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*J Immunol* 2005; 175:909-916; ;  
doi: 10.4049/jimmunol.175.2.909  
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The American Association of Immunologists, Inc.,  
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# In Vivo Assessment of the Relative Contributions of Deletion, Anergy, and Editing to B Cell Self-Tolerance<sup>1</sup>

Keli L. Hippen,<sup>2\*</sup> Brian R. Schram,\* Lina E. Tze,<sup>3\*</sup> Kathryn A. Pape,<sup>†</sup> Marc K. Jenkins,<sup>†</sup> and Timothy W. Behrens<sup>2\*</sup>

**In normal B cell development, a large percentage of newly formed cells bear receptors with high levels of self-reactivity that must be tolerized before entry into the mature B cell pool. We followed the fate of self-reactive B cells expressing high affinity anti-hen egg lysozyme (HEL) Ag receptors exposed in vivo to membrane HEL in a setting in which the anti-HEL L chain was “knocked-in” at the endogenous L chain locus. These mice demonstrated extensive and efficient L chain receptor editing responses and had B cell numbers comparable to those found in animals lacking membrane Ag. BrdU labeling indicated that the time required for editing in response to membrane HEL was ~6 h. In mice transgenic for soluble HEL, anti-HEL B cells capable of editing showed evidence for both editing and anergy. These data identify receptor editing as a major physiologic mechanism by which highly self-reactive B cells are tolerized to membrane and soluble self-Ags. *The Journal of Immunology*, 2005, 175: 909–916.**

**B** lymphocyte development is characterized by an ordered rearrangement of Ig genes, beginning with H chain recombination in early pro-B cells (1). Cells that undergo a productive H chain rearrangement clonally expand at the late pro-B stage, and then initiate further rearrangements at L chain loci ( $\kappa$  and  $\lambda$ ) during the pre-B stage. A key developmental decision point occurs when B cells first express a functional surface Ig molecule (H and L chain) following successful L chain rearrangement at the pre-B stage (2). First, the new BCR must be capable of transducing a signal from the plasma membrane to turn off *Rag1* and *Rag2*, which prevents further rearrangements at Ig loci. This results in allelic exclusion, whereby individual cells express only a single H chain and a single L chain. Second, signaling through the nascent BCR in immature B cells is thought to be required for positive selection, whereby cells progress in development and gain competence to leave the bone marrow. These BCR quality control measures help ensure that once cells reach the periphery they will be able to transduce activation signals through the BCR following contact with pathogens. A third key event at the early immature B stage is the tolerization of cells carrying BCRs that are functional but recognize self-Ags with high affinity or avidity. Recent data suggest that up to half of all newly formed B cells may show self-reactivity to nuclear Ags (3); thus, the problem of maintaining self-tolerance early in B cell development is significant.

Central tolerance mechanisms for B cells include anergy, deletion, and receptor editing (4, 5). Anergy can be defined as the

functional inactivation of B cells that chronically bind self-Ag to further signals through the BCR, whereas deletion generally refers to the clonal elimination of cells that bind multivalent Ags or that express “useless” BCRs. Receptor editing is characterized by the induction of new rearrangements at L chain loci, and less commonly at H chain loci, that serve to alter the specificity of the BCR. Editing in the bone marrow can be induced by self-reactivity of a BCR (5), and likely also by the expression of a receptor that delivers insufficient basal Ig signaling (6). L chain editing at  $\kappa$  inactivates the initial L chain  $V\kappa J\kappa$  rearrangement either by deletion or inversion events on the original rearranged allele, followed by new L chain rearrangements into downstream  $J\kappa$  segments on the original allele, on the other  $\kappa$  allele, or at one of the two  $\lambda$  L chain loci. Each mouse B cell has the opportunity for as many as eight rearrangements at  $\kappa$  and four rearrangements at  $\lambda$  (5).

Clonal deletion in the B lineage has been elegantly demonstrated in two conventional transgenic (Tg)<sup>4</sup> systems in which expression cassettes for pre-rearranged H and L chain receptors are integrated in the genome. Nemazee et al. (7) showed that cells bearing anti-class I MHC Abs (derived from the anti-class I 3–83 mAb) were deleted efficiently in hosts bearing an appropriate MHC class I ligand. Goodnow and colleagues (8, 9) showed that B cells carrying a conventional Tg BCR with specificity for hen egg lysozyme (HEL) underwent developmental arrest and clonal deletion in mice expressing membrane-tethered HEL (mHEL) driven by a class I MHC promoter. An important limitation of these studies was the random integration of the H and L chain transgenes in the genome, which abrogated the possibility of efficient receptor editing.

In previous experiments, we showed that a B cell restricted Bcl-x<sub>L</sub> transgene allowed conventional MD4 HEL-Ig B cells to escape deletion in mHEL mice (10). The B cells that emerged in the periphery were anergic, and could not respond to BCR stimulation by proliferating or secreting anti-HEL Abs. When the escaped B cells were examined, they were found to express significant levels of *Rag1* and *Rag2* and showed evidence for new endogenous L chain locus rearrangements by PCR, despite all cells

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Received for publication December 8, 2004. Accepted for publication May 9, 2005.

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<sup>1</sup> This work was supported by an Arthritis Foundation Investigator Award (to K.L.H.) and grants from the National Institutes of Health (to T.W.B.).

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<sup>4</sup> Abbreviations used in this paper: Tg, transgenic; HEL, hen egg lysozyme; mHEL, membrane HEL; sHEL, soluble HEL; HSA, heat stable Ag; hC $\kappa$ , human C $\kappa$ ; mC $\kappa$ , mouse C $\kappa$ ; MZ, marginal zone.

expressing the anti-HEL Tg BCR (10). This suggested that escaped B cells in this system were undergoing “frustrated” receptor editing, and were attempting to edit away from self-reactivity, but were unable to inactivate the conventional Tg BCR. Based on these data, we were interested in monitoring the development of anti-HEL B cells in both mHEL and sHEL environments in a setting in which a pre-rearranged anti-HEL L chain was “knocked-in” to its proper location in the  $\kappa$  locus and was fully capable of being inactivated by receptor editing. Our data indicate that membrane-bound Ag initiates very efficient receptor editing of self-reactive B cells, whereas soluble Ag in this system initiates both anergy and editing. These data suggest that receptor editing is a dominant central tolerance mechanism for self-reactive B cells with specificity for membrane Ags.

## Materials and Methods

### Mice

MD4 (anti-HEL H chain and L chain) conventional Tg mice, ML5 soluble HEL (sHEL), and KLK4 membrane HEL (mHEL) Tg mice (8, 11) were originally obtained from Dr. C. Goodnow (Australian National University, Canberra, ACT, Australia). MD2 (anti-HEL H chain alone) conventional Tg mice were generated in the Goodnow laboratory (12), and were kindly supplied by Dr. J. Kearney (University of Alabama, Birmingham, AL). The anti-HEL L chain knockin ( $LC^{KI/+}$ ) and human  $C\kappa$  knockin ( $hC\kappa$ ) mice were previously described (13), and were kindly provided by Dr. M. Nussenzweig (Rockefeller University, New York, NY). MD2 H chain Tg mice were first crossed with HEL  $LC^{KI}$  mice to generate MD2/ $LC^{KI/+}$  animals. These mice were then bred to mice homozygous for the mHEL transgene, the sHEL transgene, or the  $hC\kappa$  knockin allele to generate MD2/ $LC^{KI/+}$ /mHEL, MD2/ $LC^{KI/+}$ /sHEL, or MD2/ $LC^{KI/hC\kappa}$  mice. MD2, sHEL, and mHEL mice were on a pure C57BL/6 (B6) background, whereas anti-HEL  $LC^{KI/+}$  and  $hC\kappa$  mice were on a mixed B6/129Sv background, backcrossed two or three generations onto B6 to fix the endogenous IgM locus to  $IgM^b$ . All mice were housed in specific pathogen-free conditions and were genotyped by PCR using published methods. Mice were generally between 8 and 12 wk of age at the time of analysis. All experiments were approved by the University of Minnesota Institutional Animal Care and Use Committee.

### Flow cytometry

Single cell populations were prepared as described (14), and were stained with PE-Cy5 anti-B220 (eBioscience), and PE anti- $IgM^a$ , FITC anti-heart stable Ag (HSA; CD24), FITC anti-CD21, FITC anti-class II MHC, PE anti-CD23, or biotinylated anti- $IgM^a$  followed by allophycocyanin-streptavidin (BD Pharmingen). HEL-binding cells were identified using a modified HEL sandwich assay as described (14). Four- and five-color flow cytometry was performed using a FACSCaliber and LSRII (BD Biosciences), respectively, and data were analyzed using Flowjo (TreeStar) and CellQuest (BD Biosciences) software.

### Serum anti-HEL $IgM$ ELISA

HEL-specific ELISAs were performed as described (14). Briefly, sera from the various mice were collected via retro-orbital puncture under anesthesia, and anti-HEL  $IgM^a$  levels were measured using dilutions of 1/500 and 1/2500 for MD4 and MD2/ $LC^{KI/+}$  animals, or 1/50 and 1/250 for the other mice. Purified anti-HEL  $IgM^a$  Abs from hybridoma supernatant (kindly provided by T. Tedder, Duke University, Durham, NC) were included in each assay to determine the absolute concentrations of anti-HEL  $IgM^a$  Abs in serum.

### BrdU labeling of pro-B cells

To label actively dividing cells, mice were injected i.p. with 1 mg of BrdU (Sigma-Aldrich), and were sacrificed at the indicated time points. For the 18- and 24-h time points, mice received a second BrdU injection at 12 h. Bone marrow and spleen cells were isolated, stained with Abs and fixed. Cells were permeabilized and stained for BrdU using a commercial kit (BD Pharmingen). Percentages of BrdU-positive cells were calculated at each time point, and linear regression analysis was used to identify the timing of the first appearance of cells within each compartment (13).

### In vitro assays

Splenic B cells were purified by negative selection using a B cell isolation kit (Miltenyi Biotec). After purification, B cells from all strain combinations were routinely  $>90\%$  B220<sup>+</sup>, with the exception of B cell preparations from MD4/mHEL mice, in which B cell numbers were low and purities were  $\sim 75\%$ . For [<sup>3</sup>H]thymidine incorporation,  $2 \times 10^5$  B cells were cultured in triplicate in 200  $\mu$ l of complete RPMI 1640 10% FCS media in the absence or presence of LPS (20  $\mu$ g/ml; Sigma-Aldrich), rIL-4 (10 ng/ml; R&D Systems) and/or anti-IgM polyclonal F(ab')<sub>2</sub> Abs (10  $\mu$ g/ml; Jackson ImmunoResearch Laboratories) or HEL (1  $\mu$ g/ml; Sigma-Aldrich) for 3 days, and [<sup>3</sup>H]thymidine incorporation measured over the final 16 h of culture. For CFSE experiments, purified B cells were washed and resuspended at  $10^7$  cells/ml in HBSS. CFSE was added to a final concentration of 1 nM, incubated for 10 min at 37°C, inverting the capped tube every 2 min. The reaction was stopped with 10 ml ice-cold media, and cells were washed once in PBS and then cultured at  $10^6$  cells/ml for 3 days alone or with the various stimuli. CFSE dye dilution was measured on gated B220<sup>+</sup> cells using flow cytometry.

### Bone marrow chimeras

Radiation chimeras were prepared as described (10). Briefly, control B6 x 129Sv F<sub>1</sub> and mHEL x 129Sv F<sub>1</sub> recipient mice were lethally irradiated with 1300 rads from a cesium source. After 3 h, animals were infused i.v. with  $2 \times 10^6$  bone marrow cells from MD2/ $LC^{KI/hC\kappa}$  animals. Chimeras were analyzed 4–8 wk after reconstitution.

### Immunofluorescence

Explanted spleens were flash-frozen in Tissue-Tek OCT compound (VWR). Six micron cryosections were cut and dehydrated in acetone, and blocked with 1% H<sub>2</sub>O<sub>2</sub>, culture supernatant containing 24G2 mAb plus 1% mouse and 1% rat serum, and avidin/biotin blocking reagents (Vector Laboratories). HEL-binding B cells were detected with 50  $\mu$ g/ml HEL (Sigma-Aldrich), followed by biotinylated anti-HEL mAbs (D1.3), HRP-conjugated streptavidin (PerkinElmer), and tyramide signal amplification-direct Cy5 (PerkinElmer).  $IgM^a$ -staining B cells were revealed with FITC-conjugated anti- $IgM^a$  mAb (BD Pharmingen), and  $IgD^a$ -staining B cells were revealed with FITC-conjugated anti- $IgD^a$  mAb (BD Pharmingen). Endogenous B220<sup>+</sup> B cells and endogenous T cells were detected with biotinylated anti-B220 mAb or biotinylated anti-Thy1.2 mAb, respectively, followed by Cy3-labeled streptavidin (Zymed Laboratories). Slides were analyzed with a confocal microscope.

### Statistical analysis

Group means were compared using the Student *t* test for paired data, and linear regression analysis was performed using SYSTAT software (Systat).

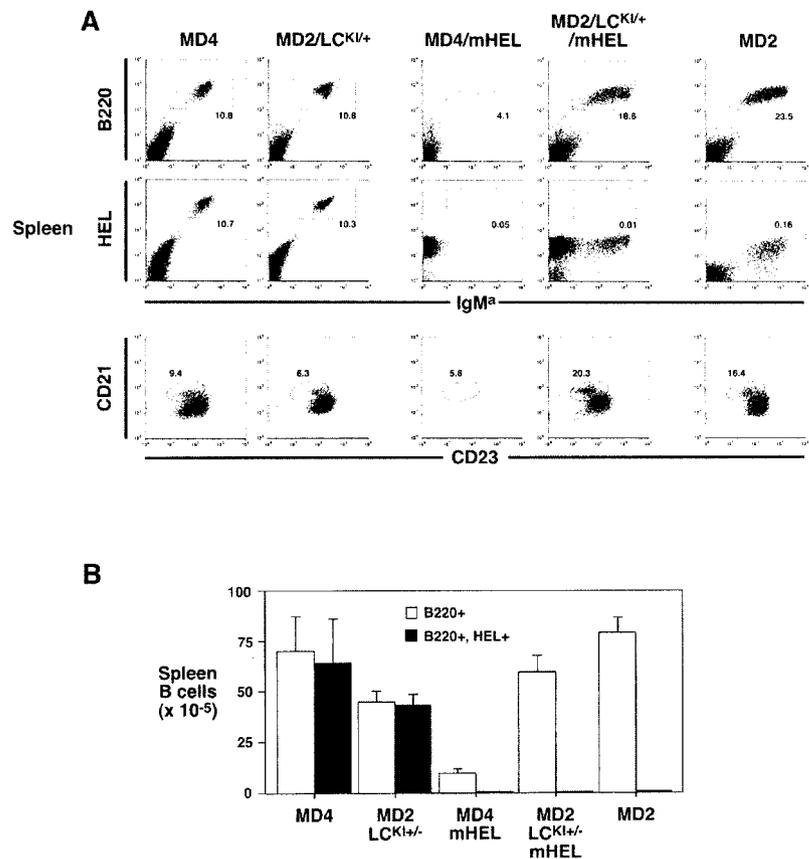
## Results

### Highly efficient BCR editing to membrane Ag

To study B cell responses to membrane-bound self-Ag in a setting in which L chain editing was possible, we bred mice carrying the MD2 anti-HEL H chain-alone transgene with animals expressing the anti-HEL L chain as a “knockin” allele ( $LC^{KI/+}$ ) at the  $\kappa$  locus (inserted into the  $J\kappa 2$  gene segment) (13), to generate MD2/ $LC^{KI/+}$  animals. Mature B cells from these mice expressed the  $IgM^a$  H chain allotype of MD2, and showed high level binding of HEL Ag in a HEL sandwich assay (Fig. 1A). B cells generated in MD2/ $LC^{KI/+}$  mice were  $IgD^{high}$ , CD5<sup>-</sup>, and expressed high levels of class II MHC (data not shown), and overall closely resembled “classical” MD4 anti-HEL conventional Tg B cells. This, together with functional experiments (see below), indicated that the knockin anti-HEL L chain paired efficiently with the MD2 H chain, and that the resulting BCR demonstrated excellent allelic exclusion at both H and L chain loci and signaled for positive selection of B cells similar to the MD4 BCR.

The MD2/ $LC^{KI/+}$  mice were then bred with mHEL Tg mice to generate MD2/ $LC^{KI/+}$ /mHEL animals. As a control, MD4/mHEL double Tg animals were studied in parallel. As previously described (9), B cells in MD4/mHEL double Tg mice were arrested in development at the pre-B stage and underwent efficient clonal deletion (see below), with few B cells reaching the periphery (Fig.

**FIGURE 1.** Efficient production of B cells in MD2/LC<sup>KI/+</sup>/mHEL mice. *A*, Isolated splenocytes from the indicated mice were analyzed by flow cytometry with labeled Abs to B220, IgM<sup>a</sup>, CD21, and CD23. HEL-binding was determined by incubating cells with 1 μg/ml HEL, followed by detection with labeled D1.3 anti-HEL Abs. The Tg and knockin alleles were as follows: MD4, anti-HEL conventional H and L chain Tg; MD2, anti-HEL conventional H chain Tg alone; LC<sup>KI/+</sup>, anti-HEL L chain knockin; mHEL, membrane HEL Tg. Double and triple Tg animals were generated by breeding. Data are representative of at least nine mice of each genotype. For B220/IgM<sup>a</sup> and HEL/IgM<sup>a</sup> stains, cells are lymphoid-gated and numbers refer to the percentage of lymphoid-gated cells. For the CD21/CD23 stains, cells are gated on B220<sup>+</sup> cells, and numbers refer to the percentage of B220<sup>+</sup> cells. *B*, Summary statistics for total B220<sup>+</sup> and B220<sup>+</sup>HEL<sup>+</sup> B cells in the spleens of the mice. Data represent mean ± SD for nine or more animals of each genotype. The numbers of B cells in MD2/LC<sup>KI/+</sup>/mHEL mice tended to be higher than observed in MD2/LC<sup>KI/+</sup> mice, but the difference was not significant ( $p = 0.058$ ).



1A). Of the small number of splenic B cells found in MD4/mHEL mice, all expressed IgM<sup>a</sup> and none showed the ability to bind HEL suggesting that these B cells expressed an endogenous L chain, and may have inactivated the Tg L chain. The low level HEL-binding exhibited by most cells in mHEL mice reflects the ubiquitous expression of mHEL, and its detection in the sandwich assay.

In contrast to the impaired development of B cells observed in MD4/mHEL mice, MD2/LC<sup>KI/+</sup>/mHEL mice had similar numbers of B cells in spleen (Fig. 1, *A* and *B*) and other secondary lymphoid organs (data not shown) as found in MD2/LC<sup>KI/+</sup> mice. The B cells in these Tg mice retained the IgM<sup>a</sup> MD2 H chain transgene and expressed a broad range of IgM levels, in contrast to the singularly high-level IgM expression observed on B cells from MD2/LC<sup>KI/+</sup> mice. Importantly, the MD2/LC<sup>KI/+</sup>/mHEL B cells no longer had the ability to bind HEL, suggesting that receptor editing was replacing the anti-HEL knockin L chain with new endogenous L chains leading to loss of affinity for HEL.

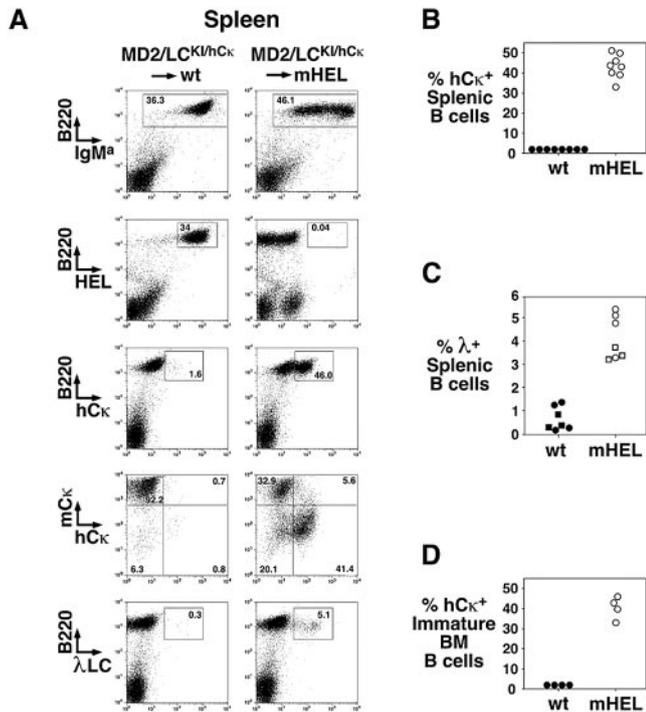
The splenic B cell phenotype of MD2/LC<sup>KI/+</sup>/mHEL animals was very similar to that of MD2 H chain-alone Tg animals, where the B cells express the MD2 H chain together with an extensive repertoire of endogenous L chains (12). MD2/LC<sup>KI/+</sup>/mHEL and MD2 mice showed an enrichment for B cells bearing surface markers characteristic of marginal zone (MZ) B cells (CD21<sup>high</sup>, CD23<sup>low</sup>) compared with either MD4 or MD2/LC<sup>KI/+</sup>. This phenotype has been previously noted in MD2 mice (15). A notable difference between the two strains was the presence of a small population of HEL-binding B cells in MD2 mice (as previously described) (12), but not in MD2/LC<sup>KI/+</sup>/mHEL mice (Fig. 1, *A* and *B*).

#### Receptor editing to mHEL in a radiation chimera model

To confirm and extend these findings, lethally irradiated mHEL or control recipient mice were reconstituted with bone marrow from

mice carrying a combination of the MD2 H chain, the anti-HEL LC<sup>KI</sup> allele and a hCκ knockin allele (MD2/LC<sup>KI/hCκ</sup>) (Fig. 2A). The hCκ allele allowed for the identification of cells that had inactivated the anti-HEL LC<sup>KI</sup> allele by receptor editing and completed a productive L chain rearrangement on the other κ allele. Although the presence of the hCκ allele indicates a cell that has undergone L chain receptor editing (13), it underestimates the extent of editing because cells can also edit the original anti-HEL L chain allele on Jκ gene segments available downstream on that same allele, and can also move to the λ L chain allele (5).

In the chimeras, donor B cells were distinguished from any endogenous recipient B cells by the absence (donor) or presence (recipient) of the allotype marker Ly9.1<sup>+</sup>, derived from the 129Sv genome in the F<sub>1</sub> recipients. MD2/LC<sup>KI/+</sup>/hCκ bone marrow introduced into wild-type recipients generated B cells that expressed high levels of HEL-binding IgM<sup>a</sup>, whereas mHEL recipients generated B cells with a broad distribution of IgM<sup>a</sup> levels, and no HEL-binding B cells (Fig. 2A). Strikingly, although there were only background levels of hCκ positive B cells in wild-type recipients, nearly half of the donor B cells in the mHEL recipients expressed the hCκ allele, indicating extensive receptor editing (Fig. 2, *A* and *B*). In these mice, expression of the mouse Cκ (mCκ) and hCκ alleles was relatively exclusive (see mCκ/hCκ plots in Fig. 2A). B cells expressing λ L chains were also significantly enriched in the spleens of mHEL recipients (and in MD2/LC<sup>KI/+</sup>/mHEL spleens) (Fig. 2, *A* and *C*). Because the remaining B cells in mHEL recipients do not bind HEL, it is likely that they have edited to downstream Jκ segments on the original anti-HEL L chain knockin allele. In parallel experiments, MD4 bone marrow was introduced into irradiated wild-type and mHEL recipients, and the fate of the B cell compartment examined. The results were as expected (8, 9), with the generation of HEL-binding B cells in

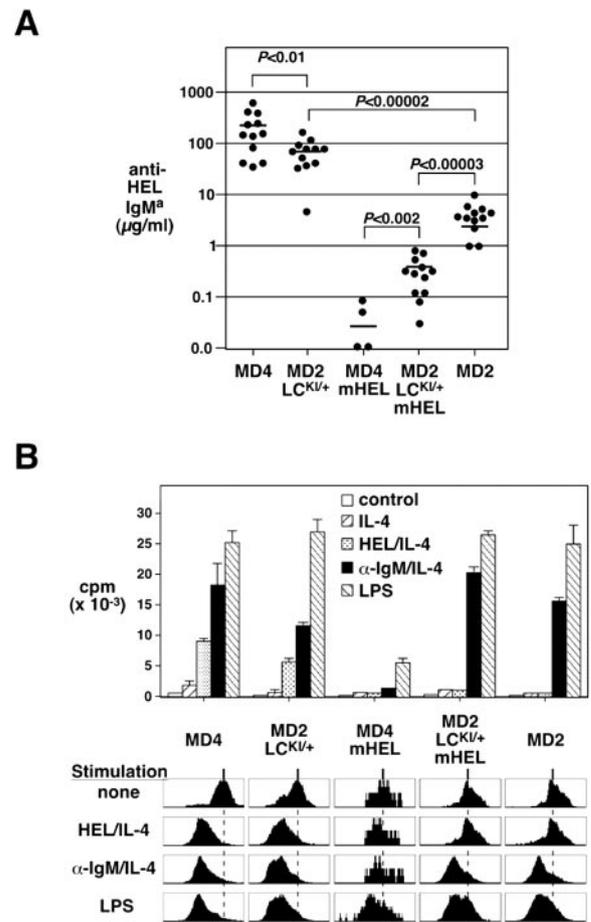


**FIGURE 2.** Self-reactive B cells undergo receptor editing in response to membrane Ag. **A**, MD2/LC<sup>KI/hCκ</sup> animals were generated by breeding. In these mice, B cells express a high affinity anti-HEL BCR comprised of the MD2 H chain Tg, the anti-HEL L chain knockin allele, and a hCκ gene segment engineered to replace mCκ gene on the other κ allele, which serves as a marker for L chain receptor editing. Bone marrow from MD2/LC<sup>KI/+hCκ</sup> mice was introduced into lethally irradiated B6 x 129Sv F<sub>1</sub> recipients (wt) or B6mHEL x 129Sv F<sub>1</sub> recipients (mHEL), and chimeras were analyzed at 4–8 wk. Splenocytes from the chimeras were stained with labeled Abs to B220, IgM<sup>a</sup>, hCκ, λ L chain (λLC) or mCκ, and analyzed by flow cytometry. HEL-binding was determined using a HEL sandwich assay. Shown are donor B cells, which are distinguished from any host B cells by the absence in the donor cells of Ly9.1 expression. In the second row showing HEL-binding, Ly9.1 gating was not performed, and the cells with low levels of (endogenous) HEL staining are host derived. The data shown are representative of four to eight mice analyzed in three independent experiments. **B–D**, Summary data indicating the percentage of splenocytes (**B** and **C**) or bone marrow (BM) immature B cells (B220<sup>int</sup>IgM<sup>+</sup>) (**D**) expressing hCκ or λ as a result of L chain receptor editing, in control (●) and mHEL (○) recipients of MD2/LC<sup>KI/hCκ</sup> bone marrow. **C**, The percentage of λ L chain-positive spleen B cells in control MD2/LC<sup>KI/+</sup> double Tg mice (■) and MD2/LC<sup>KI/+</sup>/mHEL triple Tg mice (□) are shown. Each point represents an individual mouse.

wild-type recipients and deletion of B cells in mHEL recipients (data not shown). In the mHEL recipients of MD2/LC<sup>KI/+hCκ</sup> bone marrow, hCκ<sup>+</sup> B cells were already abundant at the immature B stage (IgM<sup>+</sup>IgD<sup>-</sup>) in bone marrow (Fig. 2D). We conclude that B cells bearing receptors with high affinity for membrane-bound self-Ag are efficiently rescued by L chain receptor editing.

#### Ab and proliferative responses of edited B cells

MD2/LC<sup>KI</sup> animals had ~3-fold lower titers of anti-HEL IgM<sup>a</sup> Abs compared with MD4 animals ( $p < 0.01$ , Fig. 3A). Titers were much lower in all the other strains. Anti-HEL Ab levels in MD2 mice were ~2% that of MD4 mice, reflecting the small number of HEL-specific B cells found in these animals that result from fortuitous pairing of an endogenous L chain with the MD2 H chain to form anti-HEL receptors (see Fig. 1). Serum anti-HEL titers in MD4/mHEL mice were near the limit of detection for this assay,



**FIGURE 3.** Edited B cells in MD2/LC<sup>KI/+</sup>/mHEL mice do not secrete anti-HEL Abs or proliferate to HEL. **A**, Shown are concentrations of anti-HEL IgM<sup>a</sup> Abs in sera of MD4, MD2/LC<sup>KI/+</sup>, MD4/mHEL, MD2/LC<sup>KI/+</sup>/mHEL, and MD2 mice, as determined by ELISA. Each point represents the Ab levels of a single mouse. Differences in levels were significant between all the groups, and  $p$  values were determined using paired Student's  $t$  test. **B**, Purified B cells from the indicated genotypes were incubated with medium alone, IL-4 (10 ng/ml), HEL (1 μg/ml)+IL-4, anti-IgM (10 μg/ml)+IL-4, or LPS (20 μg/ml), and [<sup>3</sup>H]thymidine incorporation of triplicate wells were measured for the last 16 h of a 3-day incubation. Parallel experiments (*bottom panels*) are shown in which B cells were labeled with CFSE at day 0, washed and incubated with the indicated stimuli, with dye dilution measured by FACS on day 3. Note that B cells from MD2/LC<sup>KI/+</sup>/mHEL mice respond to anti-IgM stimulation but not HEL, indicating a loss of HEL-binding by the edited BCRs.

and the edited B cells in MD2/LC<sup>KI</sup>/mHEL mice showed levels of anti-HEL Abs that were also very low, and intermediate between those found in MD2 and MD4 mHEL animals.

Splenic B cells were purified from individual mice, and stimulated with IL-4, HEL plus IL-4, anti-IgM plus IL-4 or LPS, followed by measurement of proliferation by [<sup>3</sup>H]thymidine uptake and CFSE dye dilution (Fig. 3B). Although MD4 and MD2/LC<sup>KI/+</sup> B cells proliferated to both HEL and anti-IgM stimulation, the edited cells in MD2/LC<sup>KI/+</sup>/mHEL spleens responded to anti-IgM but not to HEL, with a pattern of response similar to that of MD2 B cells. Thus, edited B cells in MD2/LC<sup>KI/+</sup>/mHEL mice remain normally responsive to LPS and nonspecific BCR cross-linking, but have lost their ability to respond to HEL and to make significant titers of anti-HEL Abs.

### Bone marrow development during L chain editing

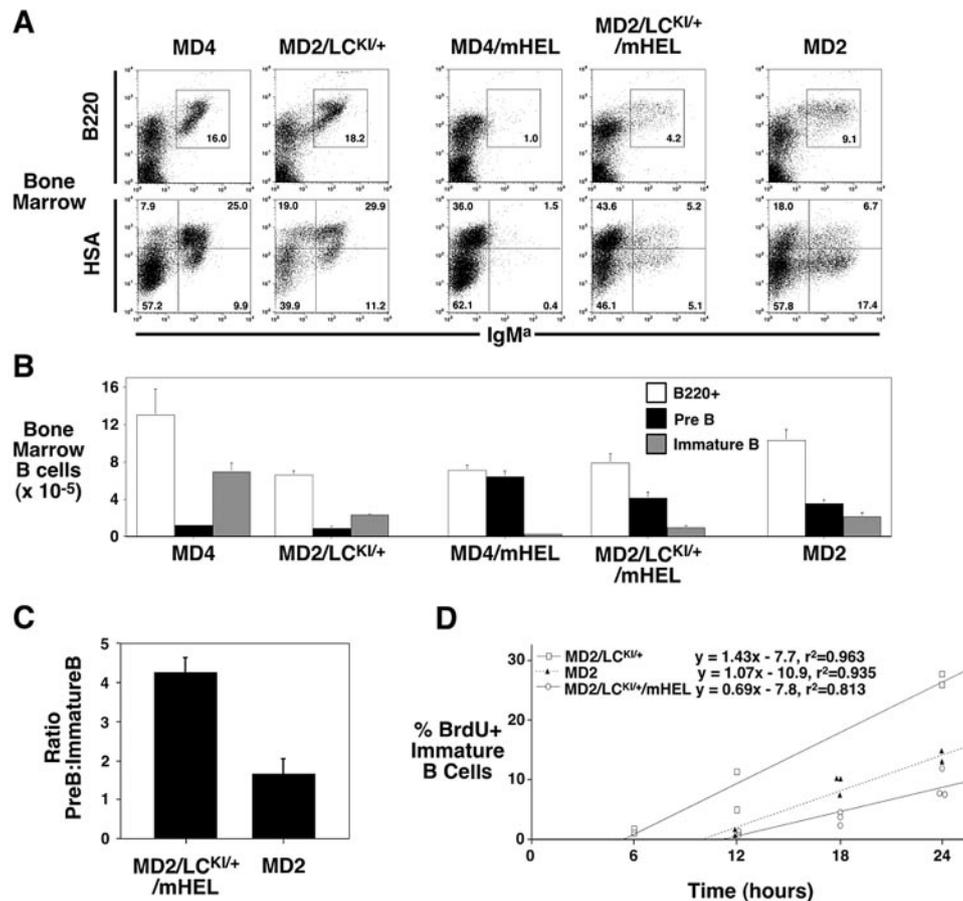
We next examined bone marrow B cell development using Abs to B220, IgM<sup>a</sup>, and HSA (CD24). These stains differentiate pro-B cells (Hardy fraction B/C: B220<sup>low</sup>, IgM<sup>-</sup>, HSA<sup>low</sup>) from pre-B cells (Hardy fraction D: B220<sup>low</sup>, IgM<sup>-</sup>, HSA<sup>high</sup>), and immature (Hardy fraction E: B220<sup>low</sup>, IgM<sup>+</sup>, HSA<sup>high</sup>) and mature (Hardy fraction F: B220<sup>high</sup>, IgM<sup>+</sup>, HSA<sup>low</sup>) B cells (Fig. 4A). B cell development was accelerated in MD4 and MD2/LC<sup>KI/+</sup> animals, with relatively fewer pre-B cells compared with the other strains (Fig. 4A). There was a striking developmental arrest in MD4/mHEL mice, with very few B220<sup>+</sup> cells progressing beyond the pre-B stage (Fig. 4, A and B) (9). Of interest, MD2/LC<sup>KI/+</sup>/mHEL mice demonstrated the highest percentage of pre-B cells of all the strains. The increased ratio of pre-B to immature B cells in editing MD2/LC<sup>KI/+</sup>/mHEL compared with MD2 mice (Fig. 4C) suggests a significant developmental delay during receptor editing.

### Kinetics of L chain receptor editing to membrane self-Ag

This system provided a unique opportunity to measure the time required for editing to membrane self-Ag in the pre-B compart-

ment. Developing B cells undergo a proliferative burst late during the pro-B stage (Hardy fraction C') before returning to G<sub>0</sub> at the pre-B stage (16). We labeled the DNA of proliferating pro-B cells in vivo by injecting MD4, MD2, MD2/LC<sup>KI/+</sup>, and MD2/LC<sup>KI/+</sup>/mHEL mice i.p. with BrdU. After 6, 12, 18, and 24 h, cohorts of mice were sacrificed and percentages of BrdU-positive cells in the various B cell subpopulations were measured by flow cytometry.

Rates of BrdU incorporation into the pro- and pre-B cell pools were similar among the four strains (data not shown). As shown in Fig. 4D, the rate of production of new immature B cells in MD2/LC<sup>KI/+</sup> mice was rapid. Low percentages of BrdU<sup>+</sup> immature B cells were first detectable at the 6-h time point, and comprised 25% of the immature pool at 24 h. Linear regression analysis suggested that labeled immature B cells first appear in MD2/LC<sup>KI/+</sup> mice at ~5.4 h. BrdU-labeled IgM<sup>+</sup> B cells in MD2 mice were not detectable at the 6-h time point (three mice examined, data not shown), and low percentages of positive cells were measurable by 12 h. Regression analysis of the 12, 18, and 24 h data suggested that labeled immature B cells in MD2 mice first appear ~10.2 h after a BrdU pulse. The IgM<sup>+</sup> edited cells in MD2/LC<sup>KI/+</sup>/mHEL



**FIGURE 4.** Kinetics of receptor editing to membrane self-Ag. *A*, Shown are bone marrow phenotypes of the various mice generated by breeding. Bone marrow cells were isolated and stained with labeled Abs to B220, IgM<sup>a</sup>, and HSA and analyzed by flow cytometry. In the plots (*bottom row*) gated by B220, B cell development proceeds from the lower left quadrant (HSA<sup>-</sup>IgM<sup>(-)</sup>, pro-B), clockwise to the upper left quadrant (pre-B), upper right (immature B), and lower right (mature B). Note the increased ratio of pre-B cells (upper left quadrant) to immature B cells (upper right quadrant) in MD2/LC<sup>KI/+</sup>/mHEL bone marrow compared with MD2 bone marrow. *B*, Summary statistics for total B, pre-B, and immature B cells in the bone marrow of the indicated mice. Data represent mean  $\pm$  SD for eight animals of each genotype. *C*, Comparison of the ratios of pre-B to immature B cells in MD2/LC<sup>KI/+</sup>/mHEL and MD2 mice ( $p < 0.001$ ). *D*, MD2, MD2/LC<sup>KI/+</sup>, and MD2/LC<sup>KI/+</sup>/mHEL mice were injected i.p. with BrdU at time 0 to label newly developing pro-B cells during their expansion phase. Groups of mice were then sacrificed at 6, 12, 18, and 24 h, and BrdU incorporation into the immature B cell pool was determined. Each point represents a single mouse. Linear regression was used to estimate the time of first emergence of labeled immature B cells. The slope of the lines reflects the rate of appearance of BrdU<sup>+</sup> cells into the immature pool. At the 6-h time point, there were no labeled IgM<sup>+</sup> cells in either MD2 or MD2/LC<sup>KI/+</sup>/mHEL bone marrow (data not shown).

mice also showed no BrdU labeling at 6 h (three mice examined, data not shown), and again low percentages of BrdU<sup>+</sup> cells were first detected at the 12 h time point. The percentages of BrdU<sup>+</sup> immature B cells were lower in MD2/LC<sup>KI/+</sup>/mHEL mice compared with MD2 mice at both 18 h and 24 h (3.7 vs 9.29% at 18 h,  $p = 0.002$  and 9.21 vs 14.0% at 24 h,  $p = 0.03$ ), and regression analysis indicated that edited BrdU<sup>+</sup> cells first appear in MD2/LC<sup>KI/+</sup>/mHEL mice at  $\sim 11.3$  h following the BrdU pulse. Thus, these data suggest that H chain receptor editing to high affinity membrane self-Ag in this model requires  $\sim 6$  h. Interestingly, there is only about a 1-h delay in the appearance of edited cells in MD2/LC<sup>KI/+</sup>/mHEL mice compared with the timing of appearance of B cells in MD2 mice.

Assuming 100% labeling of pro-B cells and no loss of IgM<sup>+</sup> cells, the slope of the regression lines reflects the rate of bone marrow production of IgM<sup>+</sup>BrdU<sup>+</sup> immature B cells in the strains. With these assumptions, edited BrdU<sup>+</sup> immature B cells were generated in MD2/LC<sup>KI/+</sup>/mHEL at  $\sim 1/2$  the rate of MD2/LC<sup>KI/+</sup> mice [(slope of the line for MD2/LC<sup>KI/+</sup>)/(slope of the line for MD2/LC<sup>KI/+</sup>/mHEL) = 1.43/0.69 = 2.07]. Given that two of three new L chain rearrangements are expected to be out-of-frame and unable to encode for protein, and that every B cell in MD2/LC<sup>KI/+</sup>/mHEL mice undergoes L chain editing, these data suggest that receptor editing in this model system is remarkably efficient.

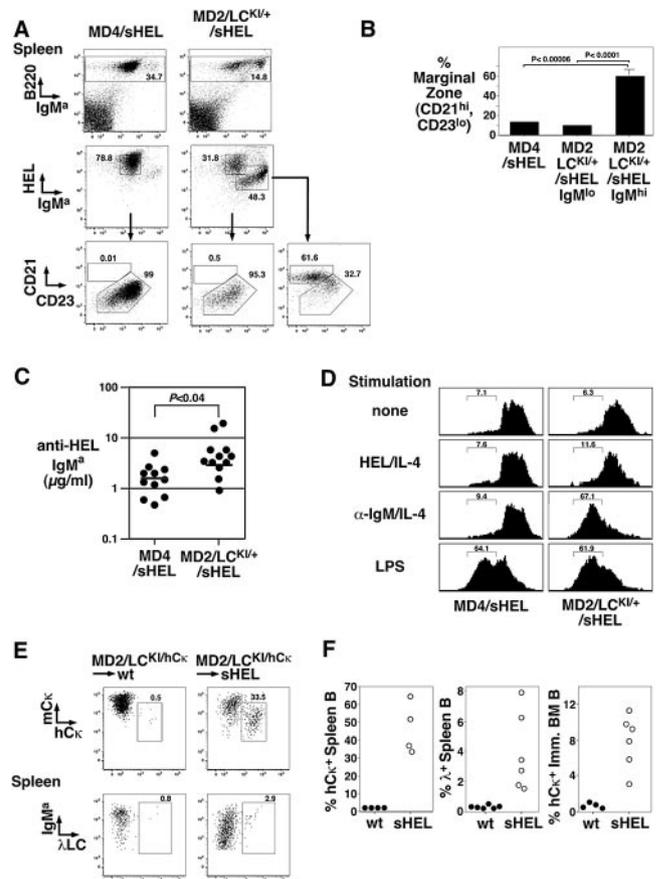
#### Soluble self-Ag induces both anergy and receptor editing

Next, we examined the fate of MD2/LC<sup>KI/+</sup> B cells in a sHEL environment. MD4 B cells developing in mice expressing sHEL undergo IgM receptor down-regulation, and enter the periphery in an anergic state, chronically binding HEL. Anergic B cells are short-lived (17) and unable to generate an efficient BCR activation response (11). Previous experiments indicated that B cells in MD4/sHEL mice showed some evidence of attempts at receptor editing, but were unable to efficiently edit the conventional MD4 Tg L chain (18).

We bred sHEL Tg mice with MD2/LC<sup>KI/+</sup> mice to generate MD2/LC<sup>KI/+</sup>/sHEL animals. Surprisingly, the spleens of these mice contained two distinct B cell populations based on IgM<sup>a</sup> expression and ability to bind HEL (Fig. 5A). One population had down-regulated IgM<sup>a</sup> levels and low-level HEL-binding characteristic of anergic B cells. The other population expressed high levels of IgM<sup>a</sup> that bound little or no HEL. The majority of IgM<sup>a(high)</sup> cells demonstrated a MZ B cell surface phenotype (CD21<sup>high</sup>CD23<sup>low</sup>), whereas the IgM<sup>a(low)</sup> population had few such cells (Fig. 5, A and B). IgM surface levels and HEL-binding in the IgM<sup>a(low)</sup> population were similar to MD4/sHEL double Tg, consistent with an anergic phenotype. In contrast, the IgM<sup>a(high)</sup> cells resembled the B cells in MD2 spleens or the edited cells present in the spleen of MD2/LC<sup>KI/+</sup>/mHEL mice.

Serum anti-HEL IgM<sup>a</sup> Ab levels were low in MD2/LC<sup>KI/+</sup>/sHEL mice indicating that the HEL-binding B cells were unable to secrete high levels of specific Abs (Fig. 5C). B cells from these mice did not proliferate in response to HEL, however some of the B cells (the edited cells) retained the ability to proliferate to anti-IgM Abs (Fig. 5D). B cells from MD4/sHEL mice could not proliferate significantly to either stimulus.

In radiation chimeras in which sHEL recipients received donor bone marrow from MD2/LC<sup>KI/+</sup>/hck animals, there were significant numbers of hC $\kappa$  and  $\lambda$  L chain-positive B cells in spleen and bone marrow 6 wk after reconstitution (Fig. 5, E and F). We conclude that there are two distinct B cell populations in MD2/LC<sup>KI/+</sup>/sHEL mice: 1) IgM<sup>a(high)</sup> B cells that have undergone receptor editing

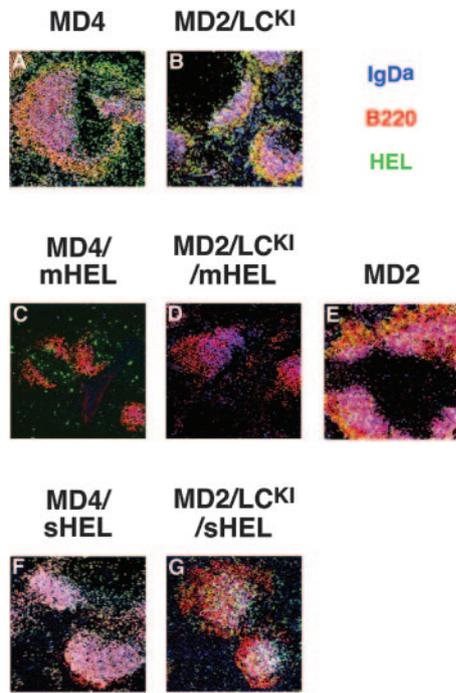


**FIGURE 5.** MD2/LC<sup>KI/+</sup> B cells show evidence for both editing and anergy induction in a sHEL environment. **A**, Surface phenotype of splenocytes in MD2/LC<sup>KI/+</sup>/sHEL and MD4/sHEL mice was determined by five-color flow cytometry using labeled Abs to B220, IgM<sup>a</sup>, CD21, and CD23. HEL-binding was determined using a HEL sandwich assay. Two distinct splenic B cell populations are evident. Note that PMTs were increased for the HEL staining to emphasize the differences in binding levels between the two B cell populations in MD2/LC<sup>KI/+</sup>/sHEL mice. The size of splenic B cell populations in these mice ( $n = 6$ ) was as follows: MD4/sHEL B cells – total B220<sup>+</sup> cells ( $23.5 \pm 8.28 \times 10^5$ ), B220<sup>+</sup>HEL<sup>+</sup> ( $21.0 \pm 6.24 \times 10^5$ ); MD2/LC<sup>KI/+</sup>/sHEL – total B220<sup>+</sup> ( $21.3 \pm 1.4 \times 10^5$ ), B220<sup>+</sup>HEL<sup>+</sup> ( $7.1 \pm 0.98 \times 10^5$ ). **B**, Many IgM<sup>a(high)</sup> cells in MD2/LC<sup>KI/+</sup>/sHEL mice adopt a MZ phenotype. Shown are percentages of MZ B cells (CD23<sup>low</sup>CD21<sup>high</sup>) in MD4/sHEL and both IgM<sup>a(low)</sup> and IgM<sup>a(high)</sup> subpopulations of MD2/LC<sup>KI/+</sup>/sHEL mice. **C**, Anti-HEL IgM<sup>a</sup> Ab levels in MD4/sHEL and MD2/LC<sup>KI/+</sup>/sHEL mice are shown (see Fig. 3 for details). Assays were run in parallel with those shown in Fig. 3, and levels can be directly compared. **D**, CFSE dye dilution of MD4/sHEL and MD2/LC<sup>KI/+</sup>/sHEL B cells in response to various stimuli (see Fig. 3 for details). **E**, Flow cytometry of spleens from control and sHEL recipients of MD2/LC<sup>KI/hC $\kappa$</sup>  bone marrow are shown. Cells are B220 gated. **F**, Percentage of hC $\kappa$  and  $\lambda$ -positive mature B cells in spleen and immature B cells in bone marrow (B220<sup>int</sup>IgM<sup>a</sup>) of MD2/LC<sup>KI/hC $\kappa$</sup> /sHEL chimeras.

that proliferate in response to anti-IgM cross-linking, and are enriched for MZ cells; and 2) IgM<sup>a(low)</sup> B cells that are anergic.

#### Splenic architecture in editing mice

Spleen sections from the various mice were then stained to visualize B cell populations in situ. As shown in Fig. 6, A and B, both MD4 and MD2/LC<sup>KI/+</sup> spleens, respectively, had well-developed B cell follicles (Fig. 6, pink) with distinct MZs (Fig. 6, yellow). In contrast, splenic follicles were poorly organized in MD4/mHEL mice (Fig. 6C), and B cells were sparse. The edited B cells in MD2/LC<sup>KI/+</sup>/mHEL (Fig. 6D) mice failed to bind HEL, and many



**FIGURE 6.** Splenic architecture in mice undergoing receptor editing to mHEL and sHEL. Spleen sections were stained for HEL (using a sandwich assay), B220, and IgD<sup>a</sup> (see *Materials and Methods*), and analyzed by confocal microscopy. Adjacent sections were stained with Thy1, B220, and IgM<sup>a</sup> to identify T cell zones and IgM<sup>a</sup>-bearing cells (data not shown). B220 is shown in red, HEL-binding in green, and IgD in blue. Follicular B cells in MD4 (A) and MD2/LC<sup>KI/+</sup> (B) mice are B220<sup>+</sup>HEL<sup>+</sup>IgD<sup>high</sup> and appear pink, and MZ B cells (B220<sup>+</sup>HEL<sup>+</sup>IgD<sup>low</sup>) appear as a yellow-colored band adjacent to the follicles. C, In MD4/mHEL spleens, B cells are sparse and do not bind HEL, and follicles are poorly developed. D, The follicles of MD2/LC<sup>KI/+</sup>/mHEL spleens show edited (HEL<sup>-</sup>) B cells, many of which adopt a MZ phenotype (B220<sup>+</sup>IgD<sup>low</sup>, red). E, MD2 spleens show a few HEL<sup>+</sup> B cells (yellow), and large MZs (red and yellow). B cell follicles in the MD2 section are pink. F, Anergic B cells in MD4/sHEL mice are IgM<sup>low</sup>HEL<sup>+</sup> (data not shown), B220<sup>+</sup>HEL<sup>+</sup>IgD<sup>high</sup> are shown in pink, and no MZ is visible. G, MD2/LC<sup>KI/+</sup>/sHEL spleens show some HEL<sup>+</sup> B cells in the follicles (pink), and a prominent HEL<sup>-</sup> MZ (B220<sup>+</sup>IgD<sup>low</sup>, red). HEL<sup>+</sup>B220<sup>-</sup>IgD<sup>-</sup> cells (green, A–C, F, and G) are likely Ab-secreting B cells. Original magnification,  $\times 100$ .

cells localized to the MZ (Fig. 6, red). The anergic B cells of MD4/sHEL mice (Fig. 6F) were almost exclusively located in follicles, had low levels of surface IgM (data not shown), and high levels of HEL-binding IgD. The spleens of MD4/LC<sup>KI/+</sup>/sHEL (Fig. 6G) mice showed two main populations of cells: 1) follicular B cells that were IgM<sup>low</sup> (data not shown), IgD<sup>high</sup>, B220<sup>+</sup>, and HEL-binding (via IgD); and 2) IgD<sup>low</sup>, B220<sup>+</sup>, and non-HEL-binding B cells localized to the MZ.

## Discussion

Current models to explain the maintenance of central tolerance in the B lineage suggest that deletion, anergy, and receptor editing are the primary mechanisms at work to prevent large numbers of self-reactive B cells from leaving the bone marrow and seeding the peripheral circulation and secondary lymphoid tissues (4, 5). Although there is evidence that all these mechanisms can operate in the normal immune system, there is little data to suggest which of these mechanisms predominates for various types of Ags. In general, anergy is thought to be a major mechanism to tolerize B cells to soluble Ags of low valency, whereas deletion and receptor editing are the major mechanisms to tolerize B cells recognizing

highly multivalent Ags such as DNA and membrane proteins, which strongly cross-link the BCR. Previous experiments in conventional Tg model systems suggest that clonal deletion of developing B cells in bone marrow is a dominant mechanism used to maintain tolerance to class I molecules, membrane-bound HEL and DNA (7, 8). However, in these original model systems receptor editing was not an efficient option due to the integration of the H and L chain transgenes outside the normal H and L chain alleles.

With the recent generation of an anti-HEL L chain knockin allele (13), we set out in the first series of experiments to test the response of anti-HEL B cells bearing an “editable” L chain following exposure to mHEL. The major finding was that membrane Ag induced very efficient editing in MD2/LC<sup>KI/+</sup>/mHEL mice, accompanied by the accumulation of B cells in the periphery comparable in numbers to those found in MD2/LC<sup>KI/+</sup> mice. The production of new B cells in MD2/LC<sup>KI/+</sup>/mHEL mice, as detected by BrdU incorporation into developing immature B cells, was only slightly delayed ( $\sim 1$  h) compared with B cells from MD2 H chain alone Tg mice. Compared with the kinetics of production of BrdU<sup>+</sup> cells in MD2/LC<sup>KI/+</sup> mice in the absence of Ag, L chain receptor editing away from HEL reactivity was found to require  $\sim 6$  h. Thus, these data suggest that receptor editing in this model system is extremely efficient, and is a major mechanism maintaining B cell tolerance to membrane Ags.

Although we observed essentially no evidence for deletion in response to exposure to mHEL Ag in this system, we cannot exclude the possibility that there is some degree of cell loss during the extended period of editing in the bone marrow of MD2/LC<sup>KI/+</sup>/mHEL mice. Apoptotic cells are rapidly cleared in the bone marrow by resident macrophages, and even in MD4/mHEL bone marrow, where the vast majority of cells are deleted at the pre-B cell stage, significant numbers of apoptotic cells cannot be detected (8–10). The normal production of edited B cells in these experiments argues that, even if deletion is occurring, it is highly compensated by the efficiency of L chain receptor editing. Clonal deletion is still likely to be an important mechanism shaping the B cell repertoire in situations in which all possible J $\kappa$  H chain gene segments have been exhausted by nonproductive secondary rearrangements, and in the setting where pairing of L chains with the rearranged H chain cannot signal for positive selection.

In MD2/LC<sup>KI/+</sup>/sHEL mice, we found two distinct populations of B cells in spleen, an IgM<sup>a(high)</sup> population that had edited the L chain, and an IgM<sup>a(low)</sup> population that was anergic. Despite the fact that about half of splenic B cells show the edited phenotype, these data suggest that L chain receptor editing occurs at a lower frequency in sHEL compared with mHEL mice. The frequency of hC $\kappa$ <sup>+</sup> immature B cells in the bone marrow of sHEL chimeras was only  $\sim 8\%$  compared with  $\sim 40\%$  in mHEL recipients. It seems likely that differential cell fate of the two populations explains the high number of edited cells in the sHEL spleens. Many of the cells that edit in response to sHEL are selected into the MZ pool (Fig. 5), which is a long-lived B cell population compared with follicular B cells, and especially compared with anergic B cells (17). MZ B cells are also known to have the potential for self-renewal (19). We hypothesize that, over time, there is a relative enrichment for edited IgM<sup>a(high)</sup> B cells due to the long lifespan of MZ B cells and the higher rate of turnover of the IgM<sup>a(low)</sup> anergic B cells. It has been shown previously that, in the presence of normal follicular B cells, anergic cells compete poorly for space in follicles and localize primarily to the T cell to B cell border (17). It is possible in this study that anergic B cells from MD2/LC<sup>KI/+</sup>/sHEL mice are found in follicles due to the fact that their “competitors” are primarily localizing to the MZ, not the follicle.

Taken together, we suggest that anergy rather than editing is the dominant tolerance mechanism in the bone marrow to sHEL Ag. Our finding of editing in response to sHEL is consistent with the identification of new endogenous L chain rearrangements in the spleen cells of MD4/sHEL mice (18), and suggests that sHEL provides a signal to developing B cells that is close to, or at, the threshold that determines whether B cells will edit or become anergic. Slight differences in the extent of self-aggregation of HEL, or issues relating to subcompartmentalization of responses in bone marrow, may contribute to the dual fate of B cells in these mice.

Very recently, experiments from Pelanda and colleagues (20) have found that receptor editing is also a major mechanism by which B cells are tolerized in the anti-class I MHC model system. B cells carrying knockin H chain and L chain 3–83 anti-class I MHC receptors underwent efficient editing when introduced into Ag containing hosts. Normal numbers of B cells were produced, and the incorporation of BrdU into wild-type and edited spleen B cells was similar, supporting the concept that editing was very efficient in this model. It was also shown that deletion, rather than editing, was the fate of L chain knockin B cells when there were no available downstream J $\kappa$  gene segments, or in Rag2-deficient animals. Those data independently support the conclusions of this report with regard to the importance of L chain editing for tolerizing B cells to membrane Ag.

In a previous study using the mice carrying the hC $\kappa$  knockin allele, BrdU-labeled IgM<sup>+</sup> cells were first observed at ~2 h following a BrdU pulse, and labeled hC $\kappa$ <sup>+</sup> cells were initially identified ~2 h later (13). Thus, the kinetics of appearance of BrdU<sup>+</sup> cells in that system was accelerated compared with the current experiments. There are important technical differences between the two studies that likely account for the differences. The experiments of Nussenzweig and colleagues (13) were performed in mice carrying a knockin L chain allele and the hC $\kappa$  allele, but lacking a Tg H chain. In their experiments, normal endogenous H chain rearrangements were required to first generate H chain protein, followed then by the further rearrangements at the L chain loci. Therefore, at the time of L chain loci rearrangements, Rag genes had already been transcriptionally activated at the pro-B stage, and the cells were likely “primed” for L chain rearrangements, as would occur in normal B cells. In the system described in this study, a pre-rearranged H chain Tg is available to each B cell, circumventing the need to rearrange H chain and engage the Rag genes at the pro-B cell stage. In MD2 mice and in MD2/LC<sup>K1/+</sup>/mHEL mice undergoing extensive editing, the pre-B cell stage is the first time that Rag genes are transcribed and translated. The data suggest that transcription of Rags and the time required to open chromatin sufficiently for L chain recombination in these B cells at the pre-B stage may be ~4 h.

The immune system has in place many layers of quality control measures to ensure the broadest possible repertoire of lymphocyte specificities, whereas at the same time diminishing the chances for strongly self-reactive cells. Receptor editing is an efficient mechanism for salvaging B cells expressing autoreactive receptors (21), providing cells with a number of opportunities to edit away from self-reactivity. Autoimmune diseases such as systemic lupus erythematosus are characterized by an increased frequency of B cells with evidence for excessive receptor editing (22). Given the important role for receptor editing in shaping the immune repertoire,

efforts to better understand the mechanisms that control this process are critical, and may translate into an improved understanding of autoimmunity.

## Acknowledgments

We thank A. Baker for excellent animal husbandry and genotyping, and C. Goodnow and M. Nussenzweig for gifts of mice.

## Disclosures

The authors have no financial conflict of interest.

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