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N-Glycan Branching Is Required for Development of Mature B Cells

Christie-Lynn Mortales,* Sung-Uk Lee,† and Michael Demetriou*,†

Galectins have been implicated in inhibiting BCR signaling in mature B cells but promoting pre-BCR signaling during early development. Galectins bind to branched *N*-glycans attached to cell surface glycoproteins to control the distribution, clustering, endocytosis, and signaling of surface glycoproteins. During T cell development, *N*-glycan branching is required for positive selection of thymocytes, inhibiting both death by neglect and negative selection via enhanced surface retention of the CD4/CD8 coreceptors and limiting TCR clustering/signaling, respectively. The role of *N*-glycan branching in B cell development is unknown. In this study, we report that *N*-glycan branching is absolutely required for development of mature B cells in mice. Elimination of branched *N*-glycans in developing B cells via targeted deletion of *N*-acetylglucosaminyl transferase I (Mgat1) markedly reduced cellularity in the bone marrow and/or spleen and inhibited maturation of pre-, immature, and transitional stage 2 B cells. Branching deficiency markedly reduced surface expression of the pre-BCR/BCR coreceptor CD19 and promoted spontaneous death of pre-B cells and immature B cells in vitro. Death was rescued by low-dose pre-BCR/BCR stimulation but exacerbated by high-dose pre-BCR/BCR stimulation as well as antiapoptotic Bcl_{xL} overexpression in pre-B cells. Branching deficiency also enhanced Nur77 induction, a marker of negative selection. Together, these data suggest that, as in T cells, *N*-glycan branching promotes positive selection of B cells by augmenting pre-BCR/BCR signaling via CD19 surface retention, whereas limiting negative selection from excessive BCR engagement. ***The Journal of Immunology*****, 2020, 205: 630–636.**

T and B cells (or lymphocytes) undergo similar developmental events and checkpoints that involve the dynamic expression of transcription factors, response to extracellular environmental cues such as cytokines, and the somatic rearrangement, expression, and screening of their Ag receptor (AgR) to generate a functionally diverse, self-tolerant lymphocyte repertoire (1–4). Abnormalities in lymphocyte development and selection give rise to congenital immunodeficiencies, leukemias/lymphomas, and autoimmune diseases (5). In the bone marrow, developing B cells rearrange Ig μ H chain and L chain genes to express surface bound IgM that complexes with ITAM containing Igα and Igβ (CD79A and CD79B) signaling proteins to form the BCR on immature B cells (6–11). BCR binding to Ag directs immature B cell fate: when the BCR is engaged by a self-antigen, immature B cells die by clonal deletion (negative selection by apoptosis) or undergo receptor editing (continuous L chain rearrangement) to reduce/eliminate autoreactivity (12). Immature B cells continue selection through transitional developmental stages 1 (T1) and 2 (T2) in the bone marrow and the spleen but predominantly maturate into follicular (FO) or marginal zone (MZ) B cells in the spleen and coexpress surface bound IgM and IgD (13). Approximately 25% of developing B cells become mature B cells in the bone marrow (14). Unengaged BCRs induce tonic signaling to drive developmental progression and survival of immature B cells in a CD19-dependent manner (15). CD19 is a transmembrane glycoprotein first expressed in pro-B cells in the bone marrow and augments BCR signaling at multiple points during B cell development, maturation, and differentiation (16–20). Mice with targeted deletion of CD19 have greatly reduced B cell numbers, impaired B cell function, and defective immune responses (21, 22).

The branching of asparagine (*N*)-linked glycans with *N*-acetylglucosamine by the *N*-acetylglucosaminyl transferases Mgat1, Mgat2, Mgat4a/b, and Mgat5 sequentially increase production of ligands for the galectin family of sugar-binding proteins (23, 24). At the cell surface, interactions of galectins with branched *N*-glycans attached to glycoproteins generates a macromolecular lattice, thereby controlling receptor localization, mobility, clustering, and surface retention to impact cell function/differentiation and disease states (25–33). Our group demonstrated that *N*-glycan branching regulates thymic positive selection by defining the upper and lower bounds of TCR affinity for peptide-MHC (33). Using a T cell–specific (Lck-driven cre) knockout of the *N*-glycan branching enzyme Mgat1, we observed markedly reduced thymic and splenic T cell numbers. Mgat1 deletion enhanced thymocyte death by neglect via decreased CD4/CD8 coreceptor surface retention (i.e., enhanced CD4/CD8 endocytosis) and associated reduced Lck-induced Erk signaling, which are important for augmenting low-affinity TCR engagement. *N*-glycan–deficient thymocytes simultaneously exhibited increased death by negative selection because of excessive Ca^{2+} flux driven by enhanced TCR clustering. Thus, during T cell development *N*-glycan branching provides a mechanism for decoupling CD4/CD8 coreceptor and TCR signaling to maintain the appropriate range of TCR signal intensity necessary for thymocyte positive selection and generation of functional circulating $CD4^+$ and $CD8^+$ T cells.

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Abbreviations used in this article: FO, follicular; L-PHA, *Phaseolus vulgaris leukoagglutinin*; MZ, marginal zone; poly-LacNAc, poly-*N*-acetyllactosamine; T1, transitional developmental stage 1; T2, transitional developmental stage 2.

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Various studies have demonstrated glycosylation to be important in B cell development, selection, and maturation. This includes the sialic acid–binding Ig-type lectin (Siglec) and B cell inhibitory coreceptor CD22 (34–37), the sialyltransferase ST6Gal1 (38), fucosylation (39, 40), and galectins (41–46). The role of *N*-glycan branching, however, has not been investigated. In this study, we provide evidence that *N*-glycan branching promotes positive selection of B cells by enhancing low-affinity BCR engagement via promoting CD19 surface levels, whereas it also reduces high-affinity BCR engagement to prevent negative selection.

Materials and Methods

Mice

Mgat1$^{f/f}$ (006891), *Mgat2*$^{f/f}$ (006892), *CD19-cre* (006785), *CD23-Cre* (028197), and *Bim*$^{-/-}$ (004525) mice were obtained from The Jackson Laboratory. Eμ-Bcl$_{xL}$ mice were transferred to us by Dr. B. Iritani from the Department of Comparative Medicine at the University of Washington (Seattle, WA). *Mgat1*$^{f/f}$*/tetO-cre/ROSA-rtTA* mice were previously described (33). Interbreeding generated all other mice. Mice used were 5–7 wk old but otherwise selected randomly for experiments and approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

Flow cytometry

Flow cytometric analysis was performed as previously described (25, 33, 47). The fluorophore-conjugated mouse-specific Abs from Thermo Fisher Scientific or eBioscience were CD43 (R2/60), IgM (II/41), B220 (RA3-6B2), CD19 (eBio1D3), CD23 (B3B4), and CD21 (8D9). For flow cytometric analysis of glycan expression, cells were stained with 2 μg/ml *Phaseolus vulgaris* leukoagglutinin (L-PHA) conjugated to FITC or biotinylated L-PHA followed by streptavidin-conjugated fluorophore (Vector Laboratories and Thermo Fisher Scientific, respectively). Fluorophore-conjugated annexin V was from BD Pharmingen, and fluorophore-conjugated Nur77 was from Cell Signaling Technology. Samples were acquired on the Attune NxT (Thermo Fisher Scientific) Flow Cytometer. Data analysis was performed using FlowJo software.

Cell culture and stimulation

For in vitro experiments, isolated bone marrow cells were cultured in RPMI 1640 media (Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS (VWR), 2 μM L-glutamine and 100 U/ml penicillin/streptomycin (Life Technologies), and 50 μM 2-ME (Life Technologies). Cells were cultured at 2.5×10^5 cells per well in a 96-well plate in triplicates without stimulation or in the presence of functional grade goat anti-mouse IgM F(ab′)$_2$ (polyclonal; Thermo Fisher Scientific) at various concentrations.

Statistical analysis

Prism 8 software was used for all statistical testing. For comparison of three or more groups, we used the nonparametric Kruskal–Wallis test with false discovery rate correction (Benjamini, Krieger, and Yekutieli) for multiple comparisons (Figs. 1E, 1F, 3D–G, 4B, 4C). The Mann–Whitney *U* test was used when comparing only two groups, with one-tailed tests in Figs. 2, 3C, and 4A as we predicted a priori the direction of the effect of *Mgat1* deletion based on our previous T cell data (33) as well as the results of Kruskal–Wallis tests in Fig. 1.

Results

N-*glycan branching is required for B cell generation*

To investigate a role for *N*-glycan branching in B cell development, we generated B cell–specific *Mgat1*- and *Mgat2*-deficient mice using *CD19-cre*. *Mgat1* deletion completely eliminates, whereas *Mgat2* deletion reduces, *N*-glycan branching (Supplemental Fig. 1A). CD19 expression is initiated at the pro-B cell stage (16, 17). The plant lectin L-PHA binds to β1,6GlcNAc-branched *N*-glycans generated by the sequential action of the Mgat1, Mgat2 and Mgat5 branching enzymes (Supplemental Fig. 1A) (25, 31, 33). The loss of L-PHA binding directly identifies cells deleted for *Mgat1*, *Mgat2* or *Mgat5* (25, 31, 33). Therefore, we used L-PHA flow cytometry to assess deletion of *Mgat1* and *Mgat2* in the bone marrow and spleen of B220$^+$ B cells (Fig. 1A). In *Mgat2*$^{f/f}$/CD19-cre$^{+/-}$ mice, pro-B cells displayed a small population with reduced L-PHA binding (L-PHA^{int}) that became a larger population of L-PHA$^{lo/-}$ cells while transitioning to the mature B cell stage and entering the periphery (Fig. 1B). In contrast, only a small proportion of L-PHA^{int} pre-B and immature B cells were observed in *Mgat1*$^{f/f}$/CD19-cre$^{+/-}$ mice, with the population largely disappearing at the mature B cell stage and in peripheral splenic B cells (Fig. 1B). The lack of L-PHA$^{lo/-}$ B cells in *Mgat1*$^{f/f}$/CD19-cre$^{+/-}$ mice does not result from L-PHA binding to a glycan structure other than β1,6GlcNAc-branched *N*-glycans, as disrupting their biosynthesis via deletion of *Mgat2* (i.e., *Mgat2*$^{f/f}$/CD19-cre$^{+/-}$ mice) results in a large population of L-PHA$^{lo/-}$ B cells. As CD19-cre has a deletion efficiency of only ∼75–80% in bone marrow (48), the population of L-PHA$^+$ B cells in *Mgat1*$^{f/f}$/CD19-cre$^{+/-}$ mice likely results from inefficient CD19-cre–mediated excision of *Mgat1* and continued differentiation/expansion of this L-PHA$^+$ cell population (Fig. 1B).

To further investigate these results, we generated *Mgat1*$^{f/f}$/CD23-cre$^+$ and *Mga2*$^{f/f}$/CD23-cre$^+$ mice, in which CD23-cre will delete *Mgat1* and *Mgat2* in immature T2 B220$^+$ B cells. These cells remain sensitive to BCR-regulated selection and apoptosis (49, 50). CD23-cre–driven deletion phenocopied the results of CD19-cre, with *Mgat1*$^{f/f}$/CD23-cre$^+$ displaying L-PHA^{int} but not L-PHA$^{lo/-}$ B220$^+$ splenic B cells, whereas ∼90% of B220$^+$ splenic B cells from *Mgat2*$^{f/f}$/CD23-cre$^+$ mice were L-PHA$^{lo/-}$ cells (Fig. 1C).

To directly examine whether *Mgat1* deletion impacts mature B cells, we examined doxycycline-treated *Mgat1*$^{f/f}$*/tetO-cre/ROSA-rtTA* adult mice (33). These mice displayed a large population of L-PHA$^{lo/-}$ B220$^+$ splenic B cells (Fig. 1D). This confirms that L-PHA detects *Mgat1*-deleted B cells and indicates that *Mgat1* deletion specifically impacts survival of developing B cells through to the T2 stage in the periphery although having little impact on mature peripheral B cell survival.

Consistent with the conclusion that the complete but not partial loss of *N*-glycan branching leads to the death of developing B cells, the total cell number in the bone marrow and/or spleen as well as the number of B220$^+$ B cells were markedly reduced in *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$ and *Mgat1*$^{f/f}$/CD23-cre$^+$ but not *Mgat2*$^{f/f}$/CD19-cre$^{+/-}$ or *Mgat2*$^{f/f}$/CD23-cre$^+$ mice (Fig. 1E, 1F). Together, these results indicate that partial *N*-glycan branching levels are permissible for B cell development, whereas complete elimination prevents production of mature B cells in the bone marrow and spleen without impacting survival of peripheral mature B cells. This parallels our observations in T cells, in which *Mgat1* deletion in thymocytes completely blocked development of mature T cells, whereas deletion in peripheral mature T cells did not impact cell death (33).

N-*glycan branching drives bone marrow development and splenic maturation of B cells*

Next, we examined at what stage loss of *N*-glycan branching impacts B cell development and maturation. Cell surface markers and gating strategy used to identify specific B cell populations were as previously described (16, 51) and are depicted in Fig. 1A. We focused on *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$ and *Mgat1*$^{f/f}$/CD23-cre$^+$ mice, as they displayed significant loss of B cells in the bone marrow and/or spleen. In the bone marrow of *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$ mice, cellularity at each developmental stage revealed little difference at the pro, pre, and immature stages but a drastic loss in the number of mature B cells in (Fig. 2A). In the spleen, T1, T2, FO, and MZ B cells are similarly reduced by *Mgat1* deficiency (Fig. 2B). In *Mgat1*$^{f/f}$/CD23-cre$^+$ mice, the number of T1 B cells

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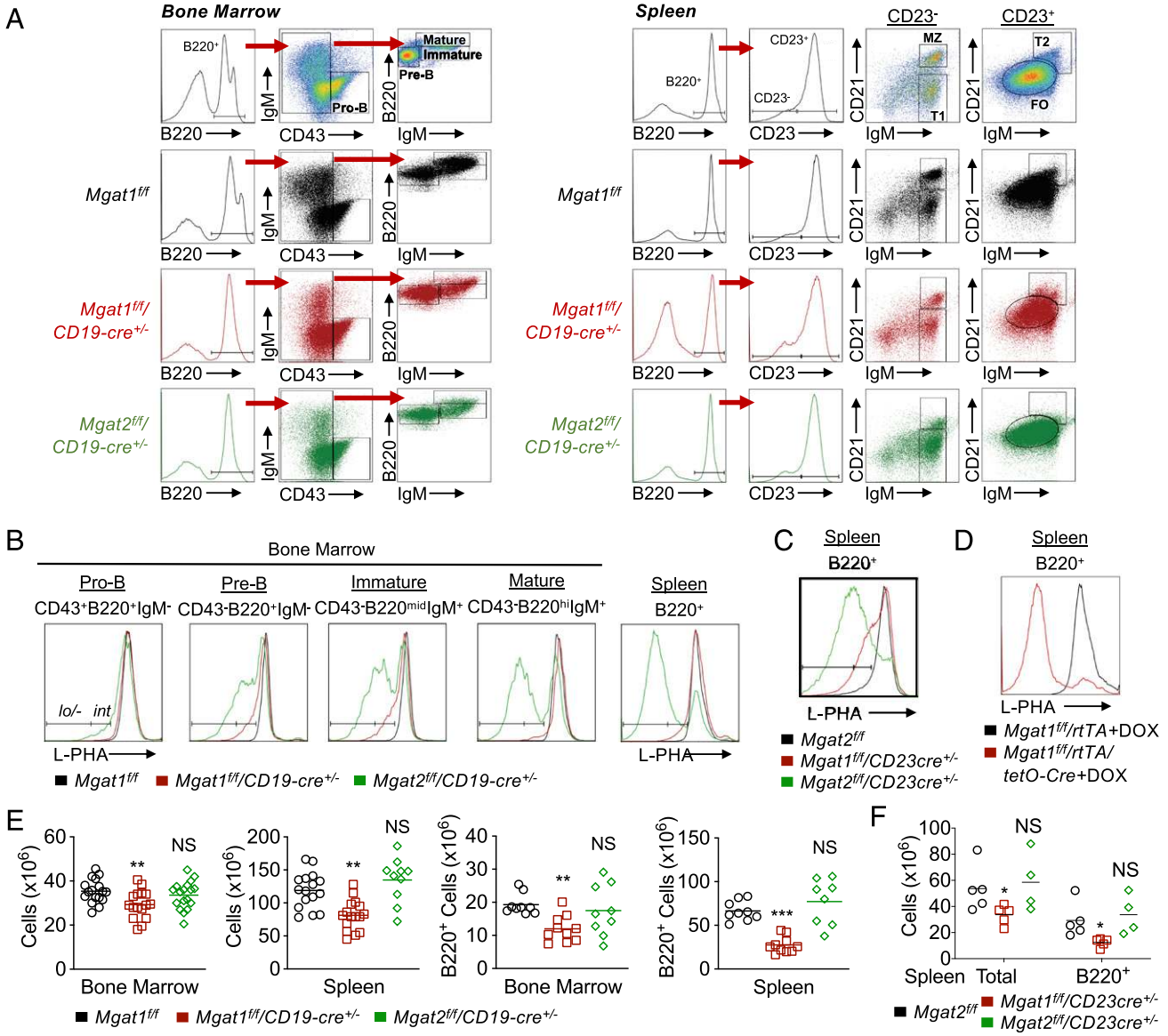

FIGURE 1. Late B cell development requires *N*-glycan branching. (**A**–**D**) Flow cytometric analysis with indicated markers on bone marrow B cell subsets and/or splenic B cells from mice of the indicated genotypes. (**E** and **F**) Total cellularity or B220$^+$ cell numbers (calculated by multiplying percentage of B220$^+$ cells acquired by flow cytometry by total cellularity) of bone marrow and/or spleens from mice of the indicated genotypes. Each dot represents one mouse. Kruskal–Wallis test with false discovery rate correction (Benjamini, Krieger, and Yekutieli) for multiple comparisons (E and F). Bars indicate mean. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

was unchanged, but there was a marked reduction in T2, FO and MZ B cells (Fig. 2C), the former consistent with CD23 expression beginning at the T2 stage. These data suggest that branching is essential for B cell development, particularly during the transition from immature and T2 to mature B cells.

Inhibiting programmed cell death fails to rescue B cell death from the loss of N*-glycan branching*

In T cells, we previously observed that the death of developing thymocytes induced by *Mgat1* deletion was rescued by overexpression of antiapoptotic Bcl_{xL} as well as targeted deletion of proapoptotic Bim (33). Therefore, we investigated whether manipulating these programmed cell death pathways [i.e., the mitochondrial apoptotic pathway (52)] could similarly rescue death of *N*-glycan branching-deficient B cells. We generated *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$*/Eμ-Bcl*$_{xL}$ to induce B cell–specific antiapoptotic Bcl_{xL} overexpression, as well as *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$*/Bim*$^{-/-}$ for deletion of proapoptotic Bim. As Mgat1 deficiency primarily altered the transition from immature to mature B cells, we focused on changes in mature B cells in the bone marrow. Neither *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$*/Eμ-Bcl*$_{xL}$, nor *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$*/Bim*$^{-/-}$ displayed an increase in the proportion of mature B cells (Fig. 2D). Thus, Bcl_{xL} overexpression and, potentially, Bim deletion fails to overcome the developmental block of *N*-glycan branching-deficient immature B cells into mature B cells.

N*-glycan branching promotes CD19-associated tonic signaling to rescue B cell death by neglect*

Pre- and immature B cells are subject to positive selection, with CD19 playing an important role in both pre-BCR signaling in pre-B cells (53) and BCR signaling in immature B cells (15). Only intermediate (i.e., tonic) pre-BCR/BCR signaling allows continued development into mature B cells. As *N*-glycan branching regulates surface retention of cell surface glycoproteins, we assessed whether *Mgat1* deficiency alters positive selection by reducing surface expression of CD19. Indeed, L-PHA^{int}B220$^+$

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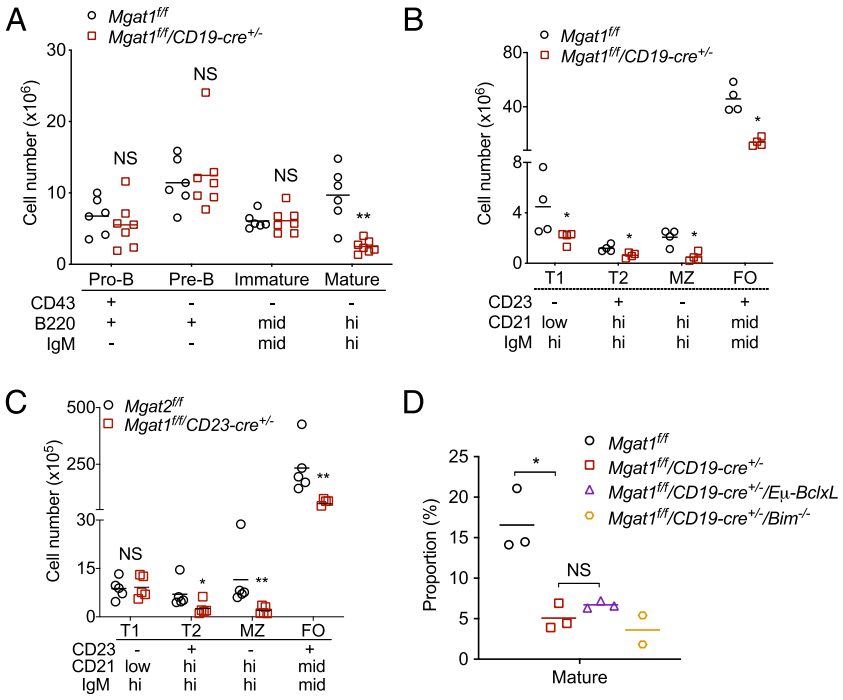

FIGURE 2. *N*-glycan branching promotes transition of immature to mature B cells in bone marrow and B cell maturation in the spleen. (**A**–**D**) Flow cytometric analysis of total number of B cell subtypes in the bone marrow (A) and spleen (B and C), using indicated markers, and proportion of mature B cells in spleen (D) from mice of the indicated genotypes. Each dot represents one mouse. Bars indicate mean. $^{**}p < 0.01$, $^{*}p < 0.05$ by unpaired one-tailed Mann–Whitney t test.

B cells from the bone marrow of *Mgat1*$^{f/f}$*/CD19-cre*$^{+/-}$ mice displayed a marked reduction in CD19 surface expression (Fig. 3A–C). To assess whether *N*-glycan branching deficiency drives death of developing B cells, we cultured bone marrow cells for 1 d in media without stimulation and assessed cell death by annexin V labeling. Both L-PHA^{int}B220^{+}IgM^{-} B cells and L-PHA^{int}B220^{+}IgM^{+} B cells from *Mgat1*$^{f/f}$/CD19-cre$^{+/-}$ mice displayed significant increases in annexin V binding relative to control cells (Fig. 3D, 3E), consistent with increased sensitivity to death from reduced CD19-dependent pre-BCR/BCR tonic signaling. Bcl_{xL} overexpression in L-PHA^{int}B220^{+}IgM^{-} cells from *Mgat1*$^{f/f}$/CD19-cre$^{+/-}$ mice reversed the increase in cell death (Fig. 3F). However, in L-PHA^{int}B220^{+}IgM^{+} B cells Bcl_{xL} overexpression displayed only a marginal and nonsignificant impact on cell death (Fig. 3E). To investigate whether increased death was from reduced pre-BCR/BCR tonic signaling, we assessed whether minimally enhancing pre-BCR/BCR signaling would reduce the death induced by *N*-glycan branching deficiency. Indeed, low doses of a polyclonal anti-IgM F(ab′)$_2$ Ab, which should activate both pre-BCR and BCR via the common H chain, partially rescued L-PHA^{int}B220^{+}IgM^{-} and L-PHA^{int}B220^{+}IgM^{+} B cells from death (Fig. 3F, 3G). Taken together, these data suggest that *N*-glycan branching promotes CD19 surface expression and associated pre-BCR/BCR tonic signaling to prevent death by neglect of developing B cells.

N-*glycan branching inhibits negative selection of developing B cells*

Low level stimulation of BCR reduced but did not fully rescue death of *N*-glycan branching-deficient developing B cells. As developing B cells are also subject to death by negative selection via strong BCR signaling from encounter with high-affinity self-antigen, this suggests that *N*-glycan branching may also regulate negative selection in immature B cells. Nur77 is a transcription factor that is expressed at higher levels in B cells subject to excessive Ag encounter and eventual deletion by negative selection (54–56). Ex vivo L-PHA^{int}B220^{+}IgM^{+} B cells displayed greater Nur77 levels compared with control (Fig. 4A). This suggests *N*-glycan branching deficiency enhances BCR engagement and negative selection in vivo. To further support this hypothesis, we examined whether providing high BCR signaling further enhances the death of B cells deficient in *N*-glycan branching. Indeed, BCR stimulation with high doses of anti-IgM F(ab′)$_2$ enhanced cell death of L-PHA^{int}B220^{+}IgM^{+} greater than L-PHA^{int}B220^{+}IgM^{-} cells, as evidenced by annexin V labeling (Fig. 4B, 4C). These data suggest that *N*-glycan branching also inhibits negative selection of immature B cells.

Discussion

In this study we report that *N*-glycan branching is required for the development but not survival of mature B cells. Loss of *N*-glycan branching impacts the survival of pre-B cells, immature B cells, and T2 transitional peripheral B cells but not mature B cells, in which only the former cells are subject to positive selection. The effects of *N*-glycan branching during B cell positive selection is similar to what we have observed in T cells (33), with *N*-glycan branching appearing to be similarly required to maintain the boundaries of pre-BCR/BCR signaling thresholds to permit positive selection by inhibiting both death by neglect and negative selection. Mechanistically, *N*-glycan branching is required for CD19 surface expression in developing B cells. CD19 is necessary for low-affinity pre-BCR/BCR engagement to maintain tonic BCR signaling and prevent death by neglect (15). The mechanism by which *N*-glycan branching inhibits death by negative selection is less clear, but may be similar to that in T cells, namely limiting Ag-induced BCR clustering to prevent excessive signaling and death by negative selection.

CD19 is coupled to both pre-BCR and BCR signaling-mediated apoptosis pathways (15, 53). For immature B cells, lack of CD19 results in loss of basal PI3K signaling, continued RAGs expression, and L chain receptor editing, thus inhibiting positive selection (15). Phosphatase and tensin homolog (PTEN) opposes PI3K activity; the absence of PTEN activity reverses the effects of CD19 loss to promote immature B cell positive selection (57, 58). Like CD19, BCR deletion results in loss of mature B cells (59, 60). Constitutive PI3K signaling was necessary and sufficient to rescue the survival of BCR-deficient B cells, whereas other interventions, such as constitutive NF-κB signaling, MAPK/ERKs

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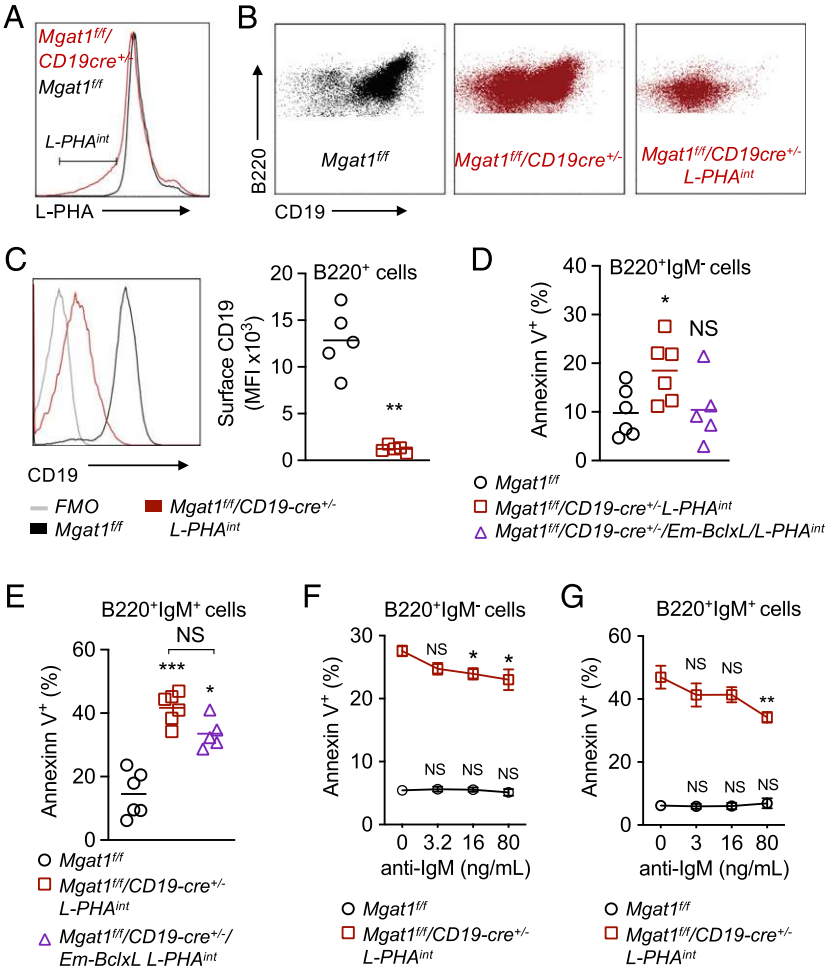

FIGURE 3. *N*-glycan branching promotes immature B cell positive selection via CD19 surface expression. (**A**–**C**) Flow cytometric analysis of ex vivo B220$^+$ bone marrow B cells for the L-PHA^{int} gate (A) and CD19 surface levels (B and C). Each dot represents one mouse., unpaired one-tailed Mann-Whitney *t* test (C). (**D**–**G**) Annexin V binding to bone marrow B cells cultured for 1 d without BCR stimulation (D and E) and with low doses of anti-IgM F(ab′)$_2$ (F and G) gated on B220$^+$IgM$^-$ (D and F) and B220$^+$IgM$^+$ (E and G) cells. Cells from control mice are gated on all B cells, whereas all others are gated on the L-PHA^{int} population as indicated below each panel ($n \geq 3$). Each dot represents one mouse (D and E). Kruskal–Wallis test with false discovery rate correction (Benjamini, Krieger, and Yekutieli) for multiple comparisons (D–G), with comparison with zero anti-IgM (F and G). Error bars indicate mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

kinase signaling, or Bcl2 overexpression, were not (59, 60). Whereas Bcl2 and Bcl$_{xL}$ are both antiapoptotic factors in programmed cell death, Bcl$_{xL}$ has been shown to be superior in promoting B cell survival (61); thus, we chose to overexpress Bcl$_{xL}$. In contrast to *N*-glycan branching-deficient thymocytes, neither Bcl$_{xL}$ overexpression nor Bim deletion significantly impacted the loss of mature B cells in vivo induced by *Mgat1* deletion. However, Bcl$_{xL}$ overexpression did rescue death of branching-deficient pre-B cells but displayed little effect on survival of immature B cells in vitro. This suggests that the failure of Bcl$_{xL}$ overexpression to rescue the generation of mature B cells in *Mgat1$^{f/f}$*/CD19-cre$^{+/-}$ mice is largely driven by the failure to rescue immature B cells rather than pre-B cells. Notably, branching deficiency induced a much greater loss of cell surface CD19 in developing B cells than CD4 and CD8 coreceptor loss induced in thymocytes (~90% versus ~25–50%, respectively) (33). Therefore, B cell *N*-glycan branching deficiency largely phenocopies CD19/BCR deficiency that could not be rescued with Bcl2 overexpression, whereas a partial loss of CD4/CD8 in thymocytes is a milder phenotype that could be rescued by altering apoptotic pathways. Future research examining mechanisms by which *N*-glycan branching deficiency affects PI3K and apoptosis in immature B cells would provide insight into how branching regulates CD19–BCR downstream signaling pathways and subsequent functional outcomes such as death.

CD19cre- and CD23cre-driven deletion of *Mgat1* both resulted in the generation of B cells with reduced (L-PHA^{int}) but not absent *N*-glycan branching (L-PHA$^{lo/-}$). Once the Golgi branching pathway is blocked (e.g., *Mgat1* deletion), there is a gradual reduction in branching at the cell surface, as 1) the enzyme is slowly lost from the Golgi, and 2) glycoproteins at the cell surface with normal glycosylation are replaced slowly by new glycoproteins lacking branched *N*-glycans. Thus, L-PHA^{int} cells represent this transition state. Further reductions in branching beyond this transition state triggers cell death, resulting in the absence of L-PHA$^{lo/-}$ B cells.

In contrast to *Mgat1* deletion, *Mgat2$^{f/f}$/CD19-cre$^{+/-}$* and *Mgat2$^{f/f}$/CD23-cre$^{+/-}$* mice were grossly comparable to controls, yet we expected them to have an intermediate phenotype due to having a single *N*-glycan branch, which should allow formation of a partially intact galectin–glycoprotein lattice. However, in T cells we recently reported that loss of branching in *Mgat2*-deficient T cells is partially compensated for by poly-*N*-acetyllactosamine (poly-LacNAc) extension of the remaining single GlcNAc branch (Supplemental Fig. 1A) (31). Thus, *N*-glycan branching loss in B cells from *Mgat2$^{f/f}$/CD19-cre$^{+/-}$* mice may be compensated for by poly-LacNAc extension to maintain lattice integrity and appropriate BCR signaling thresholds for B cell positive selection. Indeed, unpublished data indicates that *Mgat2*-deficient peripheral B cells display the same increase in poly-LacNAc extension that we observed in *Mgat2$^{-/-}$* T cells (C.-L. Mortales and M. Demetriou, submitted for publication).

Our recent work on *N*-glycan branching in thymocytes (33) and now B cell positive selection gives great emphasis to the role branching plays in maintaining central tolerance. Future research in delineating the role of *N*-glycan branching in B cell tolerance

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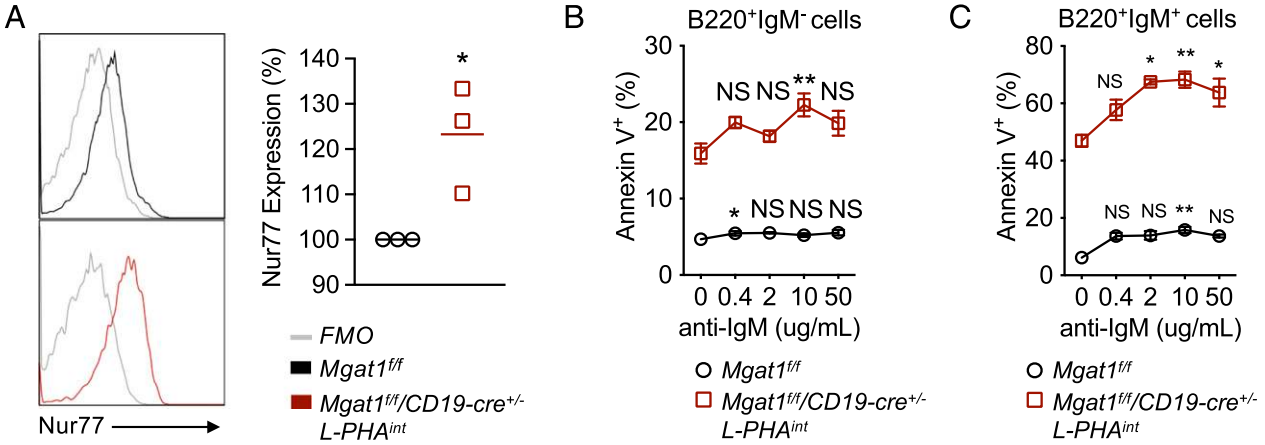

FIGURE 4. *N*-glycan branching limits immature B cell negative selection by limiting BCR engagement. (**A**) Flow cytometric analysis of ex vivo $B220^+IgM^+$ bone marrow B cells for intracellular staining of Nur77. Each dot represents one mouse of the indicated genotypes. $*p = 0.05$ by unpaired one-tailed Mann–Whitney *U* test. (**B** and **C**) Annexin V binding to bone marrow B cells following a 1-d culture with high doses of anti-IgM $F(ab')_2$ gated on $B220^+IgM^-$ (B) and $B220^+IgM^+$ (C) cells ($n = 3$). Error bars indicate mean ± SEM. $*p < 0.05$ by Kruskal–Wallis test with false discovery rate correction (Benjamini, Krieger, and Yekutieli method) for multiple comparisons, with comparison with zero anti-IgM for each genotype.

may provide new insight on the role of B cells in diseases such as systemic lupus erythematosus, rheumatoid arthritis, and multiple sclerosis.

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Disclosures

The authors have no financial conflicts of interest.

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