Immunology and Cell Biology (2008) 86, 31–39

www.nature.com/icb

REVIEW

Visualizing the effects of antigen affinity on T-dependent B-cell differentiation

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Burnet's original description of the clonal selection hypothesis of antibody production included many prescient predictions of how 'lymphocytes carrying reactive sites' for foreign antigens might respond during immune responses. Somatic mutation, plasma cell differentiation and transition into memory cells were all described as potential fates for the 'variety of descendents' derived from proliferative expansion of antigen-reactive clones. After 50 years much is known about the molecular controls that drive these various processes. Comparatively little insight has been gained, however, into why particular daughter cells progress down one response pathway versus another. In this article, we briefly describe the evolution of the genetic technologies that now allow us to visualize the very processes predicted by Burnet. An in-depth description of the recently developed SW_{HEL} mouse model and its utility for tracking *in vivo* B-cell responses to various forms of hen-egg lysozyme (HEL) is also provided. Recent data obtained with this system indicate that antigen-dependent variables play a critical role in regulating the differentiation of responding B cells into antibody-secreting plasma cells.
Immunology and Cell Biology (2008) **86,** 31–39; doi:10.1038/sj.icb.7100143; published online 20 November 2007

Keywords: B cells; antigen; affinity; germinal centres; plasma cells; antibodies

With the benefit of 50 years hindsight, the concepts outlined in Burnet's description of the clonal selection hypothesis of antibody production[1] seem axiomatic to modern immunologists. Indeed, it is almost inconceivable now to think that alternative theories for the basis of immunity were entertained.[2] In the twenty-first century, the selection and proliferative expansion of antigen-binding lymphocyte clones predicted by Burnet are readily observable through *ex vivo* analysis of processed lymphoid tissues and, more recently, by direct intravital microscopic analysis of intact tissue. More than ever, the events that unfold after the initial contact between lymphocyte and antigen are open to scrutiny. This, however, was not always the case.

In the years following the publication of Burnet's manuscript, those attempting to prove (or disprove!) the clonal selection hypothesis were faced with the prospect of isolating and studying single lymphocytes in order to verify whether these cells did indeed express only a single type of antibody. In the days before flow cytometry, this necessitated the development of a variety of ingenious approaches to reveal the properties of single lymphocytes.[3] Despite great technological challenges, the efforts of these researchers resulted in the clonal selection hypothesis being widely accepted by the late 1960s. During the 1970s, the development of monoclonal antibody technology and the elucidation of V(D)J gene rearrangement vindicated the basic tenet of the clonal selection hypothesis. These advances also paved the way for somewhat of a revolution in immunology during the 1980s when the advent of transgenesis provided a new approach for studying antigen-specific lymphocyte responses.

IMMUNOGLOBULIN TRANSGENIC MICE

Even after the general acceptance of the clonal selection hypothesis, the inherent diversity of the immune repertoire that it successfully predicted continued to provide a major obstacle to research. Thus the frequencies of lymphocytes capable of recognizing any given antigen within the normal primary repertoire are so exceedingly low as to make it virtually impossible to analyse their responses to initial antigenic encounter. Anti-immunoglobulin (anti-Ig) antibodies that bind to the constant regions of B-cell antigen receptors (BCRs) proved valuable in elucidating many of the acute responses of B cells to BCR stimulation. Ultimately, however, it was necessary to determine how the immune system deals with *bona fide* antigens that interact with the clonal complementarity-determining regions of the BCR variable regions. With the advent of multi-parameter flow cytometry, antigen-specific B cells could be identified *ex vivo*, but these were only readily visualized after immunization and were inevitably derived from multiple clones with varying initial affinities for the immunogen.[4] Analysis of antigen-specific lymphocytes from the normal primary repertoire remained problematic.

As it happened, mice carrying stably integrated gene constructs encoding Ig light and heavy chains were among the first transgenic

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Received 12 October 2007; accepted 13 October 2007; published online 20 November 2007

mice generated during the early 1980s.[4] It became apparent that, as well as achieving the desired expression of transgenic Ig molecules in B cells, the stable integration of Ig transgenes carrying the appropriate promoter and enhancer elements usually prevented expression of the endogenous Ig genes. We now know that this to be due to the feedback inhibition of V(D)J recombination that occurs after membrane expression of μ heavy chains—the process that in fact underpins the allelic exclusion of Ig expression that maintains the 'one cell: one antibody' rule predicted by Burnet. From here it was a short step to the realization that co-introduction into the mouse germ line of rearranged Ig heavy- and light-chain transgenes should result in the expression of a monoclonal antigen specificity by virtually all B cells in that Ig-transgenic mouse.[4]

The majority of the early work performed using Ig-transgenic mice was aimed at determining the fate of self-reactive B cells generated in the primary repertoire. To this end, Ig-transgenic mice were produced in which B cells expressed monoclonal specificities against self-components such as DNA, major histocompatibility complex (MHC) molecules and erythrocyte antigens.[4] A system that provided some of the most significant insights into the mechanisms of self-tolerance involved mice expressing a high-affinity BCR directed against the foreign protein hen-egg lysozyme (HEL). In this case, the B cells were not self-reactive until they were mated with separate line of transgenic mice that expressed HEL as a 'neo-self' antigen.[5] One of the triumphs of this system was the demonstration that the same self-antigen:BCR combination could result in different fates for the self-reactive B cell (that is, anergy versus deletion) depending on variables associated with self-antigen expression (that is, soluble versus membrane-bound respectively).[5,6] Similar results were subsequently obtained in a number of different systems[4] and the predictions of these Ig-transgenic models were subsequently vindicated by molecular analysis of the developing human B-cell repertoire.[7,8]

VDJ 'KNOCK-IN' MICE

The impact of molecular biology on all spheres of biological research grew dramatically during the 1980s. By the close of the decade, basic transgenesis was to a certain degree eclipsed by a sophisticated new technology that facilitated the introduction of defined and inheritable modifications into murine genes. Appropriately, the key developers of the technology were awarded the 2007 Nobel Prize in Physiology or Medicine almost 50 years to the day after the original publication of Burnet's clonal selection hypothesis (http://nobelprize.org/nobel_prizes/medicine/laureates/2007/index.html). The primary use of this technology has been to facilitate the 'reverse genetics' approach of determining gene function through the phenotypic analysis of mice carrying homozygous inactivating mutations in specific genetic loci. However, several groups also recognized the potential for targeted gene modification to overcome a major drawback associated with the use of Ig-transgenic mice.

In contrast to the profound insight they provide into the control of B cells by self-antigen, Ig-transgenic mice were of limited use in analysing productive immune responses to foreign antigens. This was primarily due to size constraints that limit Ig heavy-chain transgenes to carrying the rearranged VDJ exon linked to only μ and δ constant region exons. Thus Ig-transgenic B cells can recapitulate the co-expression of IgM and IgD during B-cell development[9] but cannot undergo the appropriate class switch recombination (CSR) to downstream Ig isotypes (IgG_3, IgG_1, IgG_{2a}, IgG_{2b}, IgE, IgA) that occurs during the majority of immune responses. The ability of gene targeting to engineer mice capable of generating monoclonal B cells capable of undergoing normal CSR was confirmed in 1993 when a rearranged VDJ exon was introduced into it's physiologically appropriate location, that is the 5′ J_H region of the mouse Ig heavy-chain locus.[10] As predicted, B cells carrying this targeted VDJ exon in their germ line were capable of both normal CSR to all isotypes and somatic hypermutation (SHM) of the targeted variable region exon. Interestingly, a significant fraction of the B cells that develop in mice heterozygous for this targeted VDJ insertion undergo recombination of upstream V_H and/or D segments into the rearranged VDJ exon during early B-cell development.[11] This phenomenon also occurs in mice carrying similarly targeted VDJ exons of different specificities[12,13] with the result that VDJ knock-in mice inevitably express the intact targeted VDJ region in some rather than all B cells, unless they are crossed onto a *Rag1*$^{-/-}$ or *Rag2*$^{-/-}$ background. As we see later, the advent of VDJ knock-in mice has significantly advanced our ability to follow the various stages of B-cell responses as they occur *in vivo*.

REGULATION OF T-DEPENDENT B-CELL RESPONSES BY ANTIGEN AFFINITY

The typical progression of a T-dependent B-cell response can be summarized as follows (Figure 1). Initial contact with antigen delivers stimulatory signals through the BCR and facilitates the internalization of antigen and subsequent presentation of peptide fragments with cell surface class II MHC molecules. BCR-stimulation drives B cells to migrate towards the T cell-rich zones of secondary lymphoid tissues where activated $CD4^+$ T cells recognizing the specific peptide:MHC conjugates on the B cell deliver helper signals that drive B-cell proliferation (Figure 1). Around days 3–4 of the response, daughter cells expanded from the original B-cell clones migrate into extrafollicular regions of the lymphoid tissue and differentiate into short-lived plasma cells that provide the initial burst of serum antibody production. Other daughter cells follow a different response pathway, migrating into the B-cell follicle to form germinal centres (GCs) in association with follicular dendritic cells and T-follicular helper cells (T_{FH}) (Figure 1). It is in GCs that B cells undergo SHM of their Ig genes. Here rare clones that acquire mutations that increase their affinity for the immunizing antigen survive preferentially (affinity maturation). GC B cells can ultimately undergo terminal differentiation into plasma cells (Figure 1). Unlike the extrafollicular plasma cells generated earlier in the response, post-GC plasma cells carry somatically mutated Ig genes and can persist for long periods in the bone marrow as a sustained source of antibody. GC B cells can also differentiate into memory B cells that persist in the body and facilitate

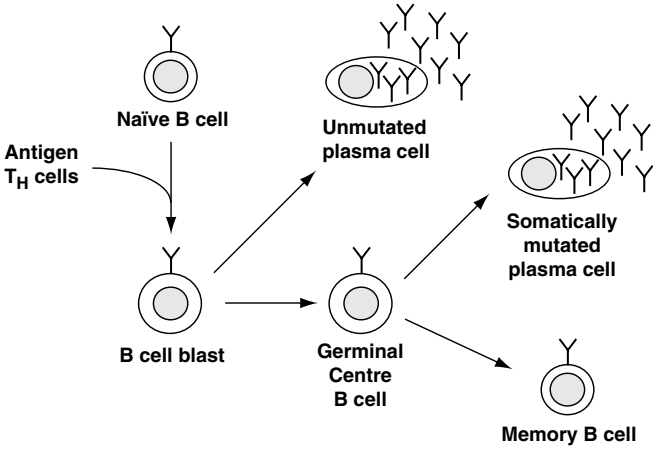


Figure 1 Schematic diagram of the basic steps in T-dependent B-cell differentiation. See text for description.

rapid recall responses upon secondary challenge with antigen (Figure 1).

Within this response framework, the affinity of the BCR for the foreign antigen is known to play a fundamental role at two key stages. The first of these is the initial recruitment of B cells into the response. Burnet explained antigen-specific immunity on proliferative expansion 'of all those clones whose reactive sites correspond to the antigenic determinants on the antigen used'. Since many BCRs are likely to bind to any given antigen with some measurable affinity, cells that 'correspond' to a given antigenic determinant must express BCRs that bind to it with sufficient affinity to ultimately recruit that B-cell clone into a proliferative response. The precise value of this 'affinity threshold' will vary for different antigens due to the influence of a variety of factors including epitope density, antigen dose, availability of co-stimuli and the relative affinities of B cells capable of competing for antigen.[14,15]

The affinity of the antigen:BCR interaction is also fundamental to the process of affinity maturation. This term was originally coined to describe the increase in the average affinity of serum antibodies over the course of T-dependent immune responses[16] but has also become synonymous with the selection of B cells that acquire increased affinity for antigen in the GC.[17,18] Antigen-based selection of B cells in GCs occurs in an environment enriched with antigen-reactive clones and so is based on competition for limiting amounts of antigen rather than being governed by a specific affinity threshold. Thus B cells that acquire increased affinity compared to surrounding clones are more likely to receive positive signals through their BCR and to present antigen to T_{FH} cells. However, these cells can be similarly outcompeted by other B cells that have acquired even higher antigen affinity.[19]

Several other phases of T-dependent B-cell responses also have the potential to be regulated by the affinity of the antigen:BCR interaction. The differentiation of B cells expanded during the initial proliferative phase into either extrafollicular plasma cells or GC B cells (Figure 1) is one of these. When mice are immunized with a chicken γ-globulin conjugate of the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP-CGG), B cells with either low or high affinity for NP are recruited into in both the extrafollicular plasma cell and early GC B-cell compartments.[20] The implication of this result is that the daughter cells of the original B-cell clones differentiate stochastically into either extrafollicular plasma cells or GC B cells independently of their affinity for the stimulating antigen. However, the early antibody responses to certain antigens are of relatively high affinity[21–23] suggesting that, at least in these cases, antigen affinity may regulate entry into the early plasma cell compartment. Data also obtained from analysis of endogenous responses to NP have suggested that the later differentiation of GC B cells into plasma cells is regulated by BCR affinity.[24,25] On the other hand, the differentiation of GC B cells into memory B cells appears to be less tightly controlled by antigen affinity.[24,26]

More recently, the role of antigen affinity has been examined using VDJ knock-in mice. One approach used was to produce two independent lines of these mice in which B cells recognize NP with relatively low ($1.25\times10^5\,M^{-1}$) or high ($5\times10^6\,M^{-1}$) affinity.[27] Independent immunization of these mice with NP-CGG gave similar responses. However, when the two types of cells were challenged in the same recipient, the higher affinity B cells dominated the response.[28] Analogous results were obtained from two similar lines of low ($3\times10^4\,M^{-1}$) and high ($1.3\times10^5\,M^{-1}$) affinity anti-NP VDJ knock-in mice[15] suggesting that relative rather than absolute affinity is the more important property of the antigen:BCR interaction in determining the fate of antigen-binding B cells in these responses. Nevertheless, different affinity thresholds do exist for the various intracellular responses triggered by BCR signalling[14,29] raising the possibility that antigen affinity can play a role in regulating cell fate decisions during T-dependent responses.

THE SW_{HEL} MODEL: A NEW SYSTEM FOR INVESTIGATING ANTIGEN AFFINITY

The success of anti-HEL Ig-transgenic mice (line MD-4) in elucidating mechanisms of self-tolerance was built to large extent on the target antigen (HEL) being a protein whose expression pattern, intracellular targeting or primary amino-acid sequence could be altered by relatively straightforward manipulations of transgenic DNA constructs.[5,6,30] Since a versatile target antigen was also likely to be advantageous in the analysis of productive immunity, we developed a heavy-chain VDJ knock-in mouse based on the original MD-4 Ig-transgenic model. This genetically modified line was designated SW_{HEL} (switching anti-HEL).[31]

SW_{HEL} mice carry two independently segregating loci. The first of these is the rearranged VDJ exon of the HyHEL10 anti-HEL hybridoma targeted to the J_H region of the endogenous Ig heavy-chain gene.[31] The second locus is a randomly integrated HyHEL10 κ light-chain transgene identical to that used to produce MD-4.[5] Mice heterozygous for both loci contain naive B cells that co-express IgM and IgD antigen receptors with the high-affinity anti-HEL specificity of HyHEL10. Due to the variable-region replacement recombination events that typically occur in VDJ knock-in lines (see above), only 10–25% of B cells express the HyHEL10 specificity in SW_{HEL} mice.[31] These anti-HEL B cells nevertheless undergo normal CSR and SHM upon challenge with immunogenic HEL.[31–34] Importantly, the SW_{HEL} line was produced and is maintained on a pure C57BL/6 genetic background.[31]

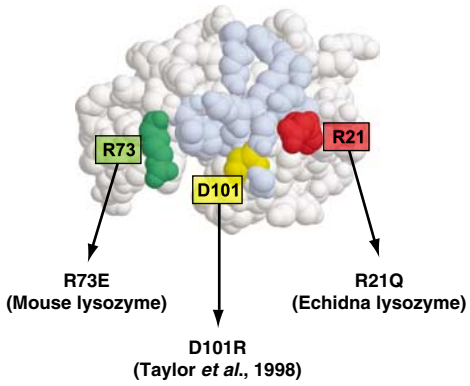
The HyHEL10 specificity expressed by SW_{HEL} B cells binds to wild-type HEL with very high-affinity ($2.0\times10^{10}\,M^{-1}$).[35] To study the effects of antigen affinity using SW_{HEL} mice, a series of recombinant HEL muteins were therefore designed to bind HyHEL10 with reduced affinity. Based on the crystal structure of the HEL:HyHEL10 complex,[35] amino-acid residues within HEL that formed direct contacts with HyHEL10 were assessed as candidates for mutagenesis. Optimal amino-acid substitutions were considered to be those that were non-conservative (to maximize the chances of destabilizing the interaction) but also unlikely to disturb the normal folding of the HEL protein. A previously characterized substitution (D101R) satisfied both these criteria.[36] Additional substitutions were identified by aligning the primary amino-acid sequence of HEL with lysozyme proteins derived from a range of avian and mammalian species. Promising naturally occurring substitutions at HyHEL10 contact residues of HEL were identified in mouse (R73E) and echidna (R21Q) lysozymes (Figure 2a). HEL cDNAs were produced that carried these three substitutions either singly or in combination.[34]

To obtain recombinant HEL proteins, wild-type and mutated HEL cDNAs were cloned into a yeast expression vector and the proteins expressed as secreted products of transformed *Pichia pastoris*. Purification of recombinant proteins from culture supernatant was achieved by nickel column affinity chromatography facilitated by a C-terminal hexa-histidine tag added to each protein. All recombinant proteins retained wild-type enzymatic activity and bound with normal affinity to the anti-HEL mAbs HyHEL5 and HyHEL9, indicating normal three-dimensional folding. As expected, however, all HEL molecules carrying one or more of these three substitutions exhibited reduced affinity for HyHEL10.

In addition to wild-type recombinant HEL (HEL^{WT}), three mutant proteins were selected for further analysis. These proteins were

designated HEL^{1X} HEL^{2X} and HEL^{3X} to reflect the number of amino-acid substitutions present in each molecule (Figure 2b). As would be predicted, the affinity of these proteins for HyHEL10 became progressively lower as the number of substitutions increased (Figure 2b).[34] The affinity of HEL^{1X} and HEL^{2X} for HyHEL10 in fact differed by only threefold. The three proteins chosen for further analysis—HEL^{WT}, HEL^{2X} and HEL^{3X}—corresponded to high-, intermediate- and low-affinity antigens respectively for the SW$_{HEL}$ BCR and covered a 10 000-fold affinity range (Figure 2b).

a



b

Recombinant HEL	HEL^{WT}	HEL^{1X}	HEL^{2X}	HEL^{3X}
Mutations		D101R	D101R R73E	D101R R73E R21Q
Affinity (M^{-1})	2.0 x 10^{10}	2.5 x 10^{8}	8.0 x 10^{7}	1.5 x 10^{6}

Figure 2 Site-directed mutagenesis of the HyHEL10-binding site of hen-egg lysozyme (HEL). (**a**) Rasmol space-filling model of HEL showing the three residues (coloured) within the HyHEL10-binding footprint (light blue) that were mutated to reduce the affinity of the HEL:HyHEL10 interaction. The specific mutations introduced were either previously published (D101R)[36] or were identified from natural variations present in either mouse (R73E) or echidna (R21Q) lysozyme. (**b**) Specific combinations of mutations in the various recombinant forms of HEL are shown together with the approximate affinities of the recombinant HEL proteins for HyHEL10 as determined by competition ELISA.[34]

To analyse the effects of antigen affinity on T-dependent B-cell responses, an adoptive transfer approach was employed that had previously been developed using commercially available wild-type HEL (Figure 3).[32] In brief, small numbers (1×10^4 to 3×10^4) of HEL-binding B cells from either the spleen or lymph nodes of a donor SW$_{HEL}$ mouse are transferred intravenously into CD45.1 congenic C57BL/6 recipient mice together with 2×10^8 sheep red blood cells covalently coupled to HEL (HEL-SRBC). In this system, anti-SRBC T-helper cells drive the response of the anti-HEL SW$_{HEL}$ B cells, since HEL fails to elicit a strong T-helper response on the C57BL/6 background.[37] By using CD45.1 congenic C57BL/6 recipients, SW$_{HEL}$ donor B cells can be identified by flow cytometry by virtue of their unique expression of the endogenous C57BL/6 CD45.2 allele as well as their ability to bind HEL (Figure 3).[32] In addition, the positioning of the responding SW$_{HEL}$ B cells within secondary lymphoid tissues is readily visualized by immunofluorescence histology.[32] Using this basic approach, we asked how the response of SW$_{HEL}$ B cells might vary when they are challenged with either HEL^{WT}, HEL^{2X} or HEL^{3X} conjugated to SRBCs.

ANTIGEN AFFINITY AND DENSITY REGULATE THE EXTRAFOLLICULAR PLASMA CELL RESPONSE

The response of SW$_{HEL}$ B cells to high-affinity antigen (HEL^{WT}-SRBC) essentially follows the schematic depicted in Figure 1. After initial proliferative expansion, daughter cells follow one of two paths, either differentiating into extrafollicular plasma cells or entering the GC reaction.[32] When SW$_{HEL}$ B cells were challenged with antigen that binds to the BCR with ~250-fold lower affinity (HEL^{2X}-SRBC) a very similar response pattern was observed, with the donor-derived B cells present after 5 days being roughly equally divided between the extrafollicular plasma cell and GC B-cell compartments.[34] A striking effect was observed, however, when antigen affinity was reduced a further 50-fold. Thus SW$_{HEL}$ B cells challenged with HEL^{3X}-SRBC generated virtually no extrafollicular plasma cell response but formed similar numbers of GC B cells to those produced in response to

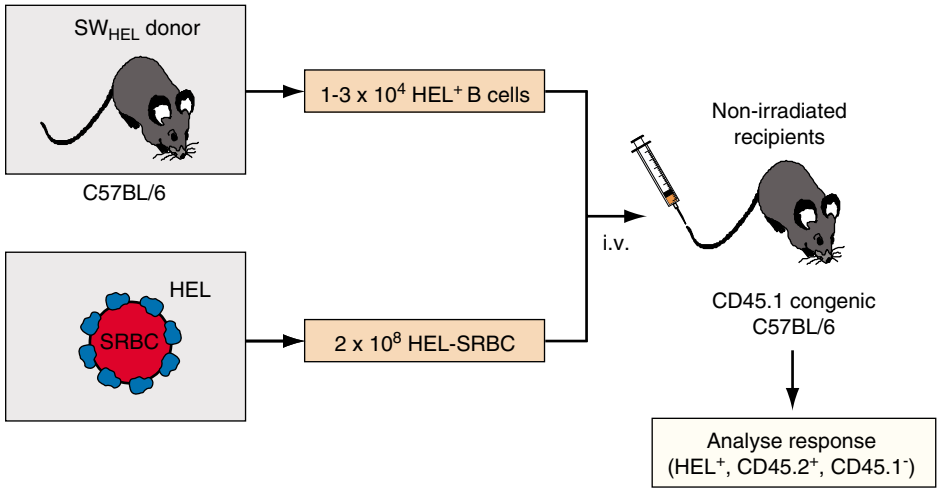


Figure 3 Schematic diagram of the basic adoptive transfer system used to analyse T-dependent responses of SW$_{HEL}$ anti-hen-egg lysozyme (HEL) B cells. Spleen and lymph node preparations from SW$_{HEL}$ mice typically contain ~10% anti-HEL (HEL$^+$) B cells, with the result that 1×10^5 to 3×10^5 unpurified SW$_{HEL}$ donor cells are transferred into each recipient mouse. SW$_{HEL}$ mice crossed onto a CD45.1 congenic C57BL/6 background can be used as a source of donor cells if recipient mice are on a CD45.2$^+$ C57BL/6 background (for example, 'gene knockout' recipients).

HEL^{WT}-SRBC and HEL^{2X}-SRBC.[34] In this system, therefore, early plasma cell differentiation but not GC B-cell differentiation required the responding B cells to exceed an antigen-affinity threshold somewhere between 8.0×10^7 and $1.5\times10^6\,M^{-1}$.

The result we obtained was consistent with previous observations that the initial phase of antibody production can be of relatively high-average affinity ($>10^7\,M^{-1}$).[21–23] Subsequent analysis of a different VDJ knock-in line has also indicated that the daughter cells of high-affinity B-cell clones preferentially differentiate into short-lived extrafollicular plasma cells.[38] Nevertheless, this result appeared to contrast with previous analyses of the endogenous anti-NP response (discussed above) that indicated that low- as well as high-affinity clones enter the early plasma cell response.[15,20,28] One reason for this discrepancy may be that the density of NP molecules on haptenated proteins such as NP-CGG is typically very high (for example, 16 NP groups per CGG monomer),[15] meaning that the antigen may still bind with relatively high avidity to cell surface BCRs even though they may have relatively low affinity for NP. In other words, BCR signal strength as opposed to affinity *per se* may be the more fundamental determinant of early plasma cell differentiation.

If this proposition is true, we reasoned that the production of early plasma cells by SW_{HEL} B cells could be altered by manipulating the density of the HEL proteins on the SRBC surface. Accordingly, it was found that SW_{HEL} B cells could make a significant early plasma cell response to low-affinity antigen (HEL^{3X}-SRBC) if the density of HEL^{3X} on the SRBC surface was increased ~threefold.[34] Similarly, the early plasma cell response to intermediate affinity antigen (HEL^{2X}-SRBC) declined as the density of HEL^{2X} was reduced.[34] It therefore appears that it is the intensity of the BCR interaction with antigen, to which both affinity and epitope density contribute, that controls the differentiation of responding B cells into short-lived extrafollicular plasma cells.

ANTIGEN AFFINITY ALSO REGULATES POST-GC PLASMA CELL DIFFERENTIATION

The SW_{HEL} model has also been use to assess the importance of antigen affinity in the differentiation of plasma cells from GC B-cell precursors. These plasma cells appear later during T-dependent responses and inevitably carry somatic mutations in their Ig variable region genes (Figure 1). It has not been previously possible to directly determine whether these later plasma cells are stochastically generated from all GC B cells or if their production might be restricted to those cells that have acquired increased affinity for the immunogen.

SW_{HEL} B cells challenged with low-affinity antigen (HEL^{3X}-SRBC) were found to undergo both serological and cellular affinity maturation. Thus, although little anti-HEL antibody was produced from the virtually non-existent early plasma cell response, anti-HEL antibody with progressively increasing affinity for HEL^{3X} subsequently arose in the sera of recipient mice.[33] This was accompanied by the accumulation of SW_{HEL} donor-derived B cells that were revealed by flow cytometry to have increased affinity for HEL^{3X}.These high-affinity anti-HEL^{3X} B cells proceeded to dominate the donor-derived B-cell population by day 15 of the response.[33] SHM analysis revealed that virtually all B cells that exhibited increased affinity for HEL^{3X} carried a canonical Ig heavy-chain somatic mutation that encoded the Y53D substitution. This substitution was confirmed to increase the affinity of HyHEL10 for HEL^{3X} by ~80-fold.[33]

Based on this information, it was possible to examine the connection between the acquisition of increased antigen affinity by GC B cells and their subsequent differentiation into plasma cells. At day 5 of the response to HEL^{3X}-SRBC, many SW_{HEL} donor-derived GC B cells had begun to accumulate somatic mutations but there was as yet no evidence of selection for B cells either carrying the Y53D mutation or exhibiting increased affinity for HEL^{3X}. By day 10, however, a significant population of B cells with increased affinity for HEL^{3X} was evident. Accordingly, ~25% of GC B cells carried the Y53D mutation at this point. Strikingly, >85% of plasma cells present at day 10 time carried the Y53D substitution,[33] indicating that the high-affinity GC B cells expressing this altered BCR were preferentially undergoing plasma cell differentiation. Consistent with this, the frequency of cells expressing the Blimp1-green fluorescent protein (GFP) plasma cell reporter marker[39] was ~10-fold higher among responding SW_{HEL} B cells that exhibited high versus low affinity for HEL^{3X}.[33] Thus the data obtained from the SW_{HEL} model indicates that antigen affinity is an important regulator of both extrafollicular and post-GC plasma cell differentiation.

HEL^{2X}-SRBC TRIGGERS EXTRAFOLLICULAR PLASMA CELL DIFFERENTIATION AND AFFINITY MATURATION BY SW_{HEL} B CELLS

The analysis of affinity maturation to HEL^{3X}-SRBC described above was performed in parallel with responses mounted against the high-affinity HEL^{WT}-SRBC antigen. The first result revealed by this analysis was that the initial SHM rate did not differ between the two responses,[33] confirming previous observations in anti-NP VDJ knock-in mice that antigen affinity does not affect SHM kinetics.[28] In contrast to the dominant selection of the Y53D mutation during the HEL^{3X}-SRBC response, SW_{HEL} B cells challenged with HEL^{WT}-SRBC showed no evidence of selection for specific somatic mutations.[33] Given the already extremely high affinity of the HEL^{WT}:HyHEL10 interaction ($2\times10^{10}\,M^{-1}$), the apparent lack of affinity maturation in this response was not surprising and was consistent with *in vitro* studies indicating that selective advantages are unlikely to ensue for affinity increases beyond a K_a of $\sim10^{10}\,M^{-1}$.[14]

We have recently carried out a more detailed analysis of the response of SW_{HEL} B cells to the intermediate affinity antigen HEL^{2X}-SRBC. This included the employment of six-colour flow cytometry in contrast to the previously published studies that used a four-colour protocol. Figure 4b shows an analysis of SW_{HEL} donor B cells 5 days after challenge with HEL^{2X}-SRBC. A gate containing donor cells ($CD45.2^+$, $CD45.1^-$) was subsequently gated further to exclude doublets and non-HEL-binding cells and reveal the responding SW_{HEL} B-cell population (Figure 4b). After applying the same gating strategy to spleen cells from recipient mice that received SW_{HEL} donor cells along with mock-conjugated SRBCs (Figure 4a), it was apparent that donor anti-HEL B cells were expanded >500-fold during the 5 day response to HEL^{2X}-SRBC. Many donor-derived B cells exhibited class switching to IgG_1 and therefore showed proportional staining for surface IgG_1 and HEL binding on these cells (Figure 4b). As shown previously,[34] approximately half of the donor-derived B cells underwent plasma cell differentiation by day 5 of the response to HEL^{2X}-SRBC. These plasma cells are evident as a $B220^{lo}$, $GL7^{lo}$, Fas^{lo} population that is clearly distinct from the $B220^{hi}$, $GL7^{hi}$, Fas^{hi} GC B cells (Figure 4b). This early extrafollicular plasma cell population contains a significant proportion of $IgG_1{}^+$ cells (Figure 4b) with the result that large amounts of anti-HEL IgG_1 are already evident in recipient sera by day 5 of the response to HEL^{2X}-SRBC (Figure 4c).

Although the affinity of HEL^{2X} for the SW_{HEL} BCR ($8.0\times10^7\,M^{-1}$) is ~50-fold higher than that of HEL^{3X} ($1.5\times10^6\,M^{-1}$), it remains well below the theoretical upper limit for affinity maturation ($\sim10^{10}\,M^{-1}$).[14] To determine whether SW_{HEL} B cells could still undergo affinity maturation in response to HEL^{2X}-SRBC, the somatic

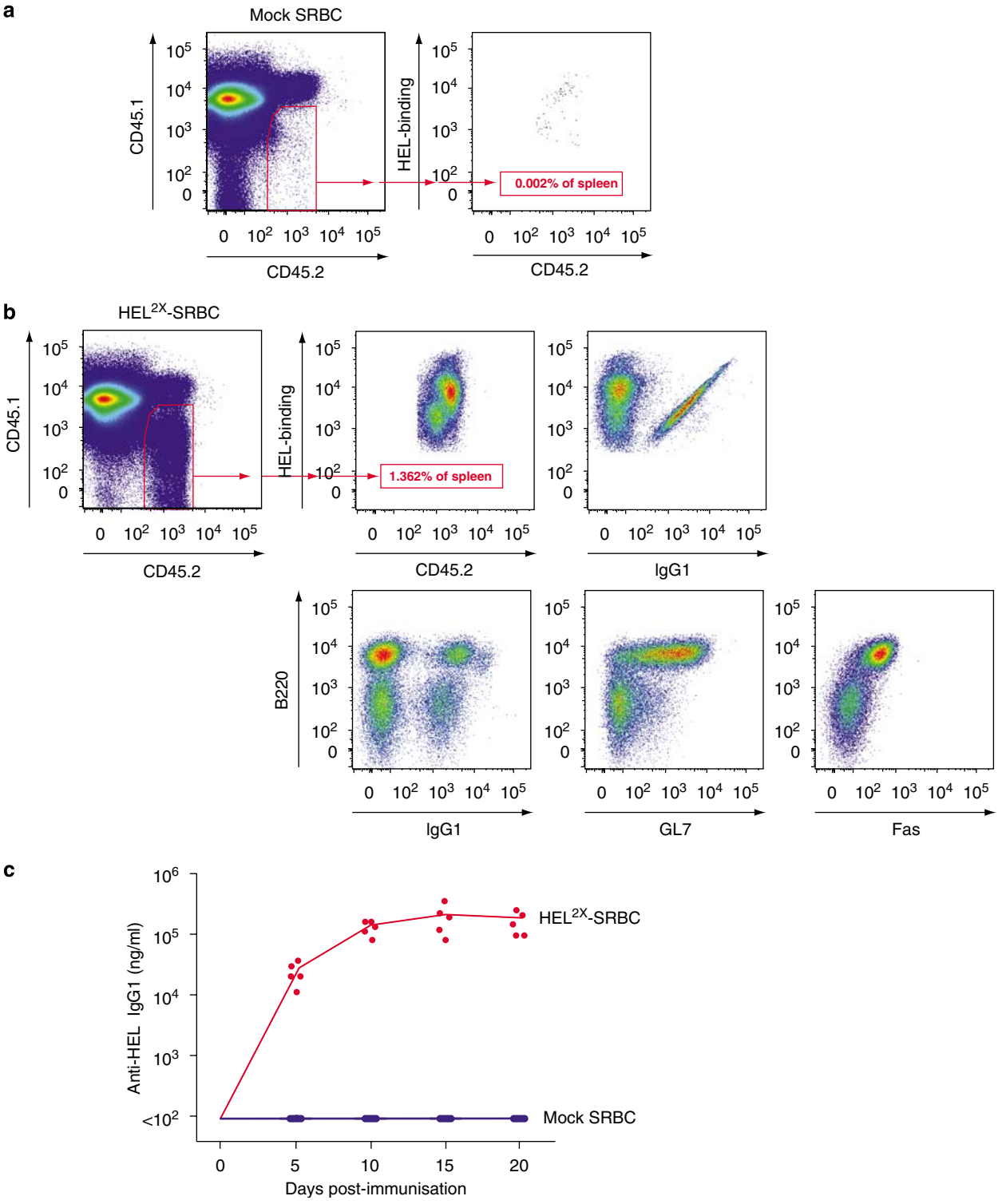


Figure 4 SW_{HEL} B cells differentiate into class-switched plasma cells and GC B cells 5 days after challenge with HEL^{2X}-SRBC. SW_{HEL} B cells were challenged with either mock-conjugated sheep red blood cells (SRBC) (**a**) or HEL^{2X}-conjugated SRBCs (**b**) as outlined in Figure 2. Recipient spleens were harvested 5 days later and stained with anti-GL7-FITC, anti-B220-PE, anti-CD45.2-PerCP/Cy5.5, anti-CD45.1-PE/Cy7 and either anti-immunoglobulin G_1 (IgG_1)-biotin or anti-Fas-biotin plus streptavidin-Pacific Blue. Data were gated sequentially for (1) resting and activated lymphocytes (FSC versus SSC), (2) donor lymphocytes (CD45.1 versus CD45.2 shown), (3) doublet exclusion (FSC-A versus FSC-H) and (4) hen-egg lysozyme (HEL) binding. Plasma cells were evident in HEL^{2X}-SRBC-immunized recipients as a $B220^{lo}$, $GL7^{lo}$, Fas^{lo} population, many of which had class switched to IgG_1. (**c**) Sera from recipient mice were assayed for anti-HEL IgG_1 by ELISA. Anti-HEL IgG_1 was already evident by day 5 of the response to HEL^{2X}-SRBC due to the production of large numbers of early IgG_1^+ plasma cells (**b**).

mutation spectrum of donor-derived GC B cells was analysed on day 10 of the response. Over 40% of the clones analysed were found to carry the same Y53D mutation that is heavily selected for in the response to HEL^{3X}-SRBC, indicating that the response to HEL^{2X}-SRBC does indeed undergo affinity maturation. Since the Y53D mutation bestows greatly improved binding of HEL^{3X} by HyHEL10,[33] we reasoned that the affinity maturation of the HEL^{2X}-SRBC response would be accompanied by the accumulation of donor-derived B cells exhibiting increased HEL^{3X} binding. This was indeed found to be the case, with HEL^{3X} binding, IgG_1^+ B cells being undetectable at day 5, making up half of the IgG_1^+ compartment at day 10, and completely dominating the response by day 15 (Figure 5a). Affinity maturation of the serum antibody response was also evident. Thus the day 5 serum anti-HEL IgG_1 derived from the initial, unmutated extrafollicular plasma cell response to HEL^{2X}-SRBC showed minimal reactivity with HEL^{3X} by enzyme-linked immunosorbent assay (ELISA), whereas the serum antibody present at day 15, some of which will have been derived from post-GC plasma cells, showed greatly increased HEL^{3X}-binding activity (Figure 5b).

These results show that, while SW_{HEL} B cells undergo affinity maturation in response to HEL^{3X}-SRBC in the absence of a significant early antibody response, their response to HEL^{2X}-SRBC includes both affinity maturation and strong early antibody production from the production of extrafollicular plasma cells. The fact that affinity maturation in each case is mediated primarily by selection for the Y53D mutation is consistent with the prediction that this amino-acid substitution both relieves a steric clash and introduces a complementary negative charge at the point of contact between HyHEL10 and the engineered D101R substitution that is common to HEL^{2X} and HEL^{3X}.[34] Nevertheless, the effect of the Y53D substitution on relative

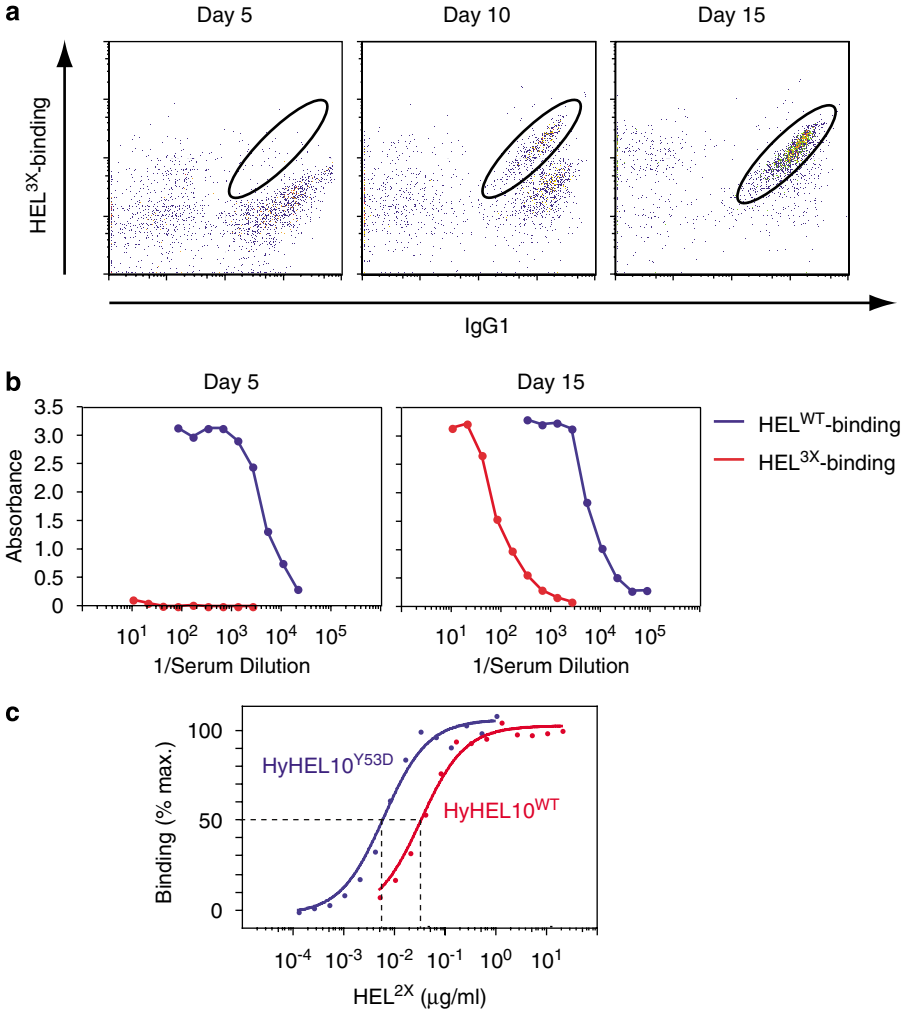


Figure 5 SW_{HEL} B cells undergo affinity maturation in response to challenge with hen-egg lysozyme (HEL^{2X})-SRBC. (**a**) SW_{HEL} B cells were challenged with HEL^{2X}-SRBC and analysed as outlined in Figure 2. Flow cytometry of spleen cells harvested from recipient mice at the indicated time points was performed as for Figure 4, except that binding of HEL^{3X} rather than HEL^{WT} was assayed. An immunoglobulin G_1 (IgG_1^+), HEL^{3X}-binding population emerged over the course of the response due to affinity maturation (the Y53D mutation selected in this response results in the SW_{HEL} BCR gaining increased affinity for both HEL^{2X} and HEL^{3X}). (**b**) Sera from recipients harvested on days 5 and 15 of the response to HEL^{2X}-SRBC were assayed for binding to ELISA plates coated with either HEL^{WT} or HEL^{3X}. Affinity maturation is evident from the specific increase in anti-HEL^{3X} IgG_1 over the course of the response. (**c**) The Y53D mutation increases the affinity of HyHEL10 for HEL^{2X} by ~6-fold. Binding of HEL^{2X} to either wild-type or Y53D-mutated HyHEL10 (IgG_1 isotypes) was assayed by ELISA using separate plates coated in the two different recombinant mAbs. A total of 50% saturation of Y53D-mutated HyHEL10 occurred at a ~6-fold lower concentration of HEL^{2X} compared to wild-type HyHEL10.

affinity of HyHEL10 for the two proteins is more pronounced in the case of HEL^{3X} ($\sim$80-fold)[33] than it is for HEL^{2X} ($\sim$6-fold) (Figure 5c).

CONCLUDING COMMENTS

In the early days of transgenesis there was a certain degree of mistrust about the applicability to 'normal' immunity of results obtained using antigen receptor transgenic lymphocytes. This view was warranted to some degree since some transgenic lines, especially those with high transgene copy numbers, occasionally exhibited somewhat bizarre phenotypes. The advent of gene targeting has allowed the genome to be modified in a far more precise and controlled manner. The ability to target a single rearranged VDJ exon to the physiologically appropriate location in the endogenous Ig heavy-chain locus not only overcomes integration site and copy number artefacts associated with transgenesis, but allows the normal processes of CSR and SHM to occur. B cells from VDJ knock-in mice are now widely accepted as valuable and reliable tools for examining antigen-specific B-cell responses *in vivo*. The generation of the SW_{HEL} mouse model has allowed these studies to include responses against the classic protein antigen HEL.

The combination of the SW_{HEL} mouse model with range of recombinant HEL proteins with differing affinities for the SW_{HEL} BCR has provided new perspectives on the role of antigen affinity in regulating T-dependent B-cell responses. The finding that antigen affinity, or more precisely the strength of the antigen:BCR interaction, plays a critical role in determining which clones enter the early plasma cell response is intriguing, given that polyclonal stimulation of B cells *in vitro* drives stochastic plasma cell differentiation in the complete absence of BCR stimulation.[40] A possible explanation for this is that BCR-dependent signals can somehow prevent B cells from migrating into the GC, since B cells that do enter the GC continue to proliferate without following a default plasma cell differentiation program.[33] A future challenge will be to determine the mechanism by which antigen:BCR interactions govern this early cell fate decision.

The requirement for the acquisition of increased affinity for the differentiation of GC B cells into plasma cells indicates that BCR signals are important for driving plasma cell differentiation both prior to and after GC formation. This suggests that the immune system places a high priority on ensuring 'quality control' of antibody production. If a strong antigen:BCR interaction is required to drive plasma cell differentiation, then the antibodies that are subsequently produced are those that are most likely to interact strongly with the antigen and thus be biologically effective. This may be of particular importance due to the limited capacity of the body to sustain plasma cells.[41,42] The 'chosen few' must therefore represent plasma cells that produce the most optimal specificities in order to ensure effective immunity.

A major role for VDJ knock-in mice has recently emerged with the development of multi-photon intravital microscopic analysis of immune responses. Dynamic visualization of GC responses occurring *in situ* within lymph node tissues has been facilitated using a transfer strategy similar to that outlined above (Figure 2) with the variation that the donor VDJ knock-in line expresses GFP in its B cells. Three recent studies demonstrated that the kinetics and direction of the migration of antigen-specific GFP^+ GC B cells can be effectively tracked using this technique.[19,43,44] It will be of great interest to see how the use of intravital microscopy develops in relation to the analysis of *in vivo* B-cell responses. For instance, will it eventually be possible to visualize affinity maturation and plasma cell differentiation using intravital microscopic analysis of the GC? The technical challenges associated with tracking these events are significant but, if overcome, would give the clearest view yet of the paths followed by the variety of descendents of the original antigen-specific B-cell clone.[1]

ACKNOWLEDGEMENTS

The work described here was funded by Program Grants 183700 and 427620 from the National Health and Medical Research Council of Australia.

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