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Positive Selection of B Cells Expressing Low Densities of Self-reactive BCRs

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Abstract

B cell tolerance or autoimmunity is determined by selective events. Negative selection of self-reactive B cells is well documented and proven. In contrast, positive selection of conventional B cells is yet to be firmly established. Here, we demonstrate that developing self-reactive B cells are not always highly sensitive to the deletion mechanisms imposed by membrane-bound self-antigens. At low amounts, membrane-bound antigens allow survival of B cells bearing a single high affinity self-reactive B cell receptor (BCR). More importantly, we show that forced allelic inclusion modifies B cell fate; low quantities of self-antigen induce the selection and accumulation of increased numbers of self-reactive B cells with decreased expression of antigen-specific BCRs. By directly measuring antigen binding by intact B cells, we show that the low amounts of self-antigen select self-reactive B cells with a lower association constant. A fraction of these B cells is activated and secretes autoantibodies that form circulating immune complexes with self-antigen. These findings demonstrate that conventional B cells can undergo positive selection and that the fate of a self-reactive B cell depends on the quantity of self-antigen, the number of BCRs engaged, and on its overall antigen-binding avidity, rather than on the affinity of individual BCRs.

Key words: B lymphocytes • self-reactivity • avidity • selection • self-antigen

Introduction

B cell repertoires are determined by different selective events (1). In transgenic mice expressing hen egg lysozyme (HEL) as a neo self-antigen, B cells transgenic for a high affinity B cell receptor (BCR) specific for HEL undergo different fates (2, 3). When HEL was expressed in membrane form, HEL-specific B cells were deleted early on ontogeny (3). When HEL was expressed in a soluble form, HEL-specific B cells were not deleted, but became unable to proliferate or secrete HEL-specific antibodies (2). Based on these results, it was postulated that B cell deletion required BCR cross-linking and could only be induced by membrane bound self-antigens. In contrast, soluble self-antigens could not induce negative selection, but only induced anergy. Studies on the fate of B cells expressing anti-MHC class I BCRs supported the role of receptor cross-linking in B cell deletion (4, 5) and showed that deletion of self-reactive B cells could also occur in the peripheral lymphoid tissues (5, 6). In contrast to T cells (7), positive selection of conventional B cells is not yet established. B cell positive selection has been shown only for a subpopulation of peritoneal B1 B cells (8). However, it is unlikely that conventional B cells do not undergo positive selection. Induced ablation of the BCR leads to rapid B cell death (9), demonstrating that B cells require BCR-mediated survival signals, either constitutive or given through their interaction with yet unknown ligands (10). The V gene usage skewing between immature BM and mature peripheral B cells further supports the role of positive selection in shaping B cell repertoires (11–13). The absence of papers demonstrating directly positive selection of conventional B cells may be due to the high affinity of the BCR interactions (compared with TCRs), which may hamper positive selection events. Recent works have shown that self-reactive B cells may escape deletion by either down-regulating the overall surface expression of BCRs (14, 15) or diluting the self-reactive BCR through the expression of two BCRs (16).

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Abbreviations used in this paper: BCR, B cell receptor; BrdU, bromodeoxyuridine; HEL, hen egg lysozyme.

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The role of the dose of antigen in B cell selection is also unknown. We may assume that to have a positive selection event, it suffices that one lymphocyte interacts with its specific ligand and receives survival signals. In contrast, to attain negative selection, that is, to expurgate a repertoire of a particular specificity, it is required that all lymphocytes bearing that specificity interact with antigen and receive death signals. Thus, the quantities of self-antigen may play a major role in the selection of B cell repertoires. Indeed, the probability of positive selection increases if the concentration of ligand favors its encounter with the rare specific cells, whereas negative selection can only be achieved if the quantities of antigen are sufficiently high to be met by all developing high affinity self-reactive cells. Previous studies have suggested that tolerance induction of self-reactive B cells was not achieved if soluble self-antigen was present below a certain concentration (17). However, the precise role of the quantity of self-antigens in B cell development and selection was not completely characterized.

To address these questions, we revisited the mouse model in which HEL-specific B cells develop in the presence of the neo self-antigen HEL (2, 3), but designed new experimental strategies to reduce self-antigen expression and/or B cell binding avidity for antigen. Our results show that membrane-bound self-antigen does not always induce B cell deletion; different types of B cell tolerance can be induced depending of the dose of self-antigen. Moreover, we estimated the antigen association constant of the intact self-reactive B cell, and showed that reduction of the B cell K_a (by forced allelic inclusion) modifies B cell fate. Low quantities of self-antigen mediate positive selection of conventional B cells and activate self-reactive B cells with a low K_a to secrete autoantibodies. In conclusion, like T cells, conventional B cells can undergo positive selection events. The quantity of self-antigen and the overall antigen-binding capacity of the cell determine the final fate of the self-reactive B cell.

Materials and Methods

Mice and Bone Marrow Chimeras. C57BL/6.Rag2-deficient mice (18) were obtained from the Centre Des Techniques Avancées, Centre Nationale de la Recherche Scientifique. The following lines of transgenic mice were used: MoMD4.Rag2$^{-/-}$ ($\alpha-$HEL; reference 2), MoSP6.Rag2$^{-/-}$ ($\alpha-$TNP; reference 19), MoMD4.MoSP6.Rag2$^{-/-}$(DIgTg; reference 20), B6.ML5.Rag2$^{-/-}$(sHEL; reference 2), and B6.KLK3.Rag2$^{-/-}$(mHEL; reference 3). All transgenic lines in the C57Bl/6.Rag2-deficient background were kept specifically pathogen-free at the Centre Des Techniques Avancées. 8-wk-old Rag2$^{-/-}$ mice were lethally irradiated with 950 rad from a ^{135}Cs source. Each host received i.v. 4×10^6 BM cells as indicated in the Results section. Donor cells were depleted of mature B cells using a Dynal MPC6 or AutoMacs (Miltenyi Biotec) magnetic sorter, and BM cells were incubated with anti-IgM biotinylated antibodies followed by streptavidin-coated Dynabeads. Chimeras were killed 6 wk after reconstitution, and the number and phenotype of BM pre–B and B cells, spleen B cells, and the serum IgM concentrations were evaluated. In the sHEL Tg donor mice, HEL is present exclusively in soluble form at ~20 ng/ml of serum. In the mixed sHEL BM chimeras, the serum amounts of HEL were below the 0.1 ng/ml detection level (not depicted) and we estimated the fraction of HEL-producing cells in each chimera by semi-quantitative PCR using primers specific for the HEL transgene (unpublished data). In the mHEL Tg donor mice, HEL (expressed under the control of the MHC class I H-2K^b promoter; reference 2) is present on the surface of all cells and shed to reach concentrations of 200–300 ng/ml in the serum (21). Thus, in the mHEL chimeras, quantification of antigen-expressing cells could be assessed either by ELISA or flow cytometry.

Flow Cytometry Analysis. Cells were stained with combinations of FITC, PE, allophycocyanin, or biotin-labeled antibodies, followed by streptavidin PerCP or streptavidin-allophycocyanin (Caltag). Monoclonal antibodies RA3-6B2 (anti-B220), S7 (anti-CD43), R6-60.5 (anti-IgM), 493, 1D3 (anti-CD19), 7G6 (anti-CD21), and B3B4 (anti-CD23) were obtained from BD Biosciences and 11-26 (anti-IgD) was obtained from Southern Biotechnology Associates, Inc. Cells expressing the α-HEL BCR Tg were identified by first incubating the cells with 500 ng/ml HEL (Sigma-Aldrich) followed by a labeled monoclonal anti-HEL mouse γ1 antibody. Lymphocytes expressing transgenic α-TNP BCR were identified with the 20.5 antibody, a BALB/c γ1 anti-SP6 idiotype that also recognizes the SP6 α-TNP IgH chain in association with other unrelated light chains. Dead cells were excluded from analysis by propidium iodide gating. All analyses were performed on a FACScalibur™ (Becton Dickinson) interfaced to a Macintosh CELLQuest™ program. Student's *t* test was used for statistical analysis of cell numbers. Probabilities of $P < 0.05$ and $P < 0.01$ were considered significant.

BrdU Labeling. Groups of mice received two daily i.p. injections of 1 mg bromodeoxyuridine (BrdU; Sigma-Aldrich) at 12-h intervals for 1 or 3 consecutive days. Surface-stained BM and spleen cells were fixed in PBS containing 1% paraformaldehyde and 0.01% Tween 20 for 48 h, and were treated with DNase I (Roche) for BrdU detection with a FITC-conjugated anti-BrdU antibody (Becton Dickinson) as described previously (22). Fixation and DNase treatment restricted the usage of certain surface staining combinations for the analysis of the BrdU-labeled cell populations.

ELISA. IgM and HEL serum concentrations were quantified by ELISA. Plates were coated with antibodies either to total IgM, IgM^a, 20.5, or HEL and saturated with PBS-5% milk. Dilutions of sera were added. After incubation (1–6 h, 37°C) and washing, biotin-labeled anti–mouse IgM or anti-HEL Abs were added. Bound antibodies were revealed with peroxidase-labeled avidin followed by the substrate *O*-phenylenediamine and H_2O_2. The reaction was stopped after 10 min by 10% SDS and the absorbance was read at 450 nm in a titertek multiscan spectrometer (Flow Laboratories). Titration of serum IgM and HEL was performed using as standard purified mouse IgM (Southern Biotechnology Associates, Inc.) or HEL (Sigma-Aldrich). Concentrations were determined by comparing the displacement of the dilution curves in the linear interval between standards at a concentration of 1 mg/ml (IgM) and 30 ng/ml (HEL), respectively, and the serum samples. For the detection of HEL–IgM immune complexes, plates were coated with antibodies to HEL and saturated. Dilutions of sera were added. After incubation (6 h, 37°C) and washing, the HEL-bound IgMs were revealed by adding biotin-labeled anti–mouse IgM antibodies followed by peroxidase-labeled avidin, the substrate *O*-phenylenediamine, and H_2O_2. The reaction was stopped as aforementioned.

[^{125}I]HEL-binding Assays. HEL was labeled with ^{125}I as described previously (23). Before each assay, the specific activity (cpm/μg of [^{125}HEL]) was measured. Saturation of the HEL-spe-

cific BCRs was studied in triplicate by incubating 10^6 spleen cells for 1 h at 4°C with increasing doses (from 0 to 250 ng) of ^{125}HEL. The plateau of radioactive HEL binding was measured and the 50% binding concentration $[^{125}HEL]_{50\%}$ was determined for each cell type. The number of HEL molecules bound by each cell and the number of BCRs per cell was evaluated. Competition assays were performed by incubating 10^6 cells with a mix of $[^{125}HEL]_{50\%}$ and increasing concentrations of unlabeled HEL. Binding data were fitted with the four-parameter logistic model of the Hill equation: $Y = Ym + (Yo - Ym)/(1 + (X/I_{50})^p)$, with a software based on a nonlinear fitting algorithm. I_{50} is the concentration of the unlabeled HEL competitor at which, at equilibrium, 50% of the binding is inhibited, and thus represents the dissociation constant Kd. The reciprocals of Kd values, $K_a = 1/Kd$, are the association constants at equilibrium. With a population of structurally heterogeneous cell receptors, this calculated K_a represents the average affinity constant of the interactions with HEL (the overall avidity of the cells for HEL). These represent an approximate quantitative measure of the interaction between intact HEL-specific B cells with HEL. These measures should be necessarily different from the affinity determinations made for the isolated purified monoclonal HEL-specific IgG antibodies used to produce MD4 Tg mice.

Results

Experimental Strategy. We designed an experimental strategy in which a homogeneous population of antigen-specific B cells develops in the presence of different amounts of self-antigen. We used lethally irradiated immune-deficient Rag2$^{-/-}$ host mice reconstituted with a mixture of BM cells containing precursors for both antigen-specific α-HEL B cells and HEL antigen-producing cells. In brief, 50% of the injected BM cells were from Rag2$^{-/-}$ donors Tg for a HEL-specific BCR (α-HEL). These BM cells from the α-HEL donors were depleted of mature B cells to ensure we were injecting only immature precursors. By keeping the fraction of HEL-specific precursor cells in the injected BM cohort constant at 50%, the number of B cell progenitors and the rate of production of the HEL-specific B cells was the same in all chimeras studied (24). Consequently, all chimeras should have the same number of HEL-specific B cells (24). The remaining 50% BM cells injected were from non-Tg Rag2$^{-/-}$ and HEL-Tg Rag2$^{-/-}$ mice producing a membrane form of HEL (mHEL; reference 3) mixed at different ratios (25). By using all host and donor mice in the Rag2$^{-/-}$ background, the only lymphocytes present in the chimeras were the HEL-specific B cells developing from the BCR Tg BM precursors. By modifying the proportions of HEL-producing cells in the remaining 50% Rag2$^{-/-}$ BM inoculum, we changed the amount of self-antigen produced. In the resulting chimeras, the fraction of mHEL-expressing hematopoietic-derived cells varied according to the fraction of HEL Tg cells present in the initial BM inoculum (unpublished data). The quantities of HEL present in the serum of the different mHEL chimeras were lower than found in the original Tg donor mice and varied between the different groups of chimeras (Table I). In these chimeras, the HEL antigen is discretely distributed and its quantities may vary with tissue localization and with time, even as a result of B cell maturation and effector functions (26) as in normal physiology. In conclusion, we have chimeras that are HEL free, chimeras with varying numbers of mHEL-producing cells, and with detectable lower or higher quantities of HEL in the serum.

Table I. *HEL Concentrations in the Serum of the Mixed Chimeras Reconstituted with a Variable Fraction of BM Cells from HEL Tg Donors*

	mHEL chimeras					
Control HEL$^-$	0.05% mHEL	0.5% mHEL	5% mHEL	10% mHEL	25% mHEL	50% mHEL
<0.1	<0.1	0.29	8.96	13.13	75.29	105.73
<0.1	<0.1	0.48	7.48	14.06	83.12	95.79
<0.1	<0.1	0.30	10.15	11.18	86.80	110.40
<0.1	<0.1	0.38	9.90	12.05	76.69	103.70
<0.1	<0.1	0.39	11.53	12.07	78.12	118.10

Non-Tg Rag2$^{-/-}$ host mice were irradiated and reconstituted with a mixture of 50% BM cells from αHEL donors and 50% BM cells from Rag2$^{-/-}$ and mHEL.Rag2$^{-/-}$ Tg donors mixed at different ratios. 6–8 wk after reconstitution, the serum HEL concentrations were measured by ELISA. Values represent the HEL concentration (ng/ml) in the serum of individual chimeras. HEL concentrations in sHEL.Rag2$^{-/-}$ and mHEL.Rag2$^{-/-}$ transgenic donors and hosts were ~26 ± 2.73 ng/ml and 285 ± 11.45 ng/ml, respectively. Control mice were non-Tg Rag2$^{-/-}$ host mice reconstituted with 50% BM cells from αHEL donors and 50% BM cells from Rag2$^{-/-}$ donors; that is, they were HEL free.

Selection of High Avidity HEL-specific B Cells in the Presence of Different Quantities of mHEL-producing Cells. We studied the development of a population of monoclonal B cells expressing the high affinity α-HEL–specific BCR in chimeras expressing different quantities of mHEL and in sHEL hosts. It was described previously that in (αHEL × mHELTg)F1 mice, the presence of mHEL deletes all α-HEL–specific B cells (3). In the mHEL hosts reconstituted with B cell–depleted BM cells from α-HEL Tg donors, where all host MHC class I$^+$ cells express HEL, the α-HEL B cells were promptly deleted (Fig. 1 A). In the chimeras injected with 50% mHEL BM, where 50% of the hematopoietic-derived cells express HEL, the number of B cells in the spleen was also considerably reduced (Fig. 1 A). However, when reducing the fraction of HEL-expressing cells, we progressively increased the number of B cells recovered in the peripheral pools. In chimeras receiving ≤5% mHEL BM cells, no B cell deletion was observed; the number of α-HEL B cells was similar to that found in chimeras not expressing the HEL self-antigen. In sHEL hosts, B cells were present in the BM (not depicted) and rare in the spleen (Fig. 1 A).

We evaluated anti-HEL IgM secretion in the same chimeras. In the mHEL hosts or in the 50% mHEL chimeras in which B cells were rare in the spleen, serum IgM was barely detectable (Fig. 1 B). However, we found that the

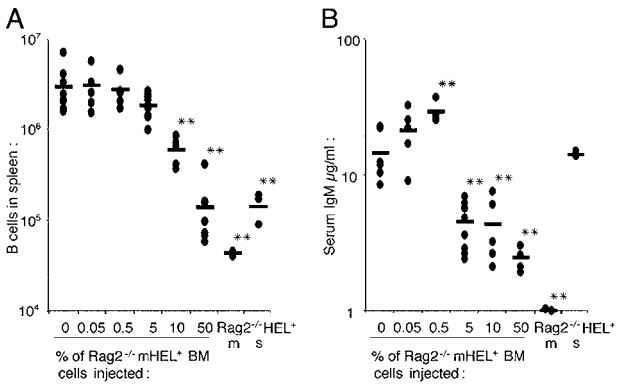

Figure 1. Number of cells recovered in mixed α-HEL/mHEL and sHEL chimeras. Non-Tg $Rag2^{-/-}$ host mice were irradiated and reconstituted with a mixture of 50% BM cells from α−HEL donors and 50% BM cells from $Rag2^{-/-}$ mixed with mHEL Tg donors (from left to right: 0, 0.05, 0.5, 5, 10, and 50% BM cells from mHEL donors). (left column) B cell development in HEL-free control chimeras, which did not receive cells from mHEL Tg donors. (right) B cell development in mHEL and sHEL hosts, respectively, reconstituted with 50% BM cells from α-HEL and 50% BM cells from $Rag2^{-/-}$ donors. (A) Number of B cells ($B220^+IgM^+$) present in the spleen of the mixed chimeras. (B) IgM concentrations (µg/ml) in the serum of the mixed chimeras. Each circle represents one individual chimera and the bar represents the arithmetic mean. p-values were compared with control HEL^-chimeras. *, $P < 0.05$. **, $P < 0.01$. Similar results were obtained in two independent experiments.

IgM levels were not always proportional to the number of B cells. This was evident in mice containing 5 or 0.5% mHEL BM-derived cells. In the 5% mHEL chimeras, B cells were not deleted, but the IgM serum levels were as low as in the 50% mHEL chimeras. These results suggest that reduced frequencies of mHEL-expressing cells can induce a state of functional anergy rather than deletion. In these chimeras, peripheral B cells were $IgM^{low}IgD^+$ (unpublished data), a phenotype compatible with a state of anergy (2). The results obtained in mice injected with 0.5% mHEL BM cells were even more surprising. In these mice, B cell numbers were normal and expressed an immature $CD23^{lo}CD21^{lo\text{-}int}$ phenotype (unpublished data), but the serum concentration of anti-HEL IgM was actually slightly increased, compared with that found in mice containing no self-antigen. In contrast, IgM levels in sHEL hosts were as in HEL-free chimeras. These results show that low levels of autoantigen-expressing cells do not induce tolerance and allow autoantibody secretion.

To summarize, our results establish a direct relationship between the amounts of self-antigen and the fate of the self-reactive B cells. They show that the developing α-HEL B cells ignore too little antigen as found in the 0.05% mHEL chimeras. When the amount of self-antigen increases, autoantibody production may even be facilitated, as found in the 0.5% chimeras. In the 5% mHEL chimeras, the α-HEL B cells are functionally inactivated (2); that is, they are present but unable to secrete Igs. At even higher quantities of HEL, the antigen-specific B cells are deleted. Our findings demonstrate that B cell fate varies according to the number of mHEL-expressing cells and HEL quantities.

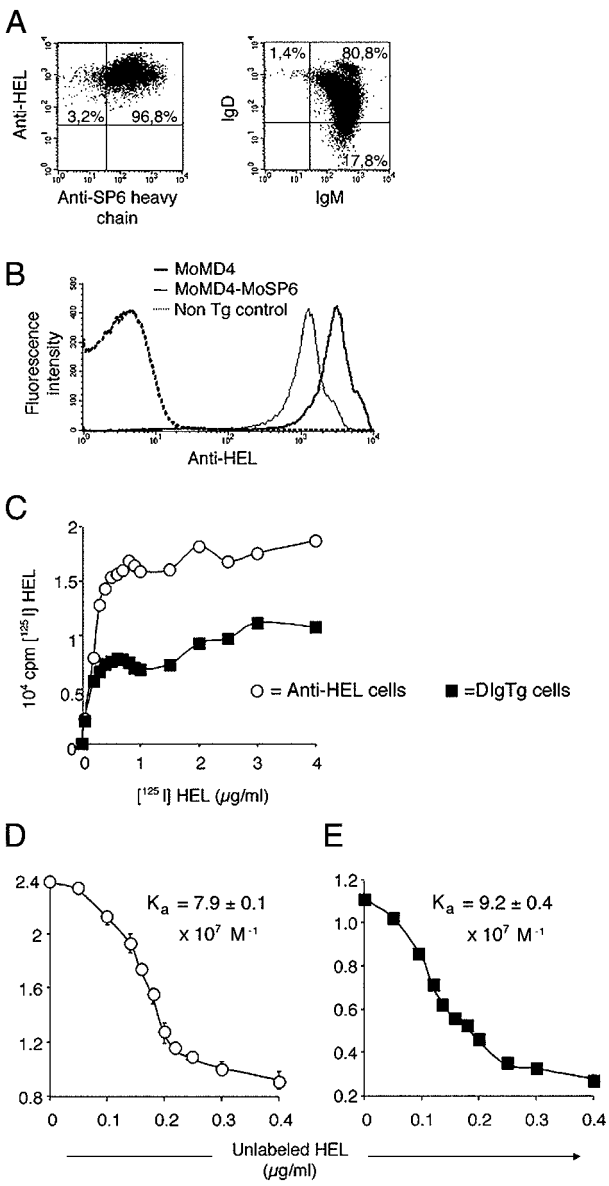

Figure 2. Expression of Ig receptors in DIgTg B cells. (A) The dot plots show the expression of α-TNP heavy chain (identified by the 20.5 Abs) and HEL binding (left) and IgD and IgM expression (right) by cells from the spleen of α-HEL.α-TNP DIgTg mice. The HEL-specific Tg encodes for both µ- and δ-chains, whereas the TNP-specific Tg only encodes for the µ-chain and the expression of the IgL and IgH chains of the two transgenes may not be comparable (reference 19). (B) The histogram shows the fluorescence intensity of the HEL binding in gated $B220^+$ spleen B cells from α-HEL (dark line), DIgTg (thin line), and control mice (dashed line). (C) HEL binding by single α-HEL and DIgTg B cells. Spleen cells from α-HEL and DIgTg mice were incubated with increasing concentrations of [^{125}I]HEL. The mean cpm is plotted as a function of the HEL concentrations. (D and E) Competition assays performed by incubating 10^6 cells with a mix of $[^{125}HEL]_{50\%}$ and increasing concentrations of unlabeled HEL for α-HEL cells (D) and DIgTg B cells (E). The value of the association constant K_a of each cell type, which represents the average affinity constant (the avidity) of the cell receptors for HEL, is indicated. Identical results were obtained in two other independent experiments.

HEL-specific Low Antigen-binding B Cells. Our findings show that membrane-bound self-antigen, when present in small quantities, not only allows the survival of self-reactive B cells but also permits autoantibody secretion. Recent papers claim that self-reactive B cells expressing two BCRs can escape deletion and survive in the peripheral pools (16). To investigate the role of allelic inclusion in B cell selection by self-antigens, we crossed the MoMD4 (α-HEL) and MoSP6 (α-TNP) Tg lines and obtained mice bearing two Ig-heavy and two Ig-light Tg chains in a $Rag2^{-/-}$ genetic background (20). In the α-HEL.α-TNP mice (DIgTg), the number of peripheral B cells (20×10^6) is similar to that of α-HEL mice (20). Although most of the B cells (>96%) bind HEL and express the α-TNP heavy chain (Fig. 2 A), the amount of HEL binding by the bulk population of DIgTg B cells was reduced to <50% of that of the α-HEL single BCR B cells (Fig. 2, B and C). The levels of surface IgM or IgD expression were retained (20). The average number of HEL molecules bound by the Ig receptors in the α-HEL B cells was estimated by [^{125}I]HEL binding to be $\sim 2.26 \times 10^5$; that is, 1.13×10^5 BCRs, whereas in the B cells from the DIgTg mice, the number of HEL-specific BCRs was reduced to $\sim 5.4 \times 10^4$ (Fig. 2 C). By using competition assays with unlabeled HEL, we measured the overall binding strength of the intact HEL-specific B cells to HEL. The estimated apparent average HEL-binding avidity of the α-HEL B cells and of the DIgTg B cell populations was $7.9 \pm 0.1 \times 10^7$ M^{-1} and $9.2 \pm 0.4 \times 10^7$ M^{-1}, respectively (Fig. 2, D and E). These values were surprising because they are well below the nanomolar range affinity of the original IgG antibody used to make the MD4 Tg mice (2). These divergences are probably due to the fact that we measured the strength of antigen binding by an intact cell, whereas former works analyzed in vitro interactions between purified antigen and antibody. It is likely that the cell surface environment and the presence of serum influence the strength of HEL binding to its IgM/D receptors in the fluid membrane phase. In summary, in DIgTg mice, the amount of signal that each individual B cell receives from the HEL-specific antigen receptor is reduced without interfering with normal B cell development because the total surface IgM per cell and the total number of B cells remains the same.

Very Low Quantities of Self-antigen Induced the Positive Selection of B Cells Expressing Low Numbers of Self-reactive BCRs. We showed that in DIgTg B cells, the level of surface expression of the HEL-specific BCR is reduced. Therefore, B cells from the DIgTg mice may be used to study the interactions of developing low antigen-binding B cells with antigen. They also permit comparison of the effects of higher (as found in single BCR B cells) with lower levels of BCR signaling (as found in DIgTg B cells) in B cell selection. We studied the development of the HEL-specific DIgTg B cells in the presence of variable quantities of HEL. In HEL^- chimeras, the number of B cells and the serum IgM levels generated from the B cell–depleted DIgTg BM precursors (Fig. 3) were identical to those obtained from single α-HEL Tg BM cells (Fig. 1). This shows that in absence of HEL, the presence of a second α-TNP Tg BCR does not interfere with B cell development and selection. In the different mHEL chimeras, we found that the fate of DIgTg B cells varied with the circulating amounts of HEL and the number of cells expressing the membrane form of the HEL self-antigen.

In mHEL hosts, dual IgTg B cells were present in the BM, but were deleted from the peripheral pool in spite of their lower HEL binding (Figs. 3, A and B). Consequently, the IgM levels were below the levels observed in HEL-free

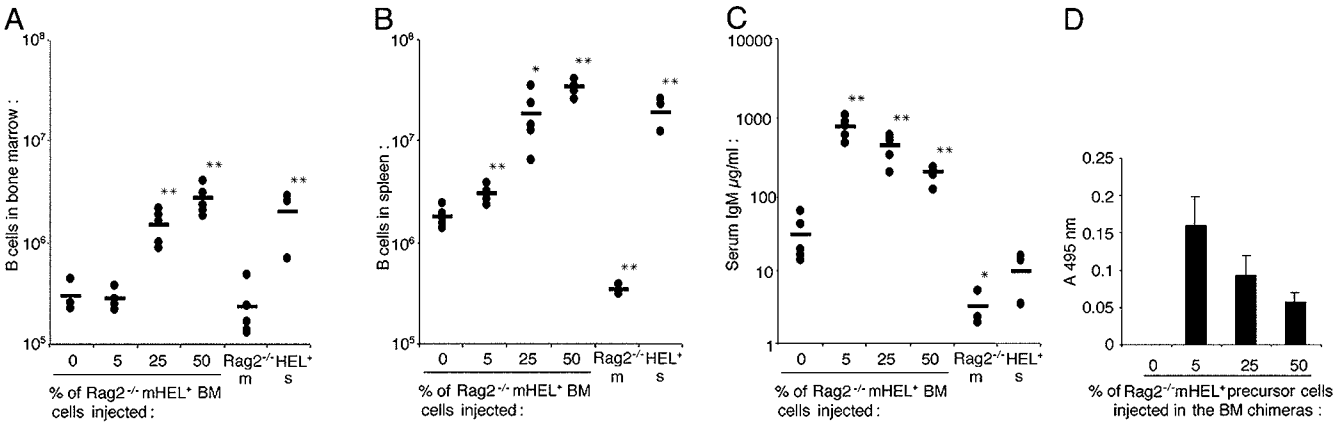

Figure 3. Number of cells recovered in mixed DIgTg/mHEL and sHEL chimeras. Non-Tg $Rag2^{-/-}$ host mice were lethally irradiated and reconstituted with a mixture of 50% BM cells from DIgTg donors and 50% BM cells from $Rag2^{-/-}$ and mHEL Tg donors mixed at different ratios (from left to right: 0, 5, 25, and 50% BM cells from mHEL donors). (left) B cell development in HEL-free control chimeras, which did not receive cells from mHEL Tg donors. (right) B cell development in mHEL and sHEL hosts, respectively, reconstituted with 50% BM cells from DIgTg and 50% BM cells from $Rag2^{-/-}$ donors. (A) Number of B cells ($B220^+IgM^+$) present in the BM of the mixed chimeras. (B) Number of B cells ($B220^+IgM^+$) present in the spleen of the mixed chimeras. (C) IgM concentrations (μg/ml) in the serum of the mixed chimeras. Each circle represents one individual chimera and the bar represents the arithmetic mean. p-values were compared with control HEL^- chimeras *, $P < 0.05$. **, $P < 0.01$. (D) Amounts of circulating HEL–IgM immune complexes detectable in the serum of the mixed DIgTg-sHEL and DIgTg-mHEL chimeras. Values expressed as arbitrary OD units. Similar results were obtained in two independent experiments.

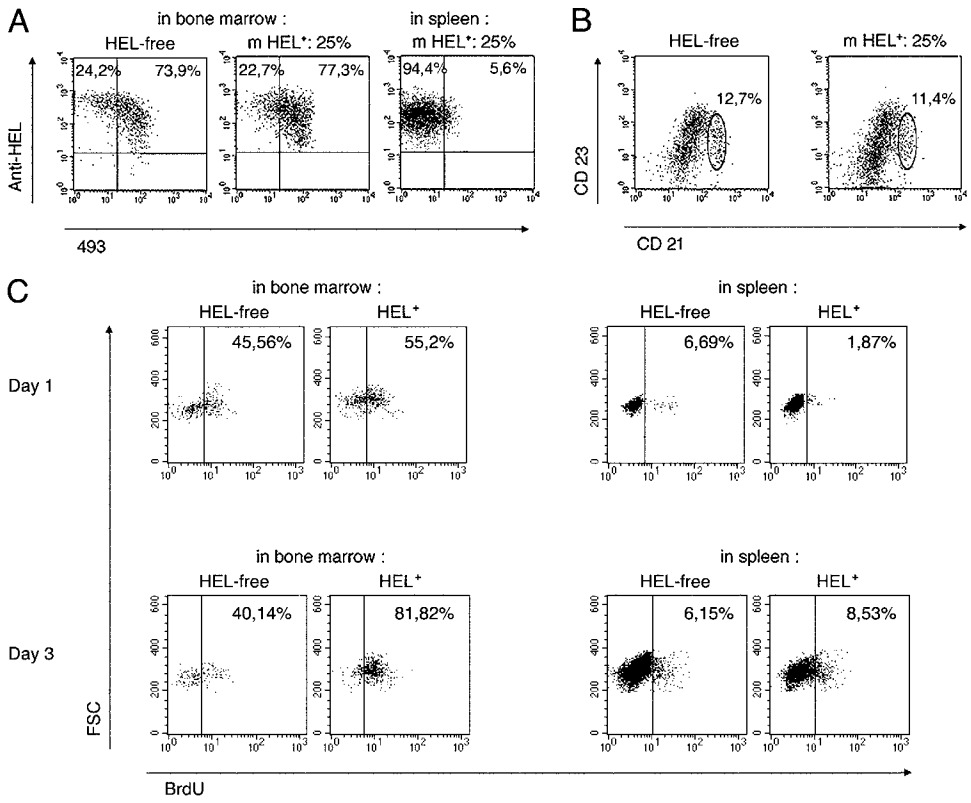

Figure 4. Phenotype of the DIgTg B cells developed in the absence or presence of small amounts of self-HEL. (A) Dot plots show HEL binding and 493 expression by gated $B220^+$ BM (left and middle) and spleen (right) cells developed in the absence (left, control) and in the presence (middle and right) of self-mHEL. (B) Dot plots show CD21 and CD23 expression by gated $B220^+$ HEL-binding spleen B cells developed in the absence (left) and presence of self-HEL (right). Total B cell numbers are shown in Fig. 3 (A and B). (C) Dot blots show the fraction of BrdU-labeled DIgTg B cells (gated $B220^+IgD^+$) in the BM and spleen 1 or 3 d after BrdU administration in HEL-free and HEL^+ chimeras. The fraction of $BrdU^+$ cells was defined using as negative controls the BrdU staining of cells from non-injected mice. Similar results were obtained in three independent chimeras in each group and each time point. It should be noted that, due to the usage of BCR Tg mice, the fraction of BM pre–B cells was negligible and all B cells express the IgD transgene. B cells were preferentially gated by IgD expression because it was found that the IgD staining resisted better to fixation and DNase treatment. These mixed BM chimeras also contained in their BM a fraction of pro-B cells from the non-Tg Rag-deficient BM inoculum.

chimeras (Fig. 3 C). In the mHEL BM chimeras with decreasing quantities of mHEL-expressing cells, the absolute number of B cells recovered in the BM and spleen and the IgM levels differed. In the chimeras reconstituted with 25–50% BM cells from mHEL Tg donors, we recovered 10–20-fold more mature B cells in the BM and spleen than in the control HEL^- chimeras (Fig. 3 B). In these chimeras, the serum IgM levels were 20-fold higher than those spontaneously secreted in the absence of HEL (Fig. 3 C). Interestingly, in sHEL hosts, the number of B cells was also increased, but the IgM levels were as in HEL-free chimeras (Fig. 3, A–C). In the chimeras reconstituted with very few mHEL Tg BM cells (5%), the number of B cells was lower, but the IgM concentrations remained high (Fig. 3 C). In the serum of the different mHEL chimeras, we also detected the presence of soluble HEL–IgM immune complexes (Fig. 3 D). In summary, these findings demonstrate that low quantities of self-antigen allow the survival and accumulation of B cells expressing lower densities of self-reactive BCRs. Moreover, these cells are functionally active because they retain anti-IgM responsiveness in vitro (unpublished data), and a fraction of these cells is activated and secretes self-reactive IgM that forms circulating immune complexes with HEL.

We found that most (>70%) of the DIgTg HEL-specific B cells present in the BM of the mHEL, sHEL, and HEL-free chimeras express 493, a surface marker that defines populations of newly produced immature B cells (Fig. 4 A; reference 27). This observation indicates that the accumulation of the HEL-specific self-reactive B cells in the BM of the HEL^+ chimeras occurs early at the immature B cell stage and is not just the result of the local BM accumulation of mature recirculating B cells that do not express 493 (27). In the spleen, the mature B cells that accumulate in the presence of small amounts of HEL are small resting cells ($CD5^-$, $CD43^-$, and $CD69^-$; not depicted), of which most lost expression of 493 (Fig. 4 A). They predominantly express a transitional $CD23^{lo}CD21^{lo\text{-}int}$ or mature $CD23^{hi}CD21^{int\text{-}hi}$ phenotype (Fig. 4 B; reference 28) that differs from the $CD23^{lo}CD21^{hi}$ phenotype characteristic of activated marginal zone B cells (29). The fraction of marginal zone type B cells was identical in the different HEL^- and HEL^+ chimeras (11–13% of the spleen B cells; Fig. 4 B).

We studied the accumulation of BrdU-labeled B cells in the BM and spleen of HEL-free and HEL^+ chimeras. At day 1 after BrdU administration, the fraction of BrdU-labeled B cells in the BM of the HEL^+ chimeras was identical to that observed in the HEL-free mice (Table II and Fig. 4 C), whereas at day 3, it was higher (Table II). Taking into account the 10-fold increase in the number of BM B cells (Ta-

Table II. *Accumulation of BrdU$^+$ DIgTg HEL-specific B Cells Developing in the Presence or Absence of HEL Neoantigen*

		BM		SPL	
		HEL free	HEL$^+$	HEL free	HEL$^+$
No. cells ($\times 10^6$)[a]		0.24 ± 0.1	3.96 ± 0.8[c]	0.5 ± 0.1	6.35 ± 2.4[d]
% BrdU$^+$[b]	day 1	44.1 ± 1.98	50.7 ± 3	6.7 ± 0.35	1.8 ± 0.07[c]
	day 3	35.7 ± 10.2	76.1 ± 13.6[c]	7.9 ± 3.3	9.5 ± 0.6

Results show the mean ± SE of three to six mice.
[a]The number of B cells in the BM and spleen of HEL-free and HEL$^+$ chimeras.
[b]The fraction of BrdU-labeled DIgTg B cells (gated B220$^+$IgD$^+$) in the BM and spleen 1 or 3 d after BrdU administration in HEL-free and HEL$^+$ chimeras.
[c]$P < 0.01$.
[d]$P < 0.05$.

ble II), this demonstrates an effective increase in the daily BM B cell production and accumulation (at day 3) in the HEL$^+$ chimeras. At day 1 after BrdU administration, 6.7% of the spleen B cells in the HEL-free chimeras were BrdU$^+$, whereas in the HEL$^+$ chimeras, only 1.8% of the B cells were BrdU$^+$ (Table II and Fig. 4 C), indicating that these cells are not actively proliferating in the presence of the self-antigen. At day 3 after BrdU administration, the fraction of BrdU-labeled spleen B cells did not differ significantly in the HEL-free and HEL$^+$ chimeras (Table II and Fig. 4 C). Considering the absolute number of spleen B cells observed in the types of chimeras, these findings suggest that the fraction of newly produced B cells that accumulate in the mature B cell pool is higher in the presence of self-HEL. In conclusion, these observations suggest that the presence of HEL promotes the production of increased B cell numbers in the BM and their subsequent accumulation in the spleen (i.e., the positive selection of early developing B cells, rather than the proliferation of mature B cells; reference 30).

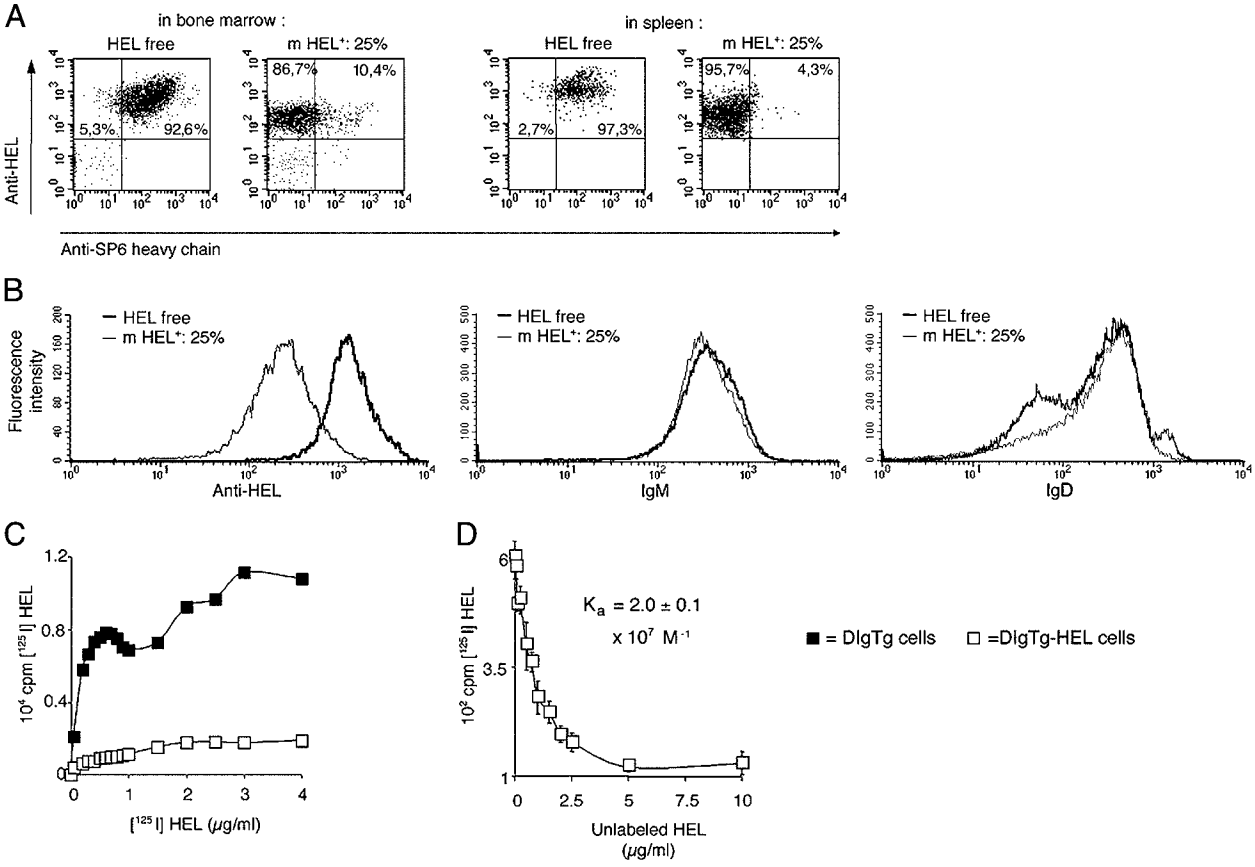

Figure 5. (A) Dot plots show HEL binding and α-TNP IgH expression by gated B220$^+$ BM (left) and spleen cells (right) from control chimeras reconstituted with BM cells from DIgTg (50%) in the absence of HEL (control) and in chimeras reconstituted with 25% BM cells from mHEL donors. (B) Histogram shows the fluorescence intensity of the HEL binding (left) and IgM (middle) and IgD (right) staining, in gated B220$^+$ spleen B cells from control (dark line) and the DIgTg-mHEL (25%) chimeras (thin line). Note that in these HEL-selected cells with lower HEL binding, the total cell surface expression of IgM receptors is retained (right histogram), which excludes BCR internalization by bound HEL. (C) HEL binding by DIgTg B cells developed in the absence or in the presence of 25% mHEL chimeras. Spleen cells were incubated with increasing concentrations of [^{125}I]HEL. Results plot the mean cpm as a function of the HEL concentrations. (D) Shows the competition assay done by incubating 10^6 DIgTg B cells selected in 25% mHEL chimeras with a mix of $[^{125}HEL]_{50\%}$ and increasing concentrations of unlabeled HEL. The value of the association constant K_a, which represents the average affinity constant (the avidity) of the cell receptors for HEL, is indicated.

We found that the selected B lymphocytes showed a unique phenotype that developed in the presence but not in the absence of self-HEL. They were all HEL-specific self-reactive cells that partially or completely lost expression of the α-TNP IgH chain (Fig. 5 A). The density of the HEL-binding receptors among the DIgTg B cells developing in the presence of self-HEL was 8.8×10^3 BCRs; that is, it was one sixth of DIgTg B cells developing in the absence of HEL (5.4×10^4; Fig. 5, B and C). Our estimate of the HEL-specific BCR number per cell was not hindered by receptor blockade by self-antigen bound in vivo because we used an indirect staining method to identify the HEL-binding $B220^+$ B cells. The cells were first incubated in the presence of soluble HEL and counterstained thereafter with a labeled anti-HEL monoclonal antibody recognizing a different HEL epitope. Thus, the staining shown in Fig. 5 B truly reflects the numbers of HEL-binding receptors/B cells because all receptors occupied by HEL either bound in vivo or after the in vitro preincubation step must be recognized by the second step anti-HEL–labeled antibody. By directly incubating the spleen cells of the different chimeras with the labeled anti-HEL Abs in the absence of the in vitro preincubation step with HEL, we also failed to stain any $B220^+$ B cells in the spleen cells (unpublished data), thus demonstrating that in vivo, HEL does not occupy their BCRs. The same technical procedure labeled the HEL-producing $B220^-$ Tg cells present in the mHEL chimeras. Moreover, the total cell surface expression of IgM and IgD receptors in the HEL-selected B cells with lower HEL-binding is retained (Fig. 5 B), which also excludes BCR internalization mediated by in vivo binding of HEL. Because we could exclude receptor blockade and internalization, we studied the inhibition of ^{125}HEL binding with cold HEL to assess the average strength of HEL binding by the population of B cells selected in presence of self-HEL. In this case, we found that the estimated apparent HEL-binding avidity of the whole DIgTg B cells is $\sim 2.0 \pm 0.1 \times 10^7\ M^{-1}$; that is, it's 20–25% that of the DIgTg B cells developing in absence of HEL (Fig. 5 D). It was previously shown that, independently of the BCR affinity, developing B cells are highly sensitive to the deletion mechanisms imposed by membrane bound self-antigens (31, 32). Our results show that this is not always the case. We found that the antigen load strictly determines the fate of the low avidity self-reactive B cells.

In conclusion, we found that low quantities of HEL self-antigen positively selected a population of self-reactive B cells that express lower densities of self-reactive BCRs and show a lower K_a binding for HEL. These self-reactive B cells accumulate in the BM and periphery, and a fraction of them is activated and secretes self-reactive IgMs that form circulating immune complexes with the HEL self-antigen.

Discussion

In this investigation, we studied the selection of homogeneous populations of self-reactive B cells differing by their capacity to bind high and low quantities of antigen in BM chimeras expressing different amounts of self-antigen. These studies revealed novel aspects of B cell selection by self-antigens. First, we demonstrated that membrane-bound self-antigens do not always lead to B cell deletion (31, 32). Different types of B cell tolerance could be induced depending of the quantity of self-antigen. Negative selection of B cells expressing a high-affinity HEL-specific self-reactive BCR was only achieved when the cells expressing membrane-bound self-antigen (mHEL) were present in high amounts. Decreasing the numbers of mHEL cells allowed the survival of the HEL-specific B cells, but these cells were functionally impaired. Finally, at even lower HEL amounts, B cells expressing high-affinity self-reactive receptors did not encounter the $mHEL^+$ hematopoietic cells, which are discretely distributed, and escaped deletion. Second, by forcing BCR allelic inclusion, we modified the fate of the self-reactive B cells. Allelic inclusion allowed B cells to escape deletion and permitted the increased accumulation (positive selection) and activation of the self-reactive B cells. Indeed, by studying the selection of DIgTg HEL-specific B cells, we observed that reducing the number of cells expressing and releasing self-antigen induced a 10–20-fold increase in the number of peripheral B cells. It also induced an increase in the serum IgM values, which were 20-fold higher than those released "spontaneously" in absence of HEL and formed detectable circulating HEL–IgM immune complexes. Interestingly, in sHEL hosts expressing exclusively soluble HEL, the positive selection of the self-reactive DIgTg B cells followed the same rules, but was not accompanied by increasing serum IgM levels.

The unique phenotype of the selected cells, and the increased number of mature B cells observed only in mice expressing but not lacking HEL, concord with a definition of positive selection in which self-reactive B cells are generated, maintained, and accumulated based on their BCR auto reactivity; that is, they are induced by the presence of self-antigen (8, 30). We found that the B cells selected by small quantities of self-antigen differed from those developing in absence of self-antigen. In absence of self-antigen, spleen DIgTg B cells express high levels of HEL-specific BCRs and coexpress the α-TNP heavy chain. In the presence of self-HEL, the selected DIgTg B cells maintained total surface IgM and IgD levels, but the density of expression of the HEL-binding receptors was one sixth that of control DIgTg B cells, and the cells did not express the α-TNP IgH chain. The average avidity of HEL binding in the population of DIgTg B cells selected in presence of self-HEL was approximately fivefold lower than that of the B cells developing in absence of HEL. This observation contradicts previous papers showing that the expression of low affinity ligands resulted always in the complete deletion of self-reactive B cells (31, 32). However, in these latter works, the development of the self-reactive B cells was investigated only in the presence of high levels of self-antigen ubiquitously distributed, and the number of the antigen-specific self-reactive BCRs was fixed (31–33). Moreover,

by comparing the fraction and the absolute number of BrdU-labeled cells in the BM and spleen, our findings show that the presence of HEL promotes an increased production of BM B cells and the accumulation of self-reactive B cells in the BM and spleen. In the BM, >70% of the HEL-specific selected DIgTg B cells retained the 493^+ phenotype characteristic of newly formed B cells (27). In the spleen of the HEL^+ chimeras, the fraction of $BrdU^+$ B cells 1 d after BrdU administration was low (<2%), indicating that they were not actively proliferating in the presence of self-antigen. Thus, the increased number of DIgTg HEL-specific B cells did not result from the proliferation of mature cells. The selected B cells were small resting noncycling $CD69^-$ cells, which showed a late transitional 493^- or mature $CD23^{hi}CD21^{int}$ phenotype (28) that differs from the $CD23^{lo}CD21^{hi}$ phenotype characteristic of the activated marginal zone B cells (29). This observation confirms that production of autoantibodies does not necessarily correlate with an increase in the fraction of marginal zone B cells (14, 16, 34).

Self-reactive B cells are generally removed at different checkpoints during ontogeny and BM B cell development (1). In the spleen of neonatal mice, there is a unique set of polyspecific self-reactive B cells expressing Vh7183 genes that is rare in adult mice (35). In adult mice, BM B cell development partially recapitulates B cell ontogeny, and immature B cells also overexpress Vh7183 genes (36). Recently, it has been shown that early immature human B cells also displayed self-reactive and polyreactive specificities, which are lost during B cell differentiation (37). Insufficient checkpoint regulation may allow complete maturation of self-reactive B cells. It has been shown that low levels of BCR surface expression and signaling allowed self-reactive B cells escape deletion (38). Reduced receptor signaling in CD45-deficient mice rescued high avidity self-reactive B cells (39). Down-regulation of surface IgM or BCR expression (14, 15) allowed survival of self-reactive B cells. Dual receptor B cells, in which the expression of a self-reactive BCR was diluted by the presence of a second BCR escaped deletion (16). In the absence of self-reactivity, B cells escape checkpoint regulation and can incorporate the immature peripheral B cell pool. On the other hand, the presence of a self-antigen has also been shown to positively select and expand a small number of self-reactive Tg PerC B1 B cells with production of self-reactive Igs (8, 40). However, PerC B cells and spleen $CD5^+$ B1 B cells were absent in the chimeras reconstituted with α-HEL or DIgTg BM cells (unpublished data). We hypothesize that the DIgTg BM precursors generate and provide a wide choice of B cells expressing different densities (numbers) of the self-reactive α-HEL receptors. HEL self-antigen, when present in low quantities, promotes the selection and expansion of the pool of B cells expressing fewer HEL-specific receptors and with a lower overall HEL-binding strength. The lack of expression of the α-TNP heavy chain by the positively selected B cells is intriguing. One may note that in the DIgTg mice, the presence of a second α-TNP Tg BCR did not interfere with B cell development and selection; the number of B cells in the DIgTg mice and BM chimeras were identical to those in the α-HEL mice and chimeras. However, MoSP6 mice lack peripheral B cells, a phenomenon that is attributed to early B cell deletion due to their anti-self DNA specificity (41). Moreover, the association of the α-TNP heavy chain with other Ig-light chains still retains an anti-DNA specificity (20). However, DIgTg B cells can dilute anti-DNA receptor by coexpressing two Tg heavy-chains, and thus escape deletion. In the presence of both DNA and HEL self-antigens, only the DIgTg B cells that express low numbers of HEL-specific receptors and simultaneously do not express a DNA-specific receptor can be selected. Cells that do not express the α-TNP heavy chain and retain the expression of the α-HEL heavy chains (μ and δ) predominantly associated with the α-TNP light chain. Thus, the selected self-reactive B cells dilute the number of HEL-specific BCRs by the coexpression of the α-TNP light chains. The presence of hybrid receptors composed of α-HEL IgH/α-HEL IgL chains and α-HEL IgH/α-TNP IgL chains may explain the lower HEL-binding avidity of the selected B cells. In summary, the positively selected self-reactive DIgTg B cells selectively reduce self-antigen signaling by diluting the self-reactive BCR, while, in contrast with the findings of previous papers (14, 15), maintaining total IgM and IgD levels. Dilution of a self-reactive BCR by light chain coexpression represents a novel form of "receptor editing" in which B cells in the presence of a self-antigen replace a self-reactive BCR by a second nonself-reactive BCR with a different specificity (42–46). The receptor editing processes described thus far (6, 42–45) require new Ig chain rearrangement, which cannot occur in the DIgTg $Rag2^{-/-}$ mice. It would be of interest to find out whether the silencing of the α-TNP IgH chain is transcriptional or translational. Overall, our findings suggest that low-avidity single BCR cells may be positively selected and activated if the number of BCRs engaged is low (i.e., when in the presence of small amounts of self-antigen).

In conclusion, the present work demonstrates for the first time that low quantities of self-antigen can promote the positive selection of conventional self-reactive B cells. By using a novel experimental strategy to study the fate of a unique population of B cells developing in BM chimeras reconstituted with a reduced fraction of B cell precursors and expressing different quantities of self-antigens, we have revealed events of B cell selection by self-antigens that are likely diluted and undetectable in normal intact mice. By directly measuring the strength of HEL binding through whole self-reactive B cells, we show that the fate of a developing B cell is dependent on the number of surface BCRs engaged and on the overall avidity of the Ag-specific cell (28, 38) rather than on the affinity of individual BCRs. The present findings have broader implications. First, they emphasize the role of BCR allelic exclusion. Compared with single BCR B cells, dual BCR B cells have a higher probability of expressing variable densities of self-reactive

receptors and, therefore, have an increased likelihood to generate cells with an avidity permissive to positive selection, activation, and production of self-reactive antibodies. Therefore, BCR allelic exclusion is required to minimize the selection of dual receptor self-reactive B cells. Second, they indicate that B cell homeostasis may be regulated by the availability of BCR ligands. By varying the quantities of HEL, we were able to modify the number of peripheral B cells, indicating that antigen may function either as a resource or as a surrogate resource; i.e., a resource not in itself in limiting supply, but which is competed for because of the access it provides to some other resource that is or may become limited in supply (10). Finally, they suggest that natural serum IgMs may be the result of the activation of low avidity self-reactive B cells, thus explaining the presence in the serum of normal individuals of a relatively high frequency of autoantibodies.

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