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Reduced Diabetes in \textit{btk}-Deficient Nonobese Diabetic Mice and Restoration of Diabetes with Provision of an Anti-Insulin IgH Chain Transgene\textsuperscript{1}

Peggy L. Kendall,\textsuperscript{2,*} Daniel J. Moore,\textsuperscript{†} Chrys Hubert,\textsuperscript{*} Kristen L. Hoek,\textsuperscript{‡} Wasif N. Khan,\textsuperscript{‡§} and James W. Thomas\textsuperscript{2*‡}

Type 1 diabetes results from T cell-mediated destruction of insulin-producing \(\beta\) cells. Although elimination of B lymphocytes has proven successful at preventing disease, modulation of B cell function as a means to prevent type 1 diabetes has not been investigated. The development, fate, and function of B lymphocytes depend upon BCR signaling, which is mediated in part by Bruton’s tyrosine kinase (BTK). When introduced into NOD mice, \(btk\) deficiency only modestly reduces B cell numbers, but dramatically protects against diabetes. In NOD, \(btk\) deficiency mirrors changes in B cell subsets seen in other strains, but also improves B cell-related tolerance, as indicated by failure to generate insulin autoantibodies. Introduction of an anti-insulin BCR H chain transgene restores diabetes in \(btk\)-deficient NOD mice, indicating that \(btk\)-deficient B cells are functionally capable of promoting autoimmune diabetes if they have a critical autoimmune specificity. This suggests that the disease-protective effect of \(btk\) deficiency may reflect a lack of autoreactive specificities in the B cell repertoire. Thus, signaling via BTK can be modulated to improve B cell tolerance, and prevent T cell-mediated autoimmune diabetes. \textit{The Journal of Immunology}, 2009, 183: 6403–6412.

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selection in the spleen, with consequent reduction in follicular (FO) B cell numbers. These mice have also been used to show that B cell proliferation depends upon signals mediated by BTK (20–23). Btk deficiency in autoimmune mouse strains, including lupus-prone MLR and NZB mice, has shown protective effects, and a dose-dependent protective effect against autoimmunity has also been shown in lyn−/− mice (24–33).

We introduced btk deficiency into NOD mice to assess the effectiveness of targeting BCR signaling pathways in the prevention of autoimmune diabetes. The data show btk deficiency affords significant protection against T1D. Failure of anti-insulin IgG to develop in btk-deficient NOD mice suggests that B cell-related tolerance is improved by loss of BTK. The protection from diabetes provided by btk deficiency is reversed by an anti-insulin Ig transgene (VH125), even though the number of anti-insulin B cells available in this model is significantly decreased by loss of BTK. Marginal zone (MZ) and B1a subsets, often implicated in this disease, are decreased in the btk-deficient model, and do not recover when disease is restored in VH125 btk-deficient NOD mice. Furthermore, regulatory T cells are not increased as they are in some models of B cell-targeted disease protection (14, 15). The overall results indicate that total B cell depletion is not necessary to prevent the development of T1D in NOD mice. Rather, for the first time, we show that targeting of BCR signaling pathways can re-inforce B cell tolerance, with downstream protection against a T cell-mediated autoimmune disease.

Materials and Methods

Mice

Btk-deficient NOD mice were generated by breeding NOD with a C57BL/6 progenitor carrying a transgenically engineered mutation in the gene encoding for BTK. This X-linked mutation results in a failure of BTK protein production, and a phenotype that mimics the sid strain (23). Offspring carrying the mutation were backcrossed with pure NOD, using a speed congenic method (9), in which breeders were selected for the presence of idd loci at the third and fourth backcrosses (N4 and N5, respectively). Table I indicates disease-associated linkage markers for which these breeders were homozygous. At the N7 and N11 generations, btk−/− NOD males were intercrossed with btk−/− NOD females to produce btk−/− female offspring for disease study. This intercross produces no btk−/− NOD females. However, a single copy of an intact btk-encoding gene is sufficient for normal B cell development and responses, as would be expected from an X-linked, recessive trait. Btk−/− females exhibit no phenotypic differences from wild type in our studies, or in studies of the original strain. Furthermore, diabetes occurred in multiple generations of btk−/− NOD beginning with the N4 female breeder, with disease penetrance and age of onset consistent with that of our wild-type NOD colony. Therefore, btk−/−/− NOD females have been used as btk-sufficient controls for their btk−/−/− NOD littermates. VH125/NOD mice were generated, as previously described (13), and are maintained by backcrossing hemizygotes to wild-type

Table I. Homozygous NOD idd loci in btk−/− NOD mice selected for breeding at the third and fourth backcrosses

<table>
<thead>
<tr>
<th>idd Locus/Chromosome</th>
<th>Linkage Marker</th>
</tr>
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<tbody>
<tr>
<td>idd1 = H2g7/17</td>
<td>D17mit34 = C4</td>
</tr>
<tr>
<td>idd3/3</td>
<td>D3mit95</td>
</tr>
<tr>
<td>idd4/11</td>
<td>D11mit320</td>
</tr>
<tr>
<td>idd5/1</td>
<td>D1mit18</td>
</tr>
<tr>
<td>idd6/6</td>
<td>D6mit339</td>
</tr>
<tr>
<td>idd7/7</td>
<td>D7mit20</td>
</tr>
<tr>
<td>idd8, Idd12/14</td>
<td>D14mit110</td>
</tr>
<tr>
<td>idd9, Idd12/14</td>
<td>D14mit222</td>
</tr>
<tr>
<td>idd10/5</td>
<td>D4mit59</td>
</tr>
<tr>
<td>idd13/2</td>
<td>D3mit103</td>
</tr>
<tr>
<td>idd14/13</td>
<td>D2mit395</td>
</tr>
<tr>
<td>idd15/5</td>
<td>D13mit64</td>
</tr>
<tr>
<td></td>
<td>D5mit48</td>
</tr>
</tbody>
</table>

NOD mice. Hemizygote VH125/NOD were crossed onto the btk-deficient NOD line at the N11 and subsequent generations. Mice are housed in specific pathogen-free conditions. The Institutional Animal Care and Use Committee of Vanderbilt University have approved all procedures.

Disease and insulitis studies

Blood glucose levels were measured weekly beginning at age 12 wk. Diabetes was diagnosed by blood glucose readings above 200 mg/dL, confirmed by readings above that level in follow-up weeks. Age of diabetes development reported is that of first reading.

Insulitis was examined both by H&E and immunofluorescent staining. For H&E staining, 5-μm sections were examined. At least 50 μm was skipped when sectioning to avoid redundant counting of individual islets. All islets on every section were imaged and scored for infiltration. Immunofluorescent images were obtained by fixing pancreata in 4% paraformaldehyde in a high phosphate buffer, sectioning, staining, and imaging, as previously described (34). Sections were stained using anti-B220 FITC and anti-CD3 PE from BD Biosciences, then pseudocolored red and blue, respectively, using Adobe Photoshop.

Flow cytometry

Lymphocytes obtained from spleen, pancreatic lymph nodes, peritoneal cavities, and pancreas, as previously described (34), were stained with fluorochrome-conjugated Abs to B220, IgM, IgD, CD21, CD23, CD86, CD80, IaK (clone 10-3-6, cross-reactive with IaG7), CD5, CD4, and CD8 from BD Pharmingen. The 7-aminocyclometinium D was used to exclude dead cells. Biotinylated insulin followed by streptavidin–fluorochrome secondary reagent was used to identify insulin-specific B cells. CD25+CD4+ Foxp3+ cells were identified using mouse regulatory T cell staining kit no. 2, by ebioscience, following manufacturer’s instructions. Data were collected on a FACSCalibur flow cytometer (BD Biosciences) or LSRII (BD Biosciences) and analyzed using WinMDI (J. Trotter, Scripps Institute, San Diego, CA) or FlowJo (Tree Star) software.

Cell stimulation assays

For proliferation studies, B cells were purified from spleen by passage through MACS columns (Miltenyi Biotech) after RBC lysis, followed by incubation with anti-CD43 beads (Miltenyi Biotech), following manufacturer’s instructions. B cells from the negative fraction (purity >85%) were cultured for 2 days, pulsed with [3H]thymidine (NEN), and harvested on day 3, as previously described (35). FIba1-2 goat anti-mouse m-chain (Jackson ImmunoResearch Laboratories), LPS, Escherichia coli B (Difco), and anti-CD40 (BD Biosciences; clone HM40-3) were used as mitogens. Scintillation counting after stimulation was used to measure [3H]thymidine uptake. Results for triplicate determinations are reported as the mean ± SD. For analysis of surface expression of Ag presentation and costimulation molecules, splenocytes were harvested for analysis by flow cytometry 16 h after plating.

CFSE labeling and anti-CD3 stimulation

CFSE labeling was performed, as previously described (36). Briefly, splenocytes were resuspended at a concentration of 10 × 106 cells/ml in serum-free DMEM at 37°C. An equal volume of a 1/350 dilution of the CFSE stock (5 mM in DMSO) was added to the cell preparation, which was subsequently incubated for 5 min at 37°C. CFSE labeling was quenched by adding an equal volume of heat-inactivated FCS, whereupon cells were washed twice and resuspended in DMEM containing 10% FCS. For stimulation, 375,000 labeled splenocytes were resuspended at a concentration of 106 cells/ml in 200 μl/well DMEM containing 10% FCS, whereupon cells were washed twice and resuspended in 100 μl/well human insulin and then washed. Mouse sera, diluted 1/100, were added before plating. Duplicate wells containing 100 ng/well human insulin for inhibition are plated in tandem. Goat anti-mouse IgG conjugated to alkaline phosphatase (Southern Biotechnology Associates), in 1:300 in veronyl-buffered saline

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Twenty-seven percent of wild-type NOD mice remained healthy (n = 31% of development in NOD mice, To understand how a defect in B cell signaling affects disease in NOD mice

**Results**

**Impaired BCR signaling protects against the development of diabetes in NOD mice**

To understand how a defect in B cell signaling affects disease development in NOD mice, btk deficiency was introgressed onto NOD, and cohorts of btk-deficient and btk-sufficient female littermates were monitored for diabetes. Because this X-linked model provides only btk+/− (btk-sufficient) littermate controls for btk−/− (btk-deficient) female offspring, wild-type NOD mice from our colony were also included as controls. As shown in Fig. 1, loss of BTK results in significant disease protection, with 83% of the study group remaining healthy to age 30 wk, compared with only 31% of btk-sufficient NOD controls (log rank, p = 0.002) and 27% of wild-type NOD controls (p = 0.001). Disease rate did not differ significantly between wild-type and btk-sufficient controls (p = 0.63). These data show that the progression of diabetes in NOD mice is significantly impaired in the absence of BTK.

**Btk deficiency in NOD mice reduces mature B cell subsets**

BTK deficiency in nonautoimmune murine strains blocks maturation of B cells through the late transitional T2 checkpoint, with a corresponding decrease in FO cells, and a 50% reduction in total numbers of splenic B cells (23). Wild-type NOD mice have alterations in peripheral B cell maturation that include increased MZ B cells and a reduction in the early transitional T1 subset (35, 40). To determine how BTK affects the fate of lymphocytes in NOD mice, the numbers and subsets of B cells in 13- to 16-wk-old prediabetic female btk-deficient NOD mice were examined and compared with btk-sufficient NOD littermates, matched for age, gender, and prediabetic status. As shown in Table II, total B cell numbers in the spleen are reduced by 18% in btk-deficient (20.6 ± 6.4 × 10^6) compared with btk-sufficient (25 ± 3.3 × 10^6) NOD mice (btk sufficient, n = 12; btk deficient, n = 8; p = 0.069 by Wilcoxon-Mann-Whitney (WMW) two-sample rank sum test). B cell numbers in draining pancreatic lymph nodes are also reduced by 19% (0.58 ± 0.29 × 10^6 in btk deficient, n = 3, vs 0.72 ± 0.27 × 10^6 in btk sufficient, n = 5; p = 1 by WMW).

Flow cytometry was also used to examine how btk deficiency alters the development of B cell subsets in the spleens of NOD mice (Fig. 2). Data for representative splenic B cell subpopulations in btk-sufficient (Fig. 2a) and btk-deficient NOD mice (Fig. 2b) show the gating patterns used for subset definitions. The results comparing total numbers of B cells in individual compartments from multiple mice are summarized in Fig. 2c, and cell proportions and total numbers are shown in Table II. Mature FO B cells (IgD+/CD23high/CD21intermediate/IgMintermediate) are substantially reduced

![Graph showing the percentage of non-diabetic individuals over weeks of age](image)

**Figure 1.** Impaired B cell signaling in NOD mice provides significant protection against diabetes. Kaplan-Meier survival curve showing percentage of diabetes-free mice plotted against age in weeks. Blood glucose levels were monitored weekly in btk-deficient female NOD (squares) and btk-sufficient female NOD littermates (circles) to the age of 30 wk. Mice were considered diabetic at the time of the first of two consecutive weekly blood glucose levels above 200 mg/dL. Because this X-linked deficiency yields no btk−/− littermate controls, conventional female NOD (wild-type (WT)) mice from our colony were also included (triangles). Eighty-three percent of wild-type NOD mice (n = 18) remained diabetes free, compared with 31% of btk-sufficient NOD mice (n = 16). Log rank, p = 0.002.

**Table II. Lymphocyte distributions in spleen and draining pancreatic lymph nodes of btk-deficient vs btk-sufficient female NOD mice**

<table>
<thead>
<tr>
<th>Subset</th>
<th>Percentage</th>
<th>No. Cells × 10^6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Btk Sufficient</td>
<td>Btk Deficient</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B cells</td>
<td>39.5% ± 4.4%</td>
<td>30.9% ± 4.3%**</td>
</tr>
<tr>
<td>FO</td>
<td>33.6% ± 6.4%</td>
<td>12.3% ± 2.2%**</td>
</tr>
<tr>
<td>MZ</td>
<td>27.1% ± 3.1%</td>
<td>21.7% ± 7.7% NS</td>
</tr>
<tr>
<td>Pre-MZ</td>
<td>5.5% ± 1.8%</td>
<td>9.5% ± 0.1%***</td>
</tr>
<tr>
<td>T2</td>
<td>8.9% ± 1.6%</td>
<td>28.4% ± 6.5%**</td>
</tr>
<tr>
<td>T1</td>
<td>6.7% ± 1.6%</td>
<td>7.6% ± 2.3% NS</td>
</tr>
<tr>
<td>CD4+</td>
<td>28.5% ± 2.1%</td>
<td>33.1% ± 2.5%**</td>
</tr>
<tr>
<td>CD8+</td>
<td>14.3% ± 1.5%</td>
<td>16.3% ± 1.9%†</td>
</tr>
<tr>
<td>Tregsb</td>
<td>2.8% ± 0.13%</td>
<td>3.3% ± 0.43% NS</td>
</tr>
<tr>
<td><strong>Pancreatic lymph nodes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B cells</td>
<td>17.3% ± 4.6%</td>
<td>12.6% ± 3.3% NS</td>
</tr>
<tr>
<td>CD4+</td>
<td>55.9% ± 2.3%</td>
<td>57.6% ± 3.1% NS</td>
</tr>
<tr>
<td>CD8+</td>
<td>23.2% ± 2.0%</td>
<td>24.9% ± 1.4% NS</td>
</tr>
<tr>
<td>Tregsb</td>
<td>4.27% ± 0.02%</td>
<td>4.9% ± 0.03% NS</td>
</tr>
</tbody>
</table>

†, p = 0.04; *, p = 0.004; **, p = 0.001; NS, p > 0.05.
bTregs, T regulatory cells.
in *btk*-deficient (3 ± 0.89 × 10^6), compared with *btk*-sufficient littermates (8.7 ± 1.98 × 10^6; p = 0.001). The percentage of late transitional T2 B cells (IgD^-/CD23^hi/CD21^int) is observed to be increased in *btk*-deficient compared with *btk*-sufficient NOD (7.06 ± 2.6 × 10^6 vs 2.28 ± 0.42 × 10^6, respectively; p = 0.004). Pre-MZ (IgD^-/CD23^hi/CD21^hi) and NOD mice (IgD^-/CD23^hi/CD21^hi) numbers are higher in *btk*-deficient compared with *btk*-sufficient NOD mice (2.32 ± 0.52 × 10^6 vs 1.41 ± 0.47 × 10^6; p = 0.004) with a corresponding decrease in mature MZ cells (IgD^-/CD23^low/CD21^hi) (5.23 ± 1.71 × 10^6 vs 6.97 ± 0.75 × 10^6; p = 0.042). (Btk-deficient NOD mice, n = 9; *btk*-deficient NOD mice, n = 5 for all B cell subset studies; p values obtained by WMW.)

As seen in nonautoimmune strains, the peritoneal B1a population (CD5^-/B220^low) is reduced by 90% in *btk*-deficient NOD mice compared with *btk*-sufficient animals, which have normal percentages (Fig. 2d). Bar chart shows averages, with SDs (btk deficient, n = 5; *btk* sufficient, n = 7; *p* = 0.003, WMW).

CD4^+^, CD8^+^, and regulatory T cell numbers are not altered by *btk* deficiency

T cell proportions and numbers were analyzed for effects of *btk* deficiency. Total numbers of CD4^+^ and CD8^+^ T cells are not significantly altered in *btk*-deficient NOD spleens or draining pancreatic lymph nodes (Table II, spleen, *btk* deficient, n = 8, and *btk* deficient, n = 6, *p* = 0.135 for CD4^+^ T cell numbers, *p* = 0.282 for CD8^+^ T cell numbers; pancreatic lymph nodes, *btk* deficient, n = 3, and *btk* sufficient, n = 5, *p* = 0.25 for both CD4^+^ and CD8^+^ cell numbers; all *p* values obtained by WMW.)

Regulatory T cell populations are expanded in some B cell-targeted disease-protective studies (14, 15). Regulatory T cell populations were therefore analyzed, using staining for CD4^+^/CD25^+^/Foxp3^+^.
CD25+/Foxp3+ in btk-deficient and btk-sufficient mice (Fig. 2, f and g, and Table II). No significant differences could be found in spleen, draining pancreatic lymph nodes, or pancreata (spleen, btk sufficient, n = 9, and btk deficient, n = 5, p = 0.112; pancreatic lymph nodes, btk sufficient, n = 5, and btk deficient, n = 3, p = 0.14; pancreas, as percentage of CD45, btk sufficient, n = 3, and btk deficient, n = 3, p = 0.7 by WMW; all mice used for T cell analysis were prediabetic 13- to 16-wk-old females). Thus, the disease protection afforded by btk-deficient B cells is not associated with substantially increased proportions of regulatory T cells.

**Insulitis develops in btk-deficient NOD mice**

B cell-targeted therapies for TID frequently result in reduced or absent insulitis (9, 14, 16). In addition, btk-deficient B cells have been shown to be defective in their chemotactic responses (41), indicating the possibility that they might fail to immigrate to inflamed islets. We therefore used H&E staining of pancreata to evaluate insulitis in 13- to 16-wk-old prediabetic female btk-deficient NOD mice, and found that, with one exception, these mice develop insulitis as efficiently as btk-sufficient littermates. Of five btk-deficient pancreata examined, one had little insulitis, with only 2 of 83 islets showing peri-insulitis, and the rest being completely healthy. This outlier was left out of the overall insulitis counts to avoid artificial skewing of the data. The other four btk-deficient mice had insulitis that was similar to btk-sufficient controls (Fig. 2e, bar chart; btk sufficient, n = 6, and islet, n = 360; btk deficient, n = 4, and islet, n = 143).

Immunofluorescent staining was used to evaluate B and T cell distributions in the islets. Fig. 2e shows typical immunofluorescent staining of a frozen pancreatic section in which B220+ B lymphocytes (red) and CD3+ (blue) T lymphocytes infiltrate pancreatic islets in btk-sufficient (left) and btk-deficient (right) NOD mice, illustrating that btk deficiency does not alter the presence of B lymphocytes in the islets, nor the typical tertiary lymphoid structure, with B lymphocytes gathered at the periphery of the T cell zone. Flow cytometry performed on lymphocytes from pancreata also failed to show alterations in B cell, CD44+, or CD8+ B cell proportions in btk-deficient vs btk-sufficient pancreata (data not shown).

**Btk-deficient NOD B lymphocytes have impaired proliferative responses to stimulation by both innate and adaptive pathways**

BTK mediates cellular proliferation induced by BCR ligation, TLR4 engagement, and CD40 stimulation (23, 42–44). Because B cells from NOD mice are characterized by heightened responses in all of these pathways, we examined the impact of btk deficiency on B cell proliferation in NOD mice (Fig. 3). B cells from btk-deficient or btk-sufficient NOD mice were purified and cultured in the presence of anti-IgM (a), LPS (b), or anti-CD40 (c), and tritiated thymidine was used to measure B lymphocyte proliferation. Btk-sufficient NOD B cells generate a robust proliferative response to anti-IgM, whereas btk-deficient B cells fail to proliferate to any dose of anti-IgM. B cell proliferation following stimulation with either LPS or anti-CD40 is detected in btk-deficient NOD, but the responses are blunted in comparison with btk-sufficient NOD B cells. These data show that BTK participates in NOD B cell proliferative responses to Ag-BCR ligation, as well as to TLR4 and CD40 stimulation. These findings do not differ from published studies of BTK-mediated cellular responses of B cells from non-autoimmune C57BL/6 mice (23, 44).
A BTK deficiency does not impair expression of costimulatory molecules necessary for T cell activation

Substantial evidence implicates Ag presentation as the mechanism of B cell contribution to T1D (11, 12). This process requires increased expression of costimulatory molecules on the surface, including CD86. Accordingly, we examined the contribution of BTK to the expression of CD86 in response to signals via the BCR, TLR4, and CD40 in NOD B cells (Fig. 3, d and e). Splenocytes from btk-sufficient or btk-deficient NOD mice were cultured overnight in the presence of anti-IgM (5 μg/ml), LPS (10 μg/ml), or anti-CD40 (0.5 μg/ml), and then harvested and analyzed by flow cytometry. CD86 expression increases to all stimuli without dependence on the presence of BTK. Bar chart (Fig. 3e) indicates the proportion of B cells that express CD86 in btk-sufficient (○) and btk-deficient (■) B cells under these conditions. B cell surface expression of CD80, MHC class II, and CD40 in response to these stimuli was also examined and likewise found to be BTK independent (data not shown). These findings indicate that btk-deficient B cells in NOD remain competent to increase key molecules necessary for B cell interactions with T cells, even though their proliferative capacity is impaired.

T cell proliferative capacity is intact in btk-deficient NOD mice, with decreased IL-10 production in response to T cell stimulation

Because B lymphocytes are required for T cell proliferation in NOD mice, we determined whether T cell activation was altered by the presence of btk-deficient B lymphocytes. To this end, we initially characterized T cell proliferation in response to mitogenic anti-CD3 by use of CFSE labeling. CFSE-labeled splenocytes from 15-wk-old btk-deficient and btk-sufficient animals were cultured for 65 h in the presence of varying concentrations of anti-CD3 and a constant concentration of 1 μg/ml anti-CD28. CD4 T cells showed capacity to proliferate in the presence and absence of BTK in B lymphocytes (Fig. 4a). Detailed examination of the proliferative response by calculation of the number of mitotic events per 10,000 CD4 T cells showed increased proliferation of CD4 T cells from btk-deficient mice, relative to btk-deficient ones, in culture conditions with anti-CD28 alone, a finding reminiscent of the previously described autoproliferation exhibited by NOD splenocytes (p = 0.015 by Mann-Whitney). Responses to titrated doses of anti-CD3 are expressed as the number of mitotic events above baseline for each genotype, and show no significant differences in proliferative ability in T cells from btk-deficient NOD mice (p < 0.9035, by ANOVA; btk deficient, n = 3, and btk sufficient, n = 5).

We next determined whether there was a change in cellular function as exhibited by cytokine production. Supernatants were collected from cultures stimulated under the same conditions described above at 65 h. Cytokine production was analyzed by cytokine bead array and comparison with a standard curve. No difference was detected in the ability of anti-CD3-stimulated splenocytes to produce IFN-γ or IL-17 (Fig. 4b). The cultured splenocytes also produced equivalent amounts of IL-6 and TNF-α. IL-4 was not appreciable in either group (data not shown). Interestingly, btk-deficient splenocytes demonstrated a reduction in their ability to produce IL-10 (p = 0.01), a cytokine that has been associated with β cell damage when produced in the pancreatic islets (45).

Anti-insulin Abs fail to develop in btk-deficient NOD mice

To determine how BTK contributes to B cell autoreactivity and B-T cell interactions in the context of T1D in NOD, we measured insulin autoantibodies in the sera of prediabetic NOD mice. Iso-type-switched, anti-insulin IgG Abs are the product of interactions between cognate autoreactive B and T cells, and these autoantibodies predict disease in both mice and humans (1, 6, 38). We therefore used ELISA to compare levels of anti-insulin IgG Abs in sera of btk-deficient and btk-sufficient littermates. Fig. 5 shows data points for individual prediabetic 14- to 16-wk-old female mice, indicating that insulin-specific IgG Abs in btk-deficient mice fail to emerge as they do in btk-sufficient mice (p = 0.001, Wilcoxon rank sum). Total IgG Ab levels in the same mice, however, differ only slightly between btk-deficient and btk-sufficient groups (Fig. 5, right panel, p = 0.20, Wilcoxon rank sum). The lack of IgG anti-insulin autoantibodies in btk-deficient NOD mice is commensurate with the disease protection shown in Fig. 1. Thus, in the absence of BTK, B-T cell interactions required for the production of insulin autoantibodies are either ineffective, or do not occur, due to a B cell repertoire shift away from anti-insulin specificities.

Anti-insulin B cell analysis shows BTK regulates B cell selection in NOD

The failure of insulin-specific Abs to emerge in btk-deficient NOD mice suggests that checkpoints imposed by the absence of BTK may shift the B cell repertoire away from autoreactivity. To test this postulate directly, we intercrossed btk-deficient NOD mice with V₄H125/NOD mice, which harbor an insulin-specific BCR H chain transgene, paired with endogenous L chains (13). This V₄H125 transgene provides a tractable population of anti-insulin B cells in a polyclonal repertoire when on the NOD, but not C57BL/6, background. As shown in Fig. 6a, loss of BTK reduces
this insulin-binding population in the spleens of 10- to 16-wk-old female NOD mice, both in total numbers (50% reduction, \( p = 0.029 \) by WMW, \( n = 4 \) per group), and as a percentage of B cells (29% reduction, \( p = 0.029 \) by WMW, \( n = 4 \) per group), indicating that BTK-mediated signaling contributes to the autoreactive B cell repertoire that emerges in NOD mice.

**Anti-insulin BCR transgene restores diabetes to btk-deficient NOD mice**

Although insulin-binding B cells in \( V_{H125} \)/NOD mice are reduced by loss of BTK, a substantial number are detected compared with wild-type NOD mice, in which fewer than 0.2% of B cells can be shown to bind insulin in the spleen (34). To determine whether these remaining \( btk \)-deficient insulin-binding B cells are functionally capable of supporting disease, we used cohorts of female NOD mice that express \( V_{H125} \) in the presence or absence of BTK. Fig. 6b shows a Kaplan-Meier curve depicting the percentage of mice that are not diabetic (y-axis) vs their age in weeks (x-axis). Seventy-one percent of \( btk \)-deficient \( V_{H125} \)/NOD mice (○) became diabetic by age 30 wk. This is significantly different from \( btk \)-deficient mice with endogenous BCRs (log rank, \( p = 0.004 \)). Consistent with previous studies, \( btk \)-sufficient mice with the \( V_{H125} \) anti-insulin BCR transgene (□) have slightly higher disease penetrance than \( btk \)-deficient \( V_{H125} \) littermates, with 90% becoming diabetic by the age of 30 wk (log rank, \( p = 0.27 \)).

Of note, B1a, FO, and late transitional (T2) B cells are not restored by the \( V_{H125} \) transgene (data not shown). B1a remain at or below 10% of normal. In the spleen, the \( V_{H125} \) transgene increases B cell surface level expression of IgM in all murine strains and genotypes, such that the T2 subset analysis by flow cytometry remains high, and FO cell subsets low, in \( V_{H125}/btk \)-deficient NOD mice.

The \( V_{H125} \) transgene also substantially increases the MZ subset proportions in all murine strains (35), a trend that is counteracted by \( btk \) deficiency, and further affected by decreased B cell numbers overall. The result is a \( V_{H125}/btk \)-deficient MZ subset that does not differ significantly in number from either \( btk \)-sufficient or \( btk \)-deficient NOD mice. Because \( V_{H125} \) transgenic (Tg) mice do not express IgD, MZ analysis was made using CD21 vs CD23 for all genotypes with the following result: \( V_{H125}/btk \)-deficient NOD, \( 7.9 \pm 2.2 \times 10^6 \) (\( n = 4 \)), \( btk \)-deficient NOD, \( 8.8 \pm 1.2 \times 10^6 \) (\( n = 9 \), \( p = 0.33 \) vs \( V_{H125}/btk \)-deficient NOD); \( btk \)-deficient NOD, \( 6.8 \pm 1.8 \times 10^6 \) (\( n = 5 \), \( p = 0.41 \) vs \( V_{H125}/btk \)-deficient NOD). As has been shown previously, anti-insulin B cells that emerge due to this transgene enter both MZ and FO compartments (35), a situation that is not changed by \( btk \) deficiency (data not shown).

Thus, introduction of a B cell repertoire that is skewed toward an important \( \beta \) cell autoantigen overcomes the protection from diabetes provided by \( btk \) deficiency, indicating that alterations of...
BCR Ag specificities, rather than perturbation of B cell functions, are responsible for disease protection in \( btk \)-deficient NOD mice.

**Discussion**

These studies reveal that BTK-mediated signaling may be targeted as a means to ameliorate autoimmune diabetes without the need for broad depletion of T or B lymphocytes. We show that the introduction of \( btk \) deficiency renders >80% of female NOD mice resistant to diabetes. Reduction in anti-insulin IgG, and of insulin-binding B cells in the setting of an anti-insulin BCR transgene, provides evidence that \( btk \) deficiency shifts the B lymphocyte repertoire away from autoreactivity. Although \( btk \) deficiency may affect APCs other than B cells, the restoration of disease by the B cell-specific anti-insulin IgH transgene implies that loss of BTK protects against disease in a B cell-specific manner, and that skewing of B cell antigenic specificities, rather than functional defects in \( btk \)-deficient B cells or other APCs, is chiefly responsible for disease protection. The ideal method to treat and prevent T1D, and other autoimmune diseases, would be to restore tolerance to the adaptive immune system, and these findings offer evidence that targeting B cell signaling may be a promising approach in pursuing that goal.

In the ongoing effort to combat the complex autoreactive milieu that results in T1D, B lymphocytes are attractive targets, because their loss results in less severe immunosuppressive effects than those conferred by elimination of T lymphocytes. In the NOD model, total depletion of B cells early in development by genetic or serological approaches, as well as targeted elimination of mature B cells using anti-CD20 and anti-CD22, verify the potential model, total depletion of B cells early in development by genetic targeting B cell signaling may be a promising approach in pursuing that goal.

The effects of \( btk \) deficiency on B cell subsets in nonautoimmune mice are well recognized, and include loss of B1a B cells and a block in the late transitional T2 stage in the spleen that leads to reduced numbers of FO B cells. In NOD mice, the B cell repertoire is disparate from normal strains, showing overrepresentation of MZ B cells and rapid transition of T1 B cells. When introduced into NOD mice, \( btk \) deficiency reduces total B cell numbers modestly (18%) compared with the usual 50% reduction in C57BL/6 mice. The reduction in NOD occurs largely in FO B cells with the anticipated block at T2, and there is also a block in the pre-MZ subset, with a slight reduction in mature MZ B cells. B1a B cells are effectively eliminated from the peritoneal cavity when BTK is absent in NOD, consistent with findings in other strains. Thus, the principal developmental phenotype of \( btk \) deficiency persists in NOD mice, although with milder effects on FO B cells, and with some reduction in the expanded NOD MZ subset not seen in C57BL/6 mice. However, it should be noted that mice in these studies were 13–16 wk old, a time point chosen for its correlation with disease progression, just before diabetes onset. The MZ subset expands with age, and published studies of \( btk \)-deficient C57BL/6 mice, without a disease agenda, may have missed subtle effects in the small MZ population in younger B6 mice.

Functional analysis of \( btk \)-deficient NOD B cells reveals that they are unable to proliferate in response to BCR stimulation and that responses to TLR4 and CD40 stimulation are impaired, all as previously documented in nonautoimmune strains. Nonetheless, \( btk \)-deficient NOD B cells retain the ability to increase costimulatory molecules such as CD86 in response to these same signals. This residual B cell function may permit T-B cell interactions that contribute to the remnant of disease in a few \( btk \)-deficient NOD mice, as well as to the restoration of disease that occurs when the anti-insulin BCR transgene is introduced.

Introduction of the anti-insulin BCR H chain transgene (V\(_{H}125\)) into \( btk \)-deficient NOD mice skews the B cell repertoire toward this B cell Ag. The representation of anti-insulin B cells that enter the repertoire of V\(_{H}125\) Tg \( btk \)-deficient NOD is reduced, but these autoreactive cells are not completely eliminated. Thus, the transgene most likely forces entry of this crucial specificity into the repertoire of \( btk \)-deficient NOD. The fact that these Tg autoreactive cells restore diabetes indicates that \( btk \)-deficient B cells are functionally able to promote disease, if even a small number are equipped with anti-insulin specificity.

This model provides insight into issues regarding the role of altered B cell subsets in disease protection in \( btk \)-deficient conventional NOD mice, including the following: 1) increased T2s, sometimes considered to be regulatory; 2) reduced FO cell numbers, with possible resultant loss of Ag presentation; 3) subtle alterations in MZ numbers; and 4) loss of the autoreactive-prone B1a subset. The fact that diabetes is restored in V\(_{H}125\) Tg \( btk \)-deficient mice indicates that none of the subset changes rendered by \( btk \) deficiency are sufficient in themselves to provide disease protection, because the changes in subsets are not revised by the transgene. The T2 subset remains significantly increased, the FO subset remains significantly decreased, MZ numbers are similar to that of conventional \( btk \)-deficient NOD, and B1a B cells are not restored in diabetes-prone V\(_{H}125\) Tg \( btk \)-deficient NOD mice. Disease restoration by the anti-insulin BCR transgene therefore suggests that \( btk \) deficiency protects against disease in mice with endogenous BCRs by altering the B cell repertoire, perhaps reducing the availability of autoreactive B cells for Ag presentation to T cells, rather than by interfering with B cell subset composition or function. This disease restoration, together with the fact that costimulatory molecules in \( btk \)-deficient B cells are up-regulated normally, implies that V\(_{H}125\) Tg \( btk \)-deficient B cells are able to act as APCs. However, a nonstatistically significant trend toward less disease penetrance in V\(_{H}125\) Tg/\( btk \)-deficient vs V\(_{H}125\) Tg/\( btk \)-sufficient NOD mice suggests a quantitative difference that requires further study.

Pilot studies using anti-insulin 125Tg (H + L) BCR transgenes in \( btk \)-deficient mice indicate that fewer than 10% of these Tg anti-insulin B cells survive, even in a noncompetitive B cell environment. This supports the concept that BTK is needed to assist in the survival of these autoreactive cells. The importance of B cell specificity is further reinforced because these few cells are nevertheless able to promote some level of disease in NOD mice (R. A. Henry, J. W. Thomas, and P. L. Kendall, manuscript in preparation).

Studies presented in this work differ in some regard from two of the most recent investigations into B cell-related therapies for T1D, which targeted the B cell surface molecules CD20 and CD22 (14, 15). Those exogenous treatments resulted in complete, or near complete, elimination of B cells for a limited time period, followed by re-emergence of B cells that appeared to have a regulatory effect over time, and also encouraged or allowed expansion of regulatory T cells, by the age of 35 wk. Insulitis was improved among responders in both cases, and reversal of hyperglycemia was also possible in 30–60% if treatment was administered early. Inflammatory cytokines such as IL-17 and IFN-\( \gamma \) were reduced in some assays using the anti-CD22 therapy. These findings contrast our studies in that insulitis is, with the exception of a single mouse, not changed in terms of cellular populations or degree of infiltration by \( btk \) deficiency. Nor do we find evidence of increases in
regulatory cell populations, at least at 13–16 wk of age. T cell production of IL-10 in response to stimulus is reduced, but IFN-γ, IL-17, and others are not changed. Nevertheless, btk deficiency is highly effective at disease prevention, with 83% of mice remaining healthy at the 30-wk time point, compared with fewer than 50% in the anti-CD20 and anti-CD22 treatment groups. Although disease outcome using genetic vs exogenous treatments is not a fair comparison for evaluating desirability of a treatment, it is notable that the disease outcomes in btk deficiency vs B cell-eliminating therapies may have disparate mechanisms, because insulitis, regulatory T cell populations, and even cytokine expressions differ. The one area in which our data agree is that the T2 population was highly effective at disease prevention, with 83% of mice remaining healthy at the 30-wk time point, compared with fewer than 50% in the anti-CD20 and anti-CD22 treatment groups. Although disease outcome using genetic vs exogenous treatments is not a fair comparison for evaluating desirability of a treatment, it is notable that the disease outcomes in btk deficiency vs B cell-eliminating therapies may have disparate mechanisms, because insulitis, regulatory T cell populations, and even cytokine expressions differ. The one area in which our data agree is that the T2 population was increased and MZ population decreased by anti-human CD20 antibodies may have disparate mechanisms, because insulitis, regulatory T cell populations, and even cytokine expressions differ. The one area in which our data agree is that the T2 population was increased and MZ population decreased by anti-human CD20 treatment, suggesting that less mature B cell populations may have some disease-protective effects, or that moderate reduction of the MZ population is helpful. If that is the case, however, theVg125Tg/btk-deficient data would again suggest that any such protective effect may be due to failure of endogenous autoreactive cells to survive the block in maturation. Thus, both approaches support the concept of intervention directed at peripheral stages of B cell maturation.

Our findings are somewhat more consistent with a study in which a murine anti-CD20 Ab was used to deplete B cells (16). This study avoids the issues associated with normal human Ig backgrounds, and disease was prevented at a rate of ~60%, with no evidence of alterations in T cell populations or emergence of regulatory T cell populations. However, insulitis was significantly improved in these mice, again contrasting our findings.

None of the above studies have attempted to analyze the effects of their treatments on BCR specificities, because this is a very difficult undertaking within a broad, endogenous repertoire. The studies presented in this work are the first in which a B cell-related treatment modality could be overridden by introduction of an autoreactive B cell specificity, indicating that shifts in BCR Ag specificity may underlie disease protection, especially because other parameters, such as insulitis, are not overtly different. We hypothesize that T cells within draining pancreatic lymph nodes or insulitis lesions of btk-deficient mice fail to encounter B cells with critical specificities, and thus lack support for disease promotion, as opposed to being down-regulated.

Another possibility is raised by our finding that splenic T cells from btk-deficient NOD mice that are stimulated by anti-CD3 and anti-CD28 fail to produce IL-10 as well as their btk-sufficient counterparts. Because IL-10 has been shown to exacerbate diabetes when expressed in vitro assays. Again, these T cell differences could conceivably be due to loss of cognate B cell interactions, if the repertoire of B cell antigenic specificities has been altered.

In humans, the abrogation of BCR-related signals through BTK inhibitor could be titrated to provide a B cell-sparing dose effect for humans that would provide a similar outcome to those shown in this study. In fact, studies in autoimmune-prone lyn−/− mice have shown that BTK dosage can be adjusted to protect against humorally mediated autoimmunity, without the level of cellular depletion induced by full btk deficiency (25). Furthermore, BTK is a multidomain molecule with multiple functions, including linker and kinase components. Current work in our laboratory is underway to determine which component of BTK is responsible for the improvement in B cell tolerance, with the hope that specific functional interventional targets may be found to eliminate autoreactive cells, without suppressing physiologic cellular functions.

Our findings establish the general principal that BCR signaling can be targeted as a means to prevent T cell-mediated autoimmune diabetes, and suggest the possibility that it may be altered to affect pathogenic B cell specificities. Although more work is needed to understand how B cell signaling contributes to impaired B cell tolerance in autoimmune disease, these studies set the stage for future efforts to specifically reduce survival of the autoreactive B cells that support T1D.

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Disclosures
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