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Natural IgM Prevents Autoimmunity by Enforcing B Cell Central Tolerance Induction

Trang T. T. Nguyen, Rebecca A. Elsner, and Nicole Baumgarth

It is unclear why selective deficiency in secreted (s)IgM causes Ab-mediated autoimmunity. We demonstrate that slgM is required for normal B cell development and selection. The CD5+ B cells that were previously shown to accumulate in body cavities of slgM−/− mice are not B-1a cells, but CD19int, CD43−, short-lived, BCR signaling-unresponsive anergic B-2 cells. Body cavity B-1 cells were >10-fold reduced, including VH11+ and phosphotidylcholine-specific B-1a cells, whereas splenic B-1 cells were unaffected and marginal zone B cells increased. Follicular B cells had higher turnover rates, survived poorly after adoptive transfer, and were unresponsive to BCR stimulation in vitro. slgM bound to B cell precursors and provided a positive signal to overcome a block at the pro/pre-B stage and during IgVH repertoire selection. Polyclonal IgM rescued B cell development and returned autoantibody levels to near normal. Thus, natural IgM deficiency causes primary autoimmune disease by altering B cell development, selection, and central tolerance induction. The Journal of Immunology, 2015, 194: 1489–1502.

IgM is produced by all jawed vertebrates. It is the first isotype produced in ontogeny and the first Ig produced in response to an insult. Its pentameric structure is also unique among the other Ig isotypes, indicating its unique contributions to immunity and the host’s interactions with its environment (1). Spontaneous “natural” IgM secretion occurs without external microbial stimulation (2, 3). Major sources of natural IgM in mice are B-1 cells situated in spleen and bone marrow, producing at least 80% of the circulating IgM (4, 5). Natural IgM-producing B-1 cells appear to be selected on self-antigens (6, 7) and exhibit dual reactivity to both self and common microbial Ags (1, 8, 9). This selection process might ensure the generation of evolutionary “useful” specificities (8). Indeed, natural Abs appear to bind particularly to “altered” self-antigens, such as Ags expressed on dead and dying cells, which is thought to allow the efficient removal of tissue debris and thereby the removal of potential autoantigens (1, 9–12).

Rapid T-independent IgM responses to systemic application of microbial components, such as LPS of Gram-negative bacteria, or polysaccharide Ags are induced by both B-1 (13, 14) and by marginal zone (MZ) B cells (15), which have a high propensity for rapid differentiation to IgM-secreting cells. Finally, most conventional B cell responses result in the initial production of IgM by early activated B cells, prior to class-switch recombination to IgG, IgA, or IgE (16). Early low-affinity IgM may facilitate Ag deposition in the developing germinal centers (17).

Selective IgM deficiency is a little studied, relatively rare primary immunodeficiency of humans, reported to occur at a prevalence rate of 0.03% (18). Selective IgM deficiency is often associated with recurrent infections (18), consistent with findings in slgM-deficient mice (μs−/−), which showed increased morbidity and mortality from various bacterial and viral infections (19–22). The data highlight the importance of both natural and Ag-induced IgM in immune protection from pathogen encounter.

Mechanistically less well understood is the observed development of autoantibodies against dsDNA (12, 23) and the increased risk of autoimmune diseases such as arthritis and systemic lupus erythematosus in a subset of humans with selective IgM deficiency and in μs−/− mice (11, 12, 18). It has been argued that this is attributable to a break of peripheral B cell tolerance due to ineffective removal of cell debris in the absence of natural Abs (1, 11, 12). This is consistent with the repertoire of self-specificities that preferentially bind to dead and dying self and other components of the altered self (24, 25). Yet, no studies to date have demonstrated such lack of self-antigen removal. Moreover, various BCR transgenic and knock-in mice have been generated during the last two decades, which express highly restricted oligoclonal or even monoclonal B cells, and often lack B-1 cells and/or B-1 cell–derived IgM (26–29). These mice do not appear to suffer from autoimmune disease, indicating that autoantibody production in IgM deficiency may have other underlying causes.

Positive and negative selection events during B cell development are critical for the elimination of self-reactive B cells. The fate of the developing B cells is strongly dependent on the strength of BCR interaction with self-antigens (30, 31). Autoreactive immature B cells may either 1) undergo L chain rearrangement, that is, change their Ag specificity, 2) become anergic, that is, unresponsive, and express the BCR inhibitory surface molecule CD5, or 3) die via apoptosis (31, 32). Overall strengths of the selecting signals appear to determine also B cell subset selection. Relatively strong signals seem to favor development of B-1 and follicular (FO) B cells, whereas weaker signals drive MZ B cell development (33, 34).

Lack of slgM may affect B cell development, possibly via expression of the recently identified FcyR (35–38). However, re-
ported alterations appeared not only subtle but also difficult to reconcile: two independently generated strains of μ−/− mice were reported to have increased numbers of MZ B and B-1 cells, but a normal FO B cell compartment (39, 40). The increased MZ B cell development in μ−/− mice may indicate that slgM may affect B cell subset selection during development, and that in its absence overall strengths of the selecting signals is reduced, resulting in increases in the MZ B cell compartment. However, this is not consistent with the reported expansion of their B-1 cell compartment (40), which one would expect to be reduced and with a lack of effects on FO B cells.

To determine the causes of autoantibody production in selective IgM deficiency we re-examined the previously generated μ−/− mice (40). Our findings demonstrate that the accumulation of autoantibodies is due to nonredundant effects of slgM on B cell development and repertoire selection. Escape from central tolerance induction in the absence of slgM explains the accumulation of anergic CD5+CD43− B-2 cells and the generation of autoantibodies and identifies slgM as a nonredundant regulator of B cell selection.

Materials and Methods

Mice

Eight- to 12-wk-old female and male mice, C57BL/6J (wild-type [WT]; CD45.2, Ig-h), B6.SJL-Ptpe−/−Pepc−/−BoyJ (CD45.1), B6.129S7-Rag2−/−/−Ighb−/−, and B6.Cg-Igha Thy1a Gpi1a−/− mice (Igh-h) were obtained from The Jackson Laboratory. Breeding pairs of B6.129S-sIgM−/− (μ−/−), CD45.2, Ig-h-a mice were a gift from Dr. Frances Lund (University of Alabama at Birmingham). Heterozygous μ−/− mice were crossed with C57BL/6J mice. Heterozygous CD45.1 × CD45.2 F1 mice were generated by crossing B6.SJL-Ptpe−/−Pepc−/−BoyJ with C57BL/6J mice. Ig-h-a × Ig-h-b mice were generated by intercrossing B6.Cg-Igha Thy1a Gpi1a−/− (Igh-h-a) with C57BL/6J mice. All mice were kept in specific pathogen-free housing, placed in filtered-air ventilated racks, and screened for the absence of 17 common mouse pathogens (list available upon request). Mice were euthanized by overexposure to carbon dioxide.

To generate mixed bone marrow irradiation chimeras, single-cell suspensions of bone marrow from the femur and tibia of WT (C57BL/6J) and μ−/− mice were injected i.v. at a 1:1 ratio into CD45.1 × CD45.2 F1 mice lethally irradiated by exposure to a gamma irradiation source (800 rad). Similarly, WT and FcγR−/− (CD45.2; provided by Dr. Hiromi Kubagawa, University of Alabama at Birmingham) mixed bone marrow chimeras were generated by injecting WT or FcγR−/− bone marrow into lethally irradiated CD45.1 mice. Chimeras were allowed to reconstitute for at least 7 wk before use in experiments. All procedures and experiments involving animals were approved by the Animal Use and Care Committee of the University of California, Davis.

B cell reconstitution after sublethal irradiation

Groups of four WT and μ−/− mice were lethally irradiated by whole-body exposure to a gamma irradiation source (350 rad). The mice were allowed to reconstitute peripheral B cell compartments for 12 d. Frequencies of transitional B cells (CD93+) in bone marrow and spleen were determined by flow cytometry.

Transfer of slgM

Groups of three or four μ−/− mice were injected i.p. with either 50 μl WT serum, containing ~200 μg IgM as assessed by ELISA, μ−/− serum, or 200 μg monoclonal IgM (Sigma-Aldrich, clone MOPC-104E, specific for α1,1,3-glucose) per mouse three times per week for 3 wk to reconstitute slgM. Control mice received PBS only.

Flow cytometry

Spleen and lymph node cells were obtained by grinding the organ between the frosted ends of glass slides. Bone marrow was obtained by flushing tibia and tibia with medium using a 23-gauge needle. Single-cell suspensions were stained as previously described (41). Briefly, after Fc receptor blocking (5 μg/ml anti-CD16/32) for 20 min on ice, cells were stained with the following fluorochrome conjugates: streptavidin (SA)–Qdot 605, SA-allophycocyanin, CD93-PE, CD80-PE, CD86-allylpllophycocyanin, CD45.1– (PerCP, C5.5-PE), CD45R−Cy7-allylophycocyanin, CD3e–Cy55-allylophycocyanin, BdvU–FITC (all BD Pharmingen), CD45.2–(Cy7-allylophycocyanin, Cy7-PE) (BioLegend), CD5–(biotin, Cy5-PE), CD45R–FITC, Cy7-allylophycocyanin, FITC–labeled, phophatidylylcholine (PC)-containing liposomes–FITC, CD21–(FITC, biotin), CD23–(FITC, allophycocyanin), CD43–(PE, Cy7-allylophycocyanin), CD19–(Cy5-PE, allophycocyanin), CD21–Cy5.5-PE, CD24–Cy5.5-PE, BP–1–allophycocyanin, IgD–Cy7-PE, and IgM–Cy7-allylophycocyanin (all in-house generated). Dead cells were excluded by Live/Dead Pacific Blue staining (Invitrogen). Staining for FcRγI was done according to the manufacturer’s protocols using a BD Pharmingen BrdU flow kit. To accurately set gates to identify CD5− and CD43− expressing cells, we used “fluorescence minus one” controls in which cells were stained with all reagents except anti-CD5 and anti-CD43, respectively.

In vitro slgM binding, slgM (SouthernBiotech, clone 11E10, specific for FcγR1 and 7-aminooctanoinocin D (7-AAA) (BD Pharmingen) overnight at 4°C. FACS analysis was done using a 15-parameter FACSAria (41) or an LSRFortessa (BD Biosciences) equipped with four lasers and optics for 22-parameter analysis. Analysis was done using FlowJo (gift of Adam Teister).

Magnetic B cell enrichment

Splenic B cells were enriched using a mixture of depleting Abs (CD90.2– biotin, Dx5-5biotin, Gr1–biotin) and anti-biotin microbeads (Miltenyi Biotech) after Fc blocking. CD23− (non-Fo) B cells in the peritoneal cavity were enriched by adding anti-CD23-biotin to the above Ab mixture. Nylon-filtered splenocytes were separated using autoMACS (Miltenyi Biotech). Purities of enriched B cells were >90% as determined by subse-quent FACS analysis.

In vitro B cell proliferation assay

FACS-purified MZ, FO, and anergic CD21+CD23− B cells from WT and μ−/− mice were labeled with 0.5 μM CFSE in PBS at a concentration of 107 cells/ml for 20 min at 37°C, washed twice with PBS, and cultured at 2.5 × 104 cells/well in the presence or absence of 20 μg/ml anti-IgM medium (RPMI 1640, 292 μg/ml l-glutamine, 100 μg/ml penicillin/streptomycin, 10% heat-inactivated FCS, 0.03 M 2-ME) in 96-well round-bottom plates for 3 d at 37°C in 5% CO2. FACS analysis was done to identify the number of dividing cells.

Testing for serum anti-nuclear Ab

The method of anti-nuclear Ab (ANA) staining was adapted from previous publications (42). 3T3 cells (A31, provided by Dr. Peter Barry, University of California, Davis) were grown on glass slides overnight and fixed with acetone for 10 min. The slides were blocked with Fc block in 10% FCS/PBS buffer for 1 h, washed twice with PBS and once with washing buffers (PBS plus 0.1% BSA, plus 0.1% normal calf serum), and then incubated with mouse serum in PBS for 2 h (1:100 dilution for 2.5-mo-old mice and 1:1000 dilution for 8-mo-old mice). Staining was revealed using goat anti-mouse IgG-biotin and SA–Alexa Fluor 594, each incubated for 1 h at room temperature. The 3T3-stained slides were washed with PBS for 5 min at least three times and mounted with Fluoromount-G (SouthernBiotech). Images were collected with an Olympus BX61 microscope with an Olympus DP72 color video camera (Olympus) and were processed by using MetaMorph (Molecular Devices) and ImageJ (National Institutes of Health) software. Differential interference contrast images were taken for outlining the cells. Compared images were collected on the same day and analyzed in the same way. The scale bars were from two images (original magnification ×20 and ×40) and applied to others.

ELISA

DNA-specific IgG was measured by ELISAs. Ninety-six-well plates (Maxisorb, Thermo Fisher Scientific) were coated with 10 μg/ml dsDNA from calf thymus or 10 μg/ml ssDNA (degraded dsDNA after heating at 94°C for 10 min in 1 N NaOH) in PBS overnight. After blocking with PBS plus 1% heat-inactivated calf serum/0.1% milk powder/0.05% Tween 20 for 1 h, 2-fold serially diluted serum in PBS was added for 2 h. Plates were washed and Ab binding was revealed using biotin-conjugated anti-IgG
DNA was isolated from sorted cells by a DNeasy kit (Qiagen) and stored in DNA storage buffer until processed. VH11 mRNA expression, total RNA from peritoneal cavity B cells was isolated with RNeasy (Qiagen) and stored in the RNA storage buffer (Ambion) at −80°C until processed. cDNA was prepared by using random hexamers (Promega) with SuperScript II reverse transcriptase (Invitrogen). Primers and probe for IG VH11 were designed using IDT software: forward primer, 5′-GGGAGACCTGGGCACGGA-3′; reverse primer, 5′-GTA-CCAGGTGCTCCCTGTCATT-3′. Relative expression was normalized to GAPDH (IDT).

Statistical analysis
Statistical analysis was done using a two-tailed Student t test. A p value <0.05 was considered statistically significant.

Results
An unusual population of CD5+CD21+CD23− B cells develops in μs−/−mice

Measurements of autoantibodies by ELISA in 10-wk-old μs−/− and age-matched WT controls confirmed earlier reports on the generation of high titers of IgG autoantibodies against dsDNA and ssDNA in μs−/− mice (Fig. 1A, 1B) (12, 23). We also found increased anti-nuclear IgG Abs, which further increased with age (Fig. 1C, 1D). Thus, selective IgM deficiency causes the development of autoantibodies against multiple self-antigens, which increase with age. Comparison of total serum IgG levels in WT and μs−/− mice as assessed by ELISA revealed no significant differences between the mouse strains at 3 mo of age and only very modest increases at 8 mo (Supplemental Fig. 1A). Similarly, the frequencies of IgG-secreting cells in the spleen as measured by ELISPOT and the frequencies of spleen CD19+CD138+ plasma cells were not significantly different between these two mouse strains (Supplemental Fig. 1B, 1C). Thus, the difference in autoantibody production in μs−/− mice cannot be explained by differences in total IgG levels and instead suggest B cell repertoire differences.

Modest increases in MZ (39) and peritoneal cavity B-1 cells in μs−/− mice (40) were previously reported. We confirmed the increased frequencies of MZ B cells and a concomitant decrease in FO B cells in μs−/− mice (Fig. 2A, 2B), but we identified multiple additional defects. First, μs−/− mice had significantly reduced numbers of CD19+ B cells, especially in lymph nodes (Fig. 2A, 2B). Moreover, we noted an unusual population of CD21hiCD23− CD19+ B cells in spleens and lymph nodes of μs−/− mice that was not present in WT mice and blunted the differentiation of MZ and FO B cells somewhat (Fig. 2A). Further analysis identified them as CD5− CD21hiCD23− CD43− CD45R−CD93+ (Fig. 2C). Despite expression of CD5 on some of these cells, they were not classical B-1a cells, as they were mostly CD43+, and CD5 expression levels of those expressing CD5 were lower and their expression of CD45R was higher compared with those of B-1a cells (Fig. 2C). They were not transitional B cells either, as they lacked expression of CD93. Based on their expression of CD80 and CD86 they appeared to be Ag experienced. In WT mice, very few B cells fell into the CD21hiCD23− B cell gate. Those that did were either B-1 (CD5−CD21hiCD23− CD43+CD45Rhi) or transitional B cells (CD5−CD21hiCD23− CD43− CD45RhiCD93+) (Fig. 2C, 2D). FO cells also showed phenotypic alterations in μs−/− mice. They expressed reduced levels of IgD and some seemed to express very low levels of CD5 based on comparison with control stains that did not include CD5 (Fig. 2D and data not shown). These dramatic changes of the peripheral B cell compartments suggested that slgM is a nonredundant component required for normal B cell development and B cell subset selection and/or maintenance. We therefore explored the relationship between the effects of IgM on the B cell compartment and the regulation of autoantibody production.

B cell development from μs−/− bone marrow precursors is rescued in the presence of slgM

First, we determined whether the B cell developmental changes in μs−/− mice could be normalized by the administration of slgM. For that we injected polyclonal IgM purified from WT serum into μs−/− mice three times per week for 3 wk. Transfer of slgM resulted in significant increases in the frequencies of FO B cells and reduced frequencies of CD21hiCD23− B cells in spleen and lymph nodes compared with mice receiving serum from μs−/− mice, a decrease that reached statistical significance in the lymph nodes (Fig. 3A). Owing to the very short half-life of IgM (2 d) (44), the levels of circulating serum slgM could not be normalized with this approach (Fig. 3B), which may explain why a more complete rescue of B cells was not achieved.

Therefore, to test for the development of B cells from precursors of μs−/− mice in the presence of normal levels of serum IgM we generated mixed bone marrow chimeras, transferring WT (CD45.1) and μs−/− (CD45.2) bone marrow at a 1:1 ratio into lethally irradiated CD45.1 × CD45.2 F1 recipients. The CD45.1/CD45.2 double-positive radio-resistant recipient cells were easily distinguished from the single-positive WT and μs−/− donor B cells in the host (Fig. 3C, second panel). Recipients of WT or mixed μs−/− WT bone marrow had similar serum IgM levels, whereas recipients of μs−/− bone marrow lacked slgM (Fig. 3E). Precursors from μs−/− mice reconstituted roughly half of each B cell compartment in the mixed bone marrow chimeras (Fig. 3C, 3D). Frequencies and phenotypes of all major B cell subsets were normal (Fig. 3C, 3D, and not shown). Furthermore, CD21hiCD23− B cells did not develop in the mixed chimeras, but they did develop in controls that received μs−/− bone marrow only and thus lacked IgM (Fig. 3C, 3E). Importantly, ANA development was greatly reduced in mixed bone marrow chimeras, compared with mice receiving μs−/− bone marrow only. The latter generated measurable levels of ANA within 10 wk after adoptive transfer (Fig. 3F). Thus, precursors of μs−/− mice have no inherent developmental defect in B cell development when raised in the presence of slgM.

B-1 cell frequencies are strongly reduced in the peritoneal cavity of μs−/− mice but are maintained at normal numbers in the spleen

Increases in peritoneal cavity CD5−CD45R− B-1 cells have been reported for μs−/− mice (40). Although our results confirmed increases in frequencies of CD5− B cells (Fig. 4A), these cells were mostly CD43−CD45Rhi and CD19+; that is, they did not represent classical CD43+CD45RhiCD19+B-1a cells of WT mice (Fig. 4A, 4B). The difference in CD45 staining shown (Fig. 4A, 4B) is highly significant (mean fluorescence intensity of 2131 ± 556 on WT CD19+IgM+ cells versus 195 ± 34 for μs−/− cells, p < 0.005). Also, the difference for CD45R expression was greatly significant (1,758 ± 259 versus 3,727 ± 115, p < 0.0001), as was the reduction in expression of CD19 (12,720 ± 1,036 versus 8,920 ± 320, p < 0.005). Therefore, CD19+CD45R−CD43+ cells,
FIGURE 1. Strong production of anti-DNA and ANA serum autoantibodies in μs−/− mice. (A and B) Shown are relative units (A) anti-dsDNA IgG and (B) anti-ssDNA IgG in WT and μs−/− mice at indicated ages as measured by ELISA. Each symbol represents values for a single mouse; horizontal bars indicate mean for the group (n = 5–6/group). (C) Detection of ANA via fluorescent staining of mouse 3T3 cells with serum from young and aged WT and μs−/− mice. Representative images are shown. Left panels shows the overlay images with differential interference contrast outlining the cells and fluorescent red staining for IgG, and right panels shows images with fluorescent red staining for IgG only. Original magnification ×20 and ×40 as indicated. (D) Shown are frequencies of cells stained with diluted sera as indicated, calculated from counting total cells and stained nuclei from randomly chosen images (n = 20–100 cells/slide). Data are representative of three (A and B) or two (C and D) independent experiments. Groupwise comparisons were conducted using a Student t test: *p < 0.05, **p < 0.0005.
FIGURE 2. Presence of unusual CD5^+CD21^{hi}CD23^{-} B cell subsets in lymph tissues of $\mu$s^{--} mice. (A) Shown are 5% contour plots with outliers of a representative spleen sample ($n = 4$) from WT (left) and $\mu$s^{--} mice (right) analyzed by flow cytometry after exclusion of dead cells. Boxes and arrows indicate gating strategy. Identified subsets among CD19^+ B cells were CD21^{hi}CD23^{-} MZ B cells, CD21^{int}CD23^{+} FO B cells, CD21^{int}CD23^{-}, an unknown B cell subset, and CD21^{lo}CD23^{lo} transitional/B-1 cells. CD21^{hi}CD23^{hi} and CD21^{lo}CD23^{lo} subsets were gated on CD43 and CD5 to identify CD43^{hi}CD5^{lo} (B-1a), CD43^{hi}CD5^{hi}. (B) Bar graphs summarize the mean frequencies ± SD and total numbers of different B cell subsets in spleen and lymph nodes of WT and $\mu$s^{--} mice ($n = 4$/group). (C and D) Overlay histograms comparing CD21^{hi}CD23^{-} B cells from $\mu$s^{--} and WT mice identifies CD21^{hi}CD23^{-} B cells as a unique cell subset not present in WT mice, as cells with that phenotype of WT mice are a mix of B-1 and immature B cells. Data are representative of three independent experiments. * $p < 0.05$, **$p < 0.005$, ***$p < 0.0005$. 
that is, classical B-1a (CD5+) and B-1b (CD5−) cells, were greatly reduced in the peritoneal cavity of ms2/2 mice (Fig. 4C). Further confirmation of a reduction in B-1 cells came from the analysis of PtC-specific cells, a B-1a cell–restricted specificity (25, 45). PtC-binding CD5+ B cells made, 1.0% of CD19+ peritoneal cavity B cells in ms2/2 mice compared with nearly 10% in C57BL/6 mice and 15% in 129 WT controls (Fig. 4D and data not shown). Because PtC-binding B-1 cells in C57BL/6 and 129 mice preferentially use VH11 (46), we determined whether the lack of B-1 cells might be the outcome of altered B cell selection. Indeed, CD232 peritoneal cavity B cells of ms2/2 mice showed a nearly complete absence of VH11 mRNA compared with the high expression seen in WT mice (Fig. 4E). The strong reduction of B cells in the ms2/2 peritoneal cavity was not due to increases in B-1 cell death (Supplemental Fig. 2). It also did not appear to be due to an increase in B-1 cell migration from the cavities to the spleen, and subsequent differentiation to Ab secretion cells, as is often seen after activation of peritoneal cavity B-1 cells, because frequencies of ASC in the spleen were comparable between WT and ms2/2 mice (Supplemental Fig. 1). Thus, lack of B-1 cells in the peritoneal cavity may suggest differences in B cell selection. In contrast, B-1 cell numbers in the spleen and lymph nodes were unaffected (Fig. 4F).

To determine whether peritoneal cavity B-1 cell development can be rescued by the presence of sIgM, we created heterozygous ms2/+ (Igh-a)3C57BL/6 (Igh-b) mice. As B cells undergo allelic exclusion, each B cell expresses IgM either from the WT or ms2/2 allele. Differences in IgM allotype expression identified their origins. The sIgM levels in ms+/2 sera were comparable to those of WT mice (data not shown), and heterozygous mice had normal frequencies of total and PtC-binding B-1 cells in the peritoneal cavity (Fig. 4G). Furthermore, ms2/2-derived (Igh-a) and WT-derived (Igh-b) B cells were present at similar frequencies in bone marrow, spleen, and peritoneal cavity (Fig. 4H). The CD5+/CD21hiCD23− B cell population observed in ms2/2 mice was absent from peritoneal cavity and spleen (data not shown), and
FIGURE 4. Lack of B-1 cells in peritoneal cavity but not spleen of μs−/− mice. (A) Shown are 5% contour plots with outliers gated on live CD19+ peritoneal cavity B cells from WT and μs−/− mice (n = 3–5 mice/group), identifying CD5+CD23− cells that differ in CD43 expression. (B) B-1 cells were further identified by gating on IgMhiIgDloCD23+CD43+ and CD5+/− (B-1a/B-1b). (C and F) Bar chart indicating mean frequencies ± SD for IgMhiIgDloCD23+CD43+CD5+ B-1 cells in peritoneal cavity (perc), spleen, and lymph nodes of indicated mice. (D) Left, 5% contour plots with outliers of live CD19+ B cells binding to PtC-containing liposomes; right, mean frequencies ± SD for PtC-binding B-1 cells. (E) Relative expression of IgHV11 of purified CD23− B cells from WT and μs−/− pleural cavity determined by quantitative RT-PCR (n = 3/group). (G) Frequencies of B-1 cells and frequencies of PtC binders in peritoneal cavity of WT, μs+/−, and μs−/− littermates (n = 4/group). (H) Representative FACS analysis of CD19+IgM+ cells in bone marrow, spleen, and peritoneal cavity of indicated mice (IgM-a, μs allele; IgM-b, WT allele). Numbers indicate mean frequencies ± SD for the group (n = 3–5). (I and J) IgG anti-dsDNA (H) and IgG anti-ssDNA (I) in sera of indicated mice as measured by ELISA. (K) ANA levels in sera of indicated mice. Data are representative of three independent experiments (A–J). *p < 0.05, **p < 0.005, ***p < 0.0005. BM, bone marrow.
IgG autoantibody levels against dsDNA (Fig. 4I), ssDNA (Fig. 4J), and ANA-specific autoantibodies (Fig. 4K) were strongly reduced in sera from 10-wk-old \( \mu s^{-/-} \) mice compared with age-matched \( \mu s^{-/-} \) controls. The slight but not statistically significant increases of autoantibodies in the heterozygous compared with the WT mice might be due to local concentrations of slgM in the immediate vicinity of B cells in the bone marrow. In summary, slgM is a non-redundant factor required for the normal development of B-2 cells and peritoneal cavity, but not splenic B-1 cells.

\( \mu s^{-/-} \) CD21\(^{hi}\)CD23\(^{-}\) B cells are anergic

CD5 is a negative regulator of BCR signaling, and its expression on conventional B cells has been previously linked to anergy (32). The expression of CD5 on some FO and CD21\(^{int}\)CD23\(^{-}\) B cells has been previously linked to anergy (33). CD5 is a negative regulator of BCR signaling, and its expression suggested the emergence of anergic B cells in \( \mu s^{-/-} \) mice. Anergic B cells are relatively short-lived and are unresponsive to BCR-mediated stimulation (47). Indeed, B cell subsets in \( \mu s^{-/-} \) mice, including MZ and FO B cells, showed enhanced turnover compared with their WT counterparts as measured by enhanced BrDu incorporation (Fig. 5A), as noted by others (48). The turnover rate of CD21\(^{hi}\)CD23\(^{-}\) B cells was similar to that of FO B cells in \( \mu s^{-/-} \) mice, but it was enhanced compared with those in WT mice (Fig. 5A), and their rate of cell death, as measured by 7-AAD-incorporation, was highest among all B cell populations (Fig. 5B).

To determine whether the higher rate of BrDu incorporation was due to reduced cell survival in \( \mu s^{-/-} \) mice, we transferred equal numbers of CFSE-labeled splenic B cells from WT (CD45.1) and \( \mu s^{-/-} \) (CD45.2) mice into WT mice. All B cell subsets from \( \mu s^{-/-} \) mice, including the CD5\(^{-}\)CD21\(^{hi}\)CD23\(^{-}\) cells, survived poorly compared with WT B cells at both 2 and 25 d after transfer (Fig. 5C, 5D). Additionally, \( \mu s^{-/-} \) mice had increased frequencies of B cells expressing the proliferation marker Ki67, as well as increased frequencies of CD19\(^{+}\) cells in the G1 phase of the cell cycle, compared with WT cells (Fig. 5E). Transfer of either slgM containing WT serum or polyclonal IgM could reverse these increases, whereas monoclonal slgM could not (Fig. 5E, 5F).

We tested whether the reduced ability of \( \mu s^{-/-} \) B cells to survive in vivo could be due to a lack of BCR-mediated signaling, as unresponsiveness to BCR signaling is a hallmark of anergic B cells, and continuous “tonic” signaling through the BCR is required for B cell survival. Indeed, all B cell subsets from \( \mu s^{-/-} \) mice showed greatly reduced proliferation in response to anti-IgM stimulation compared with their WT counterparts. The CD21\(^{hi}\)CD23\(^{-}\)CD5\(^{-}\) B cells appeared least responsive (Fig. 5G). Thus, B cells from \( \mu s^{-/-} \) mice have a shortened lifespan and are hyporesponsive to BCR stimulation, suggesting that a lack of slgM leads to the development of anergic B cells.

Lack of slgM causes alterations in B cell development

Given that the transfer of mature \( \mu s^{-/-} \) B cells into a slgM-complete environment could not rescue \( \mu s^{-/-} \) B cell survival (Fig. 5D), slgM might affect very early stages of B cell development. Analysis of bone marrow precursors showed that early pro–B cells (Hardy scheme, fraction A) and pro–B cells (fraction B) were increased in \( \mu s^{-/-} \) compared with WT mice, but they were reduced after the pre–B cell stage (Fig. 6A, Supplemental Fig. 3A). Staining for CD93 expression on Hardy fraction E further demonstrated a strong reduction of immature B cells in the bone marrow (Fig. 6A). Because reduced BCR expression might affect identification of immature B cells (usually done via IgM/IgD staining), we added staining for CD93 to the analysis, which confirmed the decrease of immature B cells in \( \mu s^{-/-} \) mice (Fig. 6A).

The rate of proliferation among pre-pro– and late pre–B cells was similar between \( \mu s^{-/-} \) and WT mice (Fig. 6B). Thus, the increases of fractions A and B were not due to their increased expansion, but were likely attributable to increased accumulation due to reduced progression through the pre–B cell stage. Consistent with the presence of reduced B cell precursors in the bone marrow, frequencies of splenic transitional B cells were reduced at transitional stage T1, but not at T2, in \( \mu s^{-/-} \) compared with WT mice (Fig. 6C, Supplemental Fig. 3B). Finally, to directly compare bone marrow output, we assessed peripheral B cell reconstitution on day 12 after sublethal irradiation of \( \mu s^{-/-} \) and WT mice. The results showed significant reductions in the frequencies (Fig. 6D) and total numbers (not shown) of immature/transitional CD19\(^{-}\) CD93\(^{+}\) B cells in the bone marrow and spleen of \( \mu s^{-/-} \) mice compared with their WT controls. We conclude that the presence of secreted IgM is required for normal bone marrow B cell development.

slgM binds to bone marrow B cells

The data thus suggest that bone marrow B cell development is dependent on the presence of slgM. To determine whether slgM can bind to bone marrow B cells, and thus to assess whether slgM/B cell precursor direct interaction may be responsible for the effect of slgM on B cell development, we measured the binding of slgM to B cells in bone marrow and periphery.

Staining for total surface IgM, which stains for bound slgM as well as for membrane-bound BCR, showed that immature B cells from \( \mu s^{-/-} \) mice had reduced IgM staining compared with WT controls (Fig. 6E). To distinguish surface IgM expression from binding of slgM to B cells via Fc or other receptors, which would not occur in \( \mu s^{-/-} \) mice, we created heterozygous Ig-ha \( \times \) Ig-hb mice, in which the expressed IgM allele and surface-bound IgM from the nonexpressed allele were differentiated by surface staining with allotype-specific anti-IgMa and anti-IgMb. All B cells stained significantly for the nonexpressed IgM allotype, with stronger surface binding on peripheral B cells compared with bone marrow precursors (Fig. 6F), which explained the reduced staining for total surface IgM in \( \mu s^{-/-} \) compared with WT mice (Fig. 6E). Also, in vitro exposure of spleen and bone marrow cells to slgM showed strong slgM binding to B cells, particularly B cells from \( \mu s^{-/-} \) mice (Fig. 6G), including immature and mature B cells, but little to other cell types (Fig. 6G, Supplemental Fig. 4). We conclude that slgM binds to B cells and their precursors in vivo.

FcμR\(^{-}\) B cells show no defect in B cell development

Of the known surface IgM receptors, only the FcμR is expressed by early B cell precursors (35, 37), and we aimed to assess its effects on B cell development. First, we tested for its contribution to slgM binding to B cells and non–B cells. For that we exposed in vitro IgM labeled with biotin to bone marrow and spleen cells from bone marrow irradiation chimeras generated by reconstituting WT C57BL/6 (CD45.1) mice with bone marrow from either WT C57BL/6 mice or FcμR\(^{-}\) mice. WT and FcμR\(^{-}\) B cells were able to bind slgM on their cell surface. The lack of the FcμR led to a measurable but only very modest reduction in slgM binding to B cells of bone marrow and spleen, whereas non–B cells showed no discernable IgM binding (Fig. 7A, 7B).

To determine whether direct binding of IgM via the Fc μR might regulate B cell development, we studied the effects of B cell development in these bone marrow irradiation chimeras. Bone marrow B cell development appeared normal (Fig. 7C), and peripheral B cell subset composition in the spleen was similar to that of WT but not \( \mu s^{-/-} \) mice. An exception was the significant reduction in MZ B cells (Fig. 7D, 7E), whereas total serum IgM levels were increased in chimeras reconstituted with FcμR\(^{-}\)-
FIGURE 5. μs<sup>−/−</sup> CD21<sup>m</sup>CD23<sup>−</sup> B cells are anergic. (A) B cell proliferation was determined in WT and μs<sup>−/−</sup> mice by flow cytometry after 7 d continuous BrdU labeling. Shown are mean frequencies ± SD BrdU<sup>+</sup> cells (n = 4/group). (B) Mean frequencies ± SD of dead cells among different B cell subsets were determined by 7-AAD staining (n = 4/group). (C) Splenic B cells from WT (CD45.1) and μs<sup>−/−</sup> (CD45.2) mice were labeled with CFSE and transferred 1:1 into WT mice (n = 4). Two and 25 d after transfer, the ratios of μs<sup>−/−</sup> B cells to WT B cells were determined by flow cytometry, gating on CFSE<sup>+</sup> CD45.2 and CD45.1 cells, respectively. Competitive index is the ratio of μs<sup>−/−</sup> to WT B cells after transfer compared with that before transfer. (D) Mature B cells from WT or μs<sup>−/−</sup> B cells were transferred i.v. into WT (CD45.1) mice. Shown are contour plots with 5% outliers after gating on live CD19<sup>+</sup> lymphocytes for frequency of CD21<sup>int</sup>CD23<sup>−</sup> and CD21<sup>int</sup>CD23+ (FO) B cells in the spleens 2 d after transfer. (E and F) Groups of μs<sup>−/−</sup> mice (n = 3 or 4) were given equivalent amounts of sIgM in form of WT serum, or monoclonal IgM (mIgM). Another group received sera from μs<sup>−/−</sup> mice; PBS-treated WT and μs<sup>−/−</sup> mice served as controls. Mean frequencies ± SD of splenic B cells in G<sub>0</sub>, G<sub>1</sub>, G<sub>2</sub>, S, M of the cell cycle as assessed by flow cytometry via Ki67 and 7-AAD staining (n = 3–4/group). Shown are representative 5% contour plots after gating on live CD19<sup>+</sup> lymphocytes for different mouse groups of treatment. (G) Shown are CFSE histograms of cells stimulated in vitro with anti-IgM (20 μg/ml) for 3 d. Numbers indicate rounds of proliferation. Data are representative of two independent experiments (A, B, and F). *p < 0.05, ***p < 0.0005.
bone marrow compared with those of WT-reconstituted mice (Fig. 7F). These findings were similar to the original reports with this strain of FcμR−/− mice (49).

In vitro responses to anti-IgM stimulation were comparable between FcμR−/− B cells and WT B cells, whereas the μs−/− B cells showed significant reduced B cell proliferation (Fig. 7G, 7H). Thus, slgM direct binding to B cells via the FcμR cannot explain the significant effects of slgM on bone marrow B cell development. Because the lack of FcμR expression reduced, but did not abrogate, slgM binding to B cells (Fig. 7A), the presence of other IgM-binding proteins on the surface of the developing B cells may regulate B cell development.

**slgM affects B cell repertoire and selection**

The observed binding of slgM onto B cell precursors may alter B cell selection at the immature B cell stage and with it the B cell repertoire. Comparison of the BCR repertoire of μs−/− and WT mice using quantitative PCR on FACS-purified B cell fractions taken at different checkpoints during bone marrow B cell development showed significant differences of VH usage among bone marrow fractions D and E from μs−/− and WT mice (Fig. 8A, 8B). Mature B cells in the bone marrow and FO B cells in the periphery showed similar repertoire differences (Fig. 8C). Comparison of IgHV usage among FO B cells between WT and μs−/− mice and the CD21hiCD23− B cells in μs−/− mice also revealed repertoire differences demonstrating the strong effects of slgM on development of all B cell compartments (Fig. 8D). Combined with the findings of increased autoantibody generation in μs−/− mice, as well as reduced VH11 gene usage among B-1 cells, the data demonstrate that the lack of slgM causes repertoire changes in both the B-1 and the B-2 cell compartments.

**Discussion**

The present study revealed profound effects of slgM on the development of most B cells and B cell subsets, thereby providing a mechanism through which slgM prevents autoantibody formation, namely by facilitating normal B cell development and enforcing negative selection of autoreactive B cells. Although the lack of autoantigen clearance may contribute to autoantibody development in μs−/− mice (1, 9–12), the observed effects of slgM on B cell development in bone marrow and periphery, as well as the reversal of autoantibody production following the re-establishment of normal B cell development, suggest that these effects are the major underlying cause for the development of autoreactive B cells in IgM deficiency.

The direct binding of slgM to B cells, which we observed at all stages of B cell development from the pre–B cell stage onward (Fig. 6E, 6F, and data not shown), may provide the nonredundant signal for B cell development and selection. Among the three known B cell slgM receptors, neither the Fco/μR nor the com-
plement receptors CR1/2 are expressed on pro-B cells, pre-B cells, and immature B cells (50–52), and thus they cannot be responsible for the observed effects of IgM deficiency. FcμR1/2 is expressed early in B cell development and was a potential candidate receptor for IgM (37, 53). Additionally, mice lacking the FcμR1/2 develop autoantibodies despite harboring normal levels serum IgM (37, 53), further suggesting a central role for IgM in regulation of B cell development rather than removal of autoantigens for the enforcement of central tolerance induction. However, studies with bone marrow FcμR1/2 chimeras (Fig. 7) failed to reveal B cell developmental defects similar to those observed in the μs−/− mice. The phenotype of the bone marrow irradiation chimera is consistent with the original description of the FcμR1/2−/− mice from which we obtained the bone marrow (49).

In those mice, overall B cell development was found to be unaffected, except for the reduction in MZ B cells and an increase in splenic B-1 cells, as well as increases in serum IgM. A second report with independently generated FcμR1/2−/− mice reported no B cell abnormalities (50). However, a third group who generated FcμR1/2−/− mice reported reduction in all B cell precursors in the bone marrow, except mature fraction F B cells cells. In contrast to our findings with the sIgM−/− mice, they found increased numbers of peritoneal cavity B-1 cells in the FcμR1/2−/− mice (38). Thus, based on published work with three different FcμR1/2−/− mice, the lack of this receptor appears to have some effects on B cell development. Those reported changes are more subtle and/

FIGURE 7. FcμR1/2−/− expression on hematopoietic cells has little effect on B cell development. Bone marrow irradiation chimeric mice were generated by reconstitution of lethally irradiated C57BL/6 (CD45.1) mice with bone marrow from either WT or μs−/− C57BL/6 (CD45.2) mice (n = 6/group). Shown are overlay histograms for sIgM binding from (A) bone marrow and (B) spleen CD19+ B cells (left panels) and non-B cells (right panels) from WT and FcμR1/2−/− chimeras after incubation with allotype-mismatched sIgM. Control stains were done with all reagents except for anti-IgM (FMO). (C) Flow cytometric assessment of B cell development in the bone marrow according to Hardy fractionation (Supplemental Fig. 3). (D and E) Comparison of splenic B cell pools from chimeras reconstituted with FcμR1/2−/− or WT bone marrows. (F) Total levels serum IgM in WT and FcμR1/2−/− chimeras as measured by ELISA. *p < 0.05. (G and H) B cells isolated from spleens of indicated chimeras, or from μs−/− mice, were stained with CFSE and stimulated with anti-IgM for 72 h. Induction of B cell proliferation was defined as reduction in CFSE staining. *p < 0.05, **p < 0.005, ***p < 0.0005.
or different from those shown in the present study with the slgM−/− mice. Most prominently, the accumulation of CD5−/− CD43− CD23− CD21int anergic B cell population in the µs−/− mice is not noted in any reports of the FcμR−/− mice, nor was it found in the FcμR−/− bone marrow chimeras we created (Fig. 7). Given the somewhat contradictory reports on the phenotype of the three independently generated FcμR−/− mice, compensatory mechanisms may make any differences on B cell development less obvious than a complete lack of slgM. Alternatively, the various approaches that were taken in targeting the FcμR might have led to the differences in the effects. A direct comparison between the different gene-targeted mice could help to resolve some of these issues.

Because natural polyclonal, but not monoclonal, IgM rescued B cell development (Fig. 5F), our data indicate that natural IgM either directly or indirectly enhances BCR signaling strengths and thereby alters B cells at central tolerance induction at the pre-B and immature B cell stage, possibly by facilitating autoantigen presentation, as natural IgM-secreting cells appear selected on and directed against self-antigens (6). However, a BCR ligand requirement for pre-B cell selection has not been documented (54). Alternatively, slgM may provide a costimulatory signal more akin to that provided by pattern recognition receptors, increasing responsiveness of the developing B cell to self-antigens. In support of a model in which slgM affects BCR signaling strengths, the lack of slgM altered the peripheral B cell VH repertoire (Fig. 8), and thus B cell selection, and it led to the accumulation of autoantibody-secreting cells as well as to the presence of CD5−/− anergic and autoreactive B cells in the periphery, possibly in an attempt to silence these autoreactive cells. Taken together, the data provide evidence that B cell signaling and negative B cell selection at the immature B cell stage are defective in the absence of slgM. The increases in MZ and decreases in FO and body cavity B-1 cells are also consistent with reduced BCR-mediated signaling in µs−/− mice (33, 34). Our findings of reduced peritoneal cavity B-1 cell frequencies in the µs−/− mice are opposite to conclusions drawn previously by others (40), but these differences can be explained by the presence of CD5−B220+ anergic B cells in the peritoneal cavity and spleen, which were likely misidentified as B-1a cells. Their lack of CD43 expression, high expression of B220, intermediate expression of CD19, lack of VH11 expression, and of PC binding are not consistent with emergence from the B-1 cell lineage. Furthermore, their turnover was among the highest of any B cells studied (Fig. 5), whereas B-1 cell turnover in the peritoneal cavity is very low (55, 56). Multiple mouse models have demonstrated that reduction in BCR signaling strength reduces peritoneal cavity B-1 cell development (7). Thus, the demonstration of curtailed B-1 cell development in the µs−/− mice further supports a model in which slgM acts as a positive costimulatory signal during B cell development.

A “chicken and egg” problem emerges from the fact that slgM is required for normal B cell development but that it is not usually transmitted from the maternal circulation to the fetus. Thus, the development of fetal IgM-secreting cells must not require IgM. Because B-1 cell numbers in the spleen were largely unaffected in µs−/− mice (Fig. 4), they might be the earliest source of natural IgM supporting further slgM-dependent B-1 and B-2 cell development. This is consistent with studies showing profound reductions in peritoneal cavity B-1 cells after splenectomy (57) and with studies indicating that B-1 cell development arises in waves (58). Splenic B-1 cells may arise as a first wave of “IgM-independent” B-1 cells during fetal development, whereas body cavity B-1 cells emerge later and develop in an slgM-dependent manner. The data indicate a new, nonredundant role for splenic B-1 cells in the regulation of B-2 cell development.

In summary, the present study demonstrates that B-1 cells produce natural IgM to regulate B cell development and selection, thereby suppressing the development of autoreactive B cells. Identification of natural IgM as a critical enforcer of central selection suggests use of these proteins as potential therapeutics against Ab-mediated autoimmune diseases.

FIGURE 8. slgM is required for BCR selection. (A–C) The relative expression of indicated IgHV regions among FACS-purified bone marrow B cell precursors in (A) fraction D, (B) fraction E, and (C) fraction F as determined by quantitative PCR after normalization to GAPDH (n = 6 mice/group). (D) Similar analysis of spleen FO B cells from WT and µS−/− and CD21intCD23− B cells from µS−/− mice. Data are representative of at least two independent experiments. *p < 0.05, ***p < 0.0005.
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Disclosures

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References


