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Negative Selection by IgM Superantigen Defines a B Cell Central Tolerance Compartment and Reveals Mutations Allowing Escape

Bao Hoa Duong,*1,2 Takayuki Ota,*1 Miyo Aoki-Ota,* Anthony Byron Cooper,* Djemel Ait-Azzouzene,*3 José Luis Vela,*4 Amanda Lee Gavin,† and David Nemazee*

To analyze B lymphocyte central tolerance in a polyclonal immune system, mice were engineered to express a superantigen reactive to IgM of allotype b (IgM^b). IgM^b mice carrying superantigen were severely B cell lymphopenic, but small numbers of B cells matured. Their sera contained low levels of IgG and occasionally high levels of IgA. In bone marrow, immature B cells were normal in number, but internalized IgM and had a unique gene expression profile, compared with those expressing high levels of surface IgM, including elevated recombinase activator gene expression. A comparable B cell population was defined in wild-type bone marrows, with an abundance suggesting that at steady state ~20% of normal developing B cells are constantly encountering autoantigens in situ. In superantigen-expressing mice, as well as in mice carrying the 3H9 anti-DNA IgH transgene, or 3H9 H^(-)-deleting element RS, IgM internalization was correlated with CD19 downmodulation. CD19^low bone marrow cells from 3H9:RS^(-/−) mice were enriched in L chains that promote DNA binding. Our results suggest that central tolerance and attendant L chain receptor editing affect a large fraction of normal developing B cells. IgH^a/b mice carrying the superantigen had a ~50% loss in follicular B cell numbers, suggesting that escape from central tolerance by receptor editing from one IgH allele to another was not a major mechanism. IgM^b superantigen hosts reconstituted with experimental bone marrow were demonstrated to be useful in revealing pathways involved in central tolerance. The Journal of Immunology, 2011, 187: 5596–5605.

Immunoglobulin gene assembly in developing B lymphocytes often initially generates self-reactive receptors. Autoreactive B cells can be regulated in several ways, including receptor editing, clonal deletion, and the induction of anergy, with attendant reduced B cell life span (1, 2). Editing is a major mechanism of central tolerance in developing bone marrow (BM) cells that in mice mainly involves secondary rearrangements on IGK loci that can eliminate one functional L chain rearrangement and exchange it for another, thus altering specificity (3–9). However, secondary L chain rearrangements also occur when surface Ig levels are insufficient to suppress recombination, Ig H/L pairing fails to occur, or signaling through the innocuous BCR is impaired (10–14). In addition, IGHV replacement might also contribute to escape from central tolerance (15, 16). Experiments in autoantibody transgenic (Tg) mice and studies involving Ab cloning from single human B cells show that autoreactivity is progressively diminished during normal B cell development, and is sometimes flawed in autoimmune-prone individuals (4, 8, 17–24). However, the frequency in the BM of B cells that are initially autoreactive, and the extent to which central tolerance and editing contribute to their control are not known.

B cells that are unable to edit efficiently might have mechanisms for altering specificity besides V(DJ) recombination. In many species, hypermutation or gene conversion can occur in developing B cells (25–27). Although these pathways are minor in the mouse (28, 29), low levels of activation-induced cytidine deaminase (AID) expression, class switching, and somatic mutation occur in normal immature B cells. AID activity can be upregulated even in pre-B cells (30, 31). B cells of μMT mice, which lack IgM membrane exons and exhibit a severe block in B cell development at the pre-B cell stage, can occasionally undergo class switch to downstream isotypes (32–35). However, what roles, if any, AID may play as a tolerance mechanism have not been investigated.

To control and visualize B cells undergoing central tolerance in a polyclonal immune system, we previously developed κ-macroself Tg mice, which express a superantigen reactive to Ck. In these mice, there was efficient κ-to-λ L chain editing in the BM, leading to significant escape of B cells carrying λ-chains (3). In this study, we generated mice expressing an IgM^b superantigen derived from mAb AF6-78 (36). We predicted that on an IgM^b background, L chain editing would be ineffectual in eliminating superantigen reactivity, and that tolerance should either promote deletion and anergy, or reveal in the surviving cells a different type of receptor selection. IgM^b-macroself mice offer a model system to determine the phenotype of developmentally blocked autoreactive B cells that are otherwise normal in their Ig gene expression and editing. The model allowed us to identify a similar population in normal

*Department of Immunology and Microbial Science, The Scripps Research Institute, La Jolla, CA 92037; and †Burnet Institute, Melbourne, Victoria, Australia 3004
B.H.D. and T.O. contributed equally to this work.
Current address: University of California, San Francisco, San Francisco, CA.
Current address: Rintat-Pflzer, South San Francisco, CA.
Current address: La Jolla Institute for Allergy and Immunology, La Jolla, CA.
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Address correspondence and reprint requests to Prof. David Nemazee, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037. E-mail address: nemaze@scripps.edu
Abbreviations used in this article: AID, activation-induced cytidine deaminase; BM, bone marrow; hCk, human Ck; IgM, intracellular IgM; LN, lymph node; mCk, mouse Ck; MZ, marginal zone; PC, peritoneal cavity; sIgM, surface IgM; SP, spleen; Tg, transgenic; WT, wild-type.
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mice that provides an estimate of the normal extent of central tolerance and receptor editing.

Materials and Methods

IgM<sup>+</sup>-macropheself construct

IgM<sup>+</sup>-specific hybridoma AF6-78 was purchased from American Type Culture Collection (Manassas, VA). The transgene encoding the IgM<sup>+</sup>-macropheself Ag was generated using methods essentially as described (37). Briefly, total RNA from AF6-78 was isolated using Trizol (Invitrogen, Carlsbad, CA), according to manufacturer’s instruction. V<sub>λ</sub> and V<sub>μ</sub> cDNA were obtained by 5′-RACE (Ambion, Austin, TX) using C<sub>x</sub> and C<sub>y</sub> antisense primers and subcloned into Zero Blunt TOPO vector (Invitrogen), and the sequence was determined. Leader (pUBF and iLAF6R), V<sub>λ</sub>(AF6VLF), and V<sub>μ</sub>(AF6VLR) encoding fragments were amplified. Purified fragments were fused using overlap PCR (pUBF and AF6VLR) and cloned into SpeI- and Xmal-digested pUlir plasmid (3). Primer sequences used were as follows: C<sub>x</sub> (5′-CTGCTCAGTGGATGTTGGAAGATGG-3′); pUBF (5′-TTTCTCGGTGCAAGGAGCCAGGATCTTG-3′); iLAF6R (5′-GTGAGAACAATTTGTGCACAGGCACCTGTAATAATATTAGGC-3′); pUBF (5′-TACAGGGGCTTGCAACAGTGGTCTCTTCA-3′); pUBF (5′-TTTCTCGGTGCAAGGAGCCAGGATCTTG-3′); iLAF6R (5′-GTGAGAACAATTTGTGCACAGGCACCTGTAATAATATTAGGC-3′); pUBF (5′-TACAGGGGCTTGCAACAGTGGTCTCTTCA-3′); pUBF (5′-TTTCTCGGTGCAAGGAGCCAGGATCTTG-3′); iLAF6R (5′-GTGAGAACAATTTGTGCACAGGCACCTGTAATAATATTAGGC-3′); pUBF (5′-TACAGGGGCTTGCAACAGTGGTCTCTTCA-3′); pUBF (5′-TTTCTCGGTGCAAGGAGCCAGGATCTTG-3′); iLAF6R (5′-GTGAGAACAATTTGTGCACAGGCACCTGTAATAATATTAGGC-3′); pUBF (5′-TACAGGGGCTTGCAACAGTGGTCTCTTCA-3′); pUBF (5′-TTTCTCGGTGCAAGGAGCCAGGATCTTG-3′); iLAF6R (5′-GTGAGAACAATTTGTGCACAGGCACCTGTAATAATATTAGGC-3′); pUBF (5′-TACAGGGGCTTGCAACAGTGGTCTCTTCA-3′); pUBF (5′-TTTCTCGGTGCAAGGAGCCAGGATCTTG-3′); iLAF6R (5′-GTGAGAACAATTTGTGCACAGGCACCTGTAATAATATTAGGC-3′); pUBF (5′-TACAGGGGCTTGCAACAGTGGTCTCTTCA-3′); pUBF (5′-TTTCTCGGTGCAAGGAGCCAGGATCTTG-3′); iLAF6R (5′-GTGAGAACAATTTGTGCACAGGCACCTGTAATAATATTAGGC-3′); pUBF (5′-TACAGGGGCTTGCAACAGTGGTCTCTTCA-3′); pUBF (5′-TTTCTCGGTGCAAGGAGCCAGGATCTTG-3′); iLAF6R (5′-GTGAGAACAATTTGTGCACAGGCACCTGTAATAATATTAGGC-3′); pUBF (5′-TACAGGGGCTTGCAACAGTGGTCTCTTCA-3′);

Mice

Eight- to 12-wk-old mice were used in most experiments. C57BL/6 were purchased from The Scripps Research Institute Institute breeding colony. Promoter injection was performed at The Scripps Research Institute Institute Mouse Genetics Core facility using (C57BL/6 x BALB/cByJ)F<sub>2</sub> mice. Three different lines (numbers 17, 68, and 72) were backcrossed to C57BL/6.Ly5a (B6.CD45.1) for further study. Based on the transgene expression, line 72 (low expresser) and line 17 (intermediate expresser) were used for most of the experiments. All IgM<sup>+</sup>-macropheself Tg mice had been bred >10 generations and maintained on C57BL/6.Ly5a background. k-macropheself Tg mice (pULix line 2) were described (3). IgH<sup>κ</sup>-mice (B6.Cg-Igh<sup>κ</sup>-Thy1<sup>1</sup>Gpl<sup>1β</sup>) were purchased from The Jackson Laboratories. Site-directed H9 H chain Tg mice on a C57BL/6 background were provided by M. Weigert (University of Chicago). Bc2 Tg mice (line Eq2-22) were provided by A. Strasser and A. Harris (Walter and Eliza Hall Institute). Human Ck (hCk)-targeted mice (38) were provided by M. Nussenzweig (The Rockefeller University). QM mice were provided by M. Cascalho (University of Michigan). All mice were maintained at our facility.

Radiation chimeras

In BM transfer experiments, each mouse host was lethally irradiated with 1000 rad 1 d prior to receiving i.v. 10<sup>7</sup> BM cells, which were isolated from both upper and lower leg bones of donors using standard protocols. Donor mice were C57BL/6.Igh<sup>κ</sup>-hogenic mice.

Serum Ig ELISAs

Nunc Maxisorp plates (Nunc, Roskilde, Denmark) were coated with 2 μg/ml Ab to mouse IgM (M41) or rat IgG-adsorbed donkey Ab to mouse IgG (H + L) (Jackson Immunoresearch Laboratories) overnight at room temperature, blocked with 1% BSA in TBS supplemented with 0.1% Tween 20 (ELISA buffer) for 1 h, and incubated for 1 h with mouse sera diluted in ELISA buffer. HRP-conjugated donkey Ab to mouse IgM, or goat Ab to mouse IgG, Fc fragment-specific (Jackson Immunoresearch Laboratories) Abs were used to detect bound mouse Ig. For IgA ELISA, plates were coated with rat Ab to mouse IgA (11-44-2; ebIsciences, San Diego, CA), and biotinylated Abs to κ and λ were used as secondary reagents, followed by incubation with streptavidin-HRP (BD Biosciences, San Diego, CA).

FIGURE 1. Negligible reactivity of IgM-reactive mAb AF6-78 to the pre-BCR and the generation of IgM<sup>+</sup>-macropheself Tg mice. A. 70Z/3 murine pre-B lymphoma cells (41) were either left unstimulated or treated with 10 μg/ml LPS for 24 h to induce expression of Igκ L chain. Cells were subsequently permeabilized and stained for total expression of IgM and Igκ. B. Affinity of mAb AF6-78 for the IgMb H chain before and after LPS induction was assessed by flow cytometric analyses of surface and total IgM expression. C. ELISA was performed to assess relative affinities of Abs to Igκ for the captured pre-BCR. Plates were coated with goat F(ab'<sub>2</sub>)<sub>2</sub> Abs to mouse IgM. The 70Z/3 lymphocytes containing the expressed pre-BCR were subsequently serially diluted and applied to each well. Detection was performed using equal concentration of each indicated Ab conjugated to biotin, and developed as described in Materials and Methods. The above experiments were repeated at least twice. D. Design and features of IgM<sup>+</sup>-macropheself Ag construct, including intron/exon structure, ubiquitin C promoter, leader exon (L), V<sub>λ</sub> and V<sub>μ</sub> codons, a linker peptide of the following sequence (GGGSGGGSGGGG), rat IgG1 hinge, C<sub>α2</sub> and C<sub>α3</sub> sequences, and transmembrane and cytoplasmic sequences from H-2K<sup>β</sup>. E. Schematic representation of the predicted protein structure of membrane-bound anti-mouse IgM<sup>+</sup>-macropheself Ag. Single-chain Fv generated from the IgM<sup>+</sup>-reactive hybridoma AF6-78 is linked to the hinge and membrane-proximal domains of rat IgG1, followed by transmembrane and cytoplasmic tail regions (Tm/Cy) of H-2K<sup>β</sup>. F. Flow cytometry analysis of superantigen expression in the tissues from Tg line 17. Comparable results were obtained for at least two additional Tg lines. Super-antigen was detected with Ab to rat IgG1 Fc.
Bound HRP was developed with Ultrasensitive TMB substrate (Millipore, Billerica, CA) per the manufacturer’s instructions. Absorbance signals were recorded at 450 nm using VersaMax microplate reader (Molecular Devices, Sunnyvale, CA).

**Flow cytometry analysis**

Flow cytometric analyses for surface markers were performed using standard protocols with appropriately diluted Abs. Intracellular proteins were stained after permeabilization using Cytofix/Cytoperm kit (BD Biosciences), according to manufacturer’s recommendations. The following mAbs were used for the experiments: B220 (RA3-6B2; Pacific Blue, FITC or allophycocyanin; eBiosciences); CD19 (1D3; PE or PE/Cy7; eBiosciences); CD21 (7E9; FITC; Biolegend, San Diego, CA); CD93 (AA4.1; FITC; Biolegend, San Diego, CA); CD11c (N418; PerCP-eFluor 710; eBiosciences); PDCA1 (PerCP-eFluor 710; eBiosciences); mAbs against mouse IgM (M41; Pacific Blue, Alexa488 or Alexa647;331.12; Alexa647); and k(187.1; Alexa647) were labeled in-house. All samples were run on a LSR-II instrument (BD) and analyzed using the FlowJo program (Tree Star, Ashland, OR).

**Cell sorting**

BM cells were harvested from three IgMb-macroself, 3H9;RS2/2 or wild-type (WT) mice. B220+ cells were enriched with CD45R (B220) MicroBeads (Miltenyi Biotec, Auburn, CA) and stained with B220-FTTC, CD19-PE, IgM-Alexa647, and dump channel mAb mixture (H57-PerCP-Cy5.5 and PDCA1-PerCP-eFluor 710). Subsequently, B220lowCD19low IgMb−, B220lowCD19lowIgMb+, and B220lowCD19highIgMb+ fractions were isolated with a FACSARia (BD) sorter.

**Quantitative PCR**

Total RNA was purified from half to 1 million sorted cells using a RNEasy Plus kit (Qiagen, Valencia, CA). Reverse transcription was performed with a QuanteTect Reverse Transcription kit (Qiagen) per the manufacturer’s protocol. Each gene was quantitated using SsoFast EvaGreen (Bio Rad, Hercules, CA) with 7900HT (Applied Biosystems, Carlsbad, CA) and normalized with ß-actin. Oligonucleotide primers for Rag1 and Rag2 were used as previously reported (37). Other primer sequences were obtained from Origene.

**FIGURE 2.** B cell deletion and hypogammaglobulinemia in IgMb-macroself Tg mice. Lymphoid tissues from WT, IgMb-macroself, or k-macroself Tg mice were analyzed by flow cytometry using the indicated Ab combinations. Plots shown were gated on TCRβ+ lymphocytes (H57). A–C, Flow cytometric analysis of B cells in BM, SP, LN, and PC using Abs to B220, IgM, and CD19. A, Top panels indicate with left, right bottom, and right top boxes B220intIgMlow pre-B/immature B cells, B220intIgM+ immature B cells, and B220highIgM+ recirculating B cells, respectively. B, Top panels indicate with left, middle, and right boxes B220lowCD19low B cells, B220intCD19int B cells, and B220highCD19high recirculating B cells, respectively. C, Analysis of PC B cells for CD19 and IgM expression (left panels) and CD19 and B220 (right panels). Analysis gates in the two right panels define B2 versus B1 cells in upper versus lower gates, respectively. D, Statistical analysis of total cell numbers in SP and LN. WT, n = 7; IgMb-macroself, n = 8. E, Abundance of B1 and B2 cells in PC expressed as percentage of viable cells. Each data point indicates the concentration in one mouse. F, Serum IgM and IgG concentrations in WT and IgMb-macroself Tg mice. WT, n = 7; IgMb-macroself, n = 8. G, Serum IgA concentrations. WT, n = 7; IgMb-macroself, n = 12. Each data point indicates the value in one mouse. Shown are means and SEM. **p < 0.005, ****p < 0.0005, *****p < 0.0001 (two-tailed Student t test). H and I, Analysis of splenic B cells of IgMb-macroself mice. Cells gated as indicated in the central panel of B were analyzed for L chain isotype (H) or CD21 and CD23 expression (I). J, Cell surface phenotype of IgMb-macroself B220low versus B220high splenic B cell subsets compared with B220high WT cells.
5′-RACE and κ-chain analysis

Total RNA was obtained from sorted cells, and κ-chain variable sequence was determined, as previously described (39). Briefly, adaptor was ligated to total RNA using 5′-RACE kit (FirstChoice RLM-RACE; Invitrogen), according to the manufacturer’s protocol, followed by reverse transcription using oligo(dT) primer with a Transcriptor High Fidelity cDNA synthesis kit (Roche, Indianapolis, IN), and PCR amplification with oligonucleotides corresponding to adapter and Cx sequences. Amplified κ-chain variable product was plasmid cloned, and the insert sequences of individual clones were determined. Obtained sequences were analyzed at the ImMunoGe-neTics Web site (www.imgt.org) (40).

Results

Anti-IgMb mAb AF6-78 fails to see the pre-BCR

To assess the ability of AF6-78 to bind to the pre-BCR, in which the μb-chain is associated with surrogate L chain components, we took advantage of a pre-B cell line (70Z/3) that expresses a pre-BCR, but which can be induced to produce κ-chain and surface IgM (sIgM) upon LPS activation (41). Flow cytometry analysis of permeabilized cells using mAb M41, an Ab reactive with μ-chain regardless of its associated L chains (42), confirmed that unstimulated 70Z/3 cells express μ-chain, but no κ-chain (Fig. 1A, left panel). By contrast, AF6-78 failed to react to unstimulated cells (Fig. 1B, solid lines), but bound avidly to LPS-stimulated 70Z/3 cells (dotted lines), indicating that AF6-78 only detects μ-chain associated with conventional L chain. To further assess the inability of AF6-78 to bind to the pre-BCR, we captured pre-BCR complexes from detergent lysates of unstimulated 70Z/3 cells on ELISA plates coated with Abs to IgM and measured binding by AF6-78, M41, and Abs to κ or the pre-BCR (Fig. 1C). Again, AF6-78 failed to bind, whereas there was clear binding by M41 or Abs to λ5 or the pre-BCR–specific Ab SL156. We conclude that AF6-78 has negligible affinity for μb-containing pre-BCRs, but binds well to IgMb.

Generation of an IgMb-reactive superantigen and expression in Tg mice

Ab genes derived from AF6-78 were then cloned and used to generate a single-chain Ab-coding construct (Fig. 1D), based on the κ-macroself construct (3), which is predicted to form a dimeric membrane protein carrying C-terminal H-2Kb transmembrane and cytoplasmic domains (Fig. 1E). Rat IgG1 H chain hinge, Cγ2 and Cγ3 domains provide flexibility and facilitate superantigen detection. Several founder lines of Tg mice expressing this construct were generated (e.g., Fig. 1F) and bred >10 generations onto the B6.CD45.1 background. All lines expressing superantigen had a similar phenotype. Studies in this work mostly involved IgMb-macroself lines 72 and 17, which gave nearly identical results.

Developmental block and Ag receptor downregulation in BM B cells

Flow cytometry was used to analyze BM, spleen (SP), lymph nodes (LN), and peritoneal cavity (PC) of IgMb-macroself mice for B cell numbers and phenotype. slgM+ cells were absent, and B cell deletion was apparent from the reduction of B220+ cells in SP and LN (Fig. 2A, center column). Staining with the pan B cell marker CD19 along with B220 (CD45R) revealed that B cell numbers were reduced to <1% of WT in secondary lymphoid organs of IgMb-macroself mice (Fig. 2B–E). By contrast, κ-macroself mice had significant numbers of B cells in the periphery owing to Ig- to-λ editing (3) (Fig. 2A, 2B, right panels). Consistent with the massive reduction of peripheral B cells, recirculating CD19+ B220high cells were absent from IgMb-macroself BM (Fig. 2A, 2B, top middle panels). Moreover, IgMb-macroself mice had greatly reduced levels of serum IgM, IgG, and IgA. However, IgG and IgA levels were clearly above background, with 2 of 12 mice expressing IgA in the normal range and the mean level of IgG at ~10 μg/ml (Fig. 2F, 2G). Similar findings of B cell deficiency and low serum Ig levels were obtained with IgMb-macroself line 17 (data not shown). Because the mice were always bred using IgMb-macroself males and WT females, some serum Ig may have been maternal. However, we could generate from LPS-stimulated splenocytes pooled from three IgMb-macroself mice several IgG-secreting hybridomas, suggesting that some IgG came from cells that had avoided deletion by class switching (data not shown). The small number of CD19+ cells in the SP of IgMb-macroself Tg mice appeared to be composed of a B220high population that had markers of follicular B cells, but little detectable surface Ig L chain (Fig. 2H, 2I, left panels) or expressed lower levels of B220 and carried L chains (right panels). This latter population had a B1-like phenotype as it expressed CD43 and CD5 and low levels of IgD (Fig. 2J). We conclude that B cells are profoundly reduced in number in IgMb-macroself Tg mice, but that some cells survive and differentiate to secretion of class-switched Abs.

BM B cells of IgMb-macroself mice were characterized in more detail. In addition to the lack of CD19+B220high recirculating cells, CD19+ B220+ cells were somewhat reduced in number compared with WT, and there was a concomitant increase in the numbers of CD19lowB220+ cells (Figs. 2B [top middle panel], 3A). The CD19lowB220+ cells appeared to be immature B cells that had downregulated slgM because they expressed high levels of intracellular μ-chain and κ-chain (Fig. 3B, 3C, right panels). More...
over, intracellular IgM (iIgM) in CD19\textsuperscript{low} B220\textsuperscript{+} cells was recognized by mAb 331.12, which requires L chain pairing (Fig. 3D). By contrast, CD19\textsuperscript{int} B220\textsuperscript{+} cells in IgM\textsuperscript{m-macroself} Tg mice were pre-B cells as indicated by their lack of cytoplasmic staining with 331.12 and Ab to \( \kappa \), and by the expression of L\( \lambda \) by a subset of cells (Fig. 3C, 3E). In all respects, comparable results were obtained in analyses of line 17 (data not shown). Immature IgM\textsuperscript{m-macroself} B cells with IgM had CD19 levels only \( \sim 1/10 \) of that of sIgM\textsuperscript{+} WT cells (Hardy fraction E), whereas WT cells that scored as IgM\textsuperscript{+}sIgM\textsuperscript{−} included a significant subset that had low CD19 levels (Fig. 3F). Thus, in two independent Tg lines, IgM\textsuperscript{m-macroself} Ag blocked B cell development at the immature B cell stage, leading to BCR and CD19 downregulation.

**Identification of a natural BM population under negative selection**

The foregoing experiments suggested that the B cells in WT BM undergoing central tolerance should have properties similar to B cells in IgM\textsuperscript{m-macroself} mice, namely IgM associated with conventional L chain, but little sIgM expression. By contrast, innocuous cells failing to see self-Ag in BM should permit surface expression. To assess this, we compared WT mice with IgM\textsuperscript{m-macroself} mice, in which we assume 100% of B cells are initially autoreactive and \( \kappa \)-macroself mice are carrying either one or two reactive IGK alleles. On an otherwise WT background, \( \kappa \)-macroself mice start out with >90% autoreactive cells (i.e., mouse \( \kappa \)\textsuperscript{+}), whereas in \( \kappa \)-macroself mice carrying one mouse Ck (mCk) allele replaced by hCk (38) only half as many cells carry mouse \( \kappa \). Using a flow cytometry strategy involving surface staining with anti-\( \mu \) mAb M41, followed by permeabilization and staining with 331.12 carrying a distinct fluorophore, we quantitated the frequency of IgM\textsuperscript{+}sIgM\textsuperscript{−} B cells (Fig. 4A). In IgM\textsuperscript{m-macroself} mice, as expected, nearly 100% of B220\textsuperscript{+}IgM\textsuperscript{+} cells lacked sIgM (Fig. 4A, second panel). As a control for innocuous cells, we chose QM mice, which are IGK deficient and express a Tg H chain that is considered to be nonautoreactive when paired with endogenous \( \lambda \) chain (43). In these mice, only 20% of IgM\textsuperscript{+} cells were sIgM\textsuperscript{−} (Fig. 4A [right panel], 4B). Assuming that this proportion represents phenotypic lag between intracellular H/L assembly and surface expression, and that 100% IgM internalization represents 100% negative selection, we assessed the percentages of IgM\textsuperscript{+} cells that were sIgM\textsuperscript{−} in WT mice and in \( \kappa \)-macroself mice that had either two mCk alleles or one mCk allele and one hCk allele (38) (Fig. 4A, 4B). In \( \kappa \)-macroself mice, \( \sim 70\% \) of IgM\textsuperscript{+} cells were sIgM\textsuperscript{−}, whereas this value was \( \sim 50\% \) in \( \kappa \)-macroself/hCk/mCk. In fully WT mice and in hCk/mCk mice lacking superantigen, the measured value was \( \sim 40\% \) of IgM\textsuperscript{+} cells that were sIgM\textsuperscript{−}. After subtracting 20% for phenotypic lag, these results suggest that \( \sim 20\% \) of normal developing B cells are undergoing central tolerance in the BM at steady state. CD19\textsuperscript{low} frequency was partly correlated with IgM internalization, being high in IgM\textsuperscript{m-macroself} mice and progressively lower in mice with fewer autoreactive cells (Fig. 4C).

However, the frequency of CD19\textsuperscript{low} cells was consistently lower than that of IgM\textsuperscript{+}sIgM\textsuperscript{−} cells. To see whether B cells undergoing negative selection had a distinct RNA expression profile, we chose to sort CD19\textsuperscript{low} cells from IgM\textsuperscript{m-macroself} mice, as this strategy does not require fixation of cells and provides a good separation from CD19\textsuperscript{high} cells, which lacked an intact IgM BCR. Negatively selecting CD19\textsuperscript{low} cells of IgM\textsuperscript{m-macroself} mice had a unique

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**FIGURE 4.** Analysis of BM B cells that internalize IgM in WT and Tg mice. A, BM cells of the indicated strains were analyzed for expression of slgM and IgM, using mAb 331.12 to detect conventional IgM expression. Cells were gated to exclude recirculating and non-B lineage cells. Note that QM B cells express only \( \lambda \)-chains, the plot demonstrates that 331.12 binds well to IgM composed of \( \mu \) and \( \lambda \)-chains. B, Analysis of the percentages of IgM\textsuperscript{+}slgM\textsuperscript{−} BM cells as a function of total IgM\textsuperscript{+} immature B cells as detected using mAb 331.12 (% IgM + slgM\textsuperscript{−} = \( 100 \times \) [slgM\textsuperscript{−}IgM\textsuperscript{+}]/[IgM\textsuperscript{+}IgM\textsuperscript{+} + slgM\textsuperscript{−}]). C, The CD19\textsuperscript{low} frequency among IgM\textsuperscript{+} immature B cells was calculated (% CD19\textsuperscript{low} = \( 100 \times \) [CD19\textsuperscript{low}]/[IgM\textsuperscript{+}IgM\textsuperscript{+} + slgM\textsuperscript{−}]). In B and C, each dot represents the value measured in an individual mouse, with the mean represented by a horizontal bar. D and E, Shown is quantitative RT-PCR expression analysis of selected mRNAs in the indicated BM B cell subsets. Expression levels found in WT slgM\textsuperscript{−} CD19\textsuperscript{high} were set to 1, and relative levels were given for other samples. **p < 0.005, ****p < 0.0001. F, Staining profiles of cells from the indicated WT or IgM\textsuperscript{m-macroself} BM cell subpopulations using Abs to the indicated cell surface markers. Data were from at least three mice per group.
transcription profile, including an intermediate level of expression of Rag1, Rag2, Foxo1, Il2ra, and Il7r; reduced expression of Cd36 and Ccnd2; and high expression of Faim3 (Fig. 4D, 4E). Surface markers correlated with these changes in the cases of Il2ra (CD25), Il7r (CD127) (Fig. 4F), and Cd36 (CD36). These patterns distinguished them from Cd19<sup>hi</sup>slgM<sup>+</sup> and Cd19<sup>lo</sup>slgM<sup>-</sup> bulk WT populations. Rag1 and Rag2 mRNA levels in negatively selecting cells were intermediate between Cd19<sup>hi</sup>slgM<sup>-</sup> and Cd19<sup>lo</sup>slgM<sup>+</sup> cells, which had high and low levels, respectively. IgM internalization thus identifies a BM population undergoing negative selection, and these cells have a distinctive gene expression profile.

To further test the validity of this approach, we analyzed additional mouse models lacking superantigen, but with altered extents of self reactivity and receptor editing, including mutants in the murine κ-deleting element RS (44) and mice carrying the 3H9 site-directed H chain transgene (4). RS<sup>-/-</sup> B cells have defective editing of a subset of autoreactive B cells, particularly those that use J<sub>IGKV</sub>6-17, J<sub>IGKV</sub>13-85, and J<sub>IGKV</sub>1-110 genes predicted to confer weak reactivity, but not to induce editing, were also enriched among Cd19<sup>lo</sup>slgM<sup>-</sup> cells (Fig. 5G, “a” versus “e,” respectively). IgKV genes predicted to confer weak reactivity, but not to induce editing, were also enriched among Cd19<sup>hi</sup>slgM<sup>-</sup> cells (marked “w”). These data demonstrate that cells internalizing IgM and CD19 are mainly composed of autoreactive cells undergoing central tolerance, not only in macroself Tg mice, but also in models that see physiological autoantigens.

**Deletion and homeostasis of B cells in IgH<sup>αβ</sup>;IgM<sup>β</sup>-macroself mice**

Analyses of the effects of superantigen on the B cell compartment in IgH<sup>αβ</sup>;IgM<sup>β</sup>-macroself mice were carried out both by introducing the line 17 transgene by breeding and by the use of IgH<sup>αβ</sup>-IgM<sup>β</sup>-macroself radiation BM chimeras. These experiments recapitulated the finding that mature IgM<sup>+</sup> cells were deleted in the presence of superantigen (data not shown), but revealed subtle features of homeostasis among the IgM<sup>+</sup> populations. In IgH<sup>αβ</sup>-IgM<sup>β</sup>-macroself mice, splenic marginal zone (MZ) and B1 cells that were IgM<sup>+</sup> developed in normal numbers, whereas B2 cell numbers were roughly half of normal (Fig. 6A–E). Interestingly, however, in IgH<sup>αβ</sup>-IgM<sup>β</sup>-macroself chimeras, similar results were obtained with B2 and B1 cells, but MZ B cell numbers were also half of normal (Fig. 6F–K). We interpret these results to mean that B2 cell number is regulated mainly by BM output, whereas non-BM (presumably fetal)–derived MZ and B1 cells are regulated by homeostatic factors independently of adult BM-derived MZ and B1 cells.

**FIGURE 5.** Increased numbers and distinct κ L chain usage in Cd19<sup>lo</sup>B220<sup>+</sup>BM cells of 3H9;RS<sup>−/−</sup> mice. A, Flow cytometry analysis of Cd19 and B220 expression of BM lymphocytes from the indicated mouse strains gated to exclude TCR<sup>+</sup> and plasmacytoid dendritic cells using H57 and PDC<sub>1</sub> mAbs, respectively. B, Quantitation of igM<sup>+</sup> and Cd19<sup>+</sup> cells among the B220<sup>+</sup>slgM<sup>+</sup> population. C, Plot showing the Cd19<sup>lo</sup>B220<sup>+</sup> frequency within the immature B cell population of the indicated strains with each point representing the value from one mouse. D and E, igM<sup>+</sup> and Cd19<sup>lo</sup> igM<sup>+</sup> frequencies, respectively, plotted as a percentage of (slgM<sup>+</sup> + slgM<sup>-</sup>) IgM<sup>+</sup> cells (WT, n = 11; RS<sup>−/−</sup>, n = 6; 3H9, n = 8; 3H9;RS<sup>−/−</sup>, n = 8). Each point represents the value obtained from an individual mouse. Horizontal lines represent means of the individual values. ns, non significant; *p < 0.05, **p < 0.005, ***p < 0.0005. F and G, Cd19<sup>+</sup>B220<sup>+</sup>slgM<sup>+</sup> and Cd19<sup>+</sup>B220<sup>+</sup>slgM<sup>-</sup> were sorted from three 3H9; RS<sup>−/−</sup> mice. RNA was extracted, and κ-chain transcripts were amplified by 5′-RACE. PCR fragments were cloned, and Vk and Jκ usages were analyzed. Total sequences analyzed were Cd19<sup>+</sup>B220<sup>+</sup>IgM<sup>+</sup>, n = 78; Cd19<sup>+</sup>B220<sup>+</sup>IgM<sup>-</sup>, n = 82.
in the peritoneum using CD19 and B220 costaining, showing gating defined by flow cytometry and scored for IgM allotype were enumerated in IgMb-macroself cells in revealing reduced B2 compartment and disproportionate percentage of MZ B maturation was evident also in the appearance in BM of cells with IgM-macroself B cells in the indicated mice. F radiation chimeras.

**FIGURE 6.** Effects of IgM superantigen on B cell homeostasis in an IgMb-macroself background. A–E, Analysis of peripheral B cell subset numbers in IgM-macroself; IgMb-macroself mice generated was skewed to an excess usage of λ, suggesting a selective escape of B cells that previously attempted to edit their receptors.

**Discussion**

Not just a poor man’s B cell knockout

We find in this study that ubiquitous expression of IgM superantigen leads to significant B cell deletion with little escape. IgH<sup>H<sub>oh</sub></sup>– IgM<sup>macroself</sup> mice are severely deficient in B cells, starting from the transitional stages of splenic B cell development, but early B cell developmental stages are unaltered because the superantigen fails to bind to the pre-BCR. Depletion of mature B cells in these mice renders them hypogammaglobulinemic. The mice are susceptible to Pneumocystis carinii infection (data not shown). Conventional receptor editing, V<sub>Q</sub> replacement, or V region hypermutation cannot rescue IgH<sup>H<sub>oh</sub></sup>- cells from reactivity to such a superantigen, potentially revealing alternative mechanisms of receptor alteration or escape. In this case, class switching appears to allow a small number of B cells to develop. In many species, developing B cells diversify their receptors through expression of AID, which is also required for initiating H chain class switch (51, 52). In mice, low, but detectable, levels of AID expression, class switching, and somatic mutation occur in immature B cells and in retrovirally infected pre-B cells (28, 31, 53). Under some circumstances, such as on the Fas<sup>+</sup> background, B cells develop in μMT/μMT mice owing to class switch recombination to downstream isotypes in pre-B cells (32–35). However, Fas mutation did not have any obvious effects on B cell development in IgM<sup>macroself</sup> mice (data not shown). Overall, apart from the exception just mentioned, the phenotype of IgM<sup>macroself</sup> mice is similar to that of μMT/μMT mice. As such, IgM<sup>macroself</sup> mice provide an alternative way to render mice substantially, but not completely, B cell deficient. In this respect, IgM<sup>macroself</sup> mice provide advantages in that the effect is genetically dominant, and deletion can be affected in the setting of radiation BM chimeras using IgM<sup>macroself</sup> hosts. Most importantly, however, IgM<sup>macroself</sup> chimeras should be particularly useful in identifying genes and mechanisms involved in regulating central tolerance.

**Downregulation of IgM and, to a lesser extent, CD19 mark editing cells in the BM**

By contrast to the deleting effects of IgM superantigen on mature peripheral B cells, immature B cells are abundant in the BM of IgM<sup>macroself</sup> mice. These cells downregulate CD19 and carry a significant level of IgM, detectable by staining permeabilized cells. Interestingly, a comparable, but less abundant, IgM<sup>slgM<sub>B220</sub></sup> population, and partly CD19<sup>+</sup>, population is apparent in normal BM. We propose that this population includes a high proportion of naturally autoreactive, editing cells. It has been suggested that such cells in humans, which have a high frequency of autoreactivity and polyreactivity, are early immature B cells, implying that they represent developmentally early B cells prior to Ag selection, although in that study they did not show the predicted skewing to reduced λ or upstream J<sub>k</sub> usage (18). Our results suggest rather that many or most such cells represent immature B cells that have internalized receptor upon BCR ligation. J<sub>k</sub> usage predominates in this population, which is clearly inconsistent with an early immature phenotype. Cells with downregulated BCR and CD19
are abundant not only in superantigen mice, but also in autoantibody Tg mice, where many cells are autoreactive (11, 54, this study). CD19 downregulation may be of some functional importance because CD19 phosphorylation and attendant PI3K activation are known to contribute to the downregulation of editing (11, 55–59). By contrast, Ig Tg mice generating innocuous receptors often have a reduced sIgM\textsuperscript{2}BM compartment owing to accelerated B cell development (54, 60, 61).

The 3H9H chain Tg mice are predicted to generate autoreactive receptors at a higher than normal frequency due to the Ab’s predisposition to bind to chromatin, DNA, and other self Ags, which leads to increased \(\lambda\) chain gene editing and bias to downstream J\(\text{\kappa}\) usage (8, 46). However, IgM internalization in immature 3H9B cells was comparable to WT, whereas the CD19\text{\textsubscript{low}} fraction was significantly increased. Similarly, RS\textsuperscript{2/2} cells appear indistinguishable from normal in IgM and CD19 downregulation, but in combination with the 3H9 gene have an increased proportion of CD19\text{\textsubscript{low}} cells. In 3H9;RS\textsuperscript{2/2}BM, autoreactive cells are highly enriched in the CD19\textsuperscript{low}sIgM\textsuperscript{2}BM compartment owing to accelerated B cell development (54, 60, 61).

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On the basis of the relative abundances in WT versus IgM\textsuperscript{2}sIgM\textsuperscript{2}BM mice of the B220\textsuperscript{2}IgM\textsuperscript{2}sIgM\textsuperscript{2}BM population (Fig. 4B), we estimate that ~20% of WT BM immature B cells are autoreactive and under negative selection, a number consistent with, or somewhat lower, than other estimates of the extent of editing or negative selection in the repertoire (18, 38, 61, 63, 64). It is important to note that in the approach taken in this study, we exclude from analysis cells that fail to undergo positive selection because of an inability to pair H and L chains, because such cells would not be scored with the 331.12 Ab. Prak and Weigert (65) and Casellas et al. (38) have shown with \(\kappa\)-chain–targeted Tg mice that functional V\(\kappa\)J\(\text{\kappa}\) exons are often displaced by editing, at frequencies ranging from 18 to 71%, but the fraction of those arising from an inability to pair with H chain was unclear. It is also possible that we might underestimate the frequency of central tolerance in the normal compartment because of the ability of cells to rapidly correct autoreactivity by editing after relatively little sIgM or CD19 downregulation. Our assumption that developing QM cells are completely nonautoreactive also most likely leads to an underestimate of as much as 15%, as some QM B cells express a non-Tg H chain and certain \(\lambda\)-chains may confer autoreactivity when paired with the QM H chain (43, 66).

**FIGURE 7.** Effect of transgene-enforced Bcl2 expression on peripheral B cell elimination by IgM\textsuperscript{2} superantigen. A and B, Flow cytometry analysis of B cell numbers in the indicated lymphoid compartments of control, Bcl2 Tg, or IgM\textsuperscript{2}-macroself single-Tg, or double-Tg (DTg) IgM\textsuperscript{2}-macroself;Bcl2 Tg mice. Data are representative of at least three independent experiments. A, Staining with Abs to B220 and CD19; B, staining with Abs to IgM and CD19. Gating was as in Fig. 2. C, Serum Ig levels measured for IgM and IgG associated with \(\kappa\) or \(\lambda\) L chains in the indicated mouse strains. Mice were 8–9 mo old.
Limited escape of IgH b B cells

Although developing B cells encountering IgM b superantigens rarely escape deletion, we often detect small numbers of residual B2-like cells in peripheral tissues of IgM b-macroself mice. In the SP, such cells are rare and have either a CD93+Slg g IgM b phenotype, which may represent the outcome of residual hematopoiesis in situ, or a B1-like phenotype. Classical anergic B cells have not been observed, probably because of the nature of the superantigen and its expression pattern in this particular model. In the peritoneum, B1 cells are occasionally present in significant numbers in older mice. Switching contributes to B cell escape because we detect low levels of IgG and IgA in the sera of most IgH b-mice carrying the IgM b-macroself Ag. We suspect that the B1 cells still present in the peritoneum of IgM b-macroself mice represent either rare class switch variants or are somatic cell mutants that can expand owing to the ability of B1 cells to self renew. In any case, these escape mechanisms seem inefficient and are unlikely to contribute significantly to normal development in mice. Our results are reminiscent of those obtained many years ago in experiments treating immature animals with γ-heavy chain Abs (67), which significantly blocked not only the production of IgM, but also that of IgG and IgA. In those studies, as in ours, low expression of IgG and IgA was often observed in a small subset of treated animals.

B cell homeostasis of IgH b cells in the presence of IgM b superantigen

In IgH b-mice, superantigen eliminates IgH b cells, resulting in a concomitant reduction in total BM output and total peripheral B2 cell numbers to ~50%. We interpret these data to indicate that BM output regulates steady state numbers of the B2 (i.e., follicular) subset. By contrast, MZ and B1 compartments were normal in size and made up of IgH b cells, indicating that these compartments are regulated independently of BM output. These results are consistent with studies in which BM B cell output was suppressed in adult mice by lack of IL-7 or induced elimination of RAG expression (68, 69). However, in IgH b→IgM m-macroself chimeras, the MZ compartment is also diminished by approximately half, suggesting that adult BM precursors give rise to MZ cells that are more limited in the capacity for self-renewal than those in intact mice. More subtly, these data appear to place limits on the potential contribution of H chain editing to the opposite allele to facilitate B cell escape from deletion, as they fail to appreciably normalize BM B cell output. However, if Vg replacement is limited only to the initially autoreactive IgH b allele, we would expect no escape from tolerance in the IgM m-macroself model, and hence our data only place limits on the extent of replacements leading to changes in allele usage.

Bcl2 overexpression in B cells in IgM m-macroself:Bcl2 Tg mice promotes a significant escape from central tolerance and peripheralization of B cells. At face value, that might be evidence that central tolerance is solely an apoptotic process. However, we have previously found that the same Bcl2 Tg line failed to promote escape in two other models, but instead facilitated receptor editing (3, 49). To explain these results, we suggest that there must be an important component of competition between innocuous versus autoreactive B cells, with autoreactive B cells at a considerable disadvantage. When editing is blocked or ineffectual, as in IgM m-macroself mice, and competing nonautoreactive B cells are rare, Bcl2 overexpression allows development to proceed. This is also consistent with our finding that Bcl2 Tg:R5−/− B cells escape tolerance when they develop in a k-macroself mice, whereas Bcl2 Tg B cells do not (44). In that model, λ B cells would provide the competition, whereas in IgH b-IgM m-macroself mice all cells are equally reactive to superantigen. R5 mutation hinders editing, which in turn reduces the abundance of competing λ cells. One interpretation of these results is that BM B cells compete for a limiting resource to enforce tolerance, a possibility with significant clinical implications. Although the nature of such a factor is unknown, we believe we can exclude BAFF as a candidate based on the inability of BAFF overexpression to rescue in this model (data not shown).

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Disclosures

The authors have no financial conflicts of interest.

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