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Bruton’s Tyrosine Kinase Synergizes with Notch2 To Govern Marginal Zone B Cells in Nonobese Diabetic Mice

James B. Case,*†,¹ Rachel H. Bonami,*†,¹ Lindsay E. Nyhoff,‡ Hannah E. Steinberg,* Allison M. Sullivan,* and Peggy L. Kendall*†

Expansion of autoimmune-prone marginal zone (MZ) B cells has been implicated in type 1 diabetes. To test disease contributions of MZ B cells in NOD mice, Notch2 haploinsufficiency (Notch2++/−) was introduced but failed to eliminate the MZ, as it does in C57BL/6 mice. Notch2++/−/NOD have MZ B cell numbers similar to those of wild-type C57BL/6, yet still develop diabetes. To test whether BCR signaling supports Notch2++/−/NOD MZ B cells, Bruton’s tyrosine kinase (Btk) deficiency was introduced. Surprisingly, MZ B cells failed to develop in Btk-deficient Notch2++/−/NOD mice. Expression of Notch2 and its transcriptional target, Hes5, was increased in NOD MZ B cells compared with C57BL/6 MZ B cells. Btk deficiency reduced Notch2++/− signaling exclusively in NOD B cells, suggesting that BCR signaling enhances Notch2 signaling in this autoimmune model. The role of BCR signaling was further investigated using an anti-insulin transgenic (Tg) BCR (125Tg). Anti-insulin B cells in 125Tg/NOD B cells, suggesting that BCR signaling enhances Notch2 signaling in this autoimmune model. The role of BCR signaling was further investigated using an anti-insulin transgenic (Tg) BCR (125Tg). Anti-insulin B cells in 125Tg/NOD B cells populate an enlarged MZ, suggesting that low-level BCR signaling overcomes reliance on Notch2. Tracking clonotypes of anti-insulin B cells in H chain–only Vh125Tg/NOD mice showed that BTK-dependent selection into the MZ depends on strength of antigenic binding, whereas Notch2-mediated selection does not. Importantly, anti-insulin B cell numbers were reduced by Btk deficiency, but not Notch2 haploinsufficiency. These studies show that 1) Notch2 haploinsufficiency limits NOD MZ B cell expansion without preventing type 1 diabetes, 2) BTK supports the Notch2 pathway in NOD MZ B cells, and 3) autoreactive NOD B cell survival relies on BTK more than Notch2, regardless of MZ location, which may have important implications for disease-intervention strategies. The Journal of Immunology, 2015, 195: 61–70.

Marginal zone (MZ) B cells have increased propensity for autoreactivity. These long-lived cells reside at the outer edges of B cell follicles, where they are poised to act as T cell–independent first responders to blood-borne Ags (1–4). The tendency of autoreactive BCRs to preferentially select into this subset, together with expanded MZs in autoimmune-prone strains of mice, drives interest in understanding their mechanisms of selection and action in autoimmune disease. In NOD mice, the MZ is enlarged and has been postulated to promote autoimmune diabetes (5–7). Notch2 is critical for MZ B cell development (8–12). Notch2 on the B cell surface interacts with its ligand at relatively low affinity in the MZ sinuses (13). MZ B cells are highly dependent on these signals, as haploinsufficiency of Notch2 (Notch2++/−) severely reduces this subset in C57BL/6 mice (12). Homozygous Notch2 deficiency is embryonic lethal, but B cell–targeted Notch2 deficiency (Notch2++/−) reproduces MZ B cell depletion seen in Notch2++/− mice, without affecting other splenic B cell compartments, localizing its primary function to this subset (8).

Autoantigen-driven selection into the MZ indicates that BCR signaling also contributes to MZ development. B cells expressing anti-insulin BCR transgens (125Tg) illustrate this concept, as they bind Ag in vivo at all developmental stages and are disproportionately distributed to the MZ in both NOD and C57BL/6 mice (14). BCR signaling strength is a factor in MZ selection, with weaker signals favoring MZ selection and stronger signals leading to follicular (FO) selection (2). However, BCR signaling pathways important for such selection have not been well defined. In particular, Bruton’s tyrosine kinase (BTK), which mediates activation signals from the BCR, is known for its role in FO B cell maturation but is generally not considered a participant in MZ development, except in rare cases involving transgenes that generate low-affinity BCRs (2, 15–19).

Factors contributing to the increased MZ B cell compartment in NOD and other autoimmune strains are not well understood. Recent work comparing gene expression in NOD versus non-autoimmune MZ compartments found differences in genes controlling cell trafficking, suggesting that factors influencing chemotaxis to the MZ also play a role (6). Components of the extracellular matrix newly discovered to contribute to MZ development are also overexpressed in the MZ of NOD mice (20). Our group has shown that polymorphisms in Igs genes contribute to increased propensity for autoreactivity in NOD B cells and that these polymorphisms are shared with systemic lupus erythematosus–prone mice (21, 22). B cell–intrinsic tolerance defects in the NOD strain also allow increased numbers of autoreactive B cells to reach maturity.
(22–24). These features in autoimmune-prone strains are likely to create a larger autoreactive repertoire available for selection into the MZ.

To better understand the role of MZ B cells in autoimmune diabetes, Notch2 haploinsufficiency was introgressed onto the NOD strain with the goal of eliminating these autoreactive-prone cells, as it does in nonautoimmune strains. However, a subset of NOD MZ B cells proved resistant to this strategy. BCR-mediated signaling was found to contribute to survival of Notch2+/−/NOD MZ B cells, as Btk deficiency eliminated them, whereas low-level BCR signaling typical of anti-insulin B cells enhanced their numbers. Furthermore, elements of the Notch2 signaling pathway had increased expression in NOD MZ B cells compared with C57BL/6 MZ B cells, and Btk deficiency reduced these levels in NOD, but not C57BL/6, B cells. Studies of anti-insulin B cell clonotypes showed that BTK-dependent MZ selection and survival depend on BCR signal strength, whereas Notch2-mediated effects do not. Finally, the ability of Notch2 haploinsufficiency to reduce autoreactive B cell selection into the MZ does not translate into overall reduction in autoreactive cell numbers, which may have important clinical implications, as suggested by the failure of this model to protect against type 1 diabetes.

Materials and Methods

Mice

Notch2+/− founder mice were kindly provided as a gift from James W. Thomas at Vanderbilt University, and C. Klug, University of Alabama at Birmingham, with permission from Hamada’s group (8, 12), who developed them. Notch2+/− mice were crossed with NOD mice and backcrossed for >10 generations. Offspring were homozygous for all NOD idd loci tested, as previously described (25) and shown in Table I. Briefly, DNA was prepared from tail biopsies using the DNeasy Blood and Tissue Kit from QIAGEN (cat. no. 69506). For PCR, DNA was initialized for 5 min at 94°C, then 40 cycles under the following conditions: 45 s at 94°C, 45 s at 53°C, and 1 min for 72°C, with the exception of idd loci 10/3 and 5/1, for which the elongation step occurred at 72°C for 2 min. A final elongation step for all loci was completed at 72°C for 7 min. All samples were run on 4% NuSieve 3:1 agarose (Lonza; cat. no. 50090) and visualized with Bio-Rad GelDoc XR+ system. The 125Tg/NOD, V H125Tg/NOD mice and Btknull deficient (4, 6, 8, 12) NOD mice were generated as previously described and shown in Table I. Offspring were homozygous for all NOD idd loci 10/3 and 5/1, for which the elongation step occurred at 72°C for 2 min. A final elongation step for all loci was completed at 72°C for 7 min. All samples were run on 4% NuSieve 3:1 agarose (Lonza; cat. no. 50090) and visualized with Bio-Rad GelDoc XR+ system. The 125Tg/NOD, V H125Tg/NOD mice and Btknull deficient (4, 6, 8, 12) NOD mice were generated as previously described (25–27). All mice were housed in specific pathogen–free conditions, with autoclaved food and water. All studies were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Disease studies

Blood glucose levels were measured weekly, and mice were considered diabetic at the first of two consecutive readings above 200 mg/dl.

Flow cytometry

Splenoctyes were isolated as isolated from wild-type (WT) or Btk-deficient NOD and C57BL/6 mice. MACS was used to deplete CD43 expressing cells using LS columns (Miltenyi Biotec). Purified cells were then stained using fluorochrome-conjugated Abs to IgD, CD21, CD23, as above, and IgM[b] (AF6-78). Alexa Fluor 700 succinimidyl ester (Life Technologies) was used to exclude dead cells. Cells were sorted for IgM[b]IgD[CD19]1/4CD21+/−/CD23+ FO, IgM[b]IgD[CD19]1/4CD21−/CD23+ MZ, IgM[b]IgD[CD21]0/CD23hi transitional 2 (T2), or IgM[b]IgD[CD19]1/4CD23hi premarginal zone (pMZ) B cells by FACS using a BD FACS Aria III cell sorter. Total RNA was isolated from each fraction using the RNeasy Micro Kit (QIAGEN) and converted to cDNA, as previously described (22). Quantitative RT-PCR (qRT-PCR) was performed using the iCycler IQ Real-Time PCR Detection System (Bio-Rad), using EXPRESS SYBR GreenER SuperMix, with premixed ROX (Invitrogen). The mRNA levels were normalized by the comparative cycle threshold (∆∆Ct) method, relative to hypoxanthine guanine phosphoribosyl transferase (HPRT) and reported as fold change compared with the WT C57BL/6 MZ B cell primer. Primers used were Hairy/enhancer of split homolog 5 (Hes5) forward 5′-GAGAGTCTGCAGCTCAAGAGGAA-3′, reverse 5′-CAGAGGCTTTGCTGTGTTCG-3′ (28); Notch 2 forward 5′-ACATCATCACAGAGGTGCC-3′, reverse 5′-CATTATTTGACACAGCTGCGC-3′ (29); and HPRT forward 5′-AGTGGCAAAGTTGCTGTGT-3′, reverse 5′-TGAAGACTTACATTATGTCAGGGGCA-3′ (Integrated DNA Technologies).

Statistical analysis

The p values were calculated using the Student t test for comparisons of cell numbers or percentages. For qRT-PCR, p values were calculated using a two-way ANOVA with Sidak correction for multiple comparisons. Log-rank comparison was used for Kaplan–Meier survival curves. Values of p < 0.05 were considered significant.

Results

Notch2 haploinsufficiency blocks MZ B cell expansion but does not eliminate the MZ in NOD mice

Past investigations using nonautoimmune C57BL/6 with B cell–targeted Notch2 deficiency, or global Notch2 haploinsufficiency (Notch2+/−), showed that both approaches eliminate MZ B cells (8, 12). Therefore, we introgressed Notch2+/− onto NOD mice to study selection and function of MZ B cells in the setting of autoimmunity. Offspring were homozygous for all idd loci listed in Table I. In contrast to studies in C57BL/6 mice, Notch2 haploinsufficiency in NOD mice does not eliminate MZ B cells (Fig. 1A–C). Rather, the MZ B cell population is reduced to levels that approach normal in nonautoimmune strains: Notch2+/−/NOD had 4.98 ± 1.14% (3.32 ± 0.49 × 10^6) MZ B cells versus 16.44% ± 0.71% (3.5 ± 1.0 × 10^7) for WT NOD (p < 0.001 for both percentage and total numbers). Of note, MZ B cell normalization had no effect on CD4 or CD8 T cell numbers (Supplemental Table I). To assess whether the MZ B cells identified by flow cytometry in Notch2+/−/NOD are anatomically positioned in the MZ, immuno-fluorescence microscopy was used to detect B cells outside the metallophilic macrophage ring that delineates the MZ. White arrows in representative images depict B cells (IgM+, green) outside the metallophilic macrophage ring (MOMA-1, red) in NOD and Notch2+/−/NOD mice (Fig. 1D). Notch2+/−/NOD images are similar to our previously published data regarding C57BL/6 MZs (14) and show that B cells retain the ability to traffic to the MZ in Notch2+/−/NOD mice.
Notch2+/− mice, whereas numbers of 125Tg/NOD MZB cells are reduced by less than half compared with NOD B cells are still highly capable of maintaining a large MZ compartment, constituting 31.5 ± 3.3% of total B cells (Fig. 2C). On the nonautoimmune, B6 background, this expanded anti-insulin MZ is significantly reduced, but not eliminated, by Notch2 insufficiency, with 8.02 ± 3.98% of B cells retaining the MZ phenotype (Fig. 2C). On the NOD background, however, 125Tg/Notch2+/−/NOD offspring were compared with WT littermates. (A) Flow cytometry gating scheme of B220+/IgM+ live lymphocytes for MZ markers (IgMhi/IgDlo/CD21hi/CD23mid) in WT (left) and Notch2+/−/NOD (right) B cells. (B and C) Average MZ B cell percentages (B) or total numbers of MZ B cells (C) in WT NOD (black bars) versus Notch2+/−/NOD (gray bars) mice. Error bars show SD for n = 7. No significant difference was noted.

FIGURE 1. Notch2 haploinsufficiency corrects the expanded NOD MZ compartment but does not protect against diabetes development. Notch2+/− was introgressed onto NOD mice and backcrossed for >10 generations. Notch2+/−/NOD offspring were compared with WT littermates. (A) Flow cytometry gating scheme of B220/IgM+ live lymphocytes for MZ markers (IgMhi/IgDlo/CD21hi/CD23mid) in WT (left) and Notch2+/−/NOD (right) B cells. (B and C) Average MZ B cell percentages (B) or total numbers of MZ B cells (C) in WT NOD (black bars) versus Notch2+/−/NOD (gray bars) mice. Error bars show SD for n = 6–8 age-matched mice per group (12–16 wk), from two experiments, **p < 0.001. (D) Immunofluorescence staining of frozen spleen sections was used to identify B cells (IgM+, green) and metallophilic macrophages (MOMA-1+, red). Representative ×10 magnification images from n = 5 NOD mice (left) and n = 4 Notch2+/−/NOD mice (right) are shown. Nondiabetic male and female mice were 14–16 wk of age. White arrows indicate examples of MZ B cells located outside the metallophilic macrophage ring that delineates the MZ. (E) Female Notch2+/−/NOD and WT NOD littermates were monitored for diabetes using weekly blood glucose levels. Kaplan–Meier survival curve shows percent nondiabetic (y-axis) versus week of age for Notch2+/−/NOD (gray line) versus WT/NOD (black line). Notch2+/−/NOD, n = 10; WT NOD, n = 7. No significant difference was noted.

Table I. NOD idd loci tested

<table>
<thead>
<tr>
<th>idd Locus/Chromosome</th>
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<tr>
<td>Idd1 = H2g7/17</td>
<td>D17Mit34 = C4</td>
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<tr>
<td>Idd3/3</td>
<td>D3Mit95</td>
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<tr>
<td>Idd4/11</td>
<td>D11Mit320</td>
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<td>D13Mit 61</td>
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<tr>
<td>Idd15/5</td>
<td>D5Mit48</td>
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idd loci were genotyped to ensure NOD homozygosity in breeding pairs used for backcrossing at the 3rd through 10th generations.

**Normalization of MZB compartment size does not protect against diabetes**

To determine whether reduction of MZ B cell numbers prevents diabetes development, we performed a disease study on female Notch2+/−/NOD and WT NOD littermates. As shown in Fig. 1E, haploinsufficiency of Notch2 does not confer disease protection, as >70% of mice in both groups become diabetic by 30 wk of age. Thus, reversal of the abnormal expansion of the MZ found in NOD mice is insufficient to protect against diabetes development.

**Insulin-specific NOD B cells maintain supranormal MZ B cell numbers in Notch2 haploinsufficiency**

Insulin specificity conferred by a transgenic (Tg) BCR (125Tg) produces an enlarged MZ B cell compartment in C57BL/6 mice, illustrating the contribution of BCR-mediated Ag selection in directing B cells to the MZ compartment. This specificity further expands the MZ B cell compartment in NOD mice (14). To examine the interplay between this autoreactive BCR specificity and Notch2 in the development of the MZ, we crossed 125Tg mice with Notch2+/− on both B6 and NOD backgrounds. As shown in Fig. 2A, 125Tg B cells bind insulin specifically (left panels), and this is not altered by haploinsufficiency of Notch2 (right panels); nor is the total number of anti-insulin B cells reduced (36.3 ± 11.1 × 10⁶ versus 42.0 ± 6.6 × 10⁶, p = 0.37). The percent of MZ B cells in 125Tg/NOD and 125Tg/B6 is 51.7 ± 3.3% and 37.4 ± 7.95% of total B cells, respectively (Fig. 2C). On the nonautoimmune, B6 background, this expanded anti-insulin MZ is significantly reduced, but not eliminated, by Notch2 insufficiency, with 8.02 ± 3.98% of B cells retaining the MZ phenotype (Fig. 2C). On the NOD background, however, 125Tg/Notch2+/−/NOD B cells are still highly capable of maintaining a large MZ compartment, constituting 31.5 ± 3.3% of total B cells (Fig. 2B, 2C). Total numbers of MZ B cells follow this same trend, as shown in Fig. 2D. Overall, B6 MZ anti-insulin B cells are more dependent on Notch2 than NOD B cells are, as 125Tg/Notch2+/−/NOD MZB cells are reduced by less than half compared with their 125Tg/NOD counterparts, whereas numbers of 125Tg/Notch2+/−/B6 MZB cells are reduced >5-fold (Fig. 2E). Thus, both autoreactive BCR specificity and the presence of an autoimmune background can override the exquisite dependence on Notch2 exhibited by MZ B cells in C57BL/6 mice with endogenous BCRs.

**BTK is required by MZ B cells in Notch2+/−/NOD mice**

The ability of autoreactive (anti-insulin) B cells to continue to enter the MZ in Notch2+/−/NOD mice suggested that BCR-mediated signaling allows some NOD MZ B cells to overcome Notch2 haploinsufficiency. To test the hypothesis that BCR-mediated signaling supports the MZ compartment that remains in Notch2+/−/NOD mice, we crossed Btk-deficient NOD mice with...
B cells, which show no statistically significant difference con-
in C57BL/6 mice. This finding contrasts with total numbers of
C for each genotype. For panels (0.05, **
Notch2+/-
identify MZ B cell populations for 125Tg B cells, which do not express IgD. (C) Percent of B cells that are MZ B cells in Notch2+/+ (black bars) versus Notch2−/− (gray bars) for 125Tg and WT (endogenous BCRs) on both NOD and C57BL/6 backgrounds. (D) Total numbers of MZ B cells for each genotype. (E) Fold change in MZ numbers conferred by Notch2 haploinsufficiency (i.e., number of MZ in Notch2+/− divided by number of MZ in Notch2−/−) for each genotype. For panels (C)-(E), n = 3–5 mice per group for NOD strains, n = 6–10 mice per group for C57BL/6 strains; all mice aged 12–13 wk. *p < 0.05, **p < 0.01, ***p < 0.001. Additional comparative significance values are available in Supplemental Table I.

**FIGURE 2.** B cells with anti-insulin BCRs (125Tg) maintain an enlarged MZ in Notch2−/−/NOD mice. Mice with B cells expressing Tg anti-insulin BCRs (125Tg) were crossed with Notch2−/− mice on both NOD and C57BL/6 backgrounds, and MZ compartments were analyzed by flow cytometry. (A) Flow cytometry showing biotinylated insulin binding by Tg IgM+ B cells from 125Tg/NOD (upper left panel) and 125Tg/Notch2−/−/NOD (upper right panel). Cells were gated on live lymphocytes. Lower panels show competitive inhibition, using 10-fold quantities of unlabeled insulin in parallel samples, to confirm insulin specificity of binding in upper panels. (B) Flow cytometry of live B220+IgM+ lymphocytes showing CD21/CD23 gating scheme used to identify MZ B cell populations for 125Tg B cells, which do not express IgD. (C) Percent of B cells that are MZ B cells in Notch2−/− (black bars) versus Notch2−/− (gray bars) for 125Tg and WT (endogenous BCRs) on both NOD and C57BL/6 backgrounds. (D) Total numbers of MZ B cells for each genotype. (E) Fold change in MZ numbers conferred by Notch2 haploinsufficiency (i.e., number of MZ in Notch2+/− divided by number of MZ in Notch2−/−) for each genotype. For panels (C)-(E), n = 3–5 mice per group for NOD strains, n = 6–10 mice per group for C57BL/6 strains; all mice aged 12–13 wk. *p < 0.05, **p < 0.01, ***p < 0.001. Additional comparative significance values are available in Supplemental Table I.

Notch2−/−/NOD mice. As shown in Fig. 3, the MZ B cell compartment in Btk-deficient Notch2−/−/NOD mice is obliterated (0.11 ± 0.04 × 10⁶ versus Notch2+/−/NOD 1.28 ± 0.48 × 10⁶, p < 0.01), similar to the effects of Notch2 haploinsufficiency alone in C57BL/6 mice. This finding contrasts with total numbers of B cells, which show no statistically significant difference conferred by the loss of BTK in Notch2−/−/NOD mice (12.1 ± 3.3 × 10⁶ versus 9.3 ± 2.3 × 10⁶, p = 0.16). These findings indicate that selection into the enlarged MZ of NOD mice relies on both Notch2 and BTK-mediated signaling. Of note, the Btk-deficient Notch2−/−/NOD model is not suitable for analysis of MZ contribution to disease outcome, as Btk-deficient NOD are already disease protected, and have other B cell abnormalities, such as signaling defects and an absent B1a compartment (25).

BTK supports elevated Notch2 expression and signaling in the NOD MZ compartment

The observation that BTK supports the development of the MZ suggests that BCR-mediated signaling may support the Notch2 signaling pathway in NOD mice. To determine the effect of BTK loss on the expression of Notch2 and its targets, we isolated B cells from WT or Btk−/− NOD mice and sorted them into FO, MZ, T2, and pMZ subsets (Fig. 4A, 4B). B cell subsets from WT and Btk−/− C57BL/6 mice were also analyzed to determine potential differences conferred by the two genetic backgrounds. Total RNA was isolated from each subset and reverse transcribed to generate cDNA. Real-time PCR was then used to analyze levels of Notch2 and its transcriptional target, Hes5. Ct values were normalized to HPRT transcript levels for each sample, and relative values for all subsets were compared. Notch2 levels were strikingly increased in WT NOD compared with WT B6 in the MZ (p < 0.0001) and pMZ (p < 0.0001) cell subsets. Hes5 expression, in parallel, was significantly increased in the WT NOD MZ compared with WT B6 MZ (p = 0.0081). Notch2 transcripts were found to be significantly decreased (p = 0.0012) in the pMZ compartment of Btknull NOD mice as compared with WT NOD controls (Fig. 4C). Accordingly, transcript levels of Hes5 were significantly decreased (p = 0.0413) in the MZ compartment of Btknull NOD mice, and a trend of decreased transcript levels is observed across all subsets compared with WT controls. In contrast, no significant difference was seen between WT B6 and Btknull B6. In addition, no difference was seen in Notch2 or Hes5 expression in FO B cells from any of the four groups (B6 versus NOD versus Btknull B6 versus Btknull NOD, not shown), nor was there a significant difference in Notch2 expression between preMZ and MZ in C56BL/6. These data suggest that BCR signaling, mediated by BTK, promotes the development of the MZ by supporting increased Notch2 expression in pMZ and MZ in C56BL/6. These data suggest that BCR signaling, mediated by BTK, promotes the development of the MZ by supporting increased Notch2 expression in pMZ NOD B cells, with downstream effects on Notch2 transcriptional activity that is most significant in MZ B cells. However, this effect is seen only in the NOD, which exhibits significantly more Notch2 transcription than the B6.

BTK-mediated support of MZ B cells depends on the quality of BCR–Ag interactions

Anti-insulin B cells expressed in the 125Tg model rely on both H chain and L chain transgenes, and are critically dependent on BTK,
comparative significance values are available in Supplemental Table II.

12- to 13-wk-old, *Notch2+/−* numbers in NOD (black bars) and analyzed for MZ numbers by flow cytometry. Upper panel *Notch2* affects expression of CD43+ cells were depleted using MACS, and in NOD, but not C57BL/6, B cells. *Hes5* mRNA. (A and B) Representative flow cytometry dotplots of WT (A) and *Btk*−/− (B) NOD mice show the cell purification scheme; IgM+IgD+B cells are gated by CD21+CD23+B for MZ cells, whereas IgD−CD23+B cells are gated by IgM−CD21+B for T2 and IgM+CD21+B for pMZ. (C and D) RNA was isolated from sorted B cells and analyzed by qRT-PCR. Ct values of *Notch2* (C) and *Hes5* (D) were normalized to *HPRT* transcript levels and shown as fold change compared with the WT B6 MZ. Mean ± SD is shown for WT B6 (black, solid), *Btk*−/− B6 (black, hatched), WT NOD (gray, solid), and *Btk*−/− NOD (gray, hatched); n = 3–4 mice per genotype. *p < 0.05, **p < 0.01, ***p < 0.001 as calculated by two-way ANOVA with a Sidak correction for multiple comparisons.

**FIGURE 3.** *Notch2+/−* NOD MZ compartment relies on BTK. Btk-deficient NOD mice were bred to *Notch2+/−* NOD mice, and offspring were analyzed for MZ numbers by flow cytometry. Upper panel, MZ B cell numbers in NOD (black bars) and *Notch2+/−* NOD (gray bars), in the presence of BTK (Btk-sufficient, left) or absence of BTK (Btk-deficient, right). Lower panel, Total B cell numbers from the same animals. Mice were 12- to 13-wk-old, n = 6–8 per group. **p < 0.01, ***p < 0.001. Additional comparative significance values are available in Supplemental Table II.

**FIGURE 4.** Btk deficiency negatively affects expression of *Notch2* and its target *Hes5* in NOD, but not C57BL/6, B cells. CD43+ cells were depleted using MACS, and B cells from WT C57BL/6 (B6) or NOD, and *Btk*−/− B6 or NOD, were sorted into MZ, T2, and pMZ cell subsets using flow cytometry and analyzed by qRT-PCR for expression of *Notch2* and *Hes5* mRNA. (A and B) Representative flow cytometry dotplots of WT (A) and *Btk*−/− (B) NOD mice show the cell purification scheme; IgM+IgD+B cells are gated by CD21+CD23+B for MZ cells, whereas IgD−CD23+B cells are gated by IgM−CD21+B for T2 and IgM+CD21+B for pMZ. (C and D) RNA was isolated from sorted B cells and analyzed by qRT-PCR. Ct values of *Notch2* (C) and *Hes5* (D) were normalized to *HPRT* transcript levels and shown as fold change compared with the WT B6 MZ. Mean ± SD is shown for WT B6 (black, solid), *Btk*−/− B6 (black, hatched), WT NOD (gray, solid), and *Btk*−/− NOD (gray, hatched); n = 3–4 mice per genotype. *p < 0.05, **p < 0.01, ***p < 0.001 as calculated by two-way ANOVA with a Sidak correction for multiple comparisons.
BTK AND Notch2 IN NOD MARGINAL ZONE B CELLS

To determine the Notch2 dependence of differentially autoreactive anti-insulin B cells, we next crossed Notch2 haploinsufficiency onto the Vκ125Tg/NOD model (Fig. 6). The percentages of both Low and High Autoreactive populations that distributed into the MZ were greatly reduced in Vκ125Tg/Notch2+/NOD (High Auto MZ: 14.7 ± 12% versus 46.7 ± 15.7%, p < 0.001; Low Auto MZ: 11.7 ± 7.4% versus 40.9 ± 22.2%, p = 0.002, Fig. 6B). Conversely, the percentages of anti-insulin B cells in the T2/FO compartment were increased in Vκ125Tg/Notch2+/NOD (High Auto T2/FO: 77.5 ± 12.5% versus 47.6 ± 12.9%, p < 0.001; Low Auto T2/FO: 77.7 ± 8.7% versus 51.4 ± 18.6% p = 0.002, Fig. 6B). The percentages of non–insulin-binding B cells in the Vκ125Tg/Notch2+/NOD mice were also reduced in the MZ compartment (10.6 ± 3.1% versus 40.9 ± 11.5%, p < 0.001) and increased in the T2/FO compartment (79.1 ± 5.0% versus 51.7 ± 8.5%, p < 0.001, Fig. 6B). The reduced MZ percentage in Vκ125Tg/Notch2+/NOD mice was reflected in decreased numbers of anti-insulin MZ B cells in both the Low and High Auto populations (High Auto MZ: 6.0 ± 4.9 × 10³ versus 24.2 ± 18.1 × 10³, p = 0.015; Low Auto MZ: 4.9 ± 3.0 × 10³ versus 29.8 ± 29.4 × 10³, p = 0.03), as well as in numbers of non–insulin-binding MZ B cells (681 ± 249 × 10³ versus 4863 ± 3441 × 10³, p = 0.004, Fig. 6C). The average numbers of High Auto anti-insulin B cells in the T2/FO compartment were increased (44.3 × 10³ versus 20.8 × 10³, p = 0.04). The average numbers of Low Auto T2/FO B cells (40.3 × 10³ versus 29.6 × 10³, p = 0.23) or non–insulin-binding T2/FO B cells (5341 × 10³ versus 5365 × 10³) were not different. Overall, total insulin-binding B cell numbers were not significantly different in Notch2+/− versus Notch2+/− Vκ125Tg/NOD mice (89.1 ± 43.0 versus 110.6 ± 61.7 × 10³, p = 0.42). These data show that Notch2 haploinsufficiency reduces insulin-binding B cells in the MZ, but does not preferentially impact the Low Autoreactive population nor decrease the overall numbers of these autoreactive cells.

Discussion

The enlarged MZ B cell compartment in NOD mice has been implicated in type 1 diabetes development, but the mechanisms underlying this expansion have not been well understood (5). The studies presented in this article show that NOD MZ B cells are surprisingly less reliant on Notch2 than their C57BL/6 counterparts. Whereas Notch2+/−/C57BL/6 mice have very few MZ B cells, Notch2+/−/NOD retain a robust MZ (Figs. 1, 2). In accordance with these findings, analysis of transcript expression of Notch2, and its downstream target Hes5, show that they are more highly expressed in NOD MZ cells than in C57BL/6 MZ cells (Fig. 4), suggesting that the single allele remaining in the Notch2+/−/NOD model provides enough signal to drive development of a MZ, commensurate in size with WT C57BL/6. This relative normalization of numbers of MZ B cells in Notch2+/−/NOD mice does not of cells in Vκ125/NOD divided by number of cells in BTKmut/Vκ125/NOD for each subset). Error bars represent SD. *p < 0.05, **p < 0.01. Additional comparative significance values are available in Supplemental Table II.
reduce diabetes, however, indicating that numeric expansion of this compartment is not a primary driving factor in diabetes development (Fig. 1). Surprisingly, we find that increased Notch2 found in NOD mice relies on BTK, as Btk deficiency reduces Notch2 overexpression in NOD cells, without having any effect in the nonautoimmune setting (Fig. 4). Likewise, Notch2+/2/NOD MZ B cells are highly dependent upon BTK, again differing from nonautoimmune mice, in which loss of BTK mainly affects the FO compartment (Ref. 16 and Fig. 3). These data provide a direct link between BTK and Notch2 in the NOD model, and suggest that BCR-mediated signaling interacts with the Notch2 pathway for selection and maintenance of NOD MZ B cells. This idea is reinforced by studies using anti-insulin B cell transgenes to show that BCR specificities contribute to MZ selection and are fine tuned in their reliance on BTK, as one VH125 clonotype is reduced by BTK deficiency, whereas the other is not (Fig. 5). Finally, these transgenes also reveal that impairment of Notch2 shifts autoreactive cells out of the MZ but does not eliminate them, leaving them available to participate in type 1 diabetes. Future studies using B cell–specific homozygous deletion of Notch2 in NOD mice would be useful in further evaluating both the dependency of NOD MZ B cells on Notch2 and their role in the disease process.

Notch2 contribution to MZ development in nonautoimmune strains has been demonstrated using both global genetic haploinsufficiency and B cell–specific homozygous deficiency. Studies using Notch2+/2/C57BL/6 mice showed near-complete elimination of the MZ (12), as did those with B cell–specific homozygous deletion, with no further effects on B cells (8). These comparable phenotypes seemed to indicate that MZ B cells are so dependent upon Notch2 that even partial depletion by haploinsufficiency can block their development as well as full genetic ablation does. However, this may not be the case in autoimmune strains. Our data are complementary to previous work showing that blockade of the Notch ligand D-like 1 eliminated the MZ in C57BL/6, whereas a similarly enlarged MZ in lupus-prone BWF mice was partially resistant to this treatment (33). The findings reported in this article suggest that Notch2 overexpression in NOD B cells, driven by BCR-mediated signaling, underlies the increased MZ in this autoimmune strain and is the most likely reason for its resistance to depletion using haploinsufficiency.

Alterations in cell trafficking have also been implicated in MZ differences between NOD and nonautoimmune strains (6). Because of this, we also tested surface levels of the homeostatic B cell chemokine receptor CXCR5 in Notch2+/2/NOD versus WT NOD
and C57BL/6, but saw no differences (Supplemental Fig. 1). Nevertheless, many other chemotactic factors and integrins could play a role and deserve future study. Of note, the same report, which relied on microarray to assess differences, did not reveal the Notch2 overexpression that we found using qRT-PCR. The reason for this is not clear but could be due to variability either between the assays, with qRT-PCR being more sensitive, or in the cell sorting method. We found that sorting into subsets that differentiated between T2 and FO, as well as pMZ and MZ, was helpful in identifying differences, whereas broader, more heterogeneous categories were less sensitive for these purposes, and may have missed nuances between subsets.

Cell signaling responses that drive MZ B cell development, selection, and support are complex and incompletely understood, even in nonautoimmune mice. Integration of signals from the BCR with Notch2, BAFFr, and various chemokine receptors contribute, as do components of the extracellular matrix (2, 6, 20). The classic model of MZ B cell selection and development suggests that low-affinity, or weak, BCR signals mediate MZ B cell selection in a BAFFr- and BTK-independent manner, whereas stronger, tonic signals mediated by BAFFr and BTK drive FO B cell development. Multiple studies have shown that MZ B cells with endogenous BCRs in nonautoimmune strains do not require BTK (16, 17). Therefore, we were surprised to find that loss of BTK almost completely eliminated the NOD MZ compartment that remained in Notch2+/−/NOD mice. Our previous studies showed a small but statistically significant decrease in MB2 cells in Btk-deficient NOD mice, with concomitant increase in pMZ2 cells, indicating a partial block in maturation (25). Our new findings show that loss of BTK also significantly reduces Notch2 transcript expression in pMZ2 B cells, and significantly decreases its transcriptional target, Hex5, in MZ2 B cells (Fig. 4), indicative of loss of function that is consistent with the block at the pMZ stage and decreased MZ, in these mice. Of interest, Hex5 expression differences lag behind Notch2 expression differences in these models, trending downward in BTK-deficient NOD pMZ but not becoming statistically significantly different from WT NOD until the MZ stage. At the same time, Notch2 expression, although still trending lower than WT NOD, loses statistical significance at the MZ stage. Because this assay measures transcripts, rather than protein, this may reflect altered surface Notch2 protein expression, or its rapid turnover in the absence of BTK. Of note, pMZ in C57BL/6 have a similar disconnect between Notch2 transcript expression, which is quite low, and Hex5, which does not differ significantly from NOD at this stage. Again, this may indicate that surface levels of the Notch2 protein are adequate, reducing further transcription in B6 pMZ2 B cells. Overall, these combined discoveries suggest that selection of some NOD B cells into the MZ depends on BTK, contrasting classic characteristics of MZ B cells in nonautoimmune strains.

Direct crosstalk between BTK-mediated pathways and those of Notch2 have not been shown before. In fact, it has been postulated that BTK might function as a negative regulator of factors that drive MZ development (34). A classic study showed lack of this compartment in mice deficient for Aiolos, a negative regulator. When BTK deficiency (xid) was used to balance Aiolos deficiency, the MZ was restored (15). However, this same strategy failed to reverse the reduced MZ compartment found in NF-κB1/p50 mice, and in fact further decreased MZ numbers, although this was not statistically significant (34). This same report showed that NF-κB1/p50 also supports expression of both Hex5 and Deltex1, transcriptional targets of Notch2. Thus, the NF-κB pathway, a downstream target of BTK-mediated signaling, seems a likely candidate for linking BTK with Notch2 in some models, and in fact, NF-κB has been shown to have increased activity in NOD B cells, further supporting this concept (35).

Of note, we also tested potential effects of Notch2 haploinsufficiency on BCR-mediated signaling, including phosphorylation of Syk, BLNK, and PLCy2, and found no differences (Supplemental Fig. 2). CD19 provides support for activating signals, is more highly expressed in NOD B cells, and has been linked to MZ development, but examination of Notch2+/−/NOD B cells showed no alteration in CD19 expression compared with WT NOD (Supplemental Fig. 1). Thus, although BTK appears to support the Notch2 pathway in NOD B cells, we did not find evidence of the reverse.

Two recent additional studies, when taken together, also support the idea that BTK may negatively regulate Notch2 via the transcription factor IFN regulatory factor 4 (IRF4) in nonautoimmune mice. One of these showed that inducible B cell–specific deletion of Irf4 increased Notch2 expression and caused accumulation of B cells in the MZ (28). A second study showed that the BTK inhibitor ibrutinib decreases IRF4 expression, which is responsive to BCR signaling effects (36). These combined findings would predict that BTK deficiency should decrease IRF4 expression, which in turn should increase Notch2 and MZ B cells, the opposite of our experimental results. Several differences in the experimental models could account for this unexpected outcome. IRF4 has various functions that are context dependent, and graded, in both normal B cells and B cell tumors. The ibrutinib studies were performed on human activated B cell–like diffuse large B cell lymphoma cell lines, in which IRF4 expression is increased in response to continuous BCR signaling resulting from mutations of proteins in the signaling pathway. These circumstances likely do not reflect events during development in normal murine B cells. Similarly, the B cell–inducible Irf4−/− model provides insight into IRF4 actions in mature C57BL/6 B cells, producing Notch2 effects within an established, mature B cell milieu. In contrast, Btk deficiency in the Notch2+/−/NOD model affects B cells throughout the life cycle and shows that decreased BTK-mediated signaling in developing and mature B cells does not induce increased MZ B cell development. Finally, NOD B cells used in this study may also have subtle differences in signaling responses that would contribute to different outcomes from those in human tumor cells or murine B cells from nonautoimmune mice.

Because BTK contributes to both BCR and BAFFr signaling (37, 38), it is possible that both pathways may be affected in this process. However, the dependence of one, but not both, anti-insulin populations on BTK in the Vγ125 model indicates that BCR-mediated signaling is a critical component. In this model, two insulin-binding populations are generated by different endogenous L chains that pair with the anti-insulin Tg H chain (Fig. 5). Anti-insulin B cells with relatively lower rodent insulin binding (22) are preferentially selected into the MZ and depend upon BTK, as their numbers are reduced by its genetic ablation (Fig. 5). This finding is in contrast to what is observed in cells with higher relative rodent insulin binding, which are evenly distributed between MZ and FO and are not numerically reduced by loss of BTK. These differing outcomes based on specificity may also contribute to a previously published model using an anti-DNA Tg BCR, 56R, which was determined to rely on BTK for MZ localization, but not survival (19). Of note, anti-insulin 125Tg B cells (in which both anti-insulin H and L chain transgenes are expressed, Fig. 2) are highly dependent on BTK, and both FO and MZ B cells are depleted by >95%, regardless of background strain (30). Thus, fine-tuned differences in BCR specificity affect reliance on BTK for MZ selection and further affect reliance on BTK for survival. B cells from autoimmune-prone mice have...
altered signaling thresholds, which reduce tolerance and generate increased numbers of BCRs with specificities that may be suitable for entry into the MZ (21, 22, 24). This feature may simply provide a numeric advantage that allows discerrmination of a partial effect of Notch2 haploinsufficiency that may be present, but less visible, in strains with smaller MZs. The effect of this property is seen in the anti-insulin BCR Tg model (Fig. 2), in which the MZ is expanded on both B6 and NOD models and shows partial resistance to elimination by Notch2 haploinsufficiency. Such a mechanism may also be represented by the TgVh3B4 model, developed from a “natural,” polyreactive Ab, which generates an enlarged MZ. These mice also maintained a small MZ in the absence of the Notch downstream transcription factor RBP-J, despite being on the C57BL/6 background (39). Thus, in both of these models, Tg B cells with autoreactive specificities were more likely to enter the MZ, and to be partially resistant to impairment in Notch2 signaling even on a nonautoimmune background, confirming that increased numbers of autoreactive B cells can affect Notch2 dependence. Importantly, the anti-insulin 125Tg studies presented in this article provide an opportunity to evaluate effects of BCR specificity and the NOD genetic background concurrently. As shown in Fig. 2, anti-insulin B cells make up >95% of splenic B cells in this model, providing a uniform population for study across models. These autoreactive cells are more likely to go to the MZ and to be partially Notch2 independent in both models, showing the importance of BCR-mediated signaling in this process. However, in NOD mice an additional effect clearly transcends BCR specificity, as 125Tg/NOD have very exaggerated MZ populations, comprising about half of B cells in NOD mice and nearly a third even in Notch2−/− NOD mice. Therefore, these studies show clearly that both BCR specificity and other aspects of the NOD background contribute to the enlarged MZ seen in NOD mice. Overall, our data show that the enlarged MZ B cell compartment in NOD mice has reduced dependence on Notch2, and an unexpected dependence on BTK, that contrasts with nonautoimmune C57BL/6. Use of anti-insulin Tg B cell models also shows that contributions of BCR-mediated signaling and BTK dependence rely on the quality of BCR interactions with Ag. Of potential clinical importance is the finding that impairment in BTK-mediated signaling reduces total numbers of anti-insulin B cells, whereas Notch2 impairment shifts cells out of the MZ but does not significantly reduce autoreactive cell numbers. These findings contribute to a growing body of literature defining the complexity underlying development of this autoimmune-prone subset of cells. Future development of methods to specifically eliminate the MZ subset in NOD mice are needed to better understand the contributions of these cells to autoimmune disease.

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References


