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J Immunol 2010; 184:4143-4158; Prepublished online 15 March 2010;
doi: 10.4049/jimmunol.0903564
http://www.jimmunol.org/content/184/8/4143

Supplementary Material http://www.jimmunol.org/content/suppl/2010/03/15/jimmunol.0903564.4.DC1

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The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Peripheral B Cell Tolerance and Function in Transgenic Mice Expressing an IgD Superantigen

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Transitional B cells turn over rapidly in vivo and are sensitive to apoptosis upon BCR ligation in vitro. However, little direct evidence addresses their tolerance sensitivity in vivo. A key marker used to distinguish these cells is IgD, which, through alternative RNA splicing of H chain transcripts, begins to be coexpressed with IgM at this stage. IgD is also expressed at high levels on naive follicular (B-2) and at lower levels on marginal zone and B-1 B cells. In this study, mice were generated to ubiquitously express a membrane-bound IgD-superantigen. These mice supported virtually no B-2 development, a greatly reduced marginal zone B cell population, but a relatively normal B-1 compartment. B cell development in the spleen abruptly halted at the transitional B cell population 1 to 2 stage, a block that could not be rescued by either Bcl-2 or BAFF overexpression. The developmentally arrested B cells appeared less mature and turned over more rapidly than nontransgenic T2 cells, exhibiting neither conventional features of anergy nor appreciable receptor editing. Paradoxically, type-2 T-independent responses were more robust in the transgenic mice, although T-dependent responses were reduced and had skewed IgL and IgH isotype usages. Nevertheless, an augmented memory response to secondary challenge was evident. The transgenic mice also had increased serum IgM, but diminished IgG, levels mirrored by the increased numbers of IgM+ plasma cells. This model should facilitate studies of peripheral B cell tolerance, with the advantages of allowing analysis of polyclonal populations, and of B cells naturally lacking IgD.  


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Received for publication November 3, 2009. Accepted for publication February 9, 2010.

This work was supported by National Institutes of Health Grant RO1AI059714 and by Training Grant T32AI007606. K.W. was supported by Grant AI74564 to M. Oldstone.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: ASC, Ab-secreting cell; BM, bone marrow; cIgM, cytoplasmic IgM; DTG, double transgenic; HEL, hen egg lysozyme; LN, lymph node; MHC II, MHC class II; MZ, marginal zone; mlg, membrane Ig; PB, Pacific Blue; PC, peritoneal cavity; p-Tyr, phosphorytose; pUb, part of the ubiquitin promoter; sIg, surface Ig; SP, spleen; T, transitional B cell population; TD, T cell-dependent; Tg, transgenic; TH2, T cell-independent type 2; TNP-Hy, trinitrophenol-hemocyanin.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.0903564
expression is correlated with developmental maturation and the loss of the ability of B cells to undergo self-Ag–induced receptor editing (46, 47). However, transitional cells might retain the capacity to edit given the appropriate microenvironment (48, 49).

Like mlgM, mlgD can transmit signals through the associated signal transducers Ig-α/β (CD79a/b) (50–52), triggering a tyrosine kinase cascade and the activation of additional signaling pathways. Early biochemical changes triggered by IgM and IgD in mature B cells are biochemically similar, although signaling through IgD may be more prolonged (53). However, upon activation by Ag, IgD expression on B cells is lost (54–56). With rare exceptions, IgD is not expressed by plasma cells, although the secretory domain of IgD is intact and conserved in most species studied. IgD is secreted at relatively high levels in rare inflammatory diseases, such as mevalonate kinase deficiency (57). Rare cells or tumors with dedicated IgD secretion can be found that have undergone a recombination event similar to classical switch recombination (58). In humans, a subset of IgD class-switched cells with unique properties exists (59), and it has been suggested that basophils carry an Fc receptor for IgD (60). Rare cells or tumors with dedicated IgD secretion can be found that have undergone a recombination event similar to classical switch recombination (58). In humans, a subset of IgD class-switched cells with unique properties exists (59), and it has been suggested that basophils carry an Fc receptor for IgD (60). However, IgD-deficient mice have been generated and shown to have only subtle defects, mainly in the kinetics of T cell–dependent (TD) Ab responses (61, 62). However, IgD-deficient mice have been generated and shown to have only subtle defects, mainly in the kinetics of T cell–dependent (TD) Ab responses (61, 62).

We recently developed a custom superantigen technology based on the expression of membrane tethered single-Abs reactive to Ab Constant regions (63–65). The goal of the current study was to assess the ability of an IgD–reactive superantigen expressed as a ubiquitously expressed transgene in mice to promote B cell tolerance and to alter B cell development and function. These studies were carried out with a view to establishing more precisely the cell lineage and precursor/product relationships among B cell subsets found in the secondary lymphoid tissues and their modes of tolerance induction.

Materials and Methods

IgDα–macroself construct

The transgene encoding IgDα–macroself Ag was generated based on a plasmid template described for IgDα–macroself (66). Total RNA from AMS9.1 hybridoma was isolated using Trizol (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions. Vι and Vα cDNA sequences were obtained by 5′ RACE (Ambion, Austin, TX) using Cα and IgG2b antisense primers and cloned into Zero Blunt TOPO vector using methods suggested by the manufacturer (Invitrogen). Using the Igs-macroself construct as template, a cdNA fragment containing part of the ubiquitin promoter (pUb) and the 5′ end of the Vι was generated by PCR using pUb F and iLAMS9 R primers. From the TOPO vector containing the Vι cdDNA, the Vι fragment was reamplified using extended primers (AMS9Vι F and AMS9Vι R) that contained the antisense sequence of the linker at its 3′ end. Similarly, the Vα fragment was reamplified using primers (AMS9Vα F and AMS9Vα R) that contained sequence for the linker at its 3′ end and part of rat IgG1 Fc at its 3′. PCRs for all three fragments were performed for 35 cycles using PrimePrimes (Invitrogen). Meltin (94˚C, allowing 55˚C, and elongation (68˚C) times were 45 s each. Subsequently, a long insert containing all three fragments was generated by nested PCR using the pUb F and iLAMS9 R primers. A total of 25 cycles were performed using the following conditions: melting (94˚C, 1 min), annealing (50˚C, 1 min), and elongation (68˚C, 1 min). The insert was subsequently gel–purified, digested with SpeI and XmaI, and cloned into the previously described Igs–macroself construct (after removing the anti-Igs cdDNA in–sent by SpeI and XmaI digestion). After linearization by Nod digestion, plasmid was sent to The Scripps Research Institute Transgenic Core (La Jolla, CA) for pronuclei injection. Primer sequences used are as follow: Cα antisense (5′-ctg ctc act gaa gga tgg tga gat gat gg–3′); IgG2b antisense (5′-ggg gag tgc taa gca gta cag aat–3′); pUb F (5′-ttt tt ctc gga cgg cgg tgc ttc ggg–3′); iLAMS9 R (5′-atc aca aca ttc tct gaa cag cca gct gta att aat aag c–3′); AMS9 Vι F (5′-tac aga ggt cag ttc aac acc tcc gaa ggt ggt–3′); and AMS9 Vα R (5′-atc acc tcc ggg tgc tgc tgt ttc aca tga cgg gag ggt ggc ggt gcg cct ccc ggc gcg ttc ggc ggt–3′).

IgDα–macroself mice

Animal studies have been reviewed and approved by the The Scripps Research Institute institutional review committee. All IgDα–macroself Tg mice described in this work had been bred and maintained on C57BL/6 background congenic for IgHα alleles (B6.Cg-Igh Thy1.1 C-kit, stock number 001317, JAX) for at least eight generations. In some experiments, they were also bred to overexpress BAFF-R or retain IgDα alleles. BAFF-R Tg mice have been described (66). To genotype for the presence of transgene in each breeding, 35 cycles of PCR were performed using AMS9.1 Vι CD3R S (5′-ccca aca tca taa tct cta caa–3′) and AMS9.1 Vι CD3R AS (5′-cag aca tcc aag tag cag tag cag ttt caa tta tct tct–3′) primers and the following conditions: melting (94˚C), annealing (58˚C), and elongation (72˚C) for 40 s each.

BM and SP cell transfers

In all BM transfer experiments, each mouse host was lethally irradiated at 300 Rad 1 d prior to receiving i.v. 106 BM cells, which were isolated from both hatching and lower leg bones of donors using standard protocols. Donor mice were either IgHα C57BL/6 congenic mice (described above) or Bcl2 Tg (67) bred onto IgHα C57BL/6 congenic background. In splenic transfer experiments, total SP cells were isolated from either non-Tg or IgDα–macroself mice by standard procedures. Subsequently, 3×105 total SP cells were injected i.v. into nonirradiated IgHα congenic mice. Because of the donor and recipient mice had been crossed onto C57BL/6 background for at least eight generations, no depletion of donor T cells was performed. I.v. injections were performed retro-orbitally.

Flow cytometry analyses

Flow cytometric analyses for surface markers were performed using standard protocols. Intracellular proteins were stained for by using Cytofix/Cytoperm Kit (BD Biosciences, San Jose, CA) according to manufacturer’s recommendations. The following Abs were used at 1:200 in all experiments described: B220 (RA-3-6B2, allophycocyanin/Alexa 750, eBio- science, San Diego, CA); CD19 (1D13; PE/Cy7; eBioscience); CD21 (TE9; FITC, allophycocyanin, or Pacific Blue [PB]; Biolegend, San Diego, CA); CD22 (OX-97; Alexa 674; AbD Serotec, Raleigh, NC); CD43 (57; PE; BD Biosciences); CD62L (MEL-14; PE; eBioscience); CD86 (GL1; PE; BD Biosciences); CD93 (AA4.1; PE/Cy7 or allophycocyanin; eBioscience); CD138 (28-1-2; PE; BD Biosciences); BAFF-R (HU216; Alexa 647; eBioscience); I-Aκ (25-9-17; FITC; BD Biosciences); IgG1 (x1-26; PE; eBioscience); IgMκ (AF6-78; FITC; Biolegend); and Igλ1,2,3 (R26-46; FITC; BD Bioscience). Abs against mouse IgM (M41; PB), IgMκ (DS-1; Alexa 647), and Igκ (187.1; Alexa 647) were labeled in-house and used at 0.5 μg (M41) or 0.1 μg (DS-1 and 187.1) per 2×107 cells (in 200 μl) for both surface and intracellular stains. To stain for intracellular IgG, FITC–labeled Abs against each isotype were purchased from eBioscience, IgG1, A85-1; IgG2a, R19-15; IgG2b, R12-3; and IgG3, R40-82 were mixed and used at 0.15 μg each per 2×107 cells in 200 μl. To gate out most T and myeloid cells, most flow analyses included a dump Ab mixture consisting of PerCP/Cy5.5-labeled Abs against CD4 (RM-4.5), CD8 (53-6-7), Gr-1 (RB6-8C5), and F4/80 (BM8) (all purchased from Biolegend), also used at 1:200 dilution each. All samples were read on an LSR-II instrument (BD Biosciences) and analyzed using the FlowJo program (Tree Star, Ashland, OR). Mean fluorescence intensity values given were calculated by geometric means of the indicated fluorophore staining of the gated cells.

Brdu labeling analyses

All mice were initially injected i.p. with 1 mg/mouse of Brdu (Sigma–Aldrich, St. Louis, MO) in PBS and immediately placed on drinking water containing 1 mg/ml Brdu for 7 d. Groups of three mice per time point were sacrificed on days 0, 1, 2, 4, and 7. Total cells harvested from their BM and SPs were subjected to intracellular Brdu staining (BD Biosciences) per manufacturer’s instruction. Surface staining was performed using the following Abs: CD93-PE (AA4.1, eBioscience); CD23/PE/Cy7 (B284, eBioscience); CD21-allophycocyanin (7E9, Biolegend); B220-allophycocyanin/Alexa 750 (RA3-b2b, eBioscience); and PerCP/Cy5.5-labeled dump Ab mixture (see above). Intracellular staining of IgM using PB-labeled clone M41 mAb was performed along with Brdu–FITC (BD Biosciences).

In vitro stimulation for phospho-protein Western analyses

Total B cells were purified from pooled mouse SPs by CD4/CD43-negative selection using the B Cell Isolation Kit (Miltenyi Biotec, Auburn, CA) per the manufacturer’s instruction. For Western analyses of phospho-protein levels, purified B cells were resuspended at 107 cells/ml in HBSS buffer...
without supplement, prewarmed to 37°C in water bath for ~10 min, and then stimulated for 10 min with 20 μg/ml of goat F(ab)² anti-mouse IgM (Jackson ImmunoResearch Laboratories, West Grove, PA). Pelleted cells were lysed in (100 μl per 10⁶ cells) TBS buffer containing 1% Nonidet P-40, Complete EDTA-Free Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN), 5 mM EDTA, 10 mM NaF, and 1 mM NaVO₄. After removal of insoluble cellular debris by centrifugation, 10 μl each lysate was run in 4–12% NuPAGE gels (Invitrogen). A total of 0.2 μm nitrocellulose membranes (Bio-Rad, Hercules, CA) were used for all Western blotting analyses. The following primary Abs were used at the indicated concentrations, diluted in TBST (TBST plus 0.1% Tween-20) containing either 5% milk or 1% BSA: anti-p53 (Y12) (1:1000 in milk; Millipore, Bedford, MA), anti-GAPDH (68C, 1:10,000 in milk; Millipore), anti-p-p53 (51D19) (1:1000 in milk; Cell Signaling Technology, Beverly, MA), anti-total RAG1 (2F2) (1:5000 in milk; gift from Dr. J. Cambier at National Jewish Health, Denver, CO), anti-total ERK1/2 (Y20 and T208) (1:1000 in BSA; Cell Signaling Technology), and anti-total RAG2 (59G12) (1:1000 in milk; gift from Dr. J. Cambier at National Jewish Health, Denver, CO). Membranes were incubated at 65°C with gentle agitation for 15–20 min in TBS buffer (pH 2.3).

In vitro proliferation assays

Two-fold serial dilutions of goat F(ab)² anti-mouse IgM (Jackson ImmunoResearch Laboratories) or LPS (055:B5, Sigma-Aldrich) in media (DMEM containing 10% FBS, 5 mM 2-ME, 1-glutamine, and penicillin-streptomycin) were prepared in 96-well plates (10 μl/well). Cells, purified from pooled lymph nodes in ELISA buffer for detection (IgG1, IgM, and IgE for IL-4 and IL-5) (Becton Dickinson), were resuspended at 2 × 10⁶ cells/ml in media and subsequently added into each well (100 μl/well). After ~24 h of stimulation, 1 μCi of [³H]thymidine (GE Healthcare, Piscataway, NJ) was added per well. Cells were allowed to label for ~3 days. All treatments were done in triplicate. The following primers were used for 1-step Ultra-TMB Substrate (Pierce) per the manufacturer’s instructions. All wells were washed 4× with TBST in between steps. Signals were recorded at 450 nm using VersaMax microplate reader (Molecular Devices, Sunnyvale, CA).

**TD and T cell-independent anti-TNP responses**

IgD-macroliss and non-Tg litttermates were bled on day 0 prior to receiving i.p. 20 μg TNP-Ficoll in PBS or 100 μg trinitrophenol-hemocyanin (TNP-Hy) in Imject Alum adjuvant (Pierce) for subsequent measurements of their T cell-independent and TD responses, respectively. Mice were bled at 7-d intervals. To assess memory responses, the latter group of mice received a secondary challenge of 100 μg TNP-Hy in PBS i.p. on day 44. Sera were collected prior to secondary challenge and 7 d later on day 51. To measure anti-TNP responses by ELISA, Nunc Maxisorp plates (Nunc) were coated with 10 μg/ml of TNP-Sep-BSA in PBS overnight, blocked with ELISA buffer for 1 h, and incubated with sera serially diluted 2× in ELISA buffer for 1 h. All subsequent detection and reporting steps were performed as described above for isotype ELISAs. Response titer at each time point per mouse was defined as the theoretical factor of serum dilution required to achieve half-maximum OD of the assay (normally at values of 1.6–1.9), extrapolated from four-parameter plots.

**Results**

**Generation of IgD⁻macroliss Tg mice**

To assess the in vivo effects of the expression of an IgD-reactive superantigen, a membrane tethered single-chain Ab gene was engineered from the variable H and L regions of anti-IgD hybridoma AMS9.1 (68) using previously described methods (63) (Supplemental Fig. 1). Tg mice that ubiquitously expressed this construct were generated (and are called IgD⁻macroliss mice hereafter). These lines with high-, intermediate-, or low-level expression were selected for further study. As the phenotypes of these mice were virtually identical, most data shown in this study involve studies carried out with mice carrying an intermediate level of macroliss expression (line #16). All IgD⁻macroliss mice subsequently analyzed had been bred onto the C57BL/6 background congenic for the IgH allele for at least eight generations.

**Altered mature B cell subsets in IgD⁻macroliss mice**

Flow cytometry analysis was used to assess the effect of superantigen expression on the numbers of B cells in different tissues. Lethally irradiated IgD⁻macroliss mice were reconstituted with IgH⁺ BM, and their B cell development was compared with that of the similarly treated non-Tg littermates. Initial analysis was carried out with CD19/B220 costaining, which identifies virtually all B cells and can reveal distinct subsets (Fig. 1A, Supplemental Table I). As expected, no significant changes were seen in immature BM B cells. By contrast, recirculating mature B-2 cells in the BM were virtually absent. This was similarly reflected by the ~96% reduction in total B cell numbers in the LNs, where the most developmentally mature recirculating B-2 cells normally reside. In the SP, B cell percentages were reduced by ~90%. A more detailed analysis for the two major mature B cell subsets in this compartment revealed that B-2 cells bearing the CD23⁻ CD93⁻ B220⁺ surface phenotype were severely reduced in frequency and number (Fig. 1B, 1E). However, CD21⁺CD1d⁻B220⁻ MZ B cells of CD93⁻ mature phenotype were clearly present, albeit at only ~15% of normal numbers (Fig. 1C, 1E, Supplemental Table I). In the peritoneum, total B cell numbers were also reduced although a significant number of CD19⁺B220⁺ B cells still remained. These cells were confirmed to be B-1 by CD43 marker expression (Fig. 1D), suggesting that B-1 cells could still be reconstituted in IgD⁻macroliss mice by adult BM. Surface IgM⁻ B cells were clearly present in all lymphoid organs tested (Supplemental Fig. 2A). However, surface IgD expression (measured using an Ab that is not affected by AMS9.1 binding) was
undetectable on these cells (Supplemental Fig. 2 B). These alterations in B cell development were reproducible in nonirradiated IgDa-macroself mice bred onto a C57BL/6 background homozygously congenic for the IgH a allele, except that normal numbers of B-1 cells were present in the peritoneum (Supplemental Fig. 3 and data not shown). Thus, presence of the IgDa-macroself Ag impaired development of all three mature B cell subsets, although B-1 cell numbers could reach the normal range in intact IgH a mice carrying the macroself Ag.

Developmental block at transitional B cell stages

To determine where developmental block occurred in IgD a-macroself mice, B cells were evaluated for expression of maturation markers. Development of mature B cells in the periphery is thought to occur via two cellular pathways, one in the BM and a second in the SP (69, 70). Recently formed CD23+CD93+ T2-like B cells in the BM of IgD a-macroself mice did not appear significantly different from those of non-Tg littermates, either quantitatively or phenotypically (Fig. 2 A, 2 D). Plots shown were gated on non-T and nonmyeloid cells. Maturity of MZ B cells was also assessed by CD93 expression, shown in lower panels of C, E. Total cell numbers for each immune tissue and the three mature B cell compartments were calculated. Shown are mean ± SD analyzed from six non-Tg and six IgD a-macroself age-matched hosts. Values of p by two-tailed Student t tests are also shown where there is statistical significance.

Increased turnover of CD23+CD93+ transitional cells

To test the hypothesis that the transitional B cells in IgD a-macroself mice were eliminated coincident with their acquisition of CD23 expression, we measured their turnover. IgD a-macroself and non-Tg mice carrying IgH a alleles were continuously labeled with BrdU, and uptake in B cells at the different developmental stages was assessed. As predicted, the splenic CD23+CD93+ transitional B cells of Tg mice exhibited a more rapid rate of turnover than those of non-Tg littermates (Fig. 3 A, third panel from left). However, no significant differences were seen at the less mature splenic CD23+CD93+ T1 or BM CD23+CD93+ T2-like stages (Fig. 3 A, first two panels on left). Interestingly, MZ B cells in IgD a-macroself mice turned over more slowly than normal, suggesting either a slower rate of homeostatic proliferation or reduced replenishment of this compartment by recently formed B cells.

Levels of sIgM appeared to be actively downregulated on some of the CD23+CD93+ T1 cells and remained downregulated upon
CD23 expression (Fig. 2B, 2C, lower panels). These IgM<sup>lo</sup> cells expressed highly elevated levels of I-A (MHC class II [MHC II]), a prominent feature of activated B cells (Fig. 3B, 3C). When isolated from SPs of BM-reconstituted IgD<sup>a</sup>-macroself mice and cultured for ~17 h in medium alone, virtually all B cells expressed elevated levels of I-A and CD86 (Supplemental Fig. 4C), although still retaining a CD21<sup>_2</sup>CD23<sup>_2</sup>CD93<sup>_+</sup> immature phenotype (Supplemental Fig. 4B). Interestingly, if relieved from IgD ligation by removal of IgD<sup>a</sup>-macroself Ag, they also began to accumulate IgD on the cell surface, whereas MZ cells remained IgD-negative (Supplemental Fig. 4A, 4B). Thus, IgD cross-linking caused transitional B cells to downregulate IgM, but also to upregulate I-A, starting at the CD21<sup>_2</sup>CD23<sup>_2</sup>CD93<sup>_+</sup> T1 stage. Subsequently, most B cells were eliminated prior to transitioning to the conventionally defined T2 stage, as reflected by their increased BrdU incorporation, except for the few MZ B cells that did not express IgD. Notably, the levels of I-A on the CD23<sup>_+</sup>CD93<sup>_+</sup> transitional B cells from IgD<sup>a</sup>-macroself mice were significantly higher than

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Developmental block of B cell maturation at CD23<sup>_+</sup>CD93<sup>_+</sup> transitional stage in IgD<sup>a</sup>-macroself mice. Lethally irradiated IgD<sup>a</sup>-macroself and non-Tg littersmates were reconstituted with syngeneic IgH<sup>a</sup> BM cells. Two months later, numbers and phenotypes were analyzed for CD23<sup>_+</sup>CD93<sup>_+</sup> T2-like cells in BM (A), splenic CD21<sup>_2</sup>CD23<sup>_2</sup>CD93<sup>_+</sup> T1 cells (B), and splenic CD23<sup>_+</sup>CD93<sup>_+</sup> transitional cells (C). Also shown are their surface IgM expression profiles (lower panels). D, Cell counts for each transitional B cell subset were compared between the two groups of mice. Shown are mean + SD analyzed from six mice per group; p values by two-tailed Student t tests. E, Surface marker phenotypes of the splenic transitional B cells in IgD<sup>a</sup>-macroself mice were compared against their counterparts in the non-Tg littersmates. All analyses were performed after gating out CD4<sup>_+</sup>CD8<sup>_+</sup>F4/80<sup>_+</sup>Gr1<sup>_+</sup> cells in the dump channel. However, in the lower panels of A–C, the dump channel cells were used to establish sIgM background (gray histograms).
those on the IgM<sup>B</sup>-CD23<sup>C</sup>-CD93<sup>C</sup> anergic T3 population of non-Tg mice as defined by Merrell et al. (71) (Fig. 3B, middle right panel). By contrast, I-A expression was not significantly elevated on MZ B cells of Tg mice and only slightly elevated on B-1 cells, suggesting that the majority of these cells were not activated. Thus, the IgD<sup>B</sup> transitional population appeared to mainly die rapidly subsequent to abortive activation, whereas remaining MZ and B-1 populations appeared stable and more quiescent.

**Absence of conventional anergy phenotype**

To determine if B cells from IgD<sup>B</sup>-macroself mice exhibited features of anergy (reviewed in Ref. 72), they were stimulated in vitro and responses assessed. Upon BCR ligation, phosphotyrosine (p-Tyr) was similar in non-Tg and IgD<sup>B</sup>-macroself B cells, except for some changes in species around ∼95 to ∼100 kDa (Fig. 4A). One of these bands proved to be CD19, which was poorly phosphorylated at Tyr531 in IgD<sup>B</sup>-macroself B cells; however, total I-A expression was not significantly increased in total MHC II when compared with non-Tg CD93<sup>C</sup>-CD23<sup>C</sup> B-2 cells (dashed line). C. Mean fluorescence intensity (MFI) of I-A<sup>+</sup> staining for each B cell subset is shown, error bars representing SD of six IgD<sup>B</sup>-macroself and four non-Tg littermates. Values of p by two-tailed Student t tests are shown for each set of comparisons where there is statistical significance.

**Altered L chain repertoire, but lack of peripheral receptor editing**

To determine if receptor editing actively occurred in peripheral B cells of IgD<sup>B</sup>-macroself mice, we assessed L chain isotype usage and RAG expression. Increased L chain usage often correlates with receptor editing (76). In BM, λ versus κ usages were not significantly different between the IgM<sup>B</sup>-immature B cell populations of non-Tg and IgD<sup>B</sup>-macroself mice (Fig. 5A, 5E). λ L chain representation, however, was significantly increased in total splenic B cells of IgD<sup>B</sup>-macroself mice (Fig. 5B, 5E). Mature peritoneal B-1 and splenic MZ B cells also had comparably increased usage of λ-chain (Fig. 5C–E). If receptor editing was responsible for increased λ usage, we predicted that increases in RAG1 and RAG2 expression would be apparent in splenic B cells. Quantitative RT-PCR analyses were performed on splenic B cells purified by CD4/CD43-negative selection, revealing that their proliferative responses to all concentrations of anti-IgM tested were severely reduced (Fig. 4C, left panel). B cells in some models of anergy have also been reported to exhibit reduced responsiveness to LPS (75). However, this was not the case with B cells from IgD<sup>B</sup>-macroself mice either, as their responses to LPS were comparable to those of wild-type B cells (Fig. 4C, right panel). Although already expressing higher levels of I-A and CD86 than non-Tg B cells when cultured in medium alone, B cells isolated from IgD<sup>B</sup>-macroself mice failed to upregulate CD86 to the extent of non-Tg B cells upon IgM cross-linking, but did fully upregulate I-A (Supplemental Fig. 4C). Altogether, these observations suggest that B cells from IgD<sup>B</sup>-macroself mice displayed a phenotype resembling immature non-Tg B cells rather than tolerized anergic cells, with the exception that they expressed higher levels of the activation markers CD86 and I-A.

**FIGURE 3.** Increased turnover rate and activation profile of transitional B cells in IgD<sup>B</sup>-macroself mice. A, IgH<sup>B</sup>-congenic C57BL/6 mice carrying or lacking the IgD<sup>B</sup>-macroself transgene were labeled with BrdU over 7 d. Cells from BM and SPs were harvested 0, 1, 2, 4, or 7 d later for analysis of BrdU incorporation by B cells. Gates used to define each transitional B cell subset were the same as those depicted in Fig. 2A–C. *p < 0.05; **p < 0.005. B, Levels of surface MHC II expression were compared between transitional B cells that expressed high and low sIgM illustrated in Fig. 2B, 2C. Note the sIgM<sup>B</sup>-CD23<sup>C</sup>-CD93<sup>C</sup> T3 population in non-Tg mice (middle right panel, shaded histogram), unlike the sIgM<sup>B</sup>-CD23<sup>C</sup>-CD93<sup>C</sup> cells in IgD<sup>B</sup>-macroself mice (solid line), did not express significantly increased levels of MHC II when compared with non-Tg CD93<sup>C</sup>-CD23<sup>C</sup> B-2 cells (dashed line). C. Mean fluorescence intensity (MFI) of I-A<sup>+</sup> staining for each B cell subset is shown, error bars representing SD of six IgD<sup>B</sup>-macroself and four non-Tg littermates. Values of p by two-tailed Student t tests are shown for each set of comparisons where there is statistical significance.
IgDa-macroself B cells had levels of RAG message, 0.4% of wild-type BM B cells (Fig. 5F). Although this level was slightly higher than that found in non-Tg B cells, it likely reflected a higher proportion of immature cells in the population because this value was less than that found in non-Tg BAFF−/− splenic B cells. Thus, induction of RAG-mediated L chain editing in SPs of IgDa-macroself mice was unlikely to be responsible for the altered λ/κ ratios observed.

Increased numbers of splenic IgM+ Ab-secreting cells

Considering that IgDa-macroself mice had a severely reduced MZ B cell compartment and virtually nonexistent B-2 cells, we next determined whether Ab-secreting cells (ASCs, defined as possessing high levels of intracellular Ig) existed at steady state. Surprisingly, more intracellular IgM+ (cytoplasmic IgM [cIgM]+) ASCs were found in SPs of IgDa-macroself mice than in non-Tg littermates. These cells expressed high levels of surface IgM (in addition to their high cIgM levels), high levels of CD138, intermediate levels of CD19, low levels of B220, and, interestingly, extremely high levels of CD93 (Supplemental Fig. 5B). This last observation is consistent with a recent report indicating that CD93 is functionally important for plasma cell maintenance (77). The frequency of IgM+ ASCs increased with age, whereas numbers of IgG+ ASCs were low but not reliably different between non-Tg and IgDa-macroself mice at all ages tested (not shown). The IgM bias may reflect the effector properties of MZ and B-1 populations (see Discussion).

Altered serum Ab levels and humoral immunity

To assess the in vivo functionality of B cells in IgDa-macroself mice, we first measured their serum Ig levels at 2 mo of age. IgM levels were significantly elevated in IgDa-macroself mice (Fig. 6A), mirroring the observed increases of splenic cIgM+ ASCs in these mice. However, the levels of IgG1 and IgG2a were reduced, and IgG2b showed a slight (although statistically nonsignificant) trend toward reduction. The levels of IgG3 and IgA were similar between the two groups of mice, but IgE was not reliably detectable in either group.

Next, we compared IgDa-macroself to non-Tg littermates for Ab responses to the T cell-independent type 2 (TI-2) Ag TNP-Ficoll. Measurement of anti-TNP titers of the two isotypes mainly
FIGURE 5. Altered ratios of κ/λ L chain representation by B cells in IgD<sup>a</sup>-macroself mice. Intracellular stains of κ and λ Ig L chains are shown for BM intracellular IgM<sup>k</sup> immature B cells (A); total splenic (SP) lymphocytes negative for CD4, CD8, Gr1, and F4/80 markers (B); peritoneal CD19<sup>+</sup>CD43<sup>+</sup> B-1 cells (C); and MZ B cells (D). E, κ/λ Ratios were calculated for each B cell subset shown in A–C (mean ± SD; n = 4 per group; p values by two-tailed Student t tests). F, Quantitative RT-PCR analyses of RAG1 and 2 expression in splenic B cells isolated from non-Tg, IgD<sup>a</sup>-macroself, and BAFF<sup>−/−</sup> mice (triplicate measurements from B cell pools of SPs from the following number of mice/group; n = 6, 6, 4, respectively). Signals were compared with those obtained from BM B cells isolated from non-Tg mice, which were set arbitrarily to 1000.

To assess TD responses, mice were primed with TNP-Hy in alum and boosted 44 d later with TNP-Hy in buffered saline. Remarkably, the primary responses of IgD<sup>a</sup>-macroself mice were also higher for IgM but subnormal for IgG1, IgG2a, and IgG2b (Fig. 6C), resembling their resting serum Ig levels. After secondary challenge, the IgM response remained higher in IgD<sup>a</sup>-macroself mice, whereas IgG1, IgG2b, and IgA responses were all significantly lower than those of non-Tg mice. Secondary IgG2a and IgE responses were comparable in the two groups. Regardless of isotype, IgD<sup>a</sup>-macroself mice were clearly capable of mounting a memory response to the secondary challenge that was significantly more robust than their primary response. These findings are consistent with our preliminary studies in which IgD<sup>a</sup>-macroself mice showed no ill effects to a primary challenge with a sublethal dose of influenza or a secondary challenge with a lethal dose of the same virus (K. Walsh, unpublished observations). Strikingly, a slight and nonstatistically significant reduction of peritoneal B-1 cells (Fig. 7B). Surface IgD was also downregulated in all compartments tested (Fig. 7C), and most B cells in the SP were CD93<sup>+</sup> (Fig. 7E). These results thus argue that tolerance mediated by the IgD superantigen can occur in fully mature follicular B cells.

Reduced competitiveness of IgH<sup>b</sup> B cells among innocuous IgH<sup>a</sup> cells

As anergic B cells have poor survival in the presence of non-energetic B cells (78, 79), we assessed the consequences of B cell competition in the IgD<sup>a</sup>-macroself model by characterizing IgH<sup>ab</sup> mice, in which only half of the B cells are predicted to express IgD<sup>a</sup>. To monitor B cells independently expressing each IgH allele, we measured the frequencies of IgM cells carrying the a and b allotypes (Fig. 6B). These experiments illustrate that despite skewed usages of L chains and H chain isotypes and reduced primary response among certain isotypes to TD Ag, IgD<sup>a</sup>-macroself mice appeared capable of mounting normal (if not exaggerated) TI-2 responses and relatively unimpaired memory responses to TD Ags.

Induction of tolerance in mature B cells by IgD superantigen

Because B cell tolerance to IgD<sup>a</sup>-macroself Ag could simply involve developmental arrest of immature B cells, we next determined whether fully mature cells could also be tolerized by the macroself Ag. Isolated SP cells from IgH<sup>b</sup> Ly5<sup>a</sup> IgD<sup>a</sup>-macroself mice were introduced into nonirradiated IgH<sup>a</sup> Ly5<sup>b</sup> mice possessing normal B cell compartments of IgH<sup>a</sup> allotype (Fig. 7A). Phenotypes of recipient B cells were assessed 7 d later. The lymphoid organs of these mice recapitulated the phenotype of IgH<sup>a</sup> IgD<sup>a</sup>-macroself mice, including severe reduction of follicular type B cells in all tissues tested (Fig. 7B, Supplemental Table II), ~80% reduction of MZ B cells in the SP (Fig. 7D), but only a slight and nonstatistically significant reduction of peritoneal B-1 cells (Fig. 7B). Surface IgD was also downregulated in all compartments tested (Fig. 7C), and most B cells in the SP were CD93<sup>+</sup> (Fig. 7E). These results thus argue that tolerance mediated by the IgD superantigen can occur in fully mature follicular B cells.

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To investigate whether deletion in the IgD α-macroself model involved a Bcl-2–inhibitable pathway, we transferred BM from IgH α-macroself mice to lethally irradiated wild-type or IgD α-macroself mice and investigated their BM overexpressing Bcl-2, which had higher numbers of IgM + ASCs in the SP, enforced Bcl-2 expression did not further increase accumulation of these cells. Interestingly, B cells in IgD α-macroself mice retained a slightly higher λ/κ ratio than those in non-Tg mice, but this trend started in the immature stage of the BM.

Accumulation of MZ B cells and plasma cells induced by BAFF overexpression

We generated double Tg mice expressing both IgD α-macroself and BAFF transgenes to assess the effects of BAFF overexpression on B cell tolerance in the IgD α-macroself model. BAFF is an important homeostatic cytokine for follicular, MZ, and plasma B cells that is known to regulate the B cell competition-dependent survival disadvantage of anergic self-reactive B cells (78, 79). However, its overexpression in the IgD α-macroself mice failed to rescue B-2 cells (Fig. 10A). The LNs of IgD α-macroself/BAFF double Tgs were nearly devoid of CD23 +CD93 - B-2 cells. However, with BAFF overexpression, the numbers of MZ B cells in IgD α-macroself mice were increased by nearly 100% (Fig. 10C), which represented ~50% of what was found in wild-type mice and ~<10% of BAFF single Tgs. In both the SP and LNs, excess BAFF promoted increased numbers of IgM + plasma cells (Fig. 10B, 10C), but not IgG + ASCs or memory cells (Supplemental Fig. 6A). Surprisingly, the numbers of CD23 + and CD23 - transitional B cells were not significantly different among IgD α-macroself mice, regardless of BAFF expression, possibly indicating that they were eliminated prior to acquisition of responsiveness to BAFF. Analysis of BAFF-R (TNFRSF13C) expression in IgD α-macroself mice revealed that MZ and both CD23 + and CD23 - populations of transitional B cells expressed lower levels than their non-Tg counterparts (Supplemental Fig. 6B). These results indicate that, unlike models of anergy in which excess BAFF could promote survival of autoreactive B cells, BAFF overexpression in the context of IgD α-macroself Ag merely supports elevated steady-state levels of MZ and IgM + plasma B cells.
Discussion

IgD<sup>+</sup>-macroself mice were generated to determine if and how polyclonal B cells can undergo tolerance at a late, post-BM developmental stage. Because IgD itself is a very useful marker for B cell maturity, the effect of IgD<sup>+</sup>-macroself Ag should by definition be restricted to a B cell that is developmentally advanced. IgD is absent from pre-B cells and is poorly expressed by fraction E immature B cells in the BM and splenic T1 cells (30, 31, 81). This delayed expression of IgD relative to IgM in turn allowed us to use IgD superantigen to further delineate B cell developmental stages and to test the prediction that self-Ags encountered at a post-BM developmental stage promote deletion or anergy, but not editing.

Studies involving treatment of animals with Abs to Ig constant regions have been instrumental in elucidating precursor/product relationships among naive, immature, and memory B lymphocyte subsets. In the developing chicken, anti-IgM H chain (m-chain) suppression revealed that expression of mIgM preceded in ontogeny the expression of other isotypes because both IgM and IgG isotypes were suppressed (82, 83). Similar studies were carried out using anti-IgM treatment in experimental animals (39, 91–104). These studies differed in many details, but suggested that anti-IgM treatment could only partly modulate Ab responses, depending upon the context of treatment, the availability of competing, Ab nonreactive B cells, and the type of immunogenic challenge. Moreover, these studies were performed before good markers of B cell subsets were available and before the process of receptor editing was well defined.

Data obtained in the current study support previous tolerance studies using conventional Ig Tg mice, suggesting that IgD can transmit tolerogenic signals (33) and that IgD<sup>+</sup> cells are largely unable to undergo receptor editing (46, 47, 105). Infusion of superantigen-carrying cells into intact mice led to deletion of follicular B cells and most MZ B cells, whereas B-1 cell numbers were only slightly affected, suggesting that cells carrying a high level of IgD were eliminated by apoptosis. Remaining splenic B cells had an immature phenotype, including CD93 expression, or were MZ B cells lacking IgD. These phenotypes were similar to those of radiation BM chimeras in which IgD<sup>+</sup>-superantigen-carrying mice were reconstituted with wild-type IgH<sup>+</sup> BM, except that B-1 cells were less numerous in BM.
chimeras. Thus, IgD superantigen can delete preformed follicular and a major subset of MZ B cells, but likely also eliminates their precursors.

Several lines of evidence from the current study indicate that B cells destined for the follicular B cell lineage undergo a developmental block and enhanced turnover at the T1-to-T2 transitional stage, where IgD is highly upregulated (30, 31). In IgDα-macroself mice, T1 cells are largely spared from deletion, probably because of a lack of sufficient IgD, whereas CD23+CD93+ transitional cells are reduced in number, increased in turnover rate, and lacked markers associated with phenotype of T2 cells. T2 cells in the SP have been defined as IgMhiCD93+CD23+ (31). In the BM of wild-type mice, CD93+CD23+ T2-like cells are also present, although they appear to be at a slightly earlier transitional stage than splenic T2 cells in that they express significantly lower levels of markers upregulated upon T1-T2 transition, including CD62L, IgD, and CD21 (30, 69). In IgDα-macroself mice, the remaining CD23+CD93+ cells present in the SP have abnormally low levels of CD21 and CD62L, thus exhibiting a phenotype similar to wild-type BM T2-like cells. Splenic T2 cells have been suggested to include a fraction that is proliferative and responsive in particular to BAFF (30, 69, 106, 107). But in IgDα-macroself mice carrying a second transgene that overexpresses BAFF, CD93+CD23+ B cells were not elevated in number. Thus, our results suggest that the T2 population in SP consists of at least two differentiative states: a less mature population reminiscent of BM T2-like cells and a developmentally more mature population that includes a BAFF-responsive subset, which is missing from IgDα-macroself mice.

We also observed B cell populations in IgDα-macroself mice that were reminiscent of T3 and T39 transitional stages (31, 71, 108), based on the following phenotypes: T3-like cells were CD93+CD23+IgMlo (but cIgM +); T39-like were CD93+CD23−sIgMlo (but cIgM +). As originally described, T3 cells (IgMloIgD+CD23+CD93+) were considered a late transitional stage derived from T2 cells (31), but have recently been viewed as anergic B cells (71), possibly similar to those generated in soluble hen egg lysozyme (HEL)/anti-HEL Tg mice (45). T3 cells differ from T2 cells in their distinctly lower sIgM levels, which presumably result from interaction with autoantigens. In IgDα-macroself mice, we found that IgM2/lo T3-like cells had intracellular IgM levels similar to splenic CD93+CD23+ cells in these mice, indicating that they internalized this receptor (not shown), but their levels of surface MHC II and turnover rates were higher than T3 cells from non-Tg controls. One caveat in distinguishing T39-like cells from T1 cells in IgDα-macroself mice is that in these mice, we failed to see sIgD on any cells, and we were unable to reliably detect intracellular IgD. Moreover, all splenic CD93+ transitional B cells in IgDα-macroself mice, regardless of CD23 expression, had identical BrdU-labeling kinetics. We suggest that the cells are T3-like and T39-like because of their IgM downregulation. In any case, these results suggest that in IgDα-macroself mice, CD93+CD23+ (presumably T2- and T3-like) cells undergo BCR activation and as
a population turn over about twice as rapidly as normal (t1/2 of 2 d versus 4 d). To our knowledge, this is the clearest evidence that transitional B cells can undergo accelerated deletion upon autoantigen encounter in vivo.

Prolonged exposure to a weakly self-reactive Ag has been shown to result in B cell anergy (109). Although details differ among the models, a common hallmark of anergic B cells is reduced Ca2+ signaling in response to IgM cross-linking (72). The T3 B cell population present in wild-type mice has been suggested to be a collection of anergic B cells also based upon this fact (71). However, in B cells isolated from IgD+macroself mice, IgM stimulation triggered robust Ca2+ signals, consistent with our interpretation that the transitional B cells in IgD+macroself mice are distinct from wild-type (anergic) T3 cells. Although upon IgM ligation, B cells from Tg mice do exhibit reduced tyrosine phosphorylation in some proteins, including CD19, this appears to be a result of their immaturity, rather than their state of unresponsiveness, as their p-Tyr profile is remarkably similar to that of B cells from BAFF-deficient mice, which are thought to be of mostly T1 type (73, 74, 110). Likewise, transitional B cells from wild-type mice have been shown to exhibit reduced proliferative responsiveness and CD86 upregulation to IgM cross-linking (106), explaining the reduced responsiveness of B cells from IgD+macroself mice to such stimuli.

Splenic B cells in IgD+macroself mice expressed extremely low levels of RAG1 and 2 mRNA. If editing occurs in this population, it is likely to be at a low level and restricted to transitional B cells. However, we cannot exclude that T2-like cells in the BM can undergo receptor editing. Thus, our data are compatible with the conclusions of Sandel et al. (48, 111), who suggested that transitional B cells provided with a BM microenvironment are competent to undergo receptor editing. Although this could in theory explain the higher frequency of λ5 B cells in IgD+macroself mice, as explained below, we rather think that this is a secondary result of the skewing in B cell subset makeup in these mice.

T3' cells are of interest because they have been suggested to be splenic B cells undergoing receptor editing. T3' cells have been defined based on experiments with 3H9H-56R anti-DNA H chain

**FIGURE 9.** Rescue of putative preplasma cells, but not total B cell loss, in IgD+macroself mice by enforced Bcl-2 expression. IgH+/Bcl2 Tg BM was used to reconstitute lethally irradiated non-Tg or IgD+macroself recipients, which were analyzed ~8 wk later. A, Shown are representative flow cytometry plots for total CD19+ B220+ cells in non-Tg and IgD+macroself mice. B, Numbers of transitional and mature B cell subsets in non-Tg and IgD+macroself mice were compared. C, Shown are representative flow plots illustrating increased representation of intracellular IgM+ CD138+ preplasma cells in IgD+macroself recipients. Also evaluated were the numbers of IgM+ ASCs (D), IgG+CD19+ putative memory B cells (D), and λ versus κ chain usages (E) in both sets of recipients. All graphs shown represent mean + SD; p values given where there is statistical significance by two-tailed Student t tests; n = 4 mice per group.
Tg mice, which have expanded numbers of a small splenic IgM<sup>+</sup>
IgD<sup>+</sup>CD93<sup>+</sup>CD23<sup>−</sup> subpopulation expressing RAG1 and 2 and
carrying dsDNA breaks at the J<sub>k</sub> locus (108) (this study also
showed some RAG expression and double-stranded breaks in T3
cells). Anergic and late immature B cells in the HEL/anti-HEL
model have also been suggested to undergo editing in vivo, but
only at the IgM<sup>+</sup>IgD<sup>−</sup> (presumably T1) stage (112). Although
splenic T3 cells, if they indeed exist in IgD<sup>+</sup>macroself mice,
should fully engage their IgD receptors, there is no evidence that
their numbers are expanded. Nor is there appreciable receptor
editing activity associated with the splenic B cells in the IgD<sup>+</sup>
macroself model, because RAG levels were at background levels
despite the presence in these samples of a relatively high pro-
portion of transitional cells. Moreover, Bcl2 Tg B cells, which are
known to be resistant to peripheral tolerance and had an enlarged
CD19<sup>+</sup>CD23<sup>−</sup>CD93<sup>+</sup> compartment, failed to show a λ<sub/>κ skewing
on the IgD<sup>+</sup>macroself background when compared with the newly
formed B cells in the BM.

If editing in semimature transitional B cells is not occurring to
a substantial extent in the SPs of IgD<sup>+</sup>macroself mice, then why do
they have elevated proportions of λ-L chain-bearing cells and
Abs? As explained by Lindsley et al. (69), a relatively high pro-
portion of newly formed and transitional B cells in normal mice
express λ (∼12–15%), and they tend to be counterselected by an
unknown process during developmental progression to the follic-
lar compartment, which has only ∼5% λ. Similar observations
have been made by Dingjan et al. (113). Consistent with this,
splenic B cells in BAFF<sup>+/−</sup> mice have elevated frequencies of λ

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**FIGURE 10.** Rescue of MZ and IgM<sup>+</sup> Ab secreting
B cells in IgD<sup>+</sup>-macroself mice by overexpression of
BAFF. A, IgD<sup>+</sup>macroself mice on IgH<sup>+</sup> congenic
C57BL/6 background were bred to overexpress BAFF
and analyzed for presence of CD23<sup>+</sup>CD93<sup>−</sup> B-2 cells. B, Shown are plots summarizing the effects of excess
BAFF on average numbers of B cells at different stages of development (+ SD) analyzed from four
mice per group; p values by two-tailed Student t tests.

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cells (data not shown). Moreover, B-1 and MZ B cells also have higher frequencies of \( \lambda \) than do follicular B cells (114, 115). Without the contribution of follicular cells, which are counter-selected in IgD\(^{-}\)-macroself mice, the remaining cells are thus enriched in \( \lambda \) by selection rather than editing.

The MZ B cell population that develops in IgD\(^{-}\)-macroself mice is of interest for several reasons. Normal MZ B cells in rodents may be heterogeneous, including a higher frequency of cells with a fetal-type repertoire (26, 115–117). They are believed to derive from a subset of CD23\(^{-}\)CD21\(^{hi}\) cells now termed MZ precursors (118). The fact that MZ B cell numbers are subnormal in IgD\(^{-}\)-macroself mice might indicate that the remaining cells represent a distinct subpopulation or lineage. Alternatively, the MZ B cells developing in IgD\(^{-}\)-macroself mice may be a transient population or rare variants that emerge and self-renew upon stringent selection. Unlike immature B cells present in IgD\(^{-}\)-macroself mice, upon in vitro culture in the absence of superantigen, MZ B cells from these mice do not upregulate IgD, indicating that the Tg environment selects for MZ B cells permanently lacking IgD. Consistent with this finding, MZ B cells in IgD\(^{-}\)-macroself mice turn over slowly and therefore are a stable population. Although these cells may represent a special subset, preliminary studies indicate that their H chain N-region length diversity is not appreciably different from that of wild-type MZ B cells (not shown). Positive selection by autoantigens has been suggested to play a role in the development of MZ B cells (26). However, in this study, the IgD\(^{-}\)-macroself autoantigen appears to solely play a role in negative selection, possibly because of the relatively high affinity of this ligand for the BCR. In any case, the fact that MZ B cells are formed at all suggests that those cells may have been derived from a precursor lacking IgD, possibly a T1 population or the early CD23\(^{-}\)CD93\(^{hi}\) T2-like stage.

By contrast, B-1 cells, which also have low but variable levels of IgD, are not reduced in number in nonirradiated IgD\(^{-}\)-macroself mice. Because B-1 cells are relatively resistant to apoptosis with the ability to self-renew, homeostatic expansion and accumulation of the rare IgD-negative B-1 cells are likely to be responsible for their normal numbers. Thus, their numbers remain low in irradiated IgD\(^{-}\)-macroself hosts reconstituted with adult BM cells, which are known to have a decreased propensity to generate B-1 cells. Rather than positive selection, our results suggest that IgD cross-linking eliminates B-1 cells that had the tendency to express high levels of IgD while sparing those that expressed lower or absent levels. Interestingly, although BAFF overexpression can expand B-1 cell number (66), this is not observed in IgD\(^{-}\)-macroself mice, which may also have elevated levels of BAFF due to reduced total number of B cells.

Consistent with the dearth of follicular B cells in IgD\(^{-}\)-macroself Ag Tg mice, their Ab responses to TD Ag given in alum were significantly reduced in both primary and secondary responses of most Ab classes, with the major exception of IgM, levels of which were elevated. It is not clear if these responses come from MZ, B-1, or transitional B cell subsets. Song and Cerny (119) assessed the ability of different B cell subsets to undergo TD Ab responses upon transfer into SCID mice along with carrier primed T cells. They found that MZ B cells are able to participate in TD Ab responses and to undergo a germinal center reaction, class switching, and somatic mutation (119). Under these conditions, immature cells could also contribute to germinal center formation. Their study also showed that MZ B cells were more effective than follicular B cells at IgM responses in the primary and secondary response. Phan et al. (120) concluded from experiments with anti-HEL Tg B cells that MZ cells were able to generate memory and class switched responses, but at a reduced efficiency compared with B-2 cells. The IgD\(^{\alpha}\)-macroself model may be useful in the study of Ab responses generated by nonfollicular B cells.

A most unexpected feature of the IgD\(^{\alpha}\)-macroself model was the presence of elevated numbers of IgM\(^{+}\) plasma cells. The simplest explanation for their abundance is that in the absence of follicular B cells, the MZ compartment undergoes more TD stimulation and attendant Ab formation, with the aforementioned skewing to the IgM class. Similarly, B-1 responses, which contribute greatly to serum IgM levels, may be enhanced in these mice and preferentially yield IgM responses. The increased numbers of IgM\(^{+}\) plasma cells may also be stabilized by a presumed increase in BAFF levels due to a total reduction in B cell numbers in these mice. Consistent with this idea, overexpression of BAFF gives rise to a further increase in their numbers. Serum IgM, but not IgG, from Tg mice also exhibited increased reactivity to dsDNA (data not shown). It remains to be determined whether this reflects abnormal selection events, as this could be a very useful model to study systemic autoimmunity.

Acknowledgments

We thank Martin L. Scott, Biogen/Idec, for providing BAFF\(^{-}\) mice and John Cambier for anti-CD19 reagent.

Disclosures

The authors have no financial conflicts of interest.

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