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Author/s:
Phan, TG;Paus, D;Chan, TD;Turner, ML;Nutt, SL;Basten, A;Brink, R

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High affinity germinal center B cells are actively selected into the plasma cell compartment

Tri Giang Phan,[1,2] Didrik Paus,[1,2] Tyani D. Chan,[1,2] Marian L. Turner,[3] Stephen L. Nutt,[3] Antony Basten,[1,2] and Robert Brink[1,2]

[1]Centenary Institute of Cancer Medicine and Cell Biology, Newtown NSW 2042, Australia
[2]Garvan Institute of Medical Research, Darlinghurst NSW 2010, Australia
[3]Walter and Eliza Hall Institute for Medical Research, Parkville VIC 3050, Australia

A hallmark of T cell–dependent immune responses is the progressive increase in the ability of serum antibodies to bind antigen and provide immune protection. Affinity maturation of the antibody response is thought to be connected with the preferential survival of germinal centre (GC) B cells that have acquired increased affinity for antigen via somatic hypermutation of their immunoglobulin genes. However, the mechanisms that drive affinity maturation remain obscure because of the difficulty in tracking the affinity-based selection of GC B cells and their differentiation into plasma cells. We describe a powerful new model that allows these processes to be followed as they occur in vivo. In contrast to evidence from in vitro systems, responding GC B cells do not undergo plasma cell differentiation stochastically. Rather, only GC B cells that have acquired high affinity for the immunizing antigen form plasma cells. Affinity maturation is therefore driven by a tightly controlled mechanism that ensures only antibodies with the greatest possibility of neutralizing foreign antigen are produced. Because the body can sustain only limited numbers of plasma cells, this "quality control" over plasma cell differentiation is likely critical for establishing effective humoral immunity.

CORRESPONDENCE
Robert Brink:
r.brink@garvan.org.au.

A major weapon used by the immune system to combat infection is the secretion of antibody molecules into bodily fluids. Antibodies, which bind to and eliminate foreign antigens, represent soluble versions of the cell surface Ig proteins that act as the B cell receptor for antigen (BCR). More than 70 yr ago, antibodies were found to alter their antigen-binding properties over the course of an immune response (1). The term "maturation of the immune response" was subsequently coined to describe the increase in antibody affinity that is now recognized to be a defining characteristic of T cell–dependent (TD) humoral immune responses (2).

To secrete antibody, antigen-activated B cells must first differentiate into plasma cells. During TD immune responses, plasma cells are initially produced in transient extrafollicular proliferative foci (3) but are subsequently derived from B cells participating in the follicular germinal center (GC) reaction (4, 5). Evidence that GCs might be connected with maturation of the serum antibody response was provided by the discovery that somatic hypermutation (SHM) of Ig genes occurs in GCs (6) and that rare mutant clones expressing BCRs with increased affinity for the immunizing antigen preferentially survive there (4, 7). Nevertheless, the precise factors that cause GC B cells to differentiate into plasma cells and, thus, drive affinity maturation of the antibody response remain unclear (8). In vitro experiments have suggested that stochastic or nonselective mechanisms are of primary importance in the regulation of plasma cell differentiation (9). On the other hand, indirect evidence suggests that plasma cell differentiation of GC B cells may be more selective, with only those cells that exceed a threshold antigen affinity contributing to the antibody response (10, 11). Distinguishing between these two possibilities has proven difficult because current experimental models do not allow affinity-based selection and plasma cell differentiation of GC B cells to be comprehensively tracked in vivo.

T.G. Phan and D. Paus contributed equally to this work.
T.G. Phan's present address is Dept. of Microbiology and Immunology, University of California, San Francisco, San Francisco, CA 94143.
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The SW_{HEL} Ig knock-in mouse model was developed to analyze TD B cell responses to the protein antigen hen egg lysozyme (HEL) conjugated to the sheep RBC (SRBC) carrier (12). SW_{HEL} B cells express the anti-HEL BCR encoded by the high affinity mAb HyHEL10 and can undergo both class switch recombination and SHM (13). Adoptive transfer of small numbers of SW_{HEL} B cells into CD45.1 congenic recipients and challenge with HEL-SRBC results in a typical TD immune response dominated by secretion of IgG1 antibodies derived from donor SW_{HEL} B cells (12). Responding SW_{HEL} B cells can be tracked with precision by virtue of their expression of the anti-HEL BCR and the CD45.2 allotypic marker. The recombinant mutant HEL protein (HEL^{3X}) binds HyHEL10 with >10,000-fold lower affinity than wild-type HEL (HEL^{WT}) (14). HEL^{3X}-SRBC triggers migration of responding SW_{HEL} B cells into GCs but is ineffective at eliciting an extrafollicular plasma cell response (14). In this report we exploit the low affinity of HEL^{3X} to develop a system in which the affinity-based selection of GC B cells and their differentiation into plasma cells can be followed. This approach revealed that affinity maturation of TD antibody responses is driven by a mechanism that permits only GC B cells that have acquired high affinity for antigen to differentiate into plasma cells.

RESULTS AND DISCUSSION

When SW_{HEL} B cells are challenged with either high affinity (HEL^{WT}-SRBC) or low affinity (HEL^{3X}-SRBC) antigen in CD45.1 congenic recipient mice, similar frequencies of donor-derived ($CD45.2^+$) GC B cells are produced at over the first 15 d of the response (14), and these cells undergo equivalent rates of class switch recombination to IgG1 (Fig. 1 A). The extent of SHM measured during the early stages of

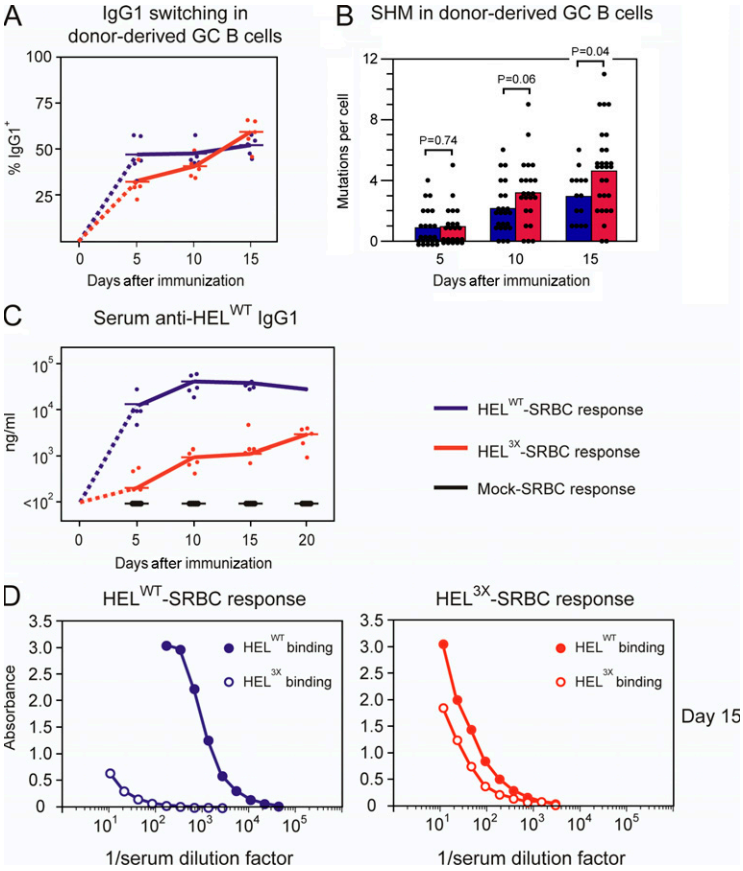


Figure 1. GC and antibody responses to low and high affinity antigens. SW_{HEL} B cells were adoptively transferred into CD45.1 congenic mice and challenged with HEL^{WT}-SRBC, HEL^{3X}-SRBC, or mock antigen. (A) Splenocytes from recipient mice were analyzed by flow cytometry for expression of CD45.2, IgG1, and the ability to bind HEL^{WT}. The percentage of donor-derived HEL^{WT}-binding GC B cells that had switched to IgG1 was determined and plotted over the course of the response. Data points represent individual recipients, and lines connect the means. (B) Splenocytes from recipient mice ($n = 3$) were pooled and stained with CD45.2, syndecan-1, and HEL^{WT}. Single $CD45.2^+$, HEL^{WT}-binding, GC (syndecan-1^-) B cells were sorted, and the targeted Ig heavy chain variable gene was PCR amplified and sequenced. Points represent the frequency of somatic mutations in individual clones (integers). A statistically significant difference in mean mutation frequency per clone (bars) was detected at day 15 and appeared close to significant at day 10 but was not significant at day 5. (C) Sera were collected from recipient mice, and the concentration of anti-HEL^{WT} IgG1 antibodies was measured by ELISA. Data points represent individual recipients, and lines connect the means. (D) Affinity maturation of the IgG1 antibody response to HEL^{3X}-SRBC. IgG1 antibodies in pooled sera from recipient mice ($n = 5$) on day 15 of the responses to HEL^{WT}-SRBC and HEL^{3X}-SRBC were analyzed for their ability to bind HEL^{WT} and HEL^{3X} in parallel ELISAs.

the GC response (day 5) also does not differ (Fig. 1 B). However, as the responses progress, GC B cells responding to the lower affinity HEL^{3X}-SRBC accumulate somatic mutations faster and by day 15 contain significantly more mutations per Ig heavy chain variable region gene than GC B cells responding to HEL^{WT}-SRBC (Fig. 1 B). These observations confirm previous analyses of TD antihapten responses showing similar rates of SHM when initial antigen affinity is high or low but enhanced selection for mutated variable regions in B cells with low initial antigen affinity (15).

SW_{HEL} B cells challenged with HEL^{3X}-SRBC do not produce the burst of extrafollicular plasma cells that typically peaks around day 5 of responses to higher affinity antigens such as HEL^{WT}-SRBC (14). As a result, the levels of both total anti-HEL antibody (14) and anti-HEL IgG1 (Fig. 1 C) present at day 5 are ~100-fold lower when SW_{HEL} B cells are challenged with HEL^{3X}-SRBC compared with HEL^{WT}-SRBC. Nevertheless, the concentration of anti-HEL IgG1 in recipient serum increases progressively from days 5 to 20 of the response to HEL^{3X}-SRBC (Fig. 1 C). This antibody is derived from SW_{HEL} donor B cells, because it is not detected in recipients receiving HEL^{3X}-SRBC alone (unpublished data). To examine whether the antibodies elicited in response to HEL^{3X} undergo affinity maturation, serum anti-HEL IgG1 present at day 15 of the two responses was tested by ELISA for binding to HEL^{WT} and HEL^{3X}. As expected, the IgG1 produced in response to HEL^{WT}-SRBC bound efficiently to HEL^{WT} but showed negligible binding to HEL^{3X} (Fig. 1 D). In contrast, the serum anti-HEL IgG1 from recipients challenged with HEL^{3X}-SRBC showed almost equivalent binding to HEL^{WT} and HEL^{3X} (Fig. 1 D), indicating that affinity maturation to HEL^{3X} had indeed occurred.

To track the appearance and ultimate fate of GC B cells acquiring increased affinity for HEL^{3X}, donor-derived B cells within the spleens of mice immunized with HEL^{3X}-SRBC were stained directly with fluorescently labeled HEL^{3X} at days 5, 10, and 15 of the response (Fig. 2). Analysis of $CD45.2^+$ donor cells revealed a progressive increase in HEL^{3X} binding in mice challenged with HEL^{3X}-SRBC but not HEL^{WT}-SRBC (Fig. 2). When HEL^{3X} binding was counterstained for surface IgG1 so that HEL^{3X} binding by $IgG1^+$ responders could be visualized independently of BCR density, a distinct population of $IgG1^+$ B cells with uniformly increased affinity for HEL^{3X} was evident by day 10 of the HEL^{3X}-SRBC response and subsequently dominated the $IgG1^+$ donor population by day 15 (Fig. 2). This population was not apparent at any stage of the response to HEL^{WT}-SRBC (Fig. 2), indicating that the cells were specifically selected because of their increased affinity for HEL^{3X}.

To determine whether B cells with high affinity for HEL^{3X} carry specific somatic mutations that increase their affinity for HEL^{3X}, single donor-derived GC B cells were sorted from immunized mice, and their Ig heavy chain variable region genes were sequenced. By day 15 of the response to HEL^{3X}-SRBC, 82% (23 out of 28) of the clones analyzed carried a specific point mutation in the tyrosine 53 codon encoding its substitution with aspartate ($Y53D_{HyHEL10}$; Fig. 3 A). No selection of any heavy chain mutation was evident in day 15 GC B cells produced in response to HEL^{WT}-SRBC (Fig. 3 A), which was consistent with the proposition that the affinity of the HEL^{WT}–HyHEL10 interaction is too high to permit further affinity maturation (16). None of the 24 clones analyzed from the day 15 HEL^{WT}-SRBC GC response contained a mutation in the $Y53_{HyHEL10}$ codon, indicating that the $Y53D_{HyHEL10}$ mutation is selected specifically in response to HEL^{3X}-SRBC and is therefore likely to increase the affinity of HyHEL10 for HEL^{3X}. This was confirmed by sorting high affinity anti-HEL^{3X} $IgG1^+$ donor B cells (see gate in Fig. 2), as subsequent sequence analysis revealed that 96% (23 out of 24) of these clones carried the $Y53D_{HyHEL10}$ mutation (unpublished data).

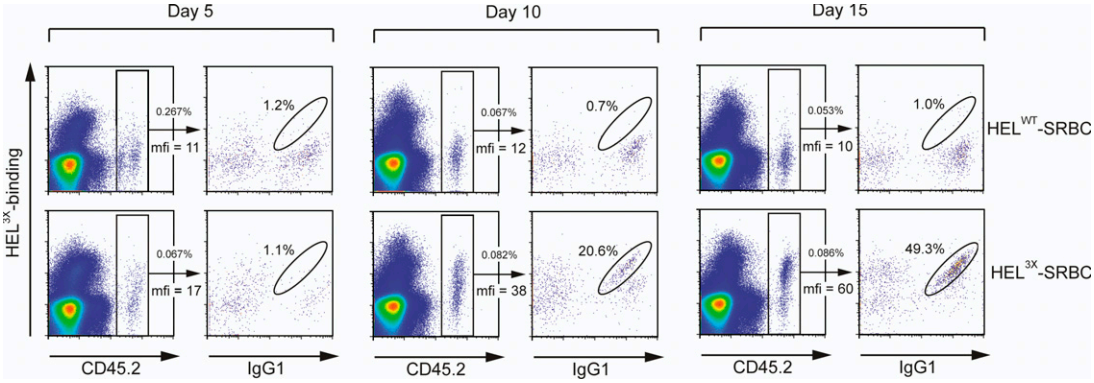


Figure 2. Tracking affinity-based selection in vivo. Splenocytes from the response shown in Fig. 1 were analyzed by flow cytometry for their expression of CD45.2, IgG1, and the ability to bind the mutant antigen HEL^{3X}, and the analogue data are presented on logarithmic axes. The frequency of donor-derived ($CD45.2^+$) cells as a proportion of total splenocytes and the mean fluorescence intensity (mfi) of HEL^{3X} binding are shown. $CD45.2^+$ cells were also analyzed for HEL^{3X} binding counterstained against IgG1 (to correct for surface Ig expression level) to show a population of high affinity HEL^{3X}-binding $IgG1^+$ cells that emerges on day 10 and dominates the response to HEL^{3X}-SRBC by day 15 (oval gate). The frequency of these high affinity cells as a proportion of donor-derived cells is shown. Concatenated data are representative of five mice in each group.

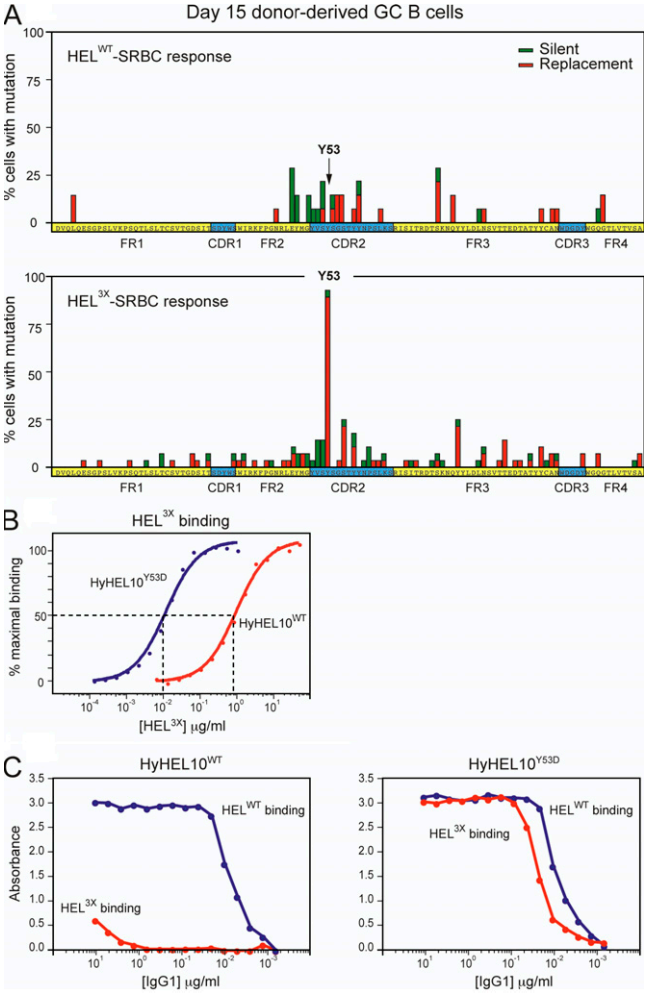


Figure 3. Selection of a high affinity Y53D somatic mutation in response to HEL^{3X}-SRBC. (A) SHM analysis of targeted Ig heavy chain loci of responding SW_{HEL} GC B cells was performed on day 15 of the responses to HEL^{WT}-SRBC and HEL^{3X}-SRBC, as described in Materials and methods. The codon encoding Y53 was found to be extensively mutated in the HEL^{3X}-SRBC response (86% of sequences) and replaced by an aspartate residue (Y53D) in the majority (82%) of cases. (B) ELISA showing the binding of soluble HEL^{3X} to plate-bound recombinant wild-type (HyHEL10^{WT}) and Y53D-mutated (HyHEL10^{Y53D}) HyHEL10 IgG1 antibodies. The Y53D mutation increased the affinity of HyHEL10 for HEL^{3X} by ~85-fold. (C) ELISAs showing the relative binding of soluble recombinant HyHEL10^{WT} and HyHEL10^{Y53D} antibodies to plate-bound HEL^{WT} versus HEL^{3X}. All four binding assays were run in parallel.

Analysis of the binding of HEL^{3X} to recombinant wild-type and Y53D-mutated HyHEL10 IgG1 antibodies showed that the $Y53D_{HyHEL10}$ mutation increases the affinity of HyHEL10 for HEL^{3X} by ~85-fold (Fig. 3 B). This affinity increase was also evident from the ability of the mutated form of HyHEL10 to recognize plate-bound HEL^{3X} efficiently in ELISA under conditions in which binding of wild-type HyHEL10 to HEL^{3X} was virtually undetectable (Fig. 3 C). Computer modeling revealed that the arginine side chain introduced at position 101 of HEL ($D101R_{HEL}$) to produce HEL^{3X} (14) is likely to cause a major steric conflict with the phenol group of $Y53_{HyHEL10}$ and that this conflict is resolved by the $Y53D_{HyHEL10}$ substitution (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20061254/DC1).

To examine the selection of the $Y53D_{HyHEL10}$ mutation over the course of the response to HEL^{3X}-SRBC, Ig heavy chain gene sequence analysis of single GC B cells and plasma cells was performed on days 5, 10, and 15. On day 5, the $Y53D_{HyHEL10}$ mutation was not detectable in either the GC or the small plasma cell compartment (Fig. 4 A), which was consistent with the absence of high affinity anti-HEL^{3X} B cells at this time point (Fig. 2). By day 10, however, this mutation was detectable in some GC B cells (22% of sequences) but was already present in the great majority of splenic plasma cells (86% of sequences). Similar domination of the GC B cell population by this mutation was not evident until day 15 (Fig. 4 A). Because hypermutated plasma cells must have derived from GC B cell precursors, this result shows that GC B cells generated in response to HEL^{3X}-SRBC do not undergo stochastic differentiation into plasma cells but instead differentiate upon acquisition of the high-affinity $Y53D_{HyHEL10}$ mutation. Accordingly, Y53D-mutated clones that had left the GC and differentiated into plasma cells by day 10 of the response had a significantly lower overall rate of SHM than the Y53D-mutated clones that remained within the GC compartment (2.2 vs. 4.6 mutations/clone, respectively; $P = 0.02$).

To monitor affinity-based regulation of post-GC plasma cell differentiation more directly, we next challenged donor B cells from $SW_{HEL} \times Blimp^{gfp/+}$ mice with HEL^{3X}-SRBC. Plasma cells generated in $Blimp^{gfp/+}$ mice express GFP under the control of the *Blimp-1* promoter and, thus, can be detected via intrinsic green fluorescence (17). By using $SW_{HEL} \times Blimp^{gfp/+}$ donor B cells in conjunction with our method for identifying somatically mutated B cells with high affinity for HEL^{3X} (Fig. 2), we directly assessed the antigen affinity of post-GC IgG1$^+$ plasma cells in the spleen. Analysis of GFP expression by low and high affinity donor-derived IgG1$^+$ B cells on day 10 of the response to HEL^{3X}-SRBC clearly demonstrated that GFP-expressing plasma cells originated almost exclusively from GC precursors that had acquired a high affinity anti-HEL^{3X} BCR (2.54% of high affinity compared with 0.27% of low affinity cells; Fig. 4 B). These GFP$^+$ cells also had low but detectable levels of surface CD45.2 and IgG1 (Fig. 4 B and not depicted), as is typical of plasma cells (14). Because >95% of high affinity clones detected by HEL^{3X} binding have the $Y53D_{HyHEL10}$ mutation, most of these GFP$^+$ plasma cells are likely to secrete antibodies carrying this amino acid change. Indeed, the serum IgG1 found in mice challenged with HEL^{3X}-SRBC shows almost identical antigen-binding characteristics to Y53D-mutated HyHEL10 (Fig. 1 D and Fig. 3 C).

The model system described in this report provides a unique window into the GC reaction by allowing the appearance, selection, and differentiation of high affinity somatically mutated B cells to be followed throughout the response. We have used this system to demonstrate that high affinity B cell specificities generated within the GC are harnessed to drive affinity maturation of the antibody response

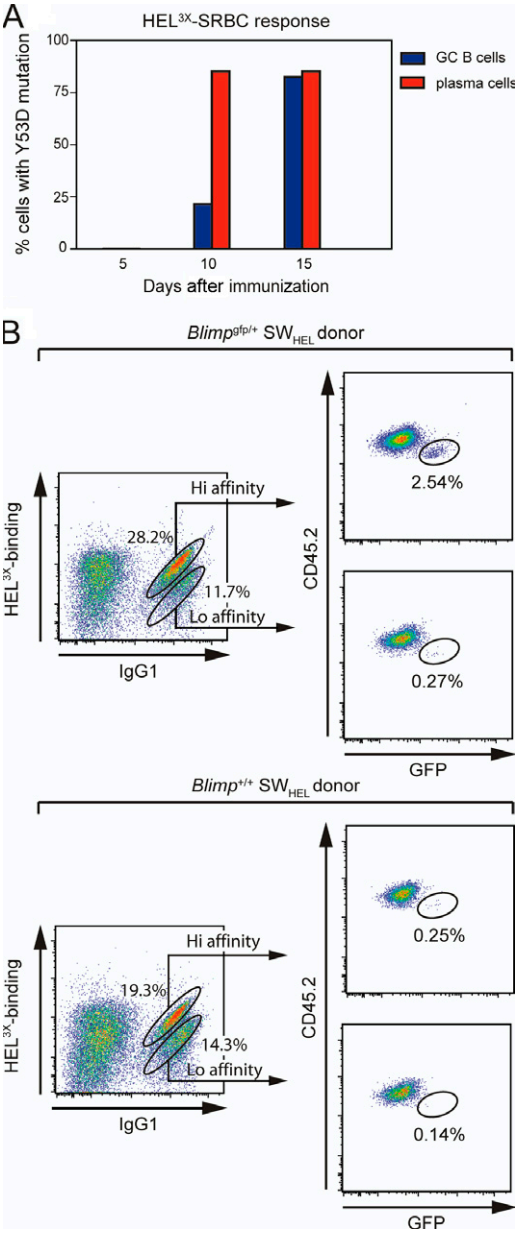


Figure 4. Affinity threshold for post-GC plasma cell differentiation. (A) SW_{HEL} B cells were adoptively transferred and challenged with HEL^{3X}-SRBC, and individual donor-derived GC B cells and plasma cells were sorted for SHM analysis, as described in Materials and methods. The proportion of donor-derived GC B cells and plasma cells with the $Y53D_{HyHEL10}$ mutation on days 5, 10, and 15 of the response are shown. From left to right, SHM frequencies were 28, 1, 98, 61, 137, and 157 mutations per 10^4 bp. (B) SW_{HEL} B cells with (SW_{HEL} × *Blimp*$^{gfp/+}$) and without the *Blimp*-GFP reporter gene were adoptively transferred and challenged with HEL^{3X}-SRBC as in Fig. 1. Splenocytes harvested on day 10 were analyzed by flow cytometry for expression of GFP, CD45.2, IgG1, and HEL^{3X} binding, and digital data are presented using biexponential axes. (left) Plots show HEL^{3X} binding versus IgG1 gated on $CD45.2^+$ donor-derived cells. (right) GFP expression by high (top oval gate) and low (bottom oval gate) affinity cells are shown. A distinct population of *Blimp*-GFP–expressing plasma cells is only evident in high affinity cells in mice that received SW_{HEL} × *Blimp*$^{gfp/+}$ B cells. Profiles represent concatenated data with equal contributions from five individual recipient mice. Similar results were obtained in two independent experiments.

by a mechanism that ensures their rapid and selective differentiation into plasma cells.

Because low affinity B cells survive within GCs without undergoing plasma cell differentiation (Fig. 4 B), it is apparent that the affinity-dependent mechanism that regulates plasma cell differentiation from GC B cell precursors operates independently of the processes that govern GC B cell survival. The existence of this mechanism was not predicted from in vitro experiments, because these show that B cells can undergo stochastic plasma cell differentiation without requiring a BCR signal (9). This would suggest that specific controls exist within the GC microenvironment that suppress plasma cell differentiation in the absence of signals from high affinity antigen.

The requirement for antigen-dependent signals to drive plasma cell differentiation from GC B cells presents an interesting parallel with the regulation of the extrafollicular plasma cell response. These early plasma cells arise independently of the GC reaction (3) but are similarly biased toward high affinity specificities or epitopes present at high density (14). Therefore, the BCR–antigen interaction appears to play a key role in regulating TD plasma cell differentiation both before and after GC formation. It is possible that BCR signaling could facilitate plasma cell differentiation via the induction of Bcl-6 degradation (18) and subsequent lifting of Blimp-1 repression (19). Alternatively, responding B cells may stochastically commence plasma cell differentiation but require BCR signals to survive beyond the very earliest stages of this process. Whatever the precise mechanism, it is apparent that the immune system places a high priority on ensuring that it devotes resources primarily to the production of relatively high affinity antibodies that are most likely to be biologically effective. The importance of this stringent regulation of plasma cell differentiation is perhaps underscored by the relatively permissive affinity requirements for GC B cells to enter the memory B cell compartment (10, 20). The emphasis on quality control of in vivo plasma cell differentiation may have been a critical development during the evolution of the immune system. Because the body is known to have only a relatively limited capacity within the specialized microenvironments that sustain plasma cells (21, 22), it can be seen that tight control over the specificities that enter the plasma cell compartment would be essential for ensuring that the antibodies that are produced provide effective immune protection.

MATERIALS AND METHODS

Mice and procedures. SW_{HEL} mice (13), *Blimp*$^{gfp/+}$ mice (17), mutant HEL proteins (14), conjugation of HEL to SRBC, and the adoptive transfer system (12) have been previously described. SW_{HEL} spleen cells were not purified before transfer. Mice were housed in a specific pathogen-free environment at Centenary Institute, and experiments were approved by the University of Sydney Animal Ethics Committee.

ELISA. Serum anti-HEL antibody levels were analyzed by ELISA as previously described (13). The specificity of serum IgG1 antibodies for HEL^{WT}

and HEL^{3X} was determined by coating the ELISA plates with the respective antigens. The relative affinities of $HyHEL10^{WT}$ and $HyHEL10^{Y53D}$ for HEL^{3X} were determined by capture ELISA. 5 μg/ml each of soluble $HyHEL10^{WT}$ and $HyHEL10^{Y53D}$ IgG1 antibodies was captured by plate-bound anti–mouse IgG1. Subsequent binding of HEL^{3X} was detected with biotinylated HyHEL9, which recognizes an epitope on HEL distinct from that bound by HyHEL10. Nonlinear regression based on a sigmoidal binding curve was performed using software (Prism; GraphPad) to find the curve-fit and calculate the half-maximal binding concentration and relative affinity of $HyHEL10^{WT}$ and $HyHEL10^{Y53D}$ for HEL^{3X}.

Flow cytometry and single cell sorting. Splenocytes were prepared, stained for surface molecules with monoclonal antibodies, and analyzed on a FACSCalibur (BD Biosciences) as previously described (14). To track affinity maturation, cells were stained with recombinant HEL^{3X} conjugated to Alexa Fluor 647 (Invitrogen). For analysis of *Blimp*-GFP expression, CD45.2 was conjugated to Pacific blue (Invitrogen) to allow identification of donor-derived cells and data acquired on a flow cytometer (LSR II; BD Biosciences). Single cells were sorted on a FACSAria (BD Biosciences) as previously described (14). The gates used for single cell sorting of GC B cells ($CD45.2^{hi}$, syndecan-1^{-}) and plasma cells ($CD45.2^{int}$, syndecan-1^{+}) were the same as those previously described (14). These gates have been verified through localization of antibody secreting activity (12) and high levels of intracellular Ig staining (14) to cells in the plasma cell gate and demonstration of high levels of GL7, PNA, and Fas on cells in the GC gate (12, 14).

SHM analysis. The HyHEL10 Ig heavy chain variable region gene was amplified from single-responding SW_{HEL} donor B cells and sequenced as previously described (14). Translated sequences were aligned with the original HyHEL10 protein sequence to determine the position and significance of the mutations, as previously described (12). An unpaired *t* test was used to calculate the probability (p-value) when comparing SHM frequency per clone in different responding populations.

Expression of wild-type and Y53D-mutated HyHEL10 IgG1 antibodies. The canonical T to G mutation encoding the $Y53D_{HyHEL10}$ substitution was introduced by PCR mutagenesis into a pcDNA3 vector (Invitrogen) encoding the HyHEL10 γ1-secreted Ig heavy chain. Wild-type and mutant heavy chain constructs were transiently expressed in Chinese hamster ovary cells along with wild-type HyHEL10 κ light chain construct, and culture supernatants were collected and concentrated.

Online supplemental material. Fig. S1 shows Rasmol wire frame representations based on the HEL–HyHEL10 complex that model the effects of the $D101R_{HEL}$ and $Y53D_{HyHEL10}$ substitutions on the interaction. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20061254/DC1.

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