transgenic CD4 T cells were adoptively transferred into naive Ly5.1+ mice. These mice were then immunized with OVA-IC, OVA-only, or PBS as a negative control. Four days after immunization Ly5.2+ donor CD4 T cells were examined by flow cytometry. A, Cell division as indicated by CFSE dilution and the expression of activation marker CD44 are shown. Columns represent different Ag treatments. B, The total number of donor CD4+ Ly5.2+ T cells per spleen is plotted. Error bars denote mean ± SEM. Data shown are representative results from two independent experiments (n = 3–5). **p < 0.01; ***p < 0.001.

controls (Fig. 4B). These data suggest that ICs can enhance activation and proliferation of naive Ag-specific CD4 T cells in vivo.

Naive B cells participate in secondary Ab responses

In a previous report we showed that naive B cells play a major role in secondary Ab responses (11). Having shown that ICs have powerful activating effects on both naive B cells and the secondary Ab response, we wanted to determine to what extent ICs stimulated naive cell participation during the secondary response. To demonstrate the ability of naive cells to respond in the environment of a secondary response, we immunized mice with CGG, allowed immunity to develop, and then immunized with NPCG. Prior to secondary immunization, these mice contained CGG-specific memory B cells and plasma cells producing anti-CGG Abs, but were completely naive to NP (Fig. 5A). However, after immunization with NPCG, the mice had a rapid anti-NP response, with large numbers of NP-specific Ab-secreting cells responding, as detected by ELISPOT 4 d postimmunization. The resulting response resulted in a greater number of cells responding much more rapidly, and it more closely resembled a secondary NP response than a primary response (Fig. 5B). To show that the enhancement is dependent on Ag specificity of the pre-existing Abs, we immunized OVA-immune mice with NPCG. As anticipated, no more NP-specific B cells than a normal primary immunization were generated in this cohort. This finding shows that the presence of ICs, generated with CGG-specific Abs, is essential for the recruitment of naive B cells in the secondary response. Unlike a normal secondary response, more of the responding NP-specific cells expressed IgM rather than IgG. Because all NP-specific cells must be naive and will have not yet undergone germinal center reactions, this is unsurprising. To confirm that this NP-specific naive B cell activation requires ICs, we blocked FcRγ binding with CD32-Ig. As expected, blocking FcRγ binding prevented naive NP-specific B cell participation in the response (Fig. 5B). As a result, we conclude that naive B cells can be enticed in to secondary responses via IC-mediated activation.
receiving CGG-primed T cells had no increase in IgM responses ($p = 0.8101$). Interestingly IgG responses were significantly increased ($p = 0.0338$). This is most likely due to enhanced class switching in the presence of memory CD4 T cells. From these data we conclude that, whereas the presence of primed CD4 T cells can enhance Ab responses modestly, they alone are not sufficient to induce an intense secondary-like Ab response.

**Discussion**

In this study, we provide evidence that the long-accepted model for secondary Ab responses lacks a key component—the participation of naive B cells. These cells are activated by ICs that are formed upon secondary Ag exposure, causing rapid and robust Ab production. This activation is dependent on the ability of ICs to bind to FcγRs, though the role of complement appears to be minimal. In addition, we show that mice lacking the γ-signaling chain of FcRs are unable to mount a secondary response to immunization, despite mounting a successful primary response.

The ability of ICs to induce potent humoral immune responses has long been known. A series of early experiments (13, 25–27) demonstrated the activating capacity of these molecules, finding them able to enhance Ab production, generate germinal centers, and stimulate memory formation with increased kinetics. Furthermore, it has been shown that passively administered Ab can provide the same effect as ICs—enhancing Ab production and cellular activity (28). Our results confirm these reports and extend the findings to include stimulating secondary Ab responses as a function of ICs.

Because ICs are able to activate naive cells with enhanced kinetics, and because ICs are present after secondary Ag encounter, it seems likely that ICs activate naive cells to participate in secondary responses. Previous studies, from our laboratory and others, have suggested that the secondary Ab response is not an entirely memory-based phenomenon. Using a mouse model in which germinal center-derived B cells were permanently marked with YFP, we found that unmarked naive B cells account for almost two thirds of the secondary Ab response (11). Experiments using adoptive transfer have also shown that naive B cells are recruited during memory responses (29, 30). In this study, we have shown that naive B cells can and do participate in secondary responses, and that Ag-specific Ab generated after primary Ag exposure is required for this participation. In our experiments, CGG-immune mice have the necessary cellular and humoral environment to stimulate a secondary response to CGG, but they lack memory B cells and Abs specific for NP. However, when given a secondary immunization of NP coupled to CGG, these mice develop a strong NP-specific response. Because these mice have no previous immunity to NP, all responding cells must be naive and all NP-specific Abs must be derived from naive cells. In other words, in the environment of a secondary response, naive B cells are activated and respond as quickly and in as great a number as memory cells.

Current understanding of naive B cell activation does not account for the rapidity of a secondary response. However, activation by membrane-bound ICs incorporates the BCR into a synapse of integrins and coreceptors that lower the signaling threshold required for B cell activation and result in more rapid Ab responses (31). Either FcγRs or complement could anchor these ICs to the cell surface, and whereas complement can also be involved in B cell costimulation through CD21, our results suggest that it is FcγRs rather than complement that give ICs their functionality in secondary Ab responses. This finding is supported by work showing that the stimulating effect of ICs is dependent on activating FcγRs (32), but does not require complement (33). In addition, it has been shown that FcγR-bound ICs can remain intact in their native form on the cell surface and, in that manner, be presented to B cells (34). That the secondary response is entirely dependent on FcγRs seems curious, as even in the absence of naive cell activation, memory and long-lived plasma cells are still present to respond to antigenic challenge. This suggests that either memory B cell activation also depends on IC interaction or perhaps memory cells are not a major component of protective immunity, as has been previously suggested (35).

Although FcγRs are present on a number of cell types, our results suggest that FcγRs using the γ-signaling chain are necessary for IC...
enrichment of secondary responses. As these activating FcγRs are not found on B cells (24), an intermediary cell must be involved in their activation. The most likely candidates are APCs, because these cells possess the necessary FcγRs and the ability to activate T cells. The identity of the APCs involved in this process warrants further investigation.

The current paradigm in humoral immunity is that immunologic memory is responsible for protective immunity, as demonstrated by robust Ab response upon repeated Ag exposure. However, we show that naive B cells are also active participants in the secondary response, and their recruitment relies on activating FcγRs and pre-existing serum Abs raised during primary Ag exposure. These data reveal a previously underappreciated positive feedback mechanism, mediated by Ag-specific serum Ab, which operates between the primary and secondary immune responses. This mechanism both accelerates secondary Ab responses via rapid CD4 T cell activation and diversifies the Ab repertoire through the recruitment of novel B cell clonotypes. Thus, the immune system retains high-affinity memory B cell responses while actively recruiting new B cell clones, thereby continuously diversifying the Ab response yet ensuring that high-affinity Abs are produced upon each Ag exposure. This process enables the immune system to stay abreast of rapidly changing pathogens.

Disclosures
The authors have no financial conflicts of interest.

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