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Btk supports autoreactive B cell development and protects against apoptosis but is expendable for antigen-presentation

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Abstract

Bruton's tyrosine kinase (Btk) propogates B cell signaling, and BTK-inhibitors are in clinical trials for autoimmune disease. While autoreactive B cells fail to develop in the absence of Btk, its role in mature cells is unknown. To address this issue, a model of conditional removal ($Btk^{flox}/Cre\text{-}ER^{T2}$) was used to excise *Btk* from mature transgenic B cells that recognize the pathophysiologic autoantigen insulin. Anti-insulin B cells escape central tolerance and promote autoimmune diabetes, mimicking human autoreactive cells. Lifelong *Btk*-deficiency was previously shown to eliminate 95% of anti-insulin B cells but in this model mature anti-insulin B cells survived for weeks after targeted *Btk* deletion, even when competing with a polyclonal repertoire. BCR-stimulated cells could still signal via Syk, PLCy2 and CD22, but failed to upregulate the anti-apoptotic protein Bcl-xl, and proliferation was impaired. Surprisingly, *Btk*-depleted anti-insulin B cells could still present antigen and activate T cells, a critical function in promoting T cell-mediated islet cell destruction. Thus, pharmacologic targeting of Btk may be most effective by blocking expansion of established autoreactive cells, and preventing emergence of new ones.

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Introduction:

Failed B cell tolerance supports autoimmune disease by producing autoantibodies and via antigen-presentation to autoreactive T cells. Central tolerance eliminates the bulk of immature autoreactive B cells, and those that escape to the periphery can be suppressed via anergy (1–3). In humans, 2.5% of B cells are estimated to be autoreactive but anergic, failing to proliferate or produce antibody in response to stimulus (4). Strong signals can reverse anergy, causing proliferation and autoantibody production, as occurs in autoimmune arthritis and SLE (5, 6). This reversal is not necessary for antigen presentation, however, an important mechanism by which B cells promote autoimmunity, particulary in autoimmune (Type 1) diabetes (7–11).

Btk is a member of the TEC family of non-receptor tyrosine kinases whose function is best understood in BCR signaling (12–20). *Btk*-deficiency prevents autoimmune disease, including Type 1 diabetes (T1D) in nonobese diabetic (NOD) mice and arthritis in the K/BxN model. It also selectively reduces anti-insulin and anti-GPI autoantibodies while preserving total IgG (18, 21–24). Further, *Btk*-deficiency reduces transgenic anergic anti-insulin B cells by 95% (25), contrasting nonautoreactive cells, which are impaired but reach mature subsets and remain able to respond to T-dependent immunizations (12, 26–28). The mechanism underlying this difference has not been understood. One possibility was that altered signaling properties of anergic autoreactive cells made them more dependent on Btk for survival after maturation. Alternatively, Btk-mediated signaling could be necessary for anergic cells to transition through the negative selective tolerance checkpoint that bridges the immature and mature states. This question is important for therapeutic interventions, as the first possibility would allow Btk-inhibitors to eliminate existing autoreactive cells, while the second would require ongoing treatment to block maturation of newly emerging autoreactive B cells.

In this study we report effects of Cre-mediated timed deletion of *Btk* in adult mice to reveal its role in anergic autoreactive B cells. $Btk^{flox}/Cre\text{-}ER^{T2}$ mice (29) were crossed with transgenic (125Tg) mice expressing anti-insulin BCRs (30). In $Btk^{flox}/Cre\text{-}ER^{T2}$ 125Tg offspring, >95% of B cells bind insulin and Btk is efficiently deleted by tamoxifen-induction of Cre recombinase activity. Surprisingly, mature anti-insulin B cells persisted for months after Btk-deletion. They could still mobilize activating and inhibitory signaling, but did not upregulate the anti-apoptotic protein Bcl-xl. Further, anti-insulin B cell proliferation, already blunted by anergy (30), was further abrogated by loss of Btk. Most importantly, these cells remain able to present antigen and activate cognate T cells.

Methods

Mice and Cre-ER^{T2} induction.

Btk^{flox} mice were developed and bred to $Cre\text{-}ER^{T2}$ mice (B6.Cg-Tg(UBC-cre/ERT2)1Ejb/1J) as previously described (29) then crossed with mice expressing anti-insulin BCR transgenes (125Tg and V_H125) (30, 31). Btk^{null} 125Tg and V_H125 B6 controls were generated as previously described (12, 18, 25, 30). Mice were bred and maintained under

specific pathogen-free conditions. Cre-activation was induced by intraperitoneal injection of 3mg of tamoxifen-free base (Sigma) in 200μL of safflower oil on days −2, −1, and 0 (29).

Flow cytometry and antibodies.

Single-cell suspensions were obtained (18) and stained using fluorochrome or biotin-conjugated antibodies against B220 (RA3–6B2), IgM (μ-chain, Life Technology), IgMa (DS-1), IgD (11–26c.2a), CD19 (1D3), CD11b (M1/70), CD11c (HL3 or N418/eBioscience), F4/80 (BM8, eBioscience), Ly6G (IA8, Tonbo), CD21 (7G6), CD23 (B3B4), CD86 (GL1), CD45 (30-F11, eBioscience), CD4 (RM4–5), CD8a (53–6.7), CD69 (H1.2F3, Tonbo), and/or IA/IE (MHC class II, M5/114.15.2). B cell specificity for insulin was confirmed using biotin-conjugated human insulin or fluorochrome-conjugated pork insulin detected with streptavidin-conjugated fluorochromes. Viability dye Violet 510 or Red 710 (Tonbo) excluded dead cells. For intracellular staining, cells were fixed using 1.6% paraformaldehyde (Electron Microscopy Sciences), permeabilized with a solution of 0.05% Triton-X-100 (SigmaUltra) or eBioscience intracellular fixation and permeablization buffer set then stained with rabbit anti-mouse Btk (D3H5, Cell Signaling) directly conjugated to fluorochrome, or followed by fluorochrome-conjugated anti-rabbit IgG(F'ab2) secondary. Cells were stained with anti-cleaved PARP (F21–852) or anti-Bcl-xL (54H6, Cell Signaling) in apoptosis assays or anti-CD22 (pY822) (12a/CD22), anti-ZAP70 (pY319)/Syk (pY352) (17A-P-ZAP70) or anti-PLCγ2 (pY759) (K86–689.37) for phospho-flow cytometry. Unless otherwise stated, antibodies were from BD Biosciences. Samples were collected on an LSRII flow cytometer (BD Biosciences) and an Aurora flow cytometer (Cytek Biosciences),and data analyzed using FlowJo software (TreeStar).

Phosphoflow cytometry.

Splenocytes were suspended at 10×10^6 cells/mL in RPMI (Gibco), rested for 30 minutes, then stimulated for 0, 4, 10, or 30 minutes with 10μg/mL goat anti-mouse IgM (μ-chain specific, Jackson Immunoresearch). Reaction was stopped with 1.6% paraformaldehyde before staining.

B cell proliferation.

Splenocytes were stained with CellTrace Violet (Life Technologies), cultured at 1×10^6 cells/mL for three days in cRPMI alone or stimulated with 10μg/mL goat anti-mouse IgM (μ-chain specific, Jackson Immunoresearch), then analyzed by flow cytometry.

B cell activation and apoptosis.

Splenocytes were cultured at 10×10^6 cells/mL for 0, 10, or 24 hours in cRPMI alone or stimulated with 10μg/mL goat anti-mouse IgM (μ-chain specific, Jackson Immunoresearch).

Antigen presentation.

Lymphocytes were purified as previously described (11). Anti-insulin B cells and OTII T cells were stained with CellTrace Violet (Life Technologies). OTII T cells were incubated in cRPMI alone or with anti-insulin B cells, with or without pork insulin conjugated to

$OVA_{323–329}$ peptide (KISQAVHC; Sigma-Genosys). Samples were harvested and analyzed on day 3. Mitotic events were calculated as previously described (11, 18, 32, 33).

Statistical analysis.

P-values were calculated using one-way or two-way ANOVAs, or multiple T-tests with Welch's correction, as appropriate, using GraphPad Prism 7.00 for Windows, (GraphPad Software, La Jolla California USA).

Results

Peripheral anti-insulin B cells do not require Btk for survival.

Ninety-five percent of transgenic anti-insulin B cells are culled from the repertoire when Btk is genetically absent (Btk^{null}) (25). To determine whether Btk is required for autoreactive B cell development versus peripheral maintenance, tamoxifen-inducible Cre recombinase was used to delete the *Btk* gene from adult mice. Anti-insulin transgenic (125Tg) $Btk^{flox/}$ $Cre\text{-}ER^{T2}$ mice were injected with tamoxifen to induce *Btk* deletion, as were 125Tg Btk^{flox} and 125Tg Btk^{null} controls. Anti-insulin B cells in bone marrow and spleen were assessed by flow cytometry five days and ten weeks later (Fig 1, Supplemental Table I). Btk deletion was highly efficient with 92.1%, 98.3%, and 94.3% of pro/pre- immature, and splenic B cells respectively Btk-negative by day five post injections (Fig 1A–C, F, G). As expected, this did not reduce numbers of pre/pro bone marrow B cells in $Btk^{flox}/Cre\text{-}ER^{T2}$ mice even 10 weeks post deletion (1.09e4±3.81e3, immature=2.28e5±1.17e5) as compared to Btk^{flox} controls (pre/pro=9.53e3±8.43e3, immature=1.20e5±7.37e4) (p=0.98) and in fact resulted in an increase in immature bone marrow B cells (Btk^{flox}=1.20e5±7.37e4, $Btk^{flox/}$ $Cre\text{-}ER^{T2}$=2.28e5±1.17e5, p=0.02) indicating that Btk is not essential for autoreactive B lymphopoiesis in bone marrow. However, mature recirculating anti-insulin B cells, which originate in lymphoid follicles, are nearly absent in Btk^{null} controls, but were not reduced in $Btk^{flox}/Cre\text{-}ER^{T2}$ (2.33e5±6.81e4) compared to Btk^{flox} controls (3.07e5±1.41e5) (p=0.425).

Splenic anti-insulin B cell numbers were strikingly preserved five days after *Btk*-deletion, contrasting their near absence in lifelong Btk-deficient Btk^{null} controls (Fig 1H). Even at ten weeks, autoreactive B cells were only reduced by 52% in spleens of $Btk^{flox}/Cre\text{-}ER^{T2}$ mice (1.23e7±3.66e6 vs 2.55e7±4.56e6 in Btk^{flox} controls). While this is a significant loss (p<0.001), it did not recapitulate the near-total absence of mature B cells seen in the Btk^{null} model. Thus, Btk-mediated signaling is not required for persistence of autoreactive mature anti-insulin B cells.

Anti-insulin B cells rely on Btk at transitional checkpoints during development

Splenic B cells that have just emerged from the bone marrow go through an additional tolerance checkpoint at transitional (T1) stage, where they are subject to deletion upon antigen encounter. Figure 2B shows that T1 B cell numbers decrease significantly as early as five days after *Btk*-deletion (1.81e5±8.17e4) and remain low at 10 weeks, (1.18e5±3.97e4), matching low numbers in Btk^{null} mice (9.81e4±3.19e4) (p=0.52, p=0.99) (Figure 2A & B and Supp Table II), suggesting that Btk supports development of autoreactive B cells that pass through peripheral tolerance mechanisms at the T1 checkpoint.

At the late transitional (T2) stage, there is an increase in anti-insulin B cells 5 days after Btk-deletion (6.48e6±1.98e6 vs 3.77e6±1.02e6 in *Btk*^{flox} controls, p<0.001), with a commensurate decrease in follicular (FO) B cells (1.83e6±1.29e6 vs 5.24e6±2.21e6 in *Btk*^{flox} controls, p<0.001) (Fig 2B, Supp Table II), reproducing a pattern typically seen in non-transgenic repertoires (12). At ten weeks post Btk loss, numbers of T2 B cells (2.42e6±7.55e5) are not reduced compared to *Btk*^{flox} controls (p=0.209). However, a block in development from T2 to FO B cells remains, resulting in a drastic reduction of FO B cells (2.01e5±1.17e5 vs 5.24e6±2.21e6 in *Btk*^{flox} controls, p<0.001) (Fig 2B, Supp Table II). The remaining few FO B cells either survived or developed from Btk-positive precursors, as the majority (57.1±23.5%) retained Btk (Fig 2C). The increase in Btk^{+} FO B cells was not due to globally increased Btk, as splenic macrophages and dendritic cells remain Btk-negative (94.8±2.32 and 96.5±2.04%, respectively) 10 weeks after tamoxifen treatment (Supp Figure 1). These findings support the idea that Btk promotes maturation of autoreactive follicular B cells by supporting their transition through the early T1 checkpoint. However, FO B cells do not appear to need Btk for survival, as they are maintained for at least five days after excision of Btk. Over time, Btk-deleted anti-insulin FO B cells likely reach the end of their lifespans and are not replenished by newly emerging cells, resulting in nearly complete loss of this compartment.

Marginal Zone anti-insulin B cells are not depleted by loss of Btk.

Most autoreactive B cells that remain 10 weeks after Btk-deletion are marginal zone (MZ) B cells, and their numbers do not differ significantly between *Btk*^{flox}*/Cre-ER*^{T2} (6.89e6±2.68e6) and *Btk*^{flox} controls (1.13e7±3.93e6e5) (p=0.152) (Fig 2B, Supp Table II). This contrasts near-complete lack of anti-insulin MZ B cells in lifelong Btk-deficiency (4.23e5±1.87e5) (p<0.001).(25) About 17% of *Btk*^{flox}*/Cre-ER*^{T2} MZ B cells are Btk^{+}, and a majority (71.5±19.6%) of the immediate precursor pre-marginal zone (pMZ) B cells are Btk^{+} (Fig 2C–D, Supp Table II). This suggests that anti-insulin B cells that retain Btk may have a competitive advantage in the pMZ compartment, and then successfully mature, joining long-lived MZ cells that survived after Btk-deletion. Thus, constitutive Btk-mediated signaling is not required for maintenance of autoreactive MZ B cells, but supports transition into this subset.

Competition with a polyclonal repertoire does not alter Btk-mediated anti-insulin B cell requirements for development and survival.

Homeostatic expansion of otherwise impaired B cells can occur in the absence of a normal repertoire. To determine whether mature anti-insulin B cells lacking Btk still survive when competing with a polyclonal repertoire, we took advantage of the V_H125 model. In this setting the same transgenic anti-insulin heavy chain pairs with dozens of endogenous light chains, of which only a small percentage confer insulin-binding (Fig 3A). In the bone marrow anti-insulin B cells make up 0.18% of the B cell population in this model (Supp Figure 2A). *Btk* deletion was highly efficient in V_H125 *Btk*^{flox}/*Cre-ER*^{T2} anti-insulin bone marrow B cells, where 91.66% were depleted of Btk by day 5 post injection (Supp Figure 2B and 2C, Supp Table III). As with 125Tg *Btk*^{flox}/*Cre-ER*^{T2} bone marrow, Btk proved unnecessary for lymphopoiesis, as total anti-insulin B cell numbers did not differ at 5 days or 10 weeks after treatment (5 days: 1.47e3±1.78e3 vs 1.98e3±2.13e3 for VH125 Btk^{flox}

controls, p=0.912; 10 weeks: 1.24e3±1.40e3 vs 1.98e3±2.13e3 for VH125 Btk^{flox} controls, p=0.8171 (Supp Figure 2D and Supp Table III).

In the spleens, 0.54% of B cells in V_H125 mice bound insulin (Supp Table III). Most enter the follicular compartment (87.4%) with a smaller proportion in marginal zones (5.9%), reflecting previously published data (30). This differs from 125Tg mice in which a higher proportion enter the marginal zone (38.% MZ, 56.96% FO) (Supp Figure 2E). The reason for this difference is unknown but may be due to slight differences in affinity between endogenous and transgenic light chains. Btk deletion was highly efficient, with 91.11% of the anti-insulin B cells Btk-negative by day 5 post injection (Fig 3C). Like 125Tg, splenic anti-insulin B cell populations were maintained 5 days post injection (Fig 3C). Anti-insulin populations were reduced 46.7% by 10 weeks post injection, although this loss was not statistically significant (8.23e4±3.63e4 vs 1.18e5±6.06e4 in V_H125 Btk^{flox} controls).

Anti-insulin B cells emerging in T1 five days after *Btk*-deletion did not demonstrate a reduction as they did in the fixed 125Tg repertoire (Fig 3E, Fig 2B). However they did move into the late T2 stage phenotype in a similar manner, (2.09e4±1.36e4 vs 5.33e3±4.52e3 in V_H125 *Btk*^{flox} controls, p=0.0136). FO anti-insulin B cells did not differ at the 5 day time point (8.76e4±3.83e4 vs 1.38e5±1.00e5 in VH125 *Btk*^{flox} controls, p=0.3542). Ten weeks after *Btk*-deletion, T2 B cells remained significantly increased (2.47e4±1.36e4 vs 5.33e3±4.52e3 in V_H125 Btk^{flox} controls, p=0.0029), with a corresponding significant decrease in FO B cells (2.26e3±3.45e3 vs 1.38e5±1.00e5 in V_H125 *Btk*^{flox} controls, p=0.0141). Comparable to 125Tg, a substantial proportion (23.63%, Figure 3F and 3G) of FO B cells expressed Btk. This supports the notion that B cells containing Btk may have a competitive advantage in the FO compartment.

There were no noticeable changes in pMZ and MZ anti-insulin B cells 5 days post Btk deletion(1.12e3±1.93e3 vs1.59e3±1.99e3 in VH125 *Btk*^{flox} controls, p=0.9229 and 4.54e3±6.93e3 vs 1.67e3±1.39e3 in VH125 *Btk*^{flox} controls, p=0.3745 respectively), nor 10 weeks post Btk deletion (1.69e3±1.13e3 vs1.59e3±1.99e3 in VH125 *Btk*^{flox} controls, p=0.9991 and 4.67e3±3.62e3 vs 1.67e3±1.39e3 in VH125 *Btk*^{flox} controls, p=0.3794 respectively) (Fig 3E, Supp Table III). Overall, these findings indicate that T2, MZ and mature FO anti-insulin B cells do not require Btk to survive, even when competing with a polyclonal repertoire.

Mature anti-insulin B cells initiate BCR proximal signaling even after Btk deletion

In non-anergic B cells, BCR-engagement activates tyrosine kinases Lyn and Syk, which phosphorylate and activate Btk and adapter protein BLNK (34–36). This recruits PLC-γ2, which is activated by Syk and Btk. Anti-insulin B cells are anergic in response to insulin stimulus but can partially respond to BCR crosslinking by anti-IgM (11, 30, 37). To assess the role of Btk in BCR-signaling of anti-insulin B cells, cells were stimulated with anti-IgM and analyzed by phospho-flow cytometry to measure phosphorylation of PLCγ2. To account for possible signaling differences between splenic B cell subsets, MZ and T2/FO B cells were analyzed separately (38). T2/FO could not be identified separately due to use of anti-IgM antibody to stimulate IgM-BCR. We also assessed activation of upstream kinase Syk and inhibitory mediator CD22 to assess overall integrity of BCR signaling

in the absence of Btk. As expected, basal and BCR-induced Syk phosphorylation were not reduced in Btk-deleted (*Btk*^{flox}/*Cre-ER*^{T2}) and Btk-sufficient (*Btk*^{flox}) cells. In fact, T2/FO Btk-deleted B cells showed increased Syk phosphorylation after BCR stimulation (*Btk*$^{+}$=2.48±0.55, *Btk*$^{-}$=3.36±0.39, p<0.001) (Fig 4A, Supplemental Table IV), possibly due to to increased surface levels of IgM that occur in the absence of Btk. Phosphorylation of CD22 was slightly enhanced at 10 minutes in Btk-negative T2/FO cells (*Btk*$^{+}$=1.95±0.43 *Btk*$^{-}$=2.51±0.35, p<0.001), but otherwise did not differ (Fig 4C, Supp Table IV). Thus, Syk activation and BCR inhibitory signaling by CD22 do not depend on Btk in mature anti-insulin B cells.

Syk phosphorylation occurs upstream of Btk while PLCγ2 is a target of Btk tyrosine phosphorylation. Surprisingly, PLCγ2 was phosphorylated even in Btk-negative *Btk*^{flox}/*Cre-ER*^{T2} T2/FO anti-insulin B cells (4min=4.41±0.93, 10min=4.85±0.76), at a higher level compared to their Btk-positive *Btk*^{flox} counterparts (4min=3.20±0.88, 10min=2.84±0.85) (p<0.01, p<0.001) (Fig 4B, Supp Table IV). Btk has been reported to phosphorylate PLCγ2, so this was unexpected.(39, 40) However, Syk can also phosphorylate PLCγ2.(40) A Syk inhibitor, R406, was used to test the hypothesis that Syk provides redundant function in PLCγ2 activation after deletion of Btk from anti-insulin B cells. Syk, CD22, and PLCγ2 all showed significantly less phosphorylation, at every stimulated time point, in the presence of R406 (Fig 4A–C, Supp Table IV). In addition, differences in signaling between Btk-positive and Btk-negative T2/FO B cells were abrogated. Because Syk has many substrates, it may mediate PLCγ2, or CD22 phosphorylation indirectly. However, inhibition shows that Syk is at least partially responsible for the ability of Btk-negative anti-insulin B cells to signal.

Btk-negative anti-insulin B cells fail to proliferate following BCR crosslinking.

Btk^{null} B cells with endogenous BCRs show reduced proliferation to anti-IgM,(12) as do mature endogenous *Btk*^{flox}/*Cre-ER*^{T2} B cells after Btk deletion (29). The anti-insulin B cells used in this report were previously shown to have reduced proliferative capacity compared with non-autoreactive cells (30, 31). As shown in Figure 5, Btk-deletion further diminishes that capacity. Despite their ability to activate proximal signaling, Btk-negative anti-insulin *Btk*^{flox}/*Cre-ER*^{T2} B cells are almost completely unable to proliferate to anti-IgM. After three days of stimulus, dye dilution analysis showed that 79.5±3.53% of live *Btk*^{flox} anti-insulin B cells had undergone at least one cycle of cell division, while only 9.40±3.98% of *Btk*^{flox}/*Cre-ER*^{T2} anti-insulin B cells did so (p<0.001, Fig 5 A–B, Supp Table V). Surviving *Btk*-negative anti-insulin B cells were also significantly less able to upregulate CD86 in response to anti-IgM. Btk-sufficient anti-insulin B cells increased expression nearly 50-fold (49.5±20.5), while the Btk-negative anti-insulin B cells only increased expression about 10-fold (9.1±5.3) (p<0.001) (Fig 5B, Supp Table V). Therefore, functional cellular responses to signaling are abnormal in anti-insulin B cells after deletion of Btk, despite intact proximal signaling, indicating that Btk contributes to additional pathways that support these autoreactive cells.

Anti-insulin B cells rely on Btk to support BCR-signaling induced anti-apoptotic mechanisms.

Btk mediates Akt activation, which regulates apoptotic pathways, including Bcl-xL, an anti-apoptotic protein important in B cell proliferation and survival (41–45). We assessed anti-insulin B cell survival and Bcl-xL expression in response to anti-IgM, with and without Btk. Immediately *ex vivo*, Btk-negative $Btk^{flox}/Cre\text{-}ER^{T2}$ and Btk-positive Btk^{flox} anti-insulin B cells showed similar low levels of cleaved PARP, a marker of apoptosis (Btk^{flox}=0.12±0.02, $Btk^{flox}/Cre\text{-}ER^{T2}$=0.10±0.01, p=0.20) (Fig 5C and D, Supp Table V). However, after 24 hours of anti-IgM stimulus, a significantly higher percentage of Btk-negative anti-insulin B cells were positive for cleaved PARP (64.4±2.15) compared to those with intact Btk (46.1±8.97) (p<0.001). Btk-negative anti-insulin B cells also showed significantly less cell viability (Btk^{flox}=43.4±11.3, $Btk^{flox}/Cre\text{-}ER^{T2}$=23.4±2.54, p=0.002) (Fig 5E, Supp Table V). Further, intracellular flow cytometry revealed that anti-insulin B cells do upregulate Bcl-xL in response to anti-IgM stimulation (4.19±0.15 at 24hr), while Btk-negative anti-insulin B cells do not (1.75±0.27 at 24hr) (p<0.001) (Fig 5G). These data suggest that strong BCR crosslinking can help break tolerance in anergic autoreactive B cells by activating Btk-mediated anti-apoptotic pathways.

Btk is not required for antigen-presentation by anti-insulin B cells.

Anergic insulin-specific B cells do not proliferate or produce antibody, but efficiently present antigen and activate cognate T cells (11). We previously showed that anti-insulin B cells can efficiently internalize antigen without Btk but the small numbers of anti-insulin B cells that remain in Btk^{null} animals used in that study were too few to allow assessment of antigen-presentation (25). Therefore, we purified anti-insulin B cells from $Btk^{flox}/Cre\text{-}ER^{T2}$ spleens five days after tamoxifen treatment. The anti-insulin B cells were then incubated with purified OT-II CD4+ T cells. Insulin was conjugated to OVA peptide to create a cognate T-B antigen, allowing anti-insulin B cells to internalize it via the insulin component, then process it and present the OVA-peptide component (11). OT-II T cells did not proliferate when incubated with OVA-insulin alone (data not shown), but significantly proliferated when Btk-negative $Btk^{flox}/Cre\text{-}ER^{T2}$ anti-insulin B cells were added (52.0±15.2%, p<0.001, Fig 6A–B, Supp Table VI). This was similar to percent proliferation induced by Btk-positive Btk^{flox} anti-insulin control B cells (61.0±15.4%, p=0.708). Both Btk-positive and Btk-negative B cells induced up to 5 cell divisions by activated T cells, although T cells incubated with $Btk^{flox}/Cre\text{-}ER^{T2}$ anti-insulin B cells underwent slightly fewer cell divisions overall. More T cells reached peak 3 and peak 5 when incubated with Btk^{flox} vs $Btk^{flox}/Cre\text{-}ER^{T2}$ anti-insulin B cells, indicating a higher number of mitotic events overall (T cells with Btk^{flox}=3.93e4±7.82e3, T cells with $Btk^{flox}/Cre\text{-}ER^{T2}$=2.25e4±7.55e3, p<0.001) (Fig 6C, Supp Table VI). This difference may not have been due to reduced antigen presenting capacity of Btk-negative $Btk^{flox}/Cre\text{-}ER^{T2}$ anti-insulin B cells however, since MHC class II was equally upregulated on Btk^{flox} (3.04±1.01) and $Btk^{flox}/Cre\text{-}ER^{T2}$ (2.88±1.26) B cells (p>0.999) (Fig 6E). Further, Btk-positive cells in culture with OT-II T cells underwent proliferation (50.1±7.69 proliferating), while Btk-negative $Btk^{flox}/Cre\text{-}ER^{T2}$ anti-insulin B cells failed to respond (14.6±1.20 proliferating) (p<0.001), resulting in fewer Btk-negative B cells remaining to help activate T cells as the days progressed (Fig 6D–E, Supp Table

VI). These data show for the first time that anergic autoreactive B cells specific for a small soluble antigen do not require Btk to present antigen and activate T cells.

Discussion

BTK-targeting, using either genetic or pharmacologic methods, has been shown to eliminate autoreactive B cells and prevent autoimmune diseases (21, 25, 46, 47). However, both methods have limitations. BTK-inhibitors affect off-target kinases, which may contribute to their efficacy, while lifelong Btk-deficiency eliminates autoreactive cells from early development, precluding study of Btk contributions to their survival and function at mature stages. Studies in this report use timed deletion to determine the checkpoints at which autoreactive B cells require Btk. The data demonstrate that Btk supports maturation through early peripheral tolerance checkpoints, but is not needed for maintenance of mature autoreactive cells (Figures 1 and 2). Importantly, these cells retain the ability to internalize autoantigen and present it to cognate T cells, an essential function for some organ-specific autoimmune diseases, exemplified by T1D (Figure 6) (11, 31). However, Btk-depletion from anti-insulin B cells renders them vulnerable to apoptosis upon BCR engagement, as they fail to upregulate the survival factor Bcl-xL, downstream of Akt, despite the fact that proximal signaling via PLCy2 remains intact (Figures 4 and 5). Thus, autoreactive clonal populations are unable to expand, even with T cell help (Figures 5 and 6). Overall, the data indicate that autoreactive B cells are most susceptible to Btk-targeting during development and upon BCR-crosslinking.

In examining developmental stages at which Btk-deletion affects autoreactive cells, the data show immediate reduction in cells emerging from the bone marrow in the early transitional (T1) stage, matching levels found in mice deficient from birth (Figure 2). This demonstrates that Btk-mediated signaling is required for autoreactive B cells to bypass this peripheral tolerance checkpoint. Normal T1 B cells undergo deletion in response to BCR crosslinking, an event thought to be useful for enforcing tolerance. However, anti-insulin B cells differ from normal B cells. Insulin is a small, soluble protein that binds BCRs with relatively low affinity. Antigen-engagement does not necessarily generate the same kind of strong signal induced by BCR crosslinking used in other experimental settings, but rather may just enhance or support constitutive levels of signaling. Their BCRs are consistently insulin-occupied *in vivo* which may support this anergic state, and at the same time may support a low level of Btk-mediated signaling necessary for them to bypass this checkpoint. Cells in the T1 compartment are in the process of moving from the bone marrow, where antigen-engagement promotes apoptosis, to more mature peripheral stages at which antigen-engagement initiates cellular activation. It may be that the T1 stage provides a narrow signaling window in which low level Btk-mediated signals are required for positive selection, while stronger signals generate negative selection. One caveat to this interpretation is that myeloid cells also express BTK, and our model does not rule out a role for these cells in supporting early transitional B cell processes, a possibility that requires further study.

Once anti-insulin B cells reach mature stages, Btk is no longer required, as evidenced by the persistence of FO cells to at least five days, and MZ cells even after 10 weeks (Figures 1 and 2). However, the loss of emerging Btk-deficient autoreactive cells through transitional

stages results in attrition of mature FO cells over time, so that by 10 weeks post-treatment those cells are nearly absent. In contrast, the long-lived MZ compartment is retained at 10 weeks. PreMZ precursors at that time are mostly Btk^+ cells that escaped deletion, as are 17% of MZ cells. Since anti-insulin B cells that are Btk-deficient from birth populate neither the FO nor the MZ compartment, these findings suggest that Btk-mediated signaling supports development of both compartments, but is not required for their persistence in the repertoire beyond transitional stages.

Btk has a well-established role in proximal signaling, particularly in phosphorylation of PLCγ2 (15). Therefore it was surprising that this pathway proved to be maintained in anti-insulin B cells after Btk was removed. In fact, PLCγ2 phosphorylation levels in T2/FO B cells were even higher in the absence of Btk, although this is most likely due to the fact that IgM surface levels are also higher in these cells, providing more contact points for signaling in response to crosslinking. Increased Syk phosphorylation is consistent with this concept, as it is downstream of the BCR but upstream of both Btk and PLCγ2. Further, inhibition of Syk abrogated PLCγ2 phosphorylation, indicating that Syk provides redundancy for Btk in this setting, either directly or indirectly. The negative regulator CD22 was also shown to rely on Syk, but not Btk. Thus, proximal signaling pathways in anergic anti-insulin B cells rely on Syk, but not Btk.

Despite intact proximal signaling, anti-insulin B cells failed to proliferate in response to BCR-crosslinking after Btk-deletion (Figure 5A,B). Even when Btk is present, anti-insulin B cells do not proliferate in response to their antigen. However strong BCR cross-linking using anti-IgM can partially break this anergy, inducing some cell division, though less than in nonautoreactive cells (30). Loss of Btk completely abrogates the ability of this strong BCR signal to break anergy. The fact that proximal signaling is still intact indicates that an additional mechanism is needed to support a functional response by anergic autoreactive B cells. These findings prompted the discovery that anergic autoreactive B cells retain the ability to protect themselves from apoptosis by upregulating Bcl-xL in response to BCR-crosslinking (Figure 5F,G). This process is Btk-dependent, as its loss abrogates Bcl-xL responses, resulting in increased PARP cleavage and cell death (Figure 5C–E). Btk activates the Akt pathway, which in turn supports Bcl-xL expression. Thus, Btk has an important role in supporting autoreactive B cells that is independent of proximal signaling via PLCγ2, but is likely mediated by the Akt pathway. In terms of pathophysiology, this pathway also appears to support anergic B cell response to T cell help, as Btk-deficient anti-insulin B cells also fail to proliferate during antigen-presentation assays using cognate T cells (Figure 6D,E).

Despite this inability to respond to T cell help, Btk-deficient anti-insulin B cells remained competent to present antigen and activate their cognate partners. This assay uses a conjugated antigen: insulin protein paired with an OVA peptide recognized by OT-II T cells (11). This approach requires internalization of the insulin component via the BCR followed by cellular processing, and presentation of the OVA peptide component to the T cells, thus testing multiple aspects of antigen-internalization, processing and presentation. We previously used this method to show that anergic B cells could process and present a physiologic autoantigen, supporting the concept that this is their primary role in Type 1

diabetes, an organ-specific, T cell-mediated disease in which autoantibodies do not appear to contribute to islet destruction (10, 31, 48). We had also shown that Btk was unnecessary for antigen-internalization by the few anti-insulin B cells that remained when Btk was deficient from birth, but there were too few cells for functional studies (25). This model overcame that limitation and shows that Btk is not necessary for antigen processing and presentation in this context. Our findings differ from two other studies showing that Btk-impaired B cells with endogenous BCRs do require Btk for antigen internalization and/or presentation (49, 50). This may be due to differences in functional capabilities of anergic versus nonanergic cells, or in differences between B cell responses to small soluble antigen such as insulin versus strong crosslinking stimulation used in those studies.

Overall, data in this report show that Btk is not required for anergic autoreactive B cells to survive *in vivo* once they have passed developmental checkpoints. Further, these cells present antigen and activate cognate T cells. However, Btk does support autoreactive B cell development, anti-apoptotic mechanisms and proliferative responses, such that drug targeting may still be effective in reducing their pathogenic contributions. These findings may help guide efforts to use BTK-inhibitors to treat or prevent autoimmune diseases, suggesting that long-term dosing at regular intervals may be more effective at preventing development of autoreactive cells than intermittent or short-term treatment.

The goal of treatment for autoimmune disease is to target autoreactive cells while leaving normal B cells intact. Human B cells rely heavily on BTK for early maturation but our work suggests that late-targeting has less effect than genetic deficiency. Indeed, early work with BTK-inhibitors suggests they do not recapitulate X-Linked Agammaglobulinemia, the disease that results from BTK-deficiency in humans (51–53). In fact, a recent case study reported that the BTK-inhibitor ibrutinib eliminates autoantibodies and reduces the need for insulin in diabetic patients, while leaving vaccine responses intact, similar to our findings in mouse models (18, 21, 54). Therefore, the approach of targeting BTK to treat autoimmune disease remains appealing, and may offer a way to act as a “rheostat,” to dial down autoreactive B cells without inducing B cell immunodeficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article:

Btk	Bruton's tyrosine kinase
Btk **flox**	Btk lox-P flanked
Cre-ER^{T2}	Tamoxifen-inducible Cre recombinase
Syk	spleen tyrosine kinase
PLCγ2	phospholipase C γ 2
SLE	systemic lupus erythematosus
T1D	type 1 diabetes
125Tg	anti-insulin VDJ$_H$-125 site directed to the native IgH locus with conventional Vκ 125 light chain
V$_H$125	anti-insulin VDJ$_H$-125 site directed to the native IgH locus
T1	transitional 1
T2	transitional 2
FO	follicular
pMZ	pre-marginal zone
MZ	marginal zone
PARP	Poly (ADP-ribose) polymerase

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Key Points:

- Btk supports autoreactive B cells through an early immune tolerance checkpoint
- Btk mediates anti-apoptotic Bcl-xL expression in anergic anti-insulin B cells
- Mature autoreactive B cells do not require Btk for survival or antigen-presentation

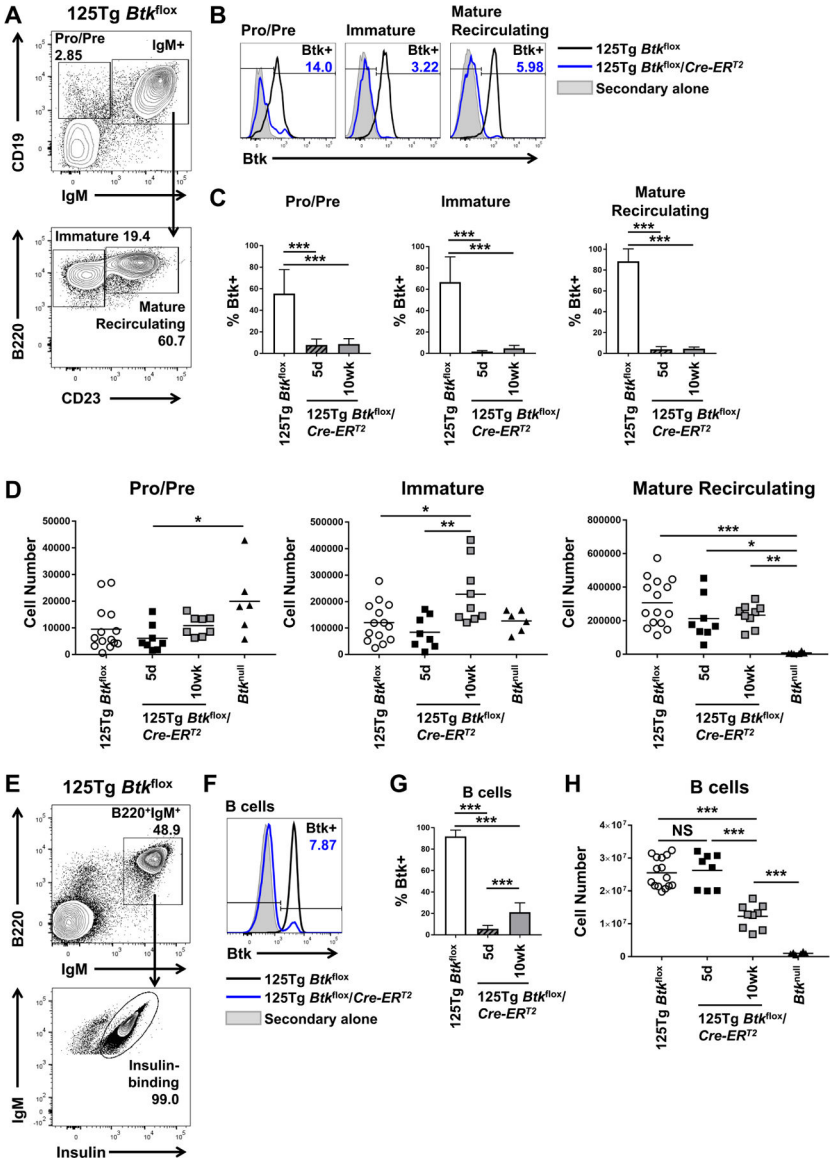

Figure 1.

Anti-insulin B cells do not require Btk for survival. Transgenic anti-insulin (Tg125) mice with *Btk*^{flox}/*Cre-ER*^{T2}, *Btk*^{flox} or *Btk*^{null} (lifelong Btk-deficient) were treated with Tamoxifen. A) Representative gating for pro/pre, immature, and mature recirculating B cells from bone marrow of a transgenic *Btk*^{flox} animal 10 weeks after tamoxifen injections. B) Representative histograms of Btk expression in pro/pre- (left), immature (middle) and mature recirculating (right) B cells, ten weeks after tamoxifen. C) Percent of pro/pre- (left), immature (middle) and mature recirculating (right) B cells that are Btk+. D) Total numbers of pro/pre- (left), immature (middle) and mature recirculating (right) B cells in the bone marrow. E) Gating scheme for splenic B cell populations analyzed ten weeks after tamoxifen treatment. Cells are pre-gated as live single as B220^{+}CD19^{+} and insulin-binding live lymphocytes, then by expression of CD19, IgM, and insulin. F, G) Representative histograms displaying Btk expression and bar graph for percent of Btk+ B cells in total

splenic B cells. H) Total numbers of B cells in the spleens of *Btk*^{flox} (circles, n=14–15), *Btk*^{flox}/*Cre-ER*^{T2} five days (black squares, n=8) or ten weeks (gray squares, n=9) after injections, or *Btk*^{null} (triangles, n=6) transgenic animals. Bar graphs show mean ± SD. $p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ by one-way ANOVA.

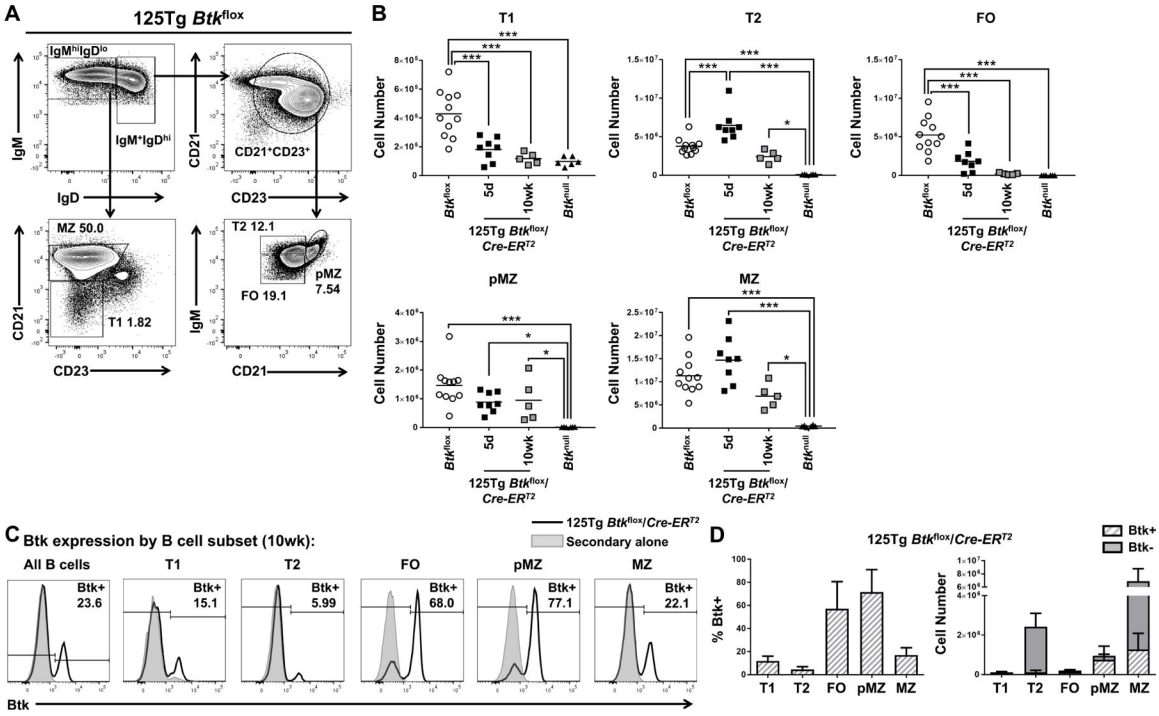

Figure 2.

Anti-insulin B cells require Btk at multiple stages of development. A) Representative gating scheme of B cell subsets in the spleen, showing *Btk*^{flox} B cells expressing heavy and light chain BCR transgenes (125Tg) 10 weeks after tamoxifen treatment. Cells are pre-gated as live, single B220^{+}IgM^{+} lymphocytes and then by expression of IgM, IgD, and CD21 into early transitional 1 (T1), late transitional 2 (T2), follicular (FO), pre-marginal zone (pMZ) and marginal zone (MZ) B cell subsets. B) Total numbers of T1, T2, FO, pMZ, and MZ B cells in the spleen of *Btk*^{flox} (circles, n=11) *Btk*^{flox}/*Cre-ER*^{T2} five days (black squares, n=8) or ten weeks (gray squares, n=5) after injections, or *Btk*^{null} (triangles, n=6) 125Tg mice. C) Representative histograms of Btk expression in all, T1, T2, FO, pMZ, and MZ B cells in *Btk*^{flox}/*Cre-ER*^{T2} (black) animals ten weeks after tamoxifen treatment. Secondary alone controls are shown in gray. D) The percentages (left) and numbers (right) of Btk-positive B cells (diagonal hatching) by B cell subset are shown in *Btk*^{flox}/*Cre-ER*^{T2} (n=5) animals ten weeks after tamoxifen treatment. Bar graphs show mean ± SD *p<0.05, ***p<0.001 by one-way ANOVA.

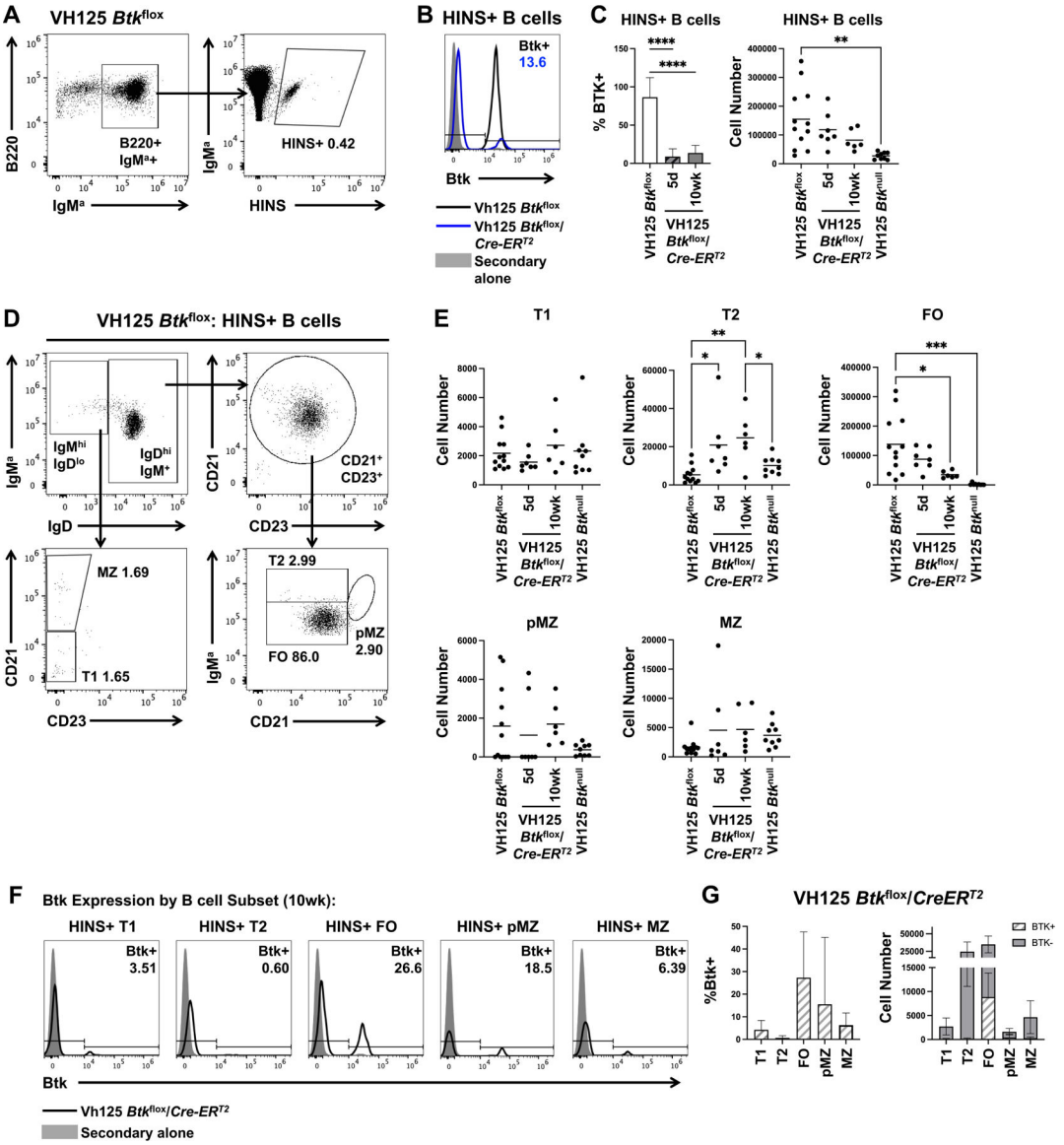

Figure 3.

Anti-insulin B cells do not require Btk when competing with a polyclonal repertoire in the periphery. Transgenic heavy chain only mice with Btk^{flox}/CreER^{T2} or Btk^{null} (lifelong Btk-deficient) were treated with Tamoxifen. A) Representative gating scheme for anti-insulin B cells in the spleen, showing VH125 Btk^{flox} B cells 10 weeks after tamoxifen treatment. Cells are pre-gated as live, single B220^{+} lymphocytes and then by expression of IgM and HINS. B) Representative histogram of Btk expression 10 weeks after injections. C) Quantification of percentage of Btk expression and total anti-insulin B cell numbers for of Btk^{flox} (n=12), Btk^{flox}/CreER^{T2} 5 days (n=7) or 10 weeks (n=6) after injections, or Btk^{null}(n=9) VH125 mice. D) Representative gating scheme of B cell subsets in the spleen, showing VH125 Btk^{flox} B cells 10 weeks after tamoxifen treatment. Cells were pre-gated as live, single B220^{+}IgM^{+} lymphocytes and then by expression of IgM, IgD and CD21 into early transitional (T1), late transitional (T2), follicular (FO), pre-marginal zone (pMZ)

and marginal zone (MZ) B cell subsets. E) Total numbers of T1, T2, FO, pMZ and MZ B cells in the spleen of Btk^{flox} (n=12), $Btk^{flox}/CreER^{T2}$ 5 days (n=7) or 10 weeks (n=6) after injections, or Btk^{null}(n=9) VH125 mice. F) Representative histograms of Btk expression in all anti-insulin, T1, T2, FO, pMZ, and MZ B cells in VH125 $Btk^{flox}/CreER^{T2}$ animals 10 weeks after tamoxifen injections. G) The percentages (left) and numbers (right) of Btk-positive B cells (diagonal hatching) by B cell subset are shown in VH125 $Btk^{flox}/CreER^{T2}$ (n=6) animals 10 weeks after tamoxifen treatment. Bar graphs show mean ± SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by one way ANOVA.

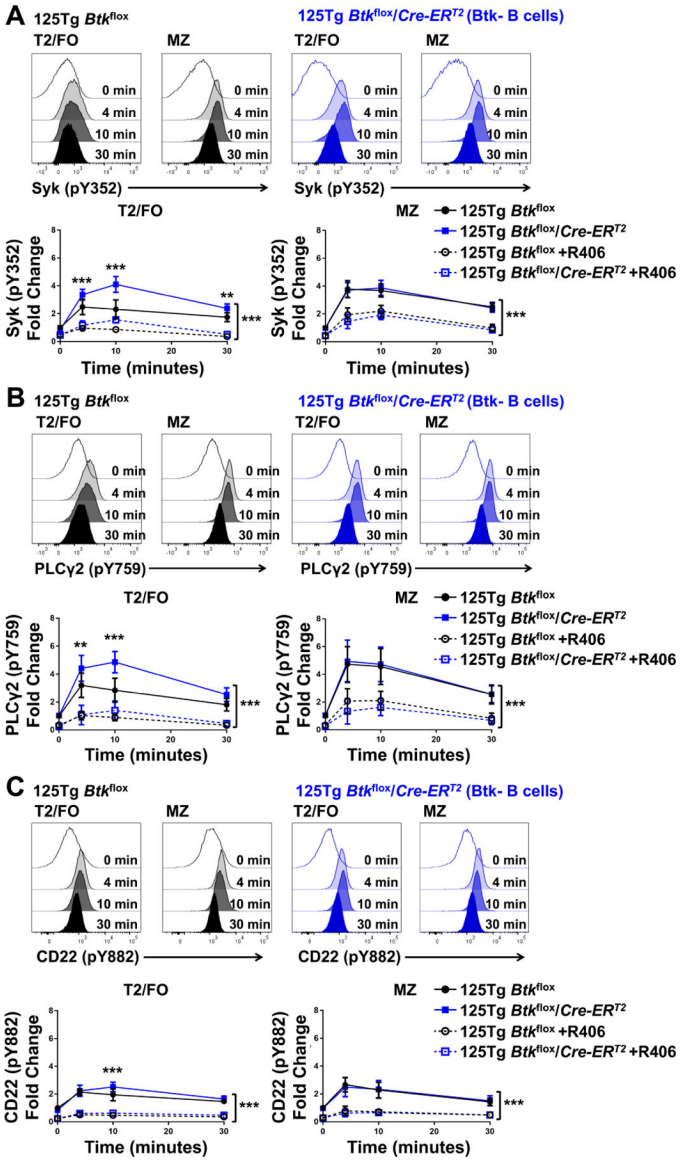

Figure 4.

Btk-deleted anti-insulin B cells signal through the BCR. A, B, C) Phosphorylation of signaling proteins Syk (A), PLCγ2 (B), and CD22 (C) in T2/FO and MZ anti-insulin B cells, after 0, 4, 10, or 30 minutes of stimulation with 10μg/mL of anti-IgM. Signaling in anti-insulin *Btk*^{flox} controls (black, n=7–11) and *Btk*^{flox}/*Cre-ER*^{T2} (blue, n=3–8) B cells is shown by representative histograms (top) and graphs (bottom). B cells from *Btk*^{flox}/*Cre-ER*^{T2} are first gated as Btk-negative and then assessed for signaling. Dashed lines show BCR signaling in presence of R406, a Syk inhibitor. Graphs show mean ± SD. **p<0.01, ***p<0.001 by two-way ANOVA.

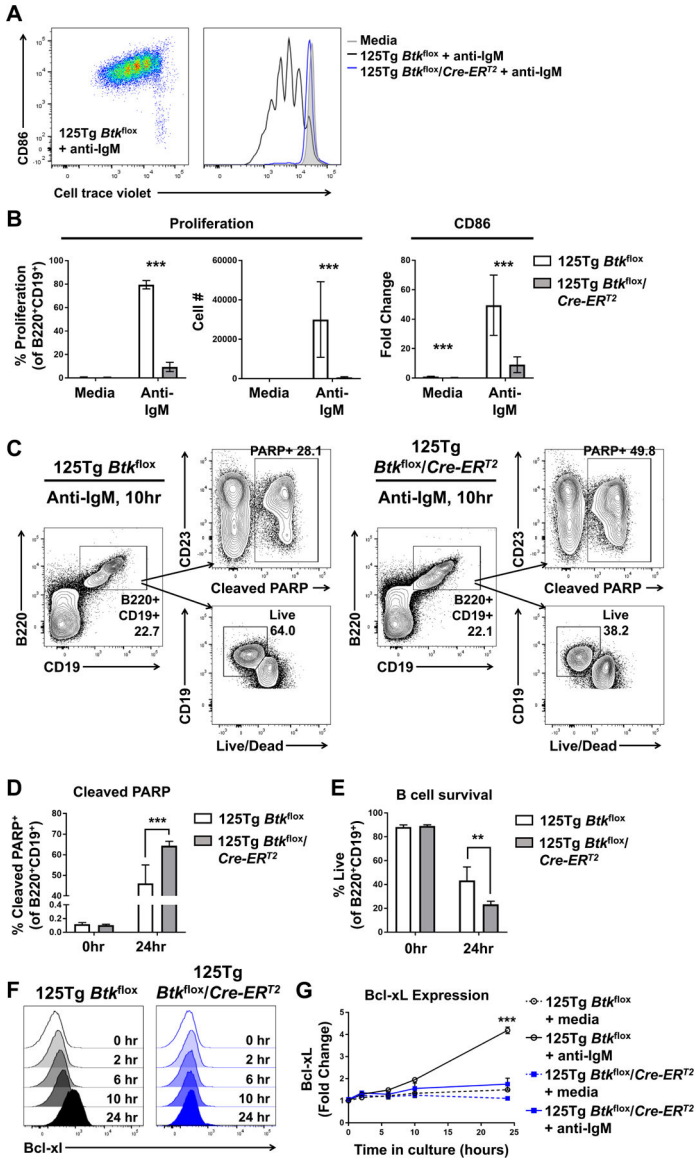

Figure 5.

Btk-negative anti-insulin B cells fail to upregulate Bcl-xl and proliferate in response to anti-IgM. A) Flow cytometric analysis of cell trace violet labeled anti-insulin B cells. Five days after tamoxifen treatment, splenocytes from *Btk*^{flox} controls (black) or *Btk*^{flox}/*Cre-ER*^{T2} (blue) mice were harvested and cultured for three days in media alone (right, gray), or with 10μg/mL anti-IgM. B) B cell proliferation and activation is quantified by percent proliferating (left), cell number proliferating (middle) and by upregulation of CD86 (right) in *Btk*^{flox} (white, n=6) and *Btk*^{flox}/*Cre-ER*^{T2} (gray, n=6). C) Representative flow plots of cell survival and PARP cleavage in *Btk*^{flox} and *Btk*^{flox}/*Cre-ER*^{T2} anti-insulin B cells. Cells were gated as single lymphocytes, then B220^{+}CD19^{+} cells were further quantified by staining for viability and PARP cleavage. D, E) Percentage of cleaved PARP-positive (D) and viable (E) *Btk*^{flox} (white, n=6) and *Btk*^{flox}/*Cre-ER*^{T2} (gray, n=6) anti-insulin B cells after 0 hours or 24 hours of anti-IgM stimulation. F) Representative histograms of Bcl-xL

expression in live B cells from *Btk*^{flox} (black) and *Btk*^{flox}/*Cre-ER*^{T2} (blue) splenocytes cultured in anti-IgM for 0, 2, 6, 10, or 24 hours. G) Fold change of Bcl-xL in live *Btk*^{flox} (black, n=3) and *Btk*^{flox}/*Cre-ER*^{T2} (blue, n=3) anti-insulin B cells cultured in media alone (solid lines) or with anti-IgM (dashed lines). Bar graphs show mean ± SD. *p<0.05, **p<0.01, ***p<0.001 by two-way ANOVA

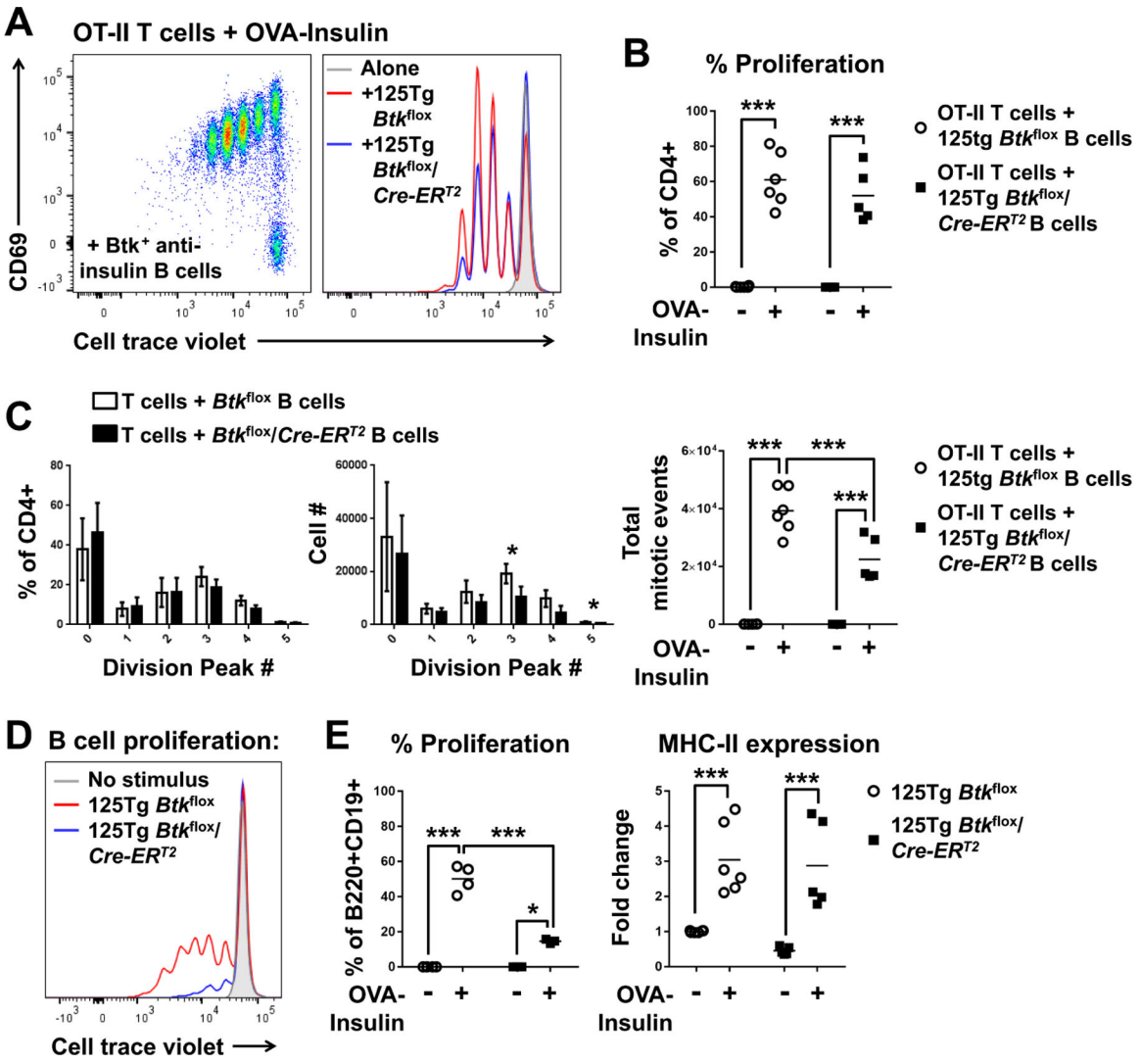

Figure 6.

Anti-insulin B cells do not require Btk to present antigen. (A, left) Cell trace violet staining and CD69 expression of $CD4^+$ OT-II T cells are shown after three days of incubation with anti-insulin B cells and 1μg/mL OVA-insulin. (A, right) Representative proliferation of $CD4^+$ OT-II T cells incubated with OVA-insulin: alone (gray), with anti-insulin B cells from Btk^{flox} control mice (red) or anti-insulin B cells from $Btk^{flox}/Cre\text{-}ER^{T2}$ animals (blue). (B) Percentage of $CD4^+$ OT-II T cells proliferating after incubation in media or 1μg/mL OVA-insulin with Btk^{flox} anti-insulin B cells (circles, n=6) or $Btk^{flox}/Cre\text{-}ER^{T2}$ anti-insulin B cells (squares, n=5). (C) Percent (left) and total number (middle) of OT-II T cells present in each proliferation peak after coculture with Btk^{flox} anti-insulin B cells (white, n=6) or $Btk^{flox}/Cre\text{-}ER^{T2}$ anti-insulin B cells (black, n=5), numbers used to calculate total mitotic events for each group (left). D) Representative histogram of proliferation of Btk^{flox} anti-insulin B cells coincubated with T cells and media (gray) or T cells and OVA-insulin (red), or $Btk^{flox}/Cre\text{-}ER^{T2}$ anti-insulin B cells with T cells and OVA-insulin (blue). E) Btk^{flox} (circles, n=4–6) or $Btk^{flox}/Cre\text{-}ER^{T2}$ (squares, n=3–5) anti-insulin B cells percent proliferating (left) or MHC-II expression (right) after incubation with T cells in media alone

or 1μg/mL OVA-insulin. Bar graphs show mean ± SD. *p<0.05, ***p<0.001, as calculated by two-way ANOVA (B, right panel C, and E), multiple-T test with Welch's correction (left and middle panels C) or by T test (D).