Antigen recognition strength regulates the choice between extrafollicular plasma cell and germinal center B cell differentiation

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B cells responding to T-dependent antigen either differentiate rapidly into extrafollicular plasma cells or enter germinal centers and undergo somatic hypermutation and affinity maturation. However, the physiological cues that direct B cell differentiation down one pathway versus the other are unknown. Here we show that the strength of the initial interaction between B cell receptor (BCR) and antigen is a primary determinant of this decision. B cells expressing a defined BCR specificity for hen egg lysozyme (HEL) were challenged with sheep red blood cell conjugates of a series of recombinant mutant HEL proteins engineered to bind this BCR over a 10,000-fold affinity range. Decreasing either initial BCR affinity or antigen density was found to selectively remove the extrafollicular plasma cell response but leave the germinal center response intact. Moreover, analysis of competing B cells revealed that high affinity specificities are more prevalent in the extrafollicular plasma cell versus the germinal center B cell response. Thus, the effectiveness of early T-dependent antibody responses is optimized by preferentially steering B cells reactive against either high affinity or abundant epitopes toward extrafollicular plasma cell differentiation. Conversely, responding clones with weaker antigen reactivity are primarily directed to germinal centers where they undergo affinity maturation.

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Abbreviations used: BCR, B cell receptor; CGG, chicken γ-globulin; GC, germinal center; HEL, hen egg lysozyme; NP, (4-hydroxy-3-nitrophenyl)acetyl; SHM, somatic hypermutation, SRBC, sheep red blood cells; TI-2, T-independent type 2.

The initial wave of antibody production after challenge with T-dependent antigen is produced by plasma cells generated from the rapid extrafollicular proliferative focus response (1). Antigen-specific B cells are initially expanded through collaboration with $CD4^+$ T helper cells at the interface between the T and B zones (2–4). After 3–4 d, activated B cells can migrate to the bridging channels and red pulp of the spleen and form extrafollicular foci. Here they differentiate into plasma cells, many of which are short lived (5) and secrete antibodies that may be either switched (e.g., IgG1) or unswitched (IgM; references 1, 6). B cells do not undergo somatic hypermutation (SHM) before or during the extrafollicular response; the resultant antibodies being comprised almost exclusively of specificities encoded within the primary repertoire (5, 7). A second wave of T-dependent plasma cell production is subsequently derived from responding B cells that, instead of colonizing extrafollicular foci, enter the primary follicle and propogate within the germinal center (GC) reaction. In GCs, responding B cells collaborate with follicular T helper and dendritic cells to undergo SHM (8) with mutant clones that recognize the immunogen with increased affinity being selectively propagated (affinity maturation; reference 9). Plasma cells that contribute to the later post-GC phase of antibody production therefore typically express somatically mutated Ig genes (10).

The decision between extrafollicular plasma cell differentiation and GC migration represents the first major branch point during a T-dependent B cell response. Nevertheless, the physiological cues responsible for directing B cells down one pathway versus the other are currently unknown (1). One theory is that responding cells

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stochastically follow either response pathway such that the original specificities recruited into the response are represented equally in both the extrafollicular and early GC populations (11, 12). Alternatively, differential recognition of antigen by B cell receptors (BCRs) expressed on individual B cells may influence their subsequent responses, in which case the specificities represented in the extrafollicular and early GC populations would differ. Evidence for the stochastic model has come from analysis of responses to the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP), where both low and high affinity clones have been detected at similar frequencies in extrafollicular foci and early GCs (11). However, the stochastic model is difficult to reconcile with data from other systems, indicating that relatively high affinity specificities can dominate the initial T-dependent antibody response (13–15). The question of whether the nature of the interaction between antigen and BCR influences cell fate during early T-dependent responses is therefore still controversial.

The two major variables that impact on the interaction of an antigen with a BCR are the affinity of the epitope for the receptor's monovalent Fab and the density or valency of the epitope on the antigen. Manipulation of either of these variables can produce profound changes in the responses of B cells to BCR stimulation in vitro (16–19) as well as to T-independent responses (20, 21) and induction of self-tolerance (22–24) in vivo. The uncertainty surrounding the impact of antigen affinity and density on early T-dependent B cell responses is due largely to the difficulty of tracking responding B cells in vivo and defining the precise nature of their initial interaction with the antigen. To overcome these problems, we have developed gene-targeted mice expressing H and L chains of HyHEL10 anti–hen egg lysozyme (HEL) mAb (SW_{HEL}), in which B cells express a defined anti-HEL BCR and are capable of normal Ig class switching and SHM (6, 25). By challenging CD45-allotyped marked SW_{HEL} B cells with HEL coupled to sheep red blood cells (SRBC) in an adoptive transfer system, T-dependent responses can be accurately tracked in vivo by both immunohistology and flow cytometry (6). In this study, SW_{HEL} B cells were challenged with a range of recombinant HEL proteins engineered to recognize the SW_{HEL} BCR over a 10,000-fold affinity range and coupled to SRBC at different antigen densities. Examination of the early T-dependent responses generated by the SW_{HEL} B cells indicated that decreasing either antigen affinity or density profoundly reduced the extrafollicular plasma cell response but had minimal impact on the GC reaction. Thus, although the requirements for GC entry are not stringent, responding B cells require a strong initial interaction with the antigen to contribute significantly to the early plasma cell response. These results reveal that the initial antigen–BCR interaction plays a fundamental role in organizing early T-dependent B cell responses.

RESULTS

Generation of HEL mutants with varying affinities for the SW_{HEL} BCR

The role played by initial antigen affinity in regulating early B cell responses was investigated by generating a panel of recombinant HEL molecules with the capacity to bind over a wide affinity range to the anti-HEL BCR expressed on SW_{HEL} B cells. SW_{HEL} mice carry a targeted Ig heavy chain variable region gene with a light chain transgene that together encode BCRs with the specificity of the HyHEL10 mAb (25). Because the affinity of WT HEL for HyHEL10 is very high (2×10^{10} M^{-1}) (26), it was necessary to create HEL mutants that bound HyHEL10 with lower affinities more typical of those found in primary B cell responses. Arginine 21, arginine 73, and aspartate 101 of HEL all form direct contacts with the Ig heavy chain of HyHEL10 (reference 26; Fig. 1 A) and were mutated to glutamine ($R21_{HEL}Q$), glutamate ($R73_{HEL}E$), and arginine ($D101_{HEL}R$) both singly and in combination. Recombinant HEL mutants were expressed in yeast and purified from culture supernatants. All retained lysozyme enzymatic activity and differed less than twofold from WT HEL (HEL^{WT}) in their affinity for the noncompetitive anti-HEL mAb HyHEL9 (unpublished data), indicating that these point mutations did not affect overall protein folding.

As expected, the affinities of the various HEL mutants for HyHEL10 varied widely. Competitive ELISA studies showed that mutant HEL containing only the $D101_{HEL}R$ substitution (HEL^{1X}) bound with ~80-fold lower affinity than HEL^{WT} to HyHEL10 (Fig. 1 B), which is in agreement with previous studies (27). Addition of $R73_{HEL}E$ to $D101_{HEL}R$ (HEL^{2X}) decreased this affinity ~3-fold, whereas subsequent addition of $R21_{HEL}Q$ (HEL^{3X}) reduced it a further ~50-fold. Because HEL^{1X} and HEL^{2X} displayed similar affinities for HyHEL10, only HEL^{WT}, HEL^{2X}, and HEL^{3X} were used in subsequent analyses. Based on previous data (18, 26, 27) and our competitive ELISA results, we estimated the affinities of the recombinant HEL proteins for HyHEL10 to be as follows: 2×10^{10} M^{-1} (HEL^{WT}), 8×10^7 M^{-1} (HEL^{2X}), and 1.5×10^6 M^{-1} (HEL^{3X}). The relative affinity measurements of these mutant proteins are also in agreement with the amount of soluble protein needed to stimulate B cells in vitro. Thus, when activation of purified splenic B cells from $SW_{HEL}.rag1^{-/-}$ (28) mice was analyzed, 10- and 1,000-fold higher concentrations of HEL^{2X} and HEL^{3X}, respectively, were required to induce comparable levels of intracellular tyrosine phosphorylation to that triggered by HEL^{WT} (Fig. 1 C). These recombinant proteins therefore provide the basis for comparing the in vivo responses of SW_{HEL} B cells to high affinity (HEL^{WT}), intermediate affinity (HEL^{2X}), and low affinity (HEL^{3X}) antigens.

SW_{HEL} B cells proliferate and form GCs in response to antigens encompassing a 10,000-fold affinity range

To compare T cell–dependent responses to the various HEL proteins, we used a previously described system in which small numbers (10^4) of anti-HEL B cells from C57BL/6 ($CD45.2^+$) SW_{HEL} donor mice are adoptively transferred into nonirradiated C57BL/6 congenic ($CD45.1^+$) recipients and challenged intravenously with HEL conjugated to SRBC (2×10^8) in the absence of additional adjuvant (6). HEL is linked to SRBC to recruit SRBC-specific T cell help to the SW_{HEL} donor B cells because HEL itself cannot elicit a T cell response

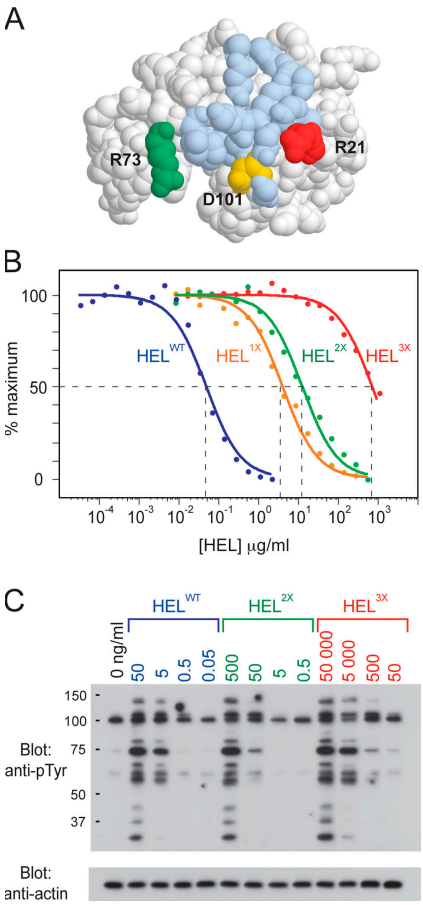

Figure 1. Comparison of the abilities of recombinant HEL proteins to bind to and signal through HyHEL10 in vitro. (A) Rasmol space-filling model of HEL showing the mutated residues (colored) within the HyHEL10-binding footprint (light blue). (B) Competitive ELISA showing relative inhibition of the binding of HEL^{WT}-biotin to HyHEL10 by varying concentrations of recombinant HEL proteins. The half-maximal inhibitory concentration for each HEL protein was used to calculate the relative affinities for HyHEL10. (C) Western blot of intracellular tyrosine phosphorylation (pTyr) after 15-min stimulation of SW_{HEL}.*rag1*$^{-/-}$ B cells with varying concentrations of recombinant HEL proteins. Blue, HEL^{WT}; orange, HEL^{1X} ($D101_{HEL}R$); green, HEL^{2X} ($R73_{HEL}E$, $D101_{HEL}R$); red, HEL^{3X} ($R21_{HEL}Q$, $R73_{HEL}E$, and $D101_{HEL}R$).

on the C57BL/6 genetic background (29). In this system, the T cell–dependent response of SW_{HEL} donor B cells can be precisely tracked by multiparameter flow cytometry based on CD45.2 and HEL-binding BCR expression (6). As the recipient mice are nonirradiated, responding B cells can navigate normally through intact peripheral lymphoid tissues where their localization can be readily visualized by immunohistology (6).

We first tested the ability of SW_{HEL} B cells to initiate a primary in vivo response when challenged with SRBC conjugates of the various recombinant HEL proteins. Adoptive transfers were performed using CFSE-labeled donor cells and proliferation of the HEL-binding donor cells was measured 64 h later. Extensive proliferation was observed in response to HEL^{WT}-SRBC, HEL^{2X}-SRBC, and HEL^{3X}-SRBC (Fig. 2). These responses were antigen specific because they did not occur upon challenge with mock-conjugated SRBC (Fig. 2). Although antigen affinity had some effect on the degree of proliferation observed (Fig. 2), SW_{HEL} B cells nevertheless responded efficiently to SRBC conjugates of all three recombinant proteins.

We next examined the ability of the SW_{HEL} donor B cells challenged with the various recombinant HEL proteins to differentiate into GC B cells. By day 5, immunohistological analysis of spleen sections confirmed that there was considerable expansion of HEL-binding B cells in response to each of the recombinant HEL proteins, whereas none was evident in mice challenged with mock-conjugated SRBCs (Fig. 3). Responding HEL-binding B cells were derived from adoptively transferred donor SW_{HEL} B cells because they were not detectable in mice that were challenged with any of the HEL-SRBC conjugates in the absence of cotransferred SW_{HEL} B cells (unpublished data). HEL-binding B cells were found within PNA^+ GCs from recipients challenged with each of the three HEL-SRBC conjugates (Fig. 3). By flow cytometry, donor-derived GC B cells were identified on the basis of

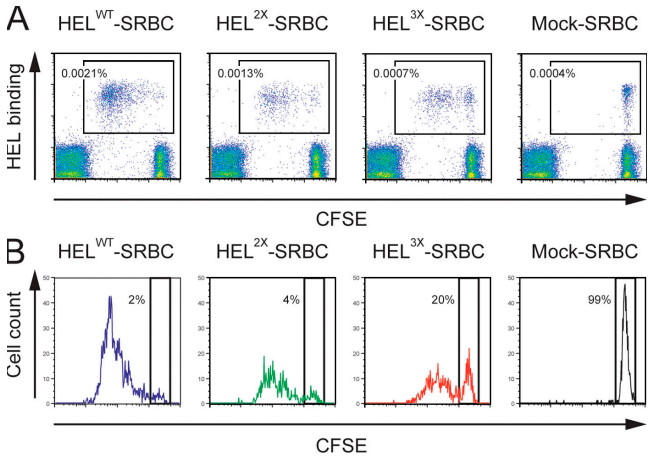

Figure 2. SW_{HEL} B cells make in vivo proliferative responses to SRBC conjugates of all three recombinant HEL proteins. SW_{HEL} B cells ($CD45.1^+$) were labeled with CFSE and challenged with various HEL-SRBC conjugates by adoptive transfer into $CD45.2^+$ congenic recipients. (A) Pseudocolor FACS plots show the CFSE profile of donor HEL-binding SW_{HEL} B cells in recipient spleens 64 h after challenge with the various HEL-SRBC conjugates. The data shown were gated from total splenocytes after staining with anti–CD45.1-PE. Numbers indicate the frequency of HEL-binding $CFSE^+$ cells among total splenocytes. Data includes a high frequency of contaminant cells (non-HEL binding and $CFSE^-$) present in this analysis as a result of background staining by the anti–CD45.1-PE antibody. Note the lack of proliferating non-HEL–binding cells, indicating that proliferation is antigen specific and that the HEL-binding stain detects all responding SW_{HEL} donor B cells. (B) CFSE profiles are shown for HEL-binding cells present in the gates shown in A. The frequencies of undivided ($CFSE^{hi}$) cells are shown. Note the increased frequency of dividing cells and their enhanced rate of proliferation (CFSE dilution) as affinity for the HEL antigen is raised.

high surface CD45.2 and HEL binding, relatively low intracellular HEL binding, and lack of syndecan-1 (CD138) expression (Fig. 4). These cells also expressed high levels of GL7 and Fas (CD95; unpublished data). Quantification of the responses to HEL^{WT}-SRBC, HEL^{2X}-SRBC, and HEL^{3X}-SRBC revealed that challenge with all three antigens elicited similar numbers of donor-derived ($CD45.2^+$) GC B cells by day 5 (Fig. 5 A). SW_{HEL} donor B cells responding to HEL-SRBC conjugates therefore proliferated and differentiated into GC B cells largely independently of the 10,000-fold variation in their initial affinity for the three recombinant HEL antigens.

Initial BCR affinity regulates the extrafollicular plasma cell response

Variation of antigen affinity, although not affecting GC B cell differentiation, did have a profound impact on the differentiation of SW_{HEL} B cells into extrafollicular proliferative focus plasma cells. Thus, histological analysis of recipient spleens at day 5 revealed that cells exhibiting a plasma cell phenotype (large size and intense cytoplasmic HEL staining) accumulated in the splenic bridging channels and red pulp of mice challenged with HEL^{WT}-SRBC and HEL^{2X}-SRBC but not in mice challenged with HEL^{3X}-SRBC (Fig. 3). By flow cytometry, these plasma cells were identifiable on the basis of lower surface CD45.2 and HEL binding compared with GC B cells, very high intracellular HEL binding, and high syndecan-1 expression (Fig. 4). At the peak of the extrafollicular response on day 5, recipients immunized with HEL^{WT}-SRBC and HEL^{2X}-SRBC contained >100-fold more splenic plasma cells than those challenged with low affinity HEL^{3X}-SRBC (Fig. 5 B). This was reflected in the relative levels of both total and IgG1 anti-HEL serum antibodies present at this time point (Fig. 5 C and not depicted). These antibodies were derived from the SW_{HEL} donor B cells because they were not detected in mice challenged with any of the HEL-SRBC conjugates in the absence of cotransferred SW_{HEL} B cells (unpublished data). Day 5 plasma cells generated in response to HEL^{WT}-SRBC and HEL^{2X}-SRBC were confirmed to have originated from extrafollicular foci by the absence of Ig heavy chain variable gene mutations (0/20 and 0/22 clones carried mutations, respectively) in contrast to the SHM already present in corresponding GC B cell populations (10/24 and 4/18 clones mutated, respectively). In this system, therefore, activated SW_{HEL} donor B cells did not undergo differentiation into extrafollicular plasma cells unless their initial

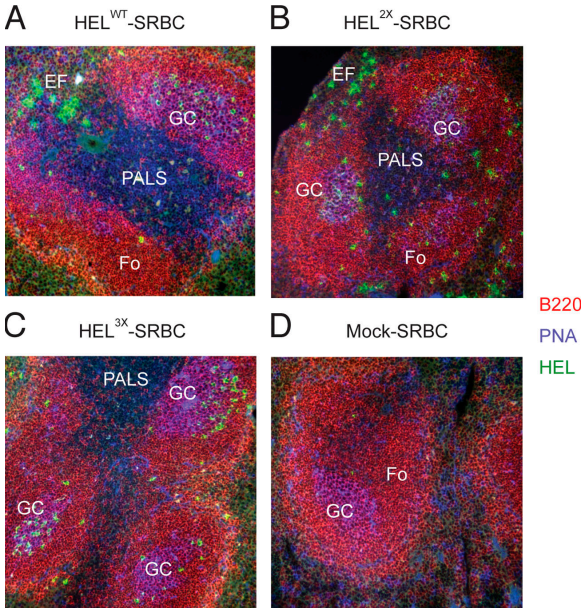

Figure 3. Antigen affinity controls the extrafollicular focus response. SW_{HEL} B cells were adoptively transferred and challenged with either HEL^{WT}-SRBC (A), HEL^{2X}-SRBC (B), HEL^{3X}-SRBC (C), or mock-conjugated SRBC (D), and the spleens were harvested at day 5 for immunofluorescence microscopy. B cells in the primary follicle are stained red with B220, GCs stained blue with peanut agglutinin, and HEL-binding Ig stained green with HEL. Anti-HEL plasma cells are evident from their intense cytoplasmic staining with HEL. While HEL-binding B cells formed GCs in response to all three HEL conjugates, no HEL-binding extrafollicular foci of plasma cells were detectable in the spleens of mice challenged with HEL^{3X}-SRBC. EF, extrafollicular focus; GC, germinal center; Fo, follicle; PALS, periarteriolar lymphatic sheath.

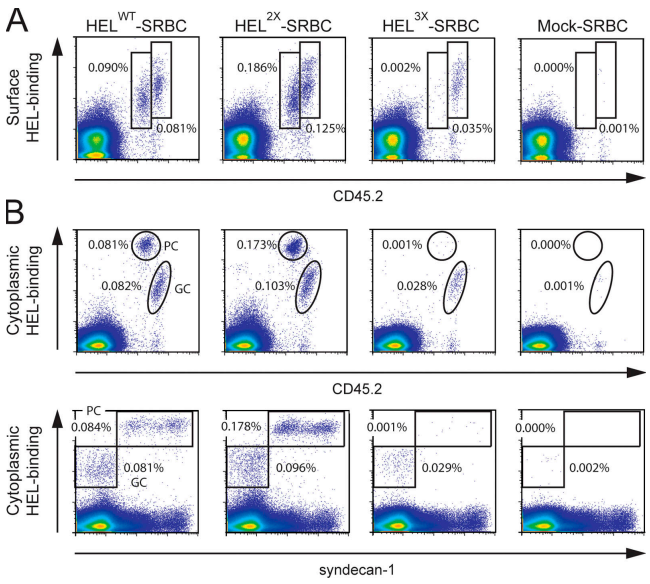

Figure 4. Antigen affinity controls the generation of early plasma cells. Recipient mice were immunized as for Fig. 3 and spleens harvested for FACS analysis on day 5. (A) Surface staining for HEL-binding BCRs and the CD45.2 congenic marker shows two populations of responding donor B cells ($CD45.2^{hi}$ HEL $binding^{hi}$ vs. $CD45.2^{int}$ HEL $binding^{int}$) in mice challenged with HEL^{WT}-SRBC and HEL^{2X}-SRBC. Only the $CD45.2^{hi}$ HEL-$binding^{hi}$ population is observed in mice challenged with HEL^{3X}-SRBC. (B) Cytoplasmic staining for HEL-binding Ig reveals the $CD45.2^{hi}$ populations as low intracellular HEL-binding, syndecan-1^- GC B cells, and the $CD45.2^{int}$ population as high intracellular HEL-binding, syndecan-1^+ plasma cells (PC). Only rare donor-derived HEL-binding B cells with a plasma cell phenotype were detectable in mice immunized with HEL^{3X}-SRBC. The proportion of spleen cells falling within the different PC and GC gates are indicated.

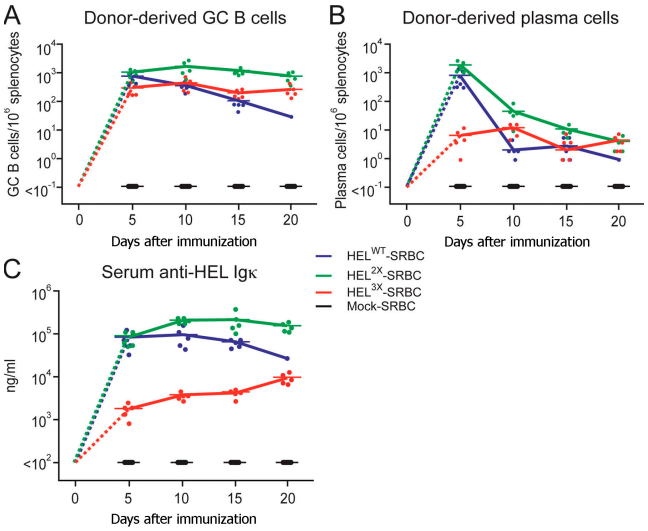

Figure 5. Intact GC but impaired extrafollicular focus and antibody responses to low affinity antigen. Recipient mice were immunized as for Fig. 3 and analyzed 5, 10, 15, and 20 d later. (A) Enumeration of donor-derived GC B cells in the spleen by flow cytometry shows similar numbers of GC B cells generated in response to all three HEL conjugates. (B) Enumeration of donor-derived plasma cells shows that the early, transient extrafollicular plasma cell response to HEL^{3X}-SRBC is ~100-fold lower than those to HEL^{WT}-SRBC and HEL^{2X}-SRBC. (C) Serum anti-HEL^{WT} Igκ antibody levels were determined by ELISA and also found to be greatly abrogated in mice immunized with HEL^{3X}-SRBC, particularly early in the response (day 5). No anti-HEL^{WT} antibody was detectable in control recipients that were immunized with any of the HEL-SRBC conjugates in the absence of cotransferred SW_{HEL} donor cells (not depicted). Each data point represents one mouse. Each data point represents one mouse, whereas bars show the mean of each group. Blue, HEL^{WT}-SRBC; green, HEL^{2X}-SRBC; red, HEL^{3X}-SRBC; black, mock-conjugated SRBC.

antigen affinity exceeded a threshold value substantially higher than that required for GC B cell differentiation.

Extrafollicular plasma cell differentiation is also regulated by epitope density

The regulation of extrafollicular plasma cell differentiation by initial antigen affinity is indicative of a pivotal role for the BCR and its interaction with antigen in determining cell fate during early T-dependent B cell responses. Although antigen affinity alone may be the critical determinant, epitope density can also have a major impact on the strength of the interaction of antigen with the BCR and on subsequent B cell responses (16, 20, 23). To test whether epitope density as well as affinity affects the decision to undergo early plasma cell versus GC B cell differentiation, SW_{HEL} B cells were challenged with SRBCs coupled to varying densities of the different recombinant HEL proteins.

Our initial experiments (Figs. 3–5) indicated an apparent affinity threshold for early plasma cell differentiation located between the affinities of HEL^{2X} and HEL^{3X} for the SW_{HEL} BCR. To test the influence of antigen density, we asked whether increasing the density of the low affinity HEL^{3X} protein on the SRBC surface might rescue the extrafollicular plasma cell response to this protein and, conversely, whether reducing antigen density might abrogate the extrafollicular response to the intermediate affinity protein HEL^{2X}. In addition to the standard SRBC conjugations using 100 μg/ml HEL^{2X} or HEL^{3X}, separate conjugations were performed using 1,000 μg/ml HEL^{3X} and 10 μg/ml HEL^{2X}. The antigen density on the different SRBC conjugates was compared by flow cytometry using the HyHEL9 mAb. The 100-μg/ml HEL^{2X} and HEL^{3X} conjugates were found to possess almost identical levels of surface antigen (intermediate density), whereas the density was increased threefold for the 1,000-μg/ml HEL^{3X} conjugate (high density) and decreased fourfold for the 10-μg/ml HEL^{2X} conjugate (low density; Fig. 6 A).

SW_{HEL} B cells were challenged with the various conjugates in adoptive transfer and their responses analyzed on day 5. When equivalent densities of HEL^{2X} and HEL^{3X} were used (intermediate densities), affinity-based regulation of the early plasma cell response was again apparent, with 20- to 30-fold more extrafollicular plasma cells and antibody being generated in response to HEL^{2X} compared with HEL^{3X} (Fig. 6, B and D–F). When the density of HEL^{3X} on the SRBC was raised threefold (high density), the production of early plasma cells and the secretion of anti-HEL antibody in response to this low affinity antigen were increased almost 10-fold (Fig. 6, B and D–F). Conversely, decreasing the density of HEL^{2X} by fourfold (low density) led to reductions of 5- to 10-fold in the plasma cell and antibody responses to this intermediate affinity antigen (Fig. 6, B and D–F). Importantly, the magnitude of the early GC response did not vary greatly when antigen density was either raised or lowered (Fig. 6, B and C). As a result, the 12-fold difference in epitope density between HEL^{2X}-SRBC (low density) and HEL^{3X}-SRBC (high density) negated the 50-fold affinity difference between these antigens and resulted in almost identical day 5 responses (Fig. 6, B and C). Collectively, these findings indicate that it is the strength of the initial antigen–BCR interaction, influenced by both epitope density and affinity, that determines the extent to which responding B cells enter the extrafollicular plasma cell response.

Affinity-based partitioning of competing B cells between the early plasma cell and GC compartments

Although the response of donor SW_{HEL} B cells occurs within the context of an endogenous anti-SRBC antibody response, the monoclonal nature of the SW_{HEL} BCR does not permit the simultaneous analysis of different B cell specificities responding to the same antigen. The prediction from our finding that the strength of the interaction between antigen and BCR directs cell fate during early T-dependent responses is that B cells expressing alternative BCRs recognizing the same epitope would be differentially represented in the extrafollicular plasma cell compartment depending on the strength of their initial interaction with the antigen. Because epitope density would in this case be fixed, our results predict that the

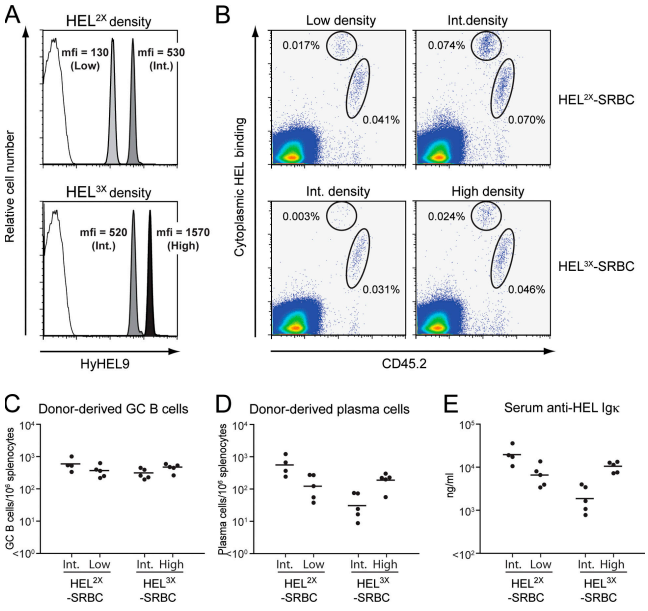

Figure 6. Epitope density also controls the extrafollicular plasma cell response and early antibody production. (A) SRBCs were conjugated to HEL^{2X} and HEL^{3X} using 10, 100, or 1,000 μg/ml of recombinant protein to produce low, intermediate, and high epitope density immunogens, respectively. The mean fluorescence intensity (mfi) of HyHEL9 staining is shown in each case. HyHEL9 recognizes both mutant HEL proteins equally well (not depicted). The open curve represents staining of mock-conjugated SRBCs. (B) Spleens from recipient mice were harvested on day 5 and analyzed by flow cytometry for cytoplasmic HEL binding and CD45.2 as for Fig. 4. Gates indicate the plasma cell ($CD45.2^{int}$, high cytoplasmic HEL binding) and GC ($CD45.2^{hi}$, low cytoplasmic HEL binding) responding populations, and the proportion of spleen cells in these gates are shown. (C–E) Donor-derived GC, plasma cells, and serum anti-HEL antibody were measured on day 5 of the different responses as for Fig. 5. Points represent data from individual recipients, whereas the bars show the mean of each group. The results show that, at a fixed antigen affinity, early plasma cell and antibody production vary directly according to antigen density, whereas GC responses are relatively unaffected.

higher affinity B cells among competing specificities will be preferentially represented in extrafollicular foci compared with early GCs.

To test this prediction, we moved out of the monoclonal SW_{HEL} model to take advantage of the fact that mice carrying the targeted heavy chain variable region gene of HyHEL10 without the light chain transgene (gene-targeted mice expressing only the H chain of HyHEL10 without the light chain transgene ($SW_{HEL(H)}$ mice) contain B cells with a range of affinities for HEL^{WT} due to pairing of the targeted heavy chain with different endogenous κ light chains (24). In particular, two B cell populations are resolvable by flow cytometry using sub-saturating levels of HEL^{WT}, including a population of relatively high affinity (0.1–0.2% of B cells) and a second population with lower mean affinity for HEL^{WT} (0.2–0.4% of B cells; reference 24; Fig. 7 A). Comparisons of BCR binding by the various recombinant HEL proteins indicated that the affinity for HEL^{WT} of the latter (lower affinity) $SW_{HEL(H)}$ B cell population was still higher than the affinity of the SW_{HEL} BCR for HEL^{3X} (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20060087/DC1). For convenience, the second $SW_{HEL(H)}$ B cell population will be referred to as having intermediate affinity for HEL^{WT}.

$SW_{HEL(H)}$ lymph node cells containing 10^4 anti-HEL B cells (high plus intermediate affinity) were adoptively transferred and challenged with HEL^{WT}-SRBC. As a control, SW_{HEL} lymph node cells containing 10^4 anti-HEL B cells (high affinity only) were similarly transferred and challenged. Because the fraction of lymph node B cells that bind HEL in SW_{HEL} mice is typically 5–15%, donor SW_{HEL} lymph node cells were supplemented with a 25-fold excess of WT C57BL/6 lymph node cells so that anti-HEL B cells comprised a similar fraction (0.2–0.6%) of the SW_{HEL} and $SW_{HEL(H)}$ donor B cell populations (Fig. 7 A). A further control group received only WT C57BL/6 lymph node donor cells and so lacked donor B cells with HEL-binding activity (Fig. 7 A). On day 5, $CD45.2^+$, $Ig\kappa^+$ donor-derived B cells (including $CD45.2^{lo}$, $Ig\kappa^{lo}$ plasma cells) were collectively analyzed by flow cytometry (Fig. 7 B). The cells were stained with a concentration of HEL^{WT} (75 ng/ml) that resolved the high and intermediate affinity $SW_{HEL(H)}$ responders. Cells were counterstained for surface Ig with anti-κ light chain to identify differences in HEL^{WT}-binding caused by differential antigen affinity rather than BCR expression level (Fig. 7 B). As expected, the anti-HEL^{WT} donor B cells evident in recipients of SW_{HEL} B cells fell almost exclusively within the high-affinity gate, whereas the only donor B cells detected in recipients of WT C57BL/6 lymph node donor cells were resting and did not bind HEL^{WT} (Fig. 7 B).

Analysis of recipients of $SW_{HEL(H)}$ cells indicated that both high and intermediate affinity anti-HEL donor B cell populations had been recruited into the response to HEL^{WT}-SRBC (Fig. 7 B). Importantly, separate analysis of the two responding populations indicated that the proportion of the responding high affinity $SW_{HEL(H)}$ donor B cells that exhibited a plasma cell phenotype was more than double that observed among the intermediate affinity responders (i.e., 45 vs. 21%; Fig. 7 C). Thus, as was predicted from our previous analysis of monoclonal T-dependent responses, the representation of competing B cells in the extrafollicular plasma cell response was greatest for those responders with the highest initial antigen affinity.

DISCUSSION

Differential recognition of antigen by BCRs has long been recognized to play a key role in two specific phases of T-dependent B cell responses. The first is when resting B cells are selected to enter the response because this requires the interaction of the BCR with the foreign antigen to exceed a critical threshold. Subsequently, somatically mutated B cells generated within GCs are only propagated if they acquire increased affinity for the antigen presented in immune complexes on follicular dendritic cells. In between these two

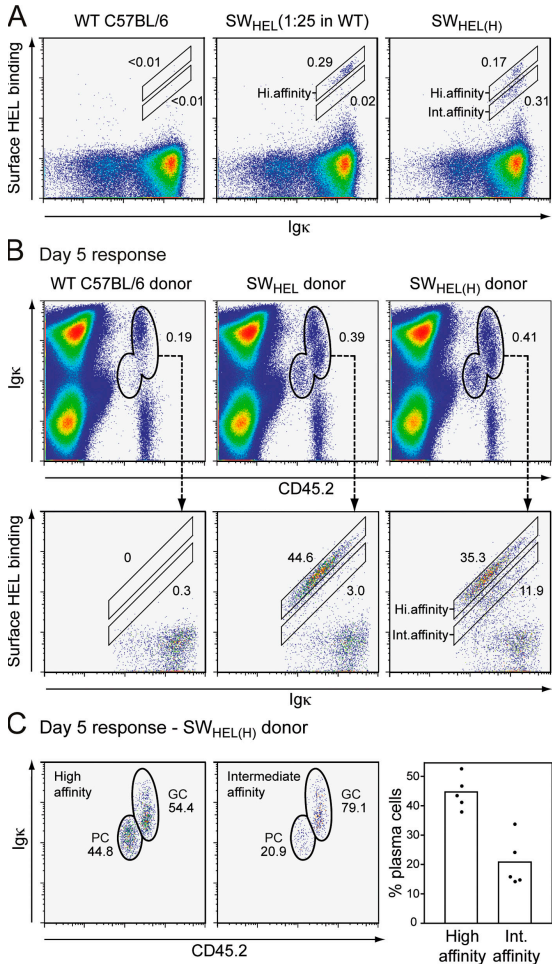

Figure 7. Competing B cells partition into the extrafollicular plasma cell response according to their relative initial antigen affinity. (A) Flow cytometric analysis of lymph node B cells from WT C57BL/6, SW$_{HEL}$, and SW$_{HEL(H)}$ mice. SW$_{HEL}$ cells were mixed with a 25-fold excess of WT C57BL/6 cells to equalize the frequency of HEL-binding cells between the SW$_{HEL}$ and SW$_{HEL(H)}$ samples. Cells were stained with 75 ng/ml HEL^{WT} followed by HyHEL9-AlexaFluor647 and then counterstained for B220 and surface Ig (anti-κ light chain). Data represent B220$^+$ cells. The diagonal gates indicate cells with proportional HEL^{WT} binding and surface Ig expression and, therefore, with similar or identical affinity for HEL^{WT}. The presence of populations with high and intermediate affinity for HEL^{WT} are evident in SW$_{HEL(H)}$ mice. (B) Lymph node cells including 10^4 HEL^{WT}-binding B cells from SW$_{HEL}$ or SW$_{HEL(H)}$ mice were adoptively transferred into CD45.1 congenic recipients and challenged with HEL^{WT}-SRBC. Before injection, SW$_{HEL}$ lymph node cells were mixed 1:25 with WT C57BL/6 lymph node cells to ensure the anti-HEL B cells from SW$_{HEL}$ and SW$_{HEL(H)}$ mice comprised similar proportions of the respective donor B cell populations (0.2–0.6%). All recipients including the controls receiving just WT C57BL/6 lymph node cells (left) were therefore injected with $\sim 2 \times 10^6$ non-HEL^{WT}–binding B cells each. On day 5, splenocytes from recipient mice were stained with 75 ng/ml HEL^{WT} as for A and for CD45.2 and Igκ. Donor B cells were identified with a combination of gates that included CD45.2^{hi}, Igκ^{hi} resting plus GC B cells, as well as CD45.2^{int}, Igκ^{int} plasma cells. High and intermediate affinity anti-HEL^{WT} B cells were identified as for A. (C) Donor B cells with high and intermediate affinity for HEL^{WT} from recipients of SW$_{HEL(H)}$ lymph node cells were analyzed separately for the plasma cell (PC) and GC phenotypes. The proportion of high affinity cells exhibiting the CD45.2^{int} Igκ^{int} plasma cell phenotype was more than double that evident among the intermediate affinity responders.

checkpoints, however, responding B cells must decide whether to enter the GC reaction or undergo rapid plasma cell differentiation in extrafollicular proliferative foci. In this study, we demonstrate that this decision is also controlled by the nature of the interaction between the BCR and antigen. Thus, we show that only responding clones that undergo a strong initial interaction with antigen can efficiently differentiate into extrafollicular plasma cells and contribute to the rapid early T-dependent antibody response.

The studies performed here required the production and purification of milligram amounts of the various recombinant HEL proteins. This was achieved using yeast expression and yielded protein preparations that were >95% pure. Nevertheless, the possibility existed that trace amounts of yeast-derived products with potential immunomodulatory effects could influence the results. We consider this to be very unlikely for several reasons. First, the response observed here after challenge with HEL^{WT}-SRBC is indistinguishable from that obtained when native, chicken egg–derived HEL is conjugated to SRBCs (6). Second, the characteristic responses obtained to each of the HEL mutants was consistent between batches of the same purified protein (unpublished data). Finally, when high and intermediate affinity B cells competitively responded to the same antigen in the same host, the predicted differences in plasma cell production were still observed (Fig. 7). Together, these observations indicate that our results were not affected by the presence of any potential yeast contaminant.

The existence of an antigen-dependent threshold for early plasma cell production was initially evident in this study from the selective absence of the extrafollicular response when SW$_{HEL}$ B cells were challenged with the low affinity (K_a of $\sim 1.5 \times 10^6$ M^{-1}) HEL^{3X}-SRBC antigen (Figs. 1, 3, and 4). This result contrasts with the findings from previous studies on T-dependent B cell responses using the chemical hapten NP conjugated to chicken γ-globulin (CGG) carrier protein (NP-CGG). In this system, B cells with a range of initial anti-NP affinities showed no differential localization to extrafollicular foci versus early GCs (11, 30, 31). Indeed affinities as low as 10^5 M^{-1} are represented equally in both extrafollicular foci and early GCs generated after NP-CGG immunization (11). The results from the anti-NP system have led to the theory that differentiation of responding B cells down the two early response pathways is essentially a stochastic process that operates independently of differences in antigen recognition (11, 32). This model predicts that the initial burst of antibody production by extrafollicular plasma cells reflects the specificities of all B cell clones recruited into the response and is therefore predominantly low affinity. Although this is indeed the case in anti-NP responses (5, 7), analysis of responses to viruses and natural protein epitopes indicates that the initial

burst of antibody production to T-dependent antigen is often of relatively high affinity ($>10^7$ M^{-1}; references 13–15).

The disparate requirements for high antigen affinity in different T-dependent antibody responses can be reconciled by recognizing that it is the strength of the interaction between antigen and BCR that is crucial for regulating this process, rather than antigen affinity per se. This notion was confirmed in the present study by showing that SW_{HEL} B cells could produce rapid plasma cell and antibody responses to the low affinity HEL^{3X}-SRBC antigen when the density of HEL^{3X} on the SRBC surface was increased (Fig. 6). The presence of low affinity clones in the extrafollicular focus response to NP-CGG is therefore readily explained by the high epitope density on this haptenated protein antigen, which typically carries 16 NP groups per CGG monomer (31). Our results suggest, therefore, that B cells that recognize low affinity epitopes can still interact sufficiently strongly with antigen to enter the extrafollicular focus response but only if the epitope is present at relatively high density.

When epitope density is held constant, it is apparent that a 50-fold reduction in antigen affinity (i.e., HEL^{2X}-SRBC to HEL^{3X}-SRBC) can remove the extrafollicular plasma cell response mounted by SW_{HEL} B cells (Figs. 3–5). Smaller quantitative shifts in the strength of the interaction between the antigen and the BCR by either lowering the density of HEL^{2X} or increasing the density of HEL^{3X} on the SRBC surface had significant but less absolute effects on the early plasma cell response (Fig. 6). Similarly, $SW_{HEL(H)}$ B cells that bound HEL^{WT} with intermediate affinity were capable of eliciting a reduced but still clearly evident plasma cell response on day 5 (Fig. 7). Collectively, these results demonstrate that there is not a sharp threshold of interaction between antigen and BCR that determines whether or not early plasma cell differentiation will occur. Rather, within a certain range of interaction strengths, the relative size of the early plasma cell response is directly related to the strength of that initial antigen interaction.

Whether a strong interaction between BCR and antigen is universally required for extrafollicular focus formation is difficult to assess without studying this question directly using other model antigens. The relatively high affinity of the early antibody response to several T-dependent antigens (13–15) is certainly consistent with BCR-dependent regulation of extrafollicular plasma cell production also occurring in these cases. Nevertheless, it is possible that adjuvant or other modifying factors alter the quantity or quality of BCR-independent signals delivered to responding B cells by T cells and/or dendritic cells and that these may in turn override the BCR-dependent controls described in the current study. It will be interesting to test this possibility in the future by challenging SW_{HEL} B cells with the different mutant HEL proteins in alternative immunogenic forms.

In contrast to T-dependent antigens, T-independent type 2 (TI-2) antigens such as NP-Ficoll typically generate an extrafollicular response only. Abortive GCs can be generated but only if antigen dose and precursor frequency are sufficiently high (33). This bias of TI-2 responses toward extrafollicular focus formation is also evident in the SW_{HEL} system. Thus, when 10^4 HEL-binding SW_{HEL} B cells are challenged with HEL^{WT}-Ficoll, short-lived extrafollicular plasma cells are generated but not GCs (unpublished data). If the observations of T-dependent responses in the current study can also be applied to TI-2 responses, it is possible to attribute the ability of TI-2 antigens to elicit a strong extrafollicular focus response to the fact that these antigens are typically highly cross-linking (34, 35) and therefore likely to interact strongly with the BCR. In this case, the failure of TI-2 antigens to efficiently generate GCs most likely reflects the importance of T cell help in potentiating GC formation. Alternatively, the strength of BCR engagement may effect T-dependent and TI-2 responses differently, potentially because of modulation in the latter case by co-stimuli delivered through CD21/35 or Toll-like receptor molecules (36).

The finding that quantitative differences in antigen recognition regulate cell fate during early T-dependent B cell responses reveals a sophisticated level of control that has presumably evolved to optimize the deployment of responding B cell clones. Linking rapid plasma cell differentiation to strong initial interaction with antigen means that the substantial resources required to support plasma cell differentiation and subsequent high level antibody production (37) are devoted only to those clones with a good chance of secreting biologically effective antibodies (38). In the case of paucivalent epitopes, this effectively means that only relatively high affinity antibodies are produced (Figs. 2–4). However, epitopes present at high density may be effectively neutralized by antibodies of lower affinity (39–41). Under these specific conditions (e.g., high density HEL^{3X}-SRBC), it makes biological sense for low affinity B cells to be permitted into the extrafollicular plasma cell response (Fig. 6). BCR-mediated regulation of early T-dependent responses also ensures that antigen-specific specificities that make minimal contribution to the initial antibody response are not discarded but specifically directed to GCs. Here they can undergo SHM and affinity maturation and may contribute new effective specificities at a later stage of the response. This is exemplified when SW_{HEL} B cells are challenged with intermediate density HEL^{3X}-SRBC, where the absence of an early (day 5) antibody response (Fig. 5 C) is followed by the subsequent accumulation of high affinity anti-HEL^{3X} antibodies produced by somatically mutated post-GC plasma cells (unpublished data).

The critical role of a strong antigen–BCR interaction in early plasma cell differentiation in vivo is intriguing given that B cells stimulated with T cell–derived signals in vitro undergo efficient proliferation-linked plasma cell differentiation without the need for BCR ligation (32). This dichotomy suggests that B cell differentiation in vivo is influenced by additional signals, which, in the absence of strong antigen recognition, suppress the default plasma cell differentiation pathway. Because responding B cells can proliferate and enter the GC response without undergoing significant plasma cell

differentiation (e.g., HEL^{3X}-SRBC response), it is possible that the GC microenvironment may inhibit this process. If this is true, a strong initial interaction between BCR and antigen may allow at least a proportion of the daughter cells of a responding clone to bypass the GC and undergo plasma cell differentiation. The specific mechanism involved is not clear but may be based on BCR-mediated alterations in the expression of chemotactic receptors that facilitate extrafollicular migration (1). Alternatively, more efficient presentation of antigen-derived peptides facilitated by stronger antigen recognition may result in quantitative or qualitative differences in the delivery of T cell help that influence the subsequent B cell response. A potential clue may lie in the fact that the progressive lowering of initial antigen affinity reduced the rate of proliferation of the anti-HEL B cells during the first 64 h of the response (Fig. 2). It is possible, therefore, that the initial proliferative response may influence the ability of responding cells to undergo rapid plasma cell differentiation. In this case it is possible that signals delivered independently of the antigen–BCR interaction but with the potential to modify B cell proliferation (e.g., Toll-like receptor ligands) may also impact on the size of the extrafollicular plasma cell response. Studies using the model described here will assist in resolving the specific mechanisms involved and further characterize the signals that shape in vivo B cell responses. Such information holds great promise for the future development of novel therapies for autoimmune diseases as well as the optimization of vaccination strategies.

MATERIALS AND METHODS

Recombinant HEL proteins. cDNAs encoding the various mutant HEL proteins were produced by site-directed mutagenesis using engineered PCR primers. Sequences encoding the mature WT and mutant HEL proteins were then cloned directly 3′ of the secretory signal sequence in the yeast expression vector pPIC9K (Invitrogen). A COOH-terminal His(6) tag (SGHHHHHH) was included in each construct. Transformation of yeast (*Pichia pastoris*) and induction of recombinant HEL expression was performed according to the manufacturer's instructions. Recombinant HEL proteins were purified from yeast culture supernatants by ion-exchange chromatography using HiTrap SP FF columns followed by Ni^{2+} affinity chromatography (both from GE Healthcare). Recombinant HEL proteins were >95% pure as determined by SDS-PAGE and staining with Coomassie blue. Yields ranged from 5–15 mg of purified protein from 600 ml of culture supernatant.

Mice and adoptive transfers. SW_{HEL} (25), $SW_{HEL}.rag1^{-/-}$ (28), and $SW_{HEL(H)}$ (24) mice have been described previously. WT C57BL/6 and CD45.1 congenic C57BL/6 mice were obtained from the Animal Resources Centre (Perth, Australia). All mice were maintained on a C57BL/6 background and housed in specific pathogen–free environment. All experimental procedures were approved by the University of Sydney Animal Ethics Committee. B cell isolation, CFSE labeling, and conjugation of HEL to SRBC were performed as described previously (6). SRBC conjugations were performed using recombinant WT or mutant HEL at 100 μg/ml unless otherwise indicated (Fig. 6). For adoptive transfers, spleen or lymph node cells from SW_{HEL} or $SW_{HEL(H)}$ donor mice containing 10^4 HEL-binding B cells were injected intravenously into male CD45.1 congenic C57BL/6 recipients together with 2×10^8 SRBC conjugated to a specific recombinant HEL protein. Mock-conjugated SRBC in which HEL was omitted from the conjugation reaction served as a control antigen. No additional adjuvant was used in the immunizations. Mice receiving only HEL-conjugated SRBCs intravenously did not produce detectable anti-HEL serum antibodies, presumably because of the low frequency of anti-HEL B cells present in the absence of transferred SW_{HEL} B cells.

ELISAs. Anti-HEL antibody levels in sera from immunized mice were analyzed by ELISA as described previously (25). Competitive ELISA was performed to measure the relative affinities of the mutant HEL for HyHEL10. For this, plates were first coated with purified HyHEL10, after which 50 ng/ml of biotinylated HEL^{WT} was added in conjunction with varying concentrations of unlabeled recombinant HEL^{WT}, HEL^{1X}, HEL^{2X}, and HEL^{3X}. Non-linear regression based on a one-site competition model was performed using GraphPad Prism software to find the curve-fit and calculate the half-maximal inhibitory concentration.

Western blotting. $SW_{HEL}.rag1^{-/-}$ B cells were isolated and stimulated with varying concentrations of recombinant HEL proteins for 15 min at 37°C, and whole cell lysates were prepared in buffer containing protease and phosphatase inhibitors as described previously (42). Proteins were separated by SDS-PAGE and membranes probed with mouse antiphosphotyrosine (Upstate Biotechnology) followed by goat anti–mouse IgG–horseradish peroxidase (Santa Cruz Biotechnology, Inc.) and developed with enhanced chemiluminescence (Perbio). Blots were stripped and reprobed with anti-actin antibody (Santa Cruz Biotechnology, Inc.) to control for protein loading.

Immunohistology. Splenic sections were stained for GCs and HEL-binding B cells as previously described (6).

FACS analysis. Splenocytes were prepared, stained for surface molecules, and analyzed on a FACSCalibur (BD Biosciences) as previously described (6). Intracellular staining to detect cytoplasmic HEL-binding Ig was performed after fixation with 10% formalin and permeabilization with 0.2% polyethylene sorbitan monolaurate. GC B cells (low intracellular HEL binding and syndecan-1 expression) and plasma cells (high intracellular HEL binding and syndecan-1 expression) were enumerated using the gates shown in Fig. 4 B. B cells in mice challenged with mock-SRBC fell in the GC B cell gate but were not counted as they had not proliferated (Fig. 2), did not express GL7 (not depicted), and were shown histologically to be absent from GC (Fig. 3 D).

SHM analysis. Single responding SW_{HEL} B cells were sorted from the spleens of recipient mice 5 d after transfer using a FACSVantage (BD Biosciences). GC and plasma cells were identified using the gates shown in Fig. S1 (available at http://www.jem.org/cgi/content/full/jem.20060087/DC1) and single cells sorted into 96-well plates containing proteinase K (Sigma-Aldrich). The variable region exon of the SW_{HEL} Ig (HyHEL10) heavy chain gene was then PCR amplified using Taq polymerase (Sigma-Aldrich) for 35 cycles with the primers GTTGTAGCCTAAAAGATGATGGTG and GATAATCTGTCCTAAAGGCTCTGAG. The primary PCR product was further amplified for 35 cycles with the nested primers TTGTAGCCTAAAAGATGATGGTGTTAAGTC and CAACTTCTCTCAGCCGGCTC. The final PCR product was sequenced with the dideoxy-chain termination method using primer TTGTAGCCTAAAAGATGATGGTGTTAAGTC at the Australian Genomic Research Foundation (Brisbane, Australia). Sequences encoding the mature VDJ region were analyzed for the presence of SHM events.

Online supplemental material. Fig. S1 shows the FACS gating strategy for sorting single cells with the GC and plasma cell phenotypes. Fig. S2 shows the binding of the various mutant HEL proteins to both SW_{HEL} and $SW_{HEL(H)}$ B cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20060087/DC1.

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