Bruton's Tyrosine Kinase Promotes Persistence of Mature Anti-Insulin B Cells

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Bruton’s Tyrosine Kinase Promotes Persistence of Mature Anti-Insulin B Cells

Rachel H. Bonami,* Allison M. Sullivan,‡ James B. Case,† Hannah E. Steinberg,‡ Kristen L. Hoek,‡ Wasif N. Khan,§ and Peggy L. Kendall‡,‡

Autoreactive B lymphocytes are essential for the development of T cell–mediated type 1 diabetes (T1D). Cytoplasmic Bruton’s tyrosine kinase (BTK) is a key component of B cell signaling, and its deletion in T1D-prone NOD mice significantly reduces diabetes. However, the role of BTK in the survival and function of autoreactive B cells is not clear. To evaluate the contributions of BTK, we used mice in which B cells express an anti-insulin BCR (125Tg) and promote T1D, despite being anergic. Crossing Btk deficiency onto 125Tg mice reveals that, in contrast to immature B cells, mature anti-insulin B cells are exquisitely dependent upon BTK, because their numbers are reduced by 95%. BTK kinase domain inhibition reproduces this effect in mature anti-insulin B cells, with less impact at transitional stages. The increased dependence of anti-insulin B cells on BTK became particularly evident in an Igα locus site-directed model, in which 50% of B cells edit their BCRs to noninsulin specificities; Btk deficiency preferentially depletes insulin binders from the follicular and marginal zone B cell subsets. The persistent few Btk-deficient anti-insulin B cells remain competent to internalize Ag and invade pancreatic islets. As such, loss of BTK does not significantly reduce diabetes incidence in 125Tg/NOD mice as it does in NOD mice with a normal B cell repertoire. Thus, BTK targeting may not impair autoreactive anti-insulin B cell function, yet it may provide protection in an endogenous repertoire by decreasing the relative availability of mature autoreactive B cells. The Journal of Immunology, 2014, 192: 000–000.
are essential APCs (8, 28–30). Interestingly, anti-insulin Abs are lost in Btk-deficient NOD mice, whereas total serum IgM is unchanged, suggesting that selective elimination of autoreactive B cells may be responsible for the disease protection observed (27). Introduction of an anti-insulin H chain BCR transgene (V_{H}125Tg), paired with endogenous L chains, revealed a role for BTK in the production or survival of anti-insulin B cells, because VH125Tg/Btk^{-/-}/NOD mice have only half as many insulin-specific B cells as do their Btk-sufficient counterparts (27). However, these are small populations of cells whose functional status is unknown.

To explore the role of BTK in tolerant, autoreactive B cells, the 125Tg/NOD model (V_{H}125V_{K}^{+}V_{\kappa}^{+}) was used to provide a uniform, well-studied population of anergic anti-insulin B cells. The findings show that anergic, anti-insulin B cells depend more heavily upon BTK than do their nonautoreactive counterparts, having <10% of the normal numbers of B cells, and that this effect is specific to mature follicular and marginal zone subsets. Use of a BTK kinase inhibitor, ibrutinib, demonstrates the dependence of mature anti-insulin B cells on the kinase function of BTK. Surviving Btk-deficient anti-insulin B cells retain their ability to internalize Ag, traffic to inflamed islets, and promote disease, although at a somewhat delayed rate. These findings suggest that protective effects in the setting of a wild-type (WT) repertoire are likely to be due to a reduction in the numbers of available autoreactive cells rather than impairment of pathogenic function.

**Materials and Methods**

**Animals and disease studies**

125Tg/NOD mice, generated as previously described (5, 7), were crossed with Btk-deficient NOD mice, also generated as previously described (27), to produce 125Tg/NOD Btk^{-/-} mice, which were backcrossed onto the NOD strain for 10 generations. Female mice were monitored for glucose levels weekly and considered diabetic at the first of two consecutive measurements. All mice were housed under specific pathogen–free conditions, and backcrossing to C57BL/6 and NOD mice and in experiments shown. VH125Tg/V_{H}125Tg/NOD mice were fed Ibrutinib (PCI-32765; Pharmacyclics [Sunnyvale, CA]; 0.24 g Ibrutinib/kg chow) or placebo chow ad libitum for 5–10 wk. All formulations were provided as a kind gift from Pharmacyclics. An average of 38 mg/kg Ibrutinib inhibitor was consumed per day, based on food weight consumed throughout the duration of the study and the number of mice/cage. This is above the dosage that ensures 99% inhibitor activity (24 mg/kg/d; personal communication, Pharmacyclics). B cell subsets were assessed by flow cytometry in freshly isolated organs, as outlined below.

**Cell isolation, flow cytometry, and Abs**

Bone marrow was eluted from long bones, and spleens and draining pancreatic lymph nodes were macerated with HBSS (Invitrogen) plus 10% FBS (HyClone). RBCs were lysed using Tris-NH_{4}Cl. Freshly isolated pancreata were digested with 3 ml 1 mg/ml collagenase P diluted in HBSS for 30 min at 37˚C, and tissue was disrupted using an 18-gauge needle. Icercold HBSS plus 10% FBS was added immediately to inhibit collagenase activity. Cells were directly analyzed by flow cytometry. Flow cytometry Ab reagents were reactive with B220 (68B2), IgM^{+} (DS-1), CD19 (I3D3), CD21 (7G6), CD23 (B3B4), CD93 (AA4.1) (BD Biosciences), or IgM (µ-light chain specific) (Life Technologies). Biotin N-hydroxysuccinimide ester was used to biotinylate human insulin (both from Sigma) at pH 8 in bicarbonate buffer. Streptavidin reagents (BD Biosciences) were used to detect biotinylated reagents. 7-Aminoactinomycin D (BD Biosciences) was used to exclude dead cells. Sample acquisition was performed using an LSR II flow cytometer (BD Biosciences), and FlowJo software (TreeStar) was used for analysis.

**Ca^{2+}-mobilization assay**

Bone marrow cells were harvested and grown in bone marrow culture media with 15 ng/ml recombinant human IL-7 (PeproTech) for 5 d and then with no IL-7 for an additional 2 d to promote differentiation, as previously described (32). Intracellular Ca^{2+} mobilization was measured by determining changes in the ratio of bound/free fura 2-AM fluorescence intensities using a FlexStation II fluorometer (Molecular Devices), as previously described (32). Basal readings were taken to stimulate cells. Cells were stimulated with 1 μg/ml anti-IgM (Fla(b')3 goat anti-mouse μ-chain; Jackson ImmunoResearch Labs), and well fluorescence was monitored at 37˚C.

**In vivo labeling of sinusaloid bone marrow B cells**

As described previously (33), lateral tail veins of mice were injected with 1 μg CD19-PB (BD Pharmingen) in 200 μl sterile 1 × PBS. Mice were sacrificed after 2 min, and bone marrow was immediately eluted from femurs. Cells were isolated, as described above, and subsequently incubated with the indicated Abss to stain cell surface markers, together with CD19-allophycocyanin to delineate all CD19+ cells, including sinusaloid B cells preferentially labeled with CD19-PB.

**Bcr internalization assay**

Ag internalization was performed as previously described (8). Briefly, freshly isolated bone marrow and spleen cells were incubated with biotinylated insulin for 30 min on ice to occupy BCR. After washing away excess biotinylated insulin, cells were incubated in complete media at 37˚C for 0–10 min, at which point cells were stained with streptavidin-fluorochrome, as well as other indicated Abs. The relative surface level of biotinylated insulin or IgM was determined by dividing the mean fluorescent intensity (MFI) at each time point by the MFI at t = 0, such that 100% represents no change in surface expression.

**Results**

**Anergic, autoreactive B cells depend upon BTK**

Btk deficiency was crossed onto 125Tg mice, on both C57BL/6 and NOD backgrounds. Fig. 1A shows representative flow cytometry dot plots from Btk-deficient 125Tg/NOD mice versus their Btk-sufficient counterparts (summarized in Table I). Btk-deficient 125Tg mice had severely reduced spleen B cell compartments (0.63 ± 0.10 × 10^{5} versus 12.6 ± 1.5 × 10^{5} cells, p < 0.001), retaining only 5% of the normal numbers of insulin-binding B cells (Fig. 1B, Table II). Results for C57BL/6 mice do not differ from those for NOD mice (data not shown). To extend
Btk deficiency reduces anti-insulin B cells and An1 cells in the spleen. (A and B) The expression of B220 and IgM and insulin reactivity were assessed in 125Tg/NOD Btk-sufficient or Btk-deficient splenocytes using flow cytometry. (A) Representative flow cytometry plots. Left panel is gated on live lymphocytes. Right panel is gated on B220⁺ IgM⁺ live lymphocytes. (B) Average number (± SEM) of B cells. (C) Splenocytes were harvested, and CD93⁺ cells were identified in B220⁺ IgM⁺ live lymphocytes (left panels); CD93⁺-gated B cells were divided into the An1 subset (IgMlo CD23+) from WT/C57BL/6 mice (right panel). This subset cannot be examined in Btk-deficient or -sufficient mice. (D) Total number of An1 cells for Btk-sufficient or Btk-deficient mice (right panel). In (A) and (B), n = 10, 8–15-wk-old male and female mice/group, n = 3 experiments. In (C) and (D), n = 7, 8–10-wk-old male and female mice/group, n = 2 experiments. All mice had blood glucose < 200 mg/dl. *p < 0.01, **p < 0.001, two-tailed t test.

FIGURE 1. Btk deficiency reduces anti-insulin B cells and An1 cells in the spleen. (A and B) The expression of B220 and IgM and insulin reactivity were assessed in 125Tg/NOD Btk-sufficient or Btk-deficient splenocytes using flow cytometry. (A) Representative flow cytometry plots. Left panel is gated on live lymphocytes. Right panel is gated on B220⁺ IgM⁺ live lymphocytes. (B) Average number (± SEM) of B cells. (C) Splenocytes were harvested, and CD93⁺ cells were identified in B220⁺ IgM⁺ live lymphocytes (left panels); CD93⁺-gated B cells were divided into the An1 subset (IgMlo CD23+) from WT/C57BL/6 Btk-sufficient or Btk-deficient mice (right panel). This subset cannot be examined in Btk-deficient or -sufficient mice. (D) Total number of An1 cells for Btk-sufficient or Btk-deficient mice (right panel). In (A) and (B), n = 10, 8–15-wk-old male and female mice/group, n = 3 experiments. In (C) and (D), n = 7, 8–10-wk-old male and female mice/group, n = 2 experiments. All mice had blood glucose < 200 mg/dl. *p < 0.01, **p < 0.001, two-tailed t test.

these findings to anergic B cells in a fully polyclonal repertoire, we also examined the effect of Btk deficiency on the anergic, autoreactive-prone An1 subset in nontransgenic mice. The An1 subset is CD93⁺/CD23⁺/IgM⁺. This subset cannot be examined in NOD mice because of technical issues with the AA4.1 (anti-CD93) Ab, so studies were performed using C57BL/6 mice. Fig. 1C shows representative dot plots of B220⁺ IgM⁺ live lymphocytes (left panels) gated on CD93⁺ cells depicting the An1 subset (CD23⁺/IgM⁺; right panels) from Btk-sufficient and -deficient mice with endogenous BCRs. Fig. 1D and Table III show that Btk-deficient mice have significantly reduced percentages and numbers of An1 B cells (p < 0.01). These data are similar to previously published findings in the Btk-deficient BALB.xid model, in which this subset, then defined as T3, also was found to be decreased (34). Thus, Btk deficiency dramatically decreases the numbers of autoreactive-prone, anergic B cells in both a naturally occurring population, as well as in a well-studied anergic, anti-insulin–transgenic model.

BTK is dispensable for development of immature anti-insulin B cells

B cell–developmental subsets were identified in freshly isolated bone marrow of Btk-sufficient or -deficient 125Tg/NOD mice using flow cytometry to detect B220, IgM⁺, and CD23 expression. Insulin-binding specificity was confirmed with biotinylated insulin staining detected by a streptavidin-fluorochrome conjugate. Representative plots are shown in Fig. 2A, and the average frequency ± SEM (Fig. 2B, Table I) or total number ± SEM (Fig. 2C, Table II) of pro and pre (B220⁺ IgM⁺⁺) immature (B220⁺ IgM⁺⁺ CD23⁻), or mature recirculating (B220⁺ IgM⁺⁺ CD23⁺) B cells is shown. Btk deficiency confers a comparable or elevated frequency and number of immature B cells in the bone marrow of 125Tg/NOD mice. In contrast, mature recirculating B cell numbers are significantly reduced (0.9 ± 0.2 × 10⁷ versus 19.0 ± 5.1 × 10⁶ cells, p = 0.008).

BCR-mediated calcium flux in immature anti-insulin B cells does not require BTK

BCR signaling is known to be impaired in mature Btk-deficient B cells (35, 36). However, the fact that immature B cell development is unimpeded in Btk-deficient 125Tg/NOD mice raises the question of whether BCR signaling in anti-insulin B cells occurs independently of BTK at this developmental stage. To test this, naïve immature 125Tg B cells were generated using IL-7–driven culture, as previously described (Materials and Methods) (32). BCR-induced calcium mobilization was measured in Btk-sufficient and -deficient immature B cells following stimulation with anti-IgM. Interestingly, comparable calcium mobilization was observed in 125Tg/NOD immature B cells, regardless of BTK status (Fig. 2D). Impaired calcium flux was observed in mature Btk-deficient 125Tg B cells, as expected (data not shown). Consistent with the above data on B cell development, these data show that Btk

| Table I. 125Tg B cell subset percentages |
|-----------------|-----------------|-----------------|-----------------|
| Organ           | B Cell Subset   | Btk Sufficient  | Btk Deficient   | p Value (t Test) |
| Bone marrow     | Total           | 2.0 ± 0.4       | 2.7 ± 0.5       | 0.35            |
| Bone marrow     | Pro/pre         | 13.2 ± 1.9      | 19.5 ± 2.9      | 0.08            |
| Bone marrow     | Immature        | 42.4 ± 6.0      | 78.3 ± 2.8      | <0.0001         |
| Spleen          | Total           | 14.0 ± 1.5      | 1.1 ± 0.1       | <0.0001         |
| Spleen          | T1              | 2.6 ± 0.5       | 16.5 ± 2.7      | <0.0001         |
| Spleen          | T2              | 10.0 ± 1.8      | 22.2 ± 0.8      | <0.0001         |
| Spleen          | Follicular      | 25.1 ± 3.2      | 3.9 ± 0.3       | <0.0001         |
| Spleen          | Premarginal zone| 4.9 ± 0.9       | 2.5 ± 0.5       | 0.02            |
| Spleen          | Marginal zone   | 57.5 ± 3.2      | 55.0 ± 4.2      | 0.604           |
| PLN             | Total           | 8.2 ± 4.0       | 0.4 ± 0.3       | <0.0001         |
| Pancreas        | Total           | 0.3 ± 0.1       | 0.01 ± 0.00     | 0.03            |

*B cell subsets identified as in Figs. 2 and 3.

bTotal indicates B lymphocyte percentage of total cells in the indicated organ.

PLN, Pancreatic lymph node.
deficiency does not impair calcium mobilization following BCR stimulation in immature 125Tg B cells, highlighting a major difference in signaling between immature and mature anti-insulin B cells.

**Btk deficiency results in loss of anti-insulin B cells at every developmental stage in the spleen**

Btk deficiency in NOD mice with nontransgenic BCRs confers an 18% reduction in splenic B cell numbers (27). However, in 125Tg/NOD mice, Btk deficiency results in >90% loss of B cells (Fig. 1). In NOD mice with endogenous BCRs, Btk deficiency causes a partial block at the T2 to follicular B cell transition, as well as a small reduction in marginal zone B cell numbers (27). To address whether Btk deficiency affects anti-insulin B cell development differently, spleen B cell subsets were compared in Btk-sufficient and -deficient 125Tg/NOD mice. Fig. 3A shows the flow cytometry gating scheme to detect early transitional T1 (CD21low CD23low), late transitional T2 (CD21med CD23high IgMhigh), follicular (CD21med CD23high IgMhigh), premarginal zone (CD21high CD23high IgMhigh), and marginal zone (CD21high CD23med) B cells. Quantification of subset proportions for each genotype (Fig. 3B, Table I) shows that both early and late transitional components are relatively overrepresented in the setting of Btk deficiency, suggesting that there is a block in maturation beyond both of these checkpoints.

However, quantification of total cell numbers shows that anti-insulin B cells are lost at all phases of development (Fig. 3C, Table II). The number of follicular B cells in 125Tg/NOD Btk-deficient mice was reduced 99% compared with 125Tg/NOD Btk-sufficient mice (2.2 ± 0.3 × 10⁶ versus 335.1 ± 79.1 × 10⁴, p < 0.001). Although the frequency of marginal zone B cells was not different, the total number of marginal zone B cells was reduced 95% in Btk-deficient 125Tg/NOD mice (36.7 ± 8.2 × 10⁴ versus 699.6 ± 72.4 × 10⁴, p < 0.001). The cell populations from which these mature subsets arise are also markedly reduced: the early transitional T1 subset is reduced by 71% (9.2 ± 1.4 × 10⁴ versus 31.9 ± 5.4 × 10⁴, p < 0.001), the late transitional T2 subset is reduced by 90% (13.0 ± 1.8 × 10⁴ versus 124.1 ± 30.3 × 10⁴, p = 0.0011), and the premarginal zone subset is reduced by 89% (1.7 ± 0.4 × 10⁴ versus 66.0 ± 16.0 × 10⁴, p < 0.001). These data show that anti-insulin B cell maturation and/or survival is more profoundly impaired by loss of BTK than is observed in the polyclonal repertoire of NOD mice with nontransgenic BCRs (27).

**Kinase inhibition of BTK reduces mature, but not transitional, anti-insulin B cells**

BTK is a complex protein with adapter, as well as kinase, functions involved in B cell development and function (37, 38). Therefore, a kinase inhibitor of BTK, Ibrutinib (PCI-32765; a kind gift from Pharmacyclics) (23, 39), was used to test the effects of kinase inhibition on anti-insulin B cell development and survival. Ibrutinib chow or placebo chow was fed to 125Tg/NOD mice for 5–10 wk. The average inhibitor dosage was 38 mg/kg/d (sufficient to elicit 99% inhibitor function; personal communication, Pharmacyclics). Flow cytometry analysis of freshly isolated splenocytes was used to identify B cell subsets, as seen in Fig. 3. These data mirror the developmental alteration seen in 125Tg/NOD Btk-deficient mice. The number of B lymphocytes was decreased in ibrutinib chow–fed mice versus placebo chow–fed mice (10 ± 3 × 10⁶ versus 22 ± 6 × 10⁶ cells, p < 0.001), whereas no difference was observed in non-B lymphocyte numbers (25 ± 9 × 10⁶ versus 29 ± 8 × 10⁶ cells, Fig. 4A). As shown in Fig. 4B, no significant difference was observed in T1 (63 ± 47 × 10⁴ versus 83 ± 32 × 10⁴ cells, p = 0.57) or T2 (49 ± 76 × 10⁴ versus 17 ± 3 × 10⁴ cells, p = 0.51) B cell subset numbers in the spleen. However, an 88% reduction in follicular (41 ± 37 × 10⁴ versus 345 ± 38 × 10⁴ cells, p < 0.001), a 60% reduction in premarginal zone (17 ± 5 × 10⁴ versus 43 ± 6 × 10⁴ cells, p = 0.0015), and a 65% reduction in marginal zone (443 ± 164 × 10⁴ versus 1283 ± 217 × 10⁴ cells, p = 0.0002) B cell numbers was observed. In contrast, no significant difference was observed in the number of B cells in the pancreatic draining lymph nodes (26 ± 9 × 10⁴ versus 21 ± 8 × 10⁴, p = 0.49) or pancreas (0.66 ± 0.80 × 10⁴ versus 5.2 ± 7.7 × 10⁴, p = 0.36, 0.51).

### Table II. 125Tg B cell subset numbers

<table>
<thead>
<tr>
<th>Organ</th>
<th>B Cell Subset</th>
<th>Btk Sufficient (× 10⁴ Cells)</th>
<th>Btk Deficient (× 10⁴ Cells)</th>
<th>p Value (t Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>Totalb</td>
<td>44.4 ± 10.6</td>
<td>73.8 ± 20.5</td>
<td>0.19</td>
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<tr>
<td>Bone marrow</td>
<td>Pro/pre</td>
<td>7.1 ± 2.8</td>
<td>14.9 ± 4.9</td>
<td>0.16</td>
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<tr>
<td>Bone marrow</td>
<td>Immature</td>
<td>17.9 ± 4.6</td>
<td>57.3 ± 15.6</td>
<td>0.013</td>
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<tr>
<td>Bone marrow</td>
<td>Mature recirculating</td>
<td>19.0 ± 5.1</td>
<td>0.9 ± 0.2</td>
<td>0.008</td>
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<tr>
<td>Spleen</td>
<td>Totalb</td>
<td>125.67 ± 152.5</td>
<td>62.8 ± 10.1</td>
<td>&lt;0.001</td>
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<tr>
<td>Spleen</td>
<td>T1</td>
<td>31.9 ± 5.4</td>
<td>9.2 ± 1.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Spleen</td>
<td>T2</td>
<td>124.1 ± 30.3</td>
<td>13.0 ± 1.8</td>
<td>0.0011</td>
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<tr>
<td>Spleen</td>
<td>Follicular</td>
<td>335.1 ± 79.1</td>
<td>2.2 ± 0.3</td>
<td>&lt;0.001</td>
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<td>Spleen</td>
<td>Premarginal</td>
<td>66.0 ± 16.0</td>
<td>1.7 ± 0.4</td>
<td>&lt;0.001</td>
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<td>Spleen</td>
<td>Marginal zone</td>
<td>699.6 ± 72.4</td>
<td>36.7 ± 8.2</td>
<td>&lt;0.001</td>
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<td>PLN</td>
<td>Totalb</td>
<td>33.9 ± 19.7</td>
<td>1.2 ± 1.1</td>
<td>&lt;0.001</td>
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<tr>
<td>Pancreas</td>
<td>Totalb</td>
<td>4.1 ± 1.3</td>
<td>0.3 ± 0.1</td>
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</table>

*B cell subsets identified as in Figs. 2 and 3.

**Table III. An1 B cell subset numbers and percentages**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Btk Sufficient (× 10⁴ Cells)</th>
<th>Btk Deficient (× 10⁴ Cells)</th>
<th>p Value (t Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>An1 subset (%)</td>
<td>7.0 ± 1.2</td>
<td>1.7 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>An1 subset (no.; × 10⁶ cells)</td>
<td>2.2 ± 0.8</td>
<td>0.3 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

An1 subset identified as B220⁺ CD93 (AA4.1)⁺ CD23⁺ IgMlow.  "Total" indicates total B cell number in the indicated organ.

PLN, Pancreatic lymph node.
Fig. 1. Inhibition of BTK kinase function impairs the maturation and/or survival of anti-insulin B lymphocytes. These data show that inhibition of BTK kinase function impairs the maturation and/or survival of anti-insulin B lymphocytes but that Ibrutinib does not block their trafficking to pancreatic draining lymph nodes or pancreas.

Anti-insulin B cells are preferentially susceptible to Btk deficiency

The dramatic reduction in mature B cell subsets in 125Tg/NOD mice compared with Btk-deficient NOD mice with endogenous BCRs suggests that autoreactive (anti-insulin) B cells rely more heavily on BTK-mediated signaling than do nonautoreactive B cells. However, the presence of a BCR transgene may have unrecognized effects that are unrelated to Ag specificity. Therefore, we used a novel BCR-transgenic model, in which an anti-insulin L chain is targeted to the Igk locus to allow receptor editing (kindly
provided by Dr. James Thomas, Vanderbilt University, Nashville, TN). This model, V<sub>H</sub>125Tg/V<sub>k</sub>125SDNeo, provides the unique opportunity to track a substantial population of anti-insulin B cells (∼50%) that develops alongside an equally large competing repertoire harboring the same BCR VH transgene (Fig. 5A, freshly isolated spleen is shown). Developmental subsets were characterized in the bone marrow and spleens of VH125Tg/V<sub>k</sub>125SDNeo/NOD Btk-deficient or -sufficient mice, as in Figs. 2 and 3, using flow cytometry (Fig. 5, Tables IV–VI).

**FIGURE 5.** Anti-insulin B cells are selectively impaired for transition into follicular and marginal zone B cells in the absence of BTK. VH125Tg/V<sub>k</sub>125SDNeo/NOD mice were developed as in Materials and Methods, and bone marrow and splenocyte B cell subsets were identified as in Figs. 2 and 3. (A) Representative flow cytometry plots of B220<sup>+</sup> IgM<sup>+</sup>–gated live lymphocytes from spleens, showing insulin binding by staining with labeled insulin. Average percentages (± SEM) (B) or total numbers (± SEM) (C) of insulin-binding B cells present in immature, T1, T2, follicular, premarginal zone, and marginal zone subsets. (D) The fold change in the number of insulin-binding B cells present in each subset from (C) was calculated by dividing the average number of B cells in the Btk-sufficient subset by the average number in the comparable subset from Btk-deficient mice (or the reverse for fold increase). The same was calculated for noninsulin-binding B cells. Dashed lines = 1 (no change). n = 9 mice, n ≥ 2 experiments. *p < 0.05, **p < 0.01, ***p < 0.001, two-tailed t test. (45%, 78%), and marginal zone (43%, 82%) subsets (Fig. 5B, 5C, Tables IV, VI).

The fold change in B cell numbers was calculated for each developmental subset, differentiating between insulin-binding and noninsulin-binding B cells from the same mice: the average number of insulin-binding Btk-sufficient follicular B cells was divided by the average number of insulin-binding Btk-deficient follicular B cells (or the reverse for fold increase). The same was also
and stained with Abs to delineate immature B cells, as well as anti-
parenchyma (33). B cells were then harvested from bone marrow
the sinusoids over those positioned away from the blood in the

Btk

sinusoidal and parenchymal populations from

Btk

deficiency does not preferentially prevent anti-insulin

Btk-deficient insulin-binding B cells are normally positioned

for bone marrow exit in the sinusoids

The Vh125Tg/Vk125SDNeo model also allows comparison of fac-
tors governing migration of insulin-binding and noninsulin-binding
B cells from bone marrow to spleen, as well as their level of depen-
dency on BTK. Because BTK was shown to support chemotactic
responses (24, 25, 40), we evaluated whether anti-insulin B cells
had altered reliance on BTK compared with nonautoactive
B cells. B lymphocytes in the bone marrow initially localize to the

B cells from reaching the pancreatic draining lymph nodes and
infiltrating the pancreas

Btk deficiency reduces anti-insulin B cells at mature stages in the

pancreatic draining lymph nodes

Pancreas Totalb 1.9 ± 0.8 0.2 ± 0.1 0.08

Table V. VH125Tg/Vk125SDNeo
B cell subset numbers

<table>
<thead>
<tr>
<th>Organ</th>
<th>B Cell Subseta</th>
<th>Btk Sufficient (× 10⁹ Cells)</th>
<th>Btk Deficient (× 10⁹ Cells)</th>
<th>p Value (t Test)</th>
</tr>
</thead>
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<tr>
<td>Bone marrow</td>
<td>Totalb</td>
<td>102.2 ± 4.9</td>
<td>132.2 ± 19.3</td>
<td>0.15</td>
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<tr>
<td>Bone marrow</td>
<td>Pro/pre</td>
<td>31.0 ± 4.3</td>
<td>49.1 ± 8.5</td>
<td>0.07</td>
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<td>Bone marrow</td>
<td>Immature</td>
<td>47.2 ± 3.8</td>
<td>66.4 ± 12.2</td>
<td>0.15</td>
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<td>Bone marrow</td>
<td>Mature recirculating</td>
<td>16.1 ± 3.5</td>
<td>9.0 ± 2.4</td>
<td>0.11</td>
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<td>Spleen</td>
<td>Totalb</td>
<td>948.7 ± 114.6</td>
<td>393.0 ± 78.2</td>
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<td>Spleen</td>
<td>T1</td>
<td>35.5 ± 7.1</td>
<td>57.6 ± 13.4</td>
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<tr>
<td>Spleen</td>
<td>T2</td>
<td>136.0 ± 16.1</td>
<td>125.0 ± 35.3</td>
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<td>Spleen</td>
<td>Follicular</td>
<td>213.0 ± 42.5</td>
<td>18.2 ± 6.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Spleen</td>
<td>Premarginal zone</td>
<td>22.4 ± 7.2</td>
<td>9.2 ± 2.5</td>
<td>0.12</td>
</tr>
<tr>
<td>Spleen</td>
<td>Marginal zone</td>
<td>516.6 ± 67.6</td>
<td>166.2 ± 32.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pancreatic draining lymph nodes</td>
<td>Totalb</td>
<td>17.4 ± 5.6</td>
<td>5.0 ± 1.0</td>
<td>0.03</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Totalb</td>
<td>1.9 ± 0.8</td>
<td>0.2 ± 0.1</td>
<td>0.08</td>
</tr>
</tbody>
</table>

aB cell subsets identified as in Fig. 5.
b“Total” indicates Total B cell number in the indicated organ.
the proportion of anti-insulin B cells at these inflammatory sites is unchanged by BTK loss. Total B cell numbers are markedly reduced in both draining pancreatic lymph nodes and pancreata of Btk-deficient V125Tg/Vk125SDNeo/NOD mice, regardless of specificity, and anti-insulin B cell numbers reflect this overall reduction (Fig. 6D, Tables V, VI). The selective reduction in mature Btk-deficient anti-insulin B cells in the spleen relative to noninsulin-binding counterparts (21-fold versus 7-fold reduction) is less apparent in pancreatic draining lymph nodes (5-fold versus 3-fold reduction), and it is not reflected at the primary site of inflammation in the pancreas (9-fold versus 10-fold reduction) (Fig. 6D, Table VI). These data show that Btk deficiency does not preferentially impede anti-insulin B cell homing to these inflammatory sites, despite bias in the mature repertoire in the spleen.

**BCR internalization of insulin is Btk independent**

To define the role of BTK in anti-insulin B cell function that promotes TID, we analyzed factors related to disease outcomes in the 125Tg/NOD model, in which the BCR repertoire specificity is essentially uniform. Anti-insulin B cells can present insulin autoantigen to T cells and are necessary for disease in NOD mice (6–8). BCR internalization is critical for autoantigen processing and presentation to T cells. Loss of BTK reduces BCR internalization following anti-IgM stimulation (41). To identify whether BTK controls BCR internalization of a small, physiologic autoantigen by anergic B cells, BCR internalization of insulin was assessed in Btk-deficient or -sufficient 125Tg/NOD mice. Surprisingly, the large majority of insulin was internalized with comparable kinetics in Btk-deficient and Btk-sufficient 125TgNOD mice within 10 min among all B cell subsets characterized (Fig. 7A). Surface IgM levels remained relatively constant during this time period in all subsets (Fig. 7A). Similar studies were performed using anti-IgMα stimulation. Consistent with previously published results (41), Btk-deficient follicular B cells showed diminished BCR internalization following stimulation with biotinylated anti-IgMα (data not shown). These data indicate that insulin internalization through the BCR is not altered by Btk deficiency and suggest that the mechanism of internalization of a small, soluble, low-affinity Ag may differ from that incurred by higher-affinity, cross-linking antigenic stimulation.

**Btk-deficient 125Tg B cells support diabetes development in NOD mice**

Btk deficiency protects against TID in NOD mice with nontransgenic BCRs (27). Disease is restored in that model when an anti-insulin BCR H chain transgene (VH125Tg) is introduced. V125Tg/NOD/Btk-deficient mice have reduced, but measurable, numbers of anti-insulin B cells, as well as a large B cell population with a broad repertoire of noninsulin-specific B cells that may include other autoantigenic specificities (27). In contrast, the 125Tg/NOD/Btk-deficient mice described in this report have very few B cells remaining; nearly all of them are specific for insulin, providing the opportunity to determine directly whether anergic, anti-insulin B cells require BTK-mediated signaling to support development of T1D. The number of anti-insulin B cells was reduced in both Btk-deficient pancreatic draining lymph nodes (1.2 ± 1.1 × 10⁴ versus 33.9 ± 19.7 × 10⁴ cells, p < 0.001) and pancreata (0.3 ± 0.1 × 10⁴ versus 4.1 ± 1.3 × 10⁶ cells, p = 0.014) (Fig. 7B, Table VI). As shown in Fig. 7C, diabetes onset is delayed in Btk-deficient 125TgNOD mice; however, by age 30 wk, the proportion with disease approaches that of their Btk-sufficient 125TgNOD littermates (60 versus 69%, p = 0.235). In contrast, despite having such low numbers of B cells, Btk-deficient 125TgNOD mice have significantly higher levels of disease than do Btk-deficient nontransgenic NOD controls expressing endogenous BCR repertoires (12%, p = 0.045). Nontransgenic/NOD Btk-deficient mice are protected from disease compared with nontransgenic/NOD Btk-sufficient mice, (p = 0.007), as previously reported (27). These data show that low numbers of residual anti-insulin B lymphocytes in Btk-deficient 125Tg/NOD mice can still support T1D.

**Discussion**

Autoreactive B cells that escape central tolerance in an anergic state have altered signaling responses compared with normal naive B cells (1, 42, 43), providing a potential opportunity to target these cells to treat or prevent autoimmune diseases. The data presented in this article show that anergic anti-insulin 125Tg B cells are exquisitely sensitive to loss of BTK. This BTK dependence of autoreactive-prone cells is mirrored in the reduction of the physiologically anergic An1 cell population. The VH125/Vk125SDNeo model, in which roughly half of peripheral B cells bind insulin, confirms that mature insulin-specific B cells depend more heavily on BTK than do nonautoreactive B cells. The BTK kinase inhibitor, brutinib, also affects mature anti-insulin B cell subsets preferentially, suggesting that activation of the kinase domain contributes to mature anti-insulin B cell development and/or survival.

Surprisingly, mature anti-insulin B cells internalize insulin autoantigen normally in the absence of BTK. Despite multiple defects, anti-insulin B cells can still home to inflamed islets and induce T1D, although with slightly delayed kinetics. This disease-related finding differs from that of BTK deficiency in NOD mice.

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Table VI. VH125Tg/Vk125SDNeo noninsulin-binding and insulin-binding B cell subset numbers

<table>
<thead>
<tr>
<th>Organ</th>
<th>B Cell Subseta</th>
<th>Btk Sufficient (× 10⁴ Cells)</th>
<th>Btk Deficient (× 10⁴ Cells)</th>
<th>p Value (t Test)</th>
<th>Btk Sufficient (× 10⁴ Cells)</th>
<th>Btk Deficient (× 10⁴ Cells)</th>
<th>p Value (t Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow</td>
<td>Immature</td>
<td>6.9 ± 1.4</td>
<td>11.1 ± 2.4</td>
<td>0.16</td>
<td>39.5 ± 4.9</td>
<td>54.1 ± 11.9</td>
<td>0.27</td>
</tr>
<tr>
<td>Bone marrow</td>
<td>Mature recirculating</td>
<td>6.8 ± 1.7</td>
<td>4.8 ± 1.5</td>
<td>0.39</td>
<td>9.1 ± 1.9</td>
<td>4.0 ± 1.0</td>
<td>0.03</td>
</tr>
<tr>
<td>Spleen</td>
<td>Totalb</td>
<td>420.0 ± 72.4</td>
<td>224.9 ± 52.4</td>
<td>0.047</td>
<td>528.7 ± 61.7</td>
<td>168.1 ± 40.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Spleen</td>
<td>T1</td>
<td>15.3 ± 3.7</td>
<td>17.1 ± 3.7</td>
<td>0.73</td>
<td>20.8 ± 3.7</td>
<td>43.7 ± 13.3</td>
<td>0.10</td>
</tr>
<tr>
<td>Spleen</td>
<td>T2</td>
<td>88.4 ± 13.9</td>
<td>77.6 ± 25.7</td>
<td>0.71</td>
<td>54.1 ± 7.0</td>
<td>57.8 ± 17.5</td>
<td>0.84</td>
</tr>
<tr>
<td>Spleen</td>
<td>Follicular</td>
<td>90.8 ± 21.4</td>
<td>12.8 ± 4.6</td>
<td>0.004</td>
<td>128.2 ± 24.6</td>
<td>6.2 ± 2.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Spleen</td>
<td>Premarginal zone</td>
<td>8.4 ± 3.2</td>
<td>6.3 ± 1.8</td>
<td>0.58</td>
<td>13.9 ± 3.9</td>
<td>3.1 ± 0.7</td>
<td>0.02</td>
</tr>
<tr>
<td>Spleen</td>
<td>Marginal zone</td>
<td>221.4 ± 40.1</td>
<td>118.5 ± 26.9</td>
<td>0.053</td>
<td>311.7 ± 39.2</td>
<td>57.2 ± 12.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pancreatic draining lymph nodes</td>
<td>Totalb</td>
<td>10.0 ± 3.6</td>
<td>3.4 ± 0.7</td>
<td>0.06</td>
<td>7.2 ± 2.1</td>
<td>1.6 ± 0.3</td>
<td>0.007</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Totalb</td>
<td>1.0 ± 0.4</td>
<td>0.1 ± 0.1</td>
<td>0.08</td>
<td>0.9 ± 0.3</td>
<td>0.1 ± 0.1</td>
<td>0.08</td>
</tr>
</tbody>
</table>

aIndividual B cell subsets identified as in Fig. 5.
bTotal indicates total B cell number in the indicated organ.
with endogenous BCRs, which significantly reduces the incidence of T1D (27). Because identifiable autoreactive B cells in the general repertoire of WT/NOD mice (as well as in humans with T1D) are rare (44), it is possible that a 95% reduction in their numbers as a result of BTK deficiency could decrease disease incidence.

In tracking the role of BTK through the developmental stages of tolerant anti-insulin 125Tg B cells, the data show that immature cells in the bone marrow develop and flux calcium normally in the absence of BTK (Fig. 2). These findings highlight a major difference in how immature and mature anti-insulin B cells signal through the BCR. Cell loss begins between the bone marrow and the early transitional T1 stage in Btk-deficient 125Tg B cells, where there is a 71% loss of cell numbers (Fig. 3, Table II). At the late transitional T2 stage, Btk-deficient 125Tg B cells, like their nontransgenic Btk-deficient counterparts with endogenous BCRs, have increased levels of surface IgM, commensurate with a block in maturation through this stage (27). However, unlike Btk-deficient mice with endogenous BCRs, there is a failure to increase T2 numbers, which is the normal result when cells destined for the follicular compartment are held back (27). Thus, it is likely that there is both a maturational block at T2 in Btk-deficient anti-insulin B cells and a loss of cells at that juncture that is more dramatic than the numbers alone would indicate.

Mature anti-insulin B cells clearly depend exquisitely on BTK, because follicular and marginal zone subsets are almost completely depleted in its absence. The V_{H}125/V_{L}125SDNeo model is especially useful in determining the role of Ag specificity at this stage, because anti-insulin follicular, premarginal zone, and marginal zone B cells are preferentially depleted compared with noninsulin-binding counterparts from the same animals (Fig. 5, Tables IV–VI). This may indicate that Ag engagement of the BCR provides an essential, positive selective signal mediated by BTK. The similar phenotype incurred by the BTK inhibitor, Ibrutinib, which blocks activation of the kinase domain while leaving the adapter function intact, further suggests that this may be the case (Fig. 4).

Of note, V_{H}125/V_{L}125SDNeo cells used in these experiments retain a neomycin-resistance cassette that enhances replacement of the anti-insulin V_{κ}κ possibly by promoter augmentation of germline transcription through the κ locus (R.H. Bonami, A.R. Rachakonda, C. Hulbert, and J.W. Thomas, manuscript in preparation). Thus, although it is useful for analyzing differences in cell fates of the resulting insulin-binding versus noninsulin-binding populations, possible effects of the cassette on receptor editing precludes conclusions regarding BTK contributions in this area. Nevertheless, V_{H}125/V_{L}125SDNeo mice still provide a unique tool for teasing out the effects of autoreactivity versus transgene effects. For example, Btk-deficient 125Tg B cells show similar maturational blocks as Btk-deficient transgenic 3-83μB cells on the nondeleting H2-K^{d} background (37). The 3-83 BCR recognizes H2-K^{d}, resulting in cellular deletion when expressed on that background but not on the H2-K^{d} background. Nevertheless, when Btk deficiency was tested in 3-83μB mice on the nondeleting background, the transgenic B cells were nearly all depleted, recapitulating many of the other findings that we report in this article for the 125Tg system, including an increased proportion of T1 cells (Fig. 3). The conclusion drawn from those findings was that a preformed BCR transgene results in faster transition out of the bone marrow (Fig. 5, Tables IV–VI). These new data suggest

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**FIGURE 6.** Btk-mediated effects on factors related to B cell trafficking in V_{H}125Tg/V_{L}125SDNeo/NOD mice. (A and B) Sinusoidal positioning for bone marrow exit: anti-CD19–PE Ab was injected i.v., and V_{H}125Tg/V_{L}125SDNeo/NOD mice were sacrificed after 2 min to preferentially label sinusoidal B cells, as in Materials and Methods. (A) Bone marrow was harvested immediately, and Abs reactive with B220, IgM, CD19, and CD23 were used to identify immature B cells (left panels) that were divided into parenchymal (CD19-PEmid; middle panels) and sinusoidal (CD19-PEhigh; right panels) B cell populations. (B) Insulin-binding B cells were further identified using labeled insulin. Representative flow cytometry plots (A) and average percentages (± SD) of insulin-binding parenchymal or sinusoidal immature B cells (B) (n = 5 mice, n = 2 experiments). Flow cytometry using labeled insulin was used to assess the proportion of anti-insulin B cells (C) and the number of insulin-binding B cells (D, top panels) or noninsulin-binding B cells (D, bottom panels) in spleen, pancreas, and draining pancreatic lymph nodes in Btk-sufficient and Btk-deficient mice (n = 6 female mice, n = 3 experiments). *p < 0.05, **p < 0.01, ***p < 0.001, two-tailed t test.
that the autoreactive specificity confers increased BTK dependency at maturation, in addition to transgene effects that may be associated with expedited bone marrow exodus. It is possible that the 3-83 BCR may recognize H2-Kd, or another unidentified autoantigen, at similar affinity to insulin; therefore, it may share some autoreactive properties with the anti-insulin 125Tg BCR, a viewpoint suggested by previous work regarding receptor editing in the 3-83 model (45).

Both of these studies contrast with a new report regarding transgenic AM14 rheumatoid factor B cells, which recognize IgG2a with low affinity ($K_d = 2.2 \times 10^6$) (46, 47). Genetic deficiency of BTK in this AM14 model depletes the transgenic autoreactive cells only to the same extent that the endogenous repertoire is depleted, further emphasizing that BTK requirements depend on the nature and affinity of the Ag (46, 47).

V_{H}125/V_{k}125^{Sدن} mice also allow analysis of insulin-specific B cell invasion of islets relative to noninsulin-binding counterparts in the same mice, which is normally a difficult task because the amount of overall islet inflammation varies significantly from mouse to mouse. Btk-deficient insulin-binding B cells are found in the pancreatic islets and draining pancreatic lymph nodes in equivalent proportions to their noninsulin-binding counterparts, regardless of BTK status, despite being preferentially culled during development in the spleen (Figs. 5, 6, Tables IV–VI), indicating that Btk-deficient insulin-binding B cells are equally adept at invading islets as are their noninsulin-binding counterparts. Of note, however, noninsulin-binding B cells in islets may differ from those in the spleen, because they are likely to be enriched for unidentified autoreactive specificities, and BTK also may support these other autoreactive B cells or may mediate responses to inflammatory cytokines, regardless of specificity.

Surprisingly, Btk-deficient anti-insulin B cells are able to internalize insulin Ag as well as their Btk-sufficient counterparts.

**FIGURE 7.** B cells deficient for BTK internalize insulin autoantigen and are capable of supporting diabetes relative to their BTK-sufficient counterparts. (A) 125Tg/NOD Btk-sufficient or Btk-deficient bone marrow or spleens were harvested, and cells were loaded with 50 ng/ml biotinylated insulin in media on ice, washed, and placed at 37°C for 0–10 min. Cells were then stained on ice with streptavidin-fluorochrome to detect remaining surface insulin, as well as with Abs reactive with B220, IgM, CD23 (bone marrow), and CD21 (spleen) to identify developmental subsets, as in Figs. 2 and 3. The MFI of insulin-biotin/streptavidin-fluorochrome (or IgM) at each time point was divided by the insulin-biotin/streptavidin-fluorochrome (or IgM) MFI at $t = 0$, such that 0 min = 100% expression for insulin (or IgM) on the cell surface. Insulin internalization (solid lines) and relative surface IgM (dashed lines) are plotted. The average ± SD is shown ($n = 4$ Btk-sufficient and $n = 6$ Btk-deficient male and female mice, 8–13 wk of age; $n = 3$ experiments). *$p < 0.05$, Btk-sufficient mice versus Btk-deficient mice, two-tailed $t$ test. (B) Flow cytometry was used to assess the proportion of total (left panels) and number of (right panels) insulin-binding B cells in draining pancreatic lymph nodes (PLN) and pancreas (live B220+ IgM+ lymphocytes) in Btk-sufficient or Btk-deficient mice ($n \geq 9$, 9–15-wk-old female nondiabetic mice). (C) Diabetes onset was monitored in 125Tg/NOD Btk-sufficient ($n = 12$), 125Tg/NOD Btk-deficient ($n = 15$), Non-Tg/NOD Btk-sufficient ($n = 9$), and Non-Tg/NOD Btk-deficient ($n = 8$) female mice. Mice were considered diabetic after two consecutive blood glucose readings $> 200$ mg/dl. *$p < 0.05$, **$p < 0.01$, log-rank test.
(Fig. 7). This is in contrast to other published work suggesting that BTK is required for Ag internalization (41). One key difference is the nature of the Ag being internalized. A small, physiologic Ag, such as insulin, may interact differently with the BCR compared with a strongly cross-linking Ag of high affinity, such as anti-IgM. We hypothesize that differences in signaling requirements exist for insulin internalization compared with large, multivalent Ags. The Kendall laboratory also found that 125Tg B cells, which are anergic, internalize Ag more efficiently than do nonanergic cells, which may also be a factor (8). Bik-deficient 125Tg/NOD mice develop diabetes (Fig. 7), despite having very few B cells, suggesting that they retain Ag-presenting function, because that is their role in the disease process (6, 8). This may have important implications for how a broad class of autoantigens is processed and presented to provoke autoimmunity and, thus, guide rationale for therapeutic targeting of this critical process.

Taken together, these data indicate that anergic, autoreactive B cells rely on BTK-mediated signaling for maturation or survival, but they can still traffic to inflamed tissues, internalize Ag, and promote cell-mediated autoimmunity without it. Thus, targeting of BTK may be most effective in reducing autoreactive cell numbers, rather than impairing their disease-related functions. This has clinical significance because a mAb that specifically depletes anti-insulin B cells was shown to prevent disease in NOD mice (44). Selectively targeting autoreactive B cells through BTK inhibition has the added advantage of not requiring prior knowledge of Ag specificity. The observation that loss of BTK in nontransgenic NOD mice dramatically reduces serum anti-insulin IgG, without altering total IgG (27), supports the concept that anti-insulin B cells can be selectively targeted by inhibiting BTK function in a fully polyclonal repertoire. Thus, the greater dependence of anti-insulin B cells on BTK relative to nonautoreactive B cells suggests that therapeutic inhibition of BTK function may hold promise for the treatment of T1D and possibly other autoimmune diseases mediated by B cells.

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Disclosures

The authors have no financial conflicts of interest.

References


