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The development of autoimmunity is correlated with heightened sensitivity of B cells to B cell Ag receptor (BCR) cross-linking. BCR signals are down-regulated by Lyn, which phosphorylates inhibitory receptors. lyn−/− mice have reduced BCR signaling thresholds and develop autoantibodies, glomerulonephritis, splenomegaly due to myeloid hyperplasia, and increased B-1 cell numbers. Bruton’s tyrosine kinase (Btk), a critical component of BCR signaling pathways, is required for autoantibody production in lyn−/− mice. It is unclear whether Btk mediates autoimmunity at the level of BCR signal transduction or B cell development, given that lyn−/−Btk−/− mice have a severe reduction in conventional B and B-1 cell numbers. To address this issue, we crossed a transgene expressing a low dosage of Btk (Btklow) in B cells to lyn−/−Btk−/− mice. Conventional B cell populations were restored to levels similar to those in lyn−/− mice. These cells were as hypersensitive to BCR cross-linking as lyn−/− B cells as measured by proliferation, Ca2+ flux, and activation of extracellular signal-regulated kinase and Akt. However, lyn−/−Btklow mice did not produce anti-ssDNA, anti-dsDNA, anti-histone, or anti-histone/DNA IgM or IgG. They also lacked B-1 cells and did not exhibit splenomegaly. Thus, B cell hyperresponsiveness is insufficient for autoimmunity in lyn−/− mice. These studies implicate B-1 and/or myeloid cells as key contributors to the lyn−/− autoimmune phenotype. The Journal of Immunology, 2003, 171: 1850–1858.

Proper function of the immune system requires the development of a B cell repertoire that recognizes a diversity of foreign Ags yet remains self-tolerant. Signals from the pre-B and B cell Ag receptors (BCR) control multiple checkpoints during B cell development. These include the proliferative expansion of pre-B cells, allelic exclusion, negative selection of autoreactive immature B cells via deletion or induction of anergy, the transition from immature to mature B cells in the periphery, and the survival of mature B cells (reviewed in Ref. 1). BCR signaling also is required for positive selection of the B-1 subpopulation of B cells (reviewed in Ref. 2). In addition, a strong correlation between the responsiveness of B cells to BCR stimulation and the development of autoimmunity in several mouse strains has suggested that the regulation of BCR signal thresholds plays a critical role in controlling B cell tolerance (reviewed in Ref. 3).

The initial events to occur in response to stimulation of the BCR is the activation of Src family kinases. Several Src kinases, including Lyn, participate in the activation of the BCR signaling cascade. Lyn also plays a unique role in down-regulating responses to BCR cross-linking by phosphorylating the immunoreceptor tyrosine-based inhibitory motifs of several inhibitory receptors including CD22 (4–7), FcγRIIb (4, 5), and PD-1 (10). B cells from lyn−/− mice are thus hyperresponsive to BCR stimulation (4, 5, 11, 12). As lyn−/− mice age, they develop several hallmarks of autoimmune disease similar to systemic lupus erythematosus (SLE), including anti-dsDNA Abs of both the IgM and IgG class and glomerulonephritis (12–14). Several studies have described an expansion of B-1 cells in these animals (12, 14, 15). Splenomegaly resulting from myeloid hyperplasia is also characteristic of aged lyn−/− animals (12–14, 16).

The mechanism for the development of autoimmunity in lyn−/− mice is not well understood. The role of Lyn in mediating B cell tolerance has been assessed in several Ig transgene models, with conflicting results. Although some studies suggest that Lyn deficiency results in increased negative selection, others demonstrate a breach of B cell tolerance in lyn−/− mice. Negative selection of autoreactive B cells is more potent in the absence of Lyn in the hen egg lysozyme (HEL) system. Soluble HEL Ag, which normally induces anergy of B cells expressing an Ig transgene specific for HEL (17), causes their deletion on a lyn−/− background (7). An alternative model that more closely mimics the breach in tolerance to nuclear Ags observed in lupus involves an IgH transgene, 3H9, which can contribute to anti-dsDNA, anti-ssDNA, and nonautoantibodies when paired with the appropriate L chains (18). In wild-type 3H9 mice, the majority of anti-dsDNA B cells are deleted (19), whereas a subpopulation of anti-dsDNA B cells identified by expression of 3H9 and endogenous A1 L chain is rendered anergic (20). Maintenance of tolerance to DNA is observed to some degree in 3H9 lyn−/− mice, because these 3H9/A1-expressing cells remain anergic. However, some anti-dsDNA IgG is present, and anti-dsDNA hybridomas can be produced in 3H9 lyn−/− mice (20), indicating that
not all B cells specific for dsDNA are tolerized in the absence of Lyn. In a third system, Lyn deficiency results in a clear breach of tolerance. Expression of an anti-RBC Ig transgene in normal mice causes the majority of peripheral B cells to have a B-1 phenotype. These cells are normally anergic but become activated in the absence of Lyn, resulting in severe autoimmune hemolytic anemia (21).

The role of Lyn in maintaining anergy of autoreactive B-1 cells in the anti-RBC Ig transgene system (21) implicates this population as an important contributor to autoimmunity in lyn−/− mice. However, two recent reports (16, 22) have contradicted earlier studies (12, 14, 15) describing an expansion of the B-1 population in lyn−/− mice. In addition, the role of B-1 cells in autoimmunity in general is controversial. There are several examples of lupus-like disease in the absence of B-1 cell expansion, including Sle1, SLE3 (23) and lpr mice (24). In the latter case, conventional B cells have been shown to be the source of anti-DNA Abs (24).

Although the mechanism by which Lyn regulates tolerance remains unclear, several lines of evidence suggest that that B cell hyperresponsiveness is likely a major contributor to the autoimmune phenotype in these animals. Like Lyn deficiency, mutations in many components of Lyn-dependent inhibitory signaling pathways result in both reduced BCR signaling thresholds and increased autoantibody levels. These include deficiency of CD22 (25, 26), FcγRIIB (27), PD-1 (28, 29), or Src homology 2 domain-containing protein tyrosine phosphatase 1 (30, 31), as well as overexpression of CD19 (32) which serves to sequester Lyn from lyn−/− mice (33). To further define the role of BCR responsiveness in autoantibody production, we and others determined whether the autoimmunity in lyn−/− mice is dependent on Bruton’s tyrosine kinase (Btk) (15, 34).

Naturally occurring mutations in Btk cause the B cell immunodeficiencies X-linked agammaglobulinemia in humans (35, 36) and X-linked immunodeficiency (xid) in mice (37, 38). Xid/mice, which have a point mutation in Btk, and Btk-deficient mice have an impaired transition from immature to mature B cells in the periphery (39–42) and lack B-1 cells (40, 43). Xid and Btk−/−B cells fail to enter the cell cycle in response to BCR cross-linking (40, 44), demonstrating that Btk is a critical component of BCR signaling pathways. Moreover, Btk is down-regulated by at least two inhibitory receptors that are phosphorylated by Lyn, FcγRIIB (45, 46), and paired Ig-like receptor-B (47). B cells from lyn−/−Btk−/− (34) and lyn−/−xid (15) mice fail to respond to BCR cross-linking, and Btk is required for the production of autoantibodies in lyn−/− mice (15, 34). Taken together, these observations suggest that Btk mediates the development of autoimmunity at the level of BCR signal transduction. However, loss of Btk function also results in a severe reduction in B cell numbers in the absence of Lyn (15, 34), making it impossible to determine whether impaired B cell development or failure of those cells that do develop to transmit BCR signals is responsible for the lack of autoimmunity in lyn−/−Btk−/− or lyn−/−xid mice.

To resolve this issue, we took advantage of a transgenic mouse strain that expresses 25% of endogenous Btk levels (Btklow) in B cells. One allele of this transgene completely rescues conventional B cell development in Btk−/−or xid mice and partially restores the response to BCR cross-linking (48). We predicted that this transgene would also restore B cell development in lyn−/−Btk−/−mice, allowing us to clearly assess the role of Btk in the B cell hyperactivity and autoimmunity characteristic of Lyn deficiency. Although similar numbers of hyperresponsive B cells were present in lyn−/− and lyn−/−Btklow mice, the latter did not develop autoantibodies, B-1 cells, or splenomegaly. Thus, the reduced threshold for BCR signaling is not the sole defect leading to autoimmunity in lyn−/− mice.

Materials and Methods

Mice

Btk−/− mice are Btk−/− mice carrying a wild-type Btk transgene driven by the Ig H chain promoter and enhancer (48). The generation of lyn−/−, lyn−/−Btk−/−, and lyn−/−Btklow mice has been described previously (34).

Because mice were of a mixed genetic background (C57BL/6 × 129), littersmates were compared directly when possible, and experiments were repeated with multiple litters. Mice used for signaling studies and flow cytometry were between 6 and 12 wk of age and showed no signs of splenomegaly. Mice assayed for autoantibodies and splenomegaly were between 4 and 8 and between 6 and 8 mo old, respectively.

Flow cytometry

Splenocytes and peritoneal wash cells were depleted of RBC by a 5-min incubation in 0.15 M NaCl, 1 mM KHCO3, 0.1 M Na2EDTA and stained with combinations of anti-IgD FITC (BD Pharmingen, San Diego, CA), anti-IgM PE (BD Pharmingen), anti-CD5 PE (BD Pharmingen), anti-CD221FITC (BD Pharmingen), and anti-CD232biont (BD Pharmingen) plus streptavidin-APC (Caltag Laboratories). Samples were run on a FACSCalibur (BD Biosciences, San Jose, CA) and analyzed with CellQuest software (BD Biosciences, San Jose, CA).

Proliferation assays

Spleenic B cells were purified by negative selection with anti-CD34 magnetic beads, positive selection with anti-B220 beads, or a combination of both using the Miltenyi MidiMACS system (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. No difference was observed in the proliferative responses of B cells purified by each method. Purified B cells were plated in well-plate plates at 106/ml in RPMI plus 10% FCS. Triplicate wells were used for each stimulation condition. Stimulation conditions included medium alone and 2 or 20 μg/ml goat anti-mouse IgM F(ab′)2 fragments (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were incubated at 37°C in a humidified incubator for 48 h. [3H]Thymidine (Amersham, Arlington Heights, IL) was added at 1 μCi/well for the final 6 h. Cells were harvested onto Beckman Ready Filters (Beckman Coulter, Fullerton, CA), and [3H]thymidine incorporation was measured with a scintillation counter.

Extracellular signal-regulated kinase (ERK) andAkt activation

B cells were purified with anti-B220 magnetic beads using the Miltenyi MACS system according to the manufacturer’s instructions. Purified B cells were resuspended at 107/ml in serum-free medium and stimulated with 0 or 2 μg/ml anti-IgM F(ab′)2 fragments (Jackson ImmunoResearch Laboratories) for 5 min. Cells were lysed by boiling in SDS sample buffer for 10 min. Total cell extracts (2 × 106 cells equivalents) were electrophoresed on a 10% SDS-PAGE gel and blotted to nitrocellulose. Blots were blocked in 5% milk in 10 mM Tris, pH 7.5, and 150 mM NaCl and probed with anti-phospho-ERK (Thr202/Tyr204) (Cell Signaling Technology, Beverly, MA), anti-ERK (Cell Signaling Technology), anti-phospho-Akt (Ser473) (Cell Signaling Technology), or anti-Akt (Cell Signaling Technology) diluted in 10 mM Tris (pH 7.5), 250 mM NaCl, 0.05% Tween 20, 0.2% sodium azide. Blots were washed in 10 mM Tris (pH 7.5), 500 mM NaCl, 0.05% Tween 20 and then incubated with HRP-conjugated goat anti-rabbit Ig (Bio-Rad, Hercules, CA) diluted in 10 mM Tris (pH 7.5), 250 mM NaCl, 0.05% Tween 20. Blots were washed in 10 mM Tris (pH 7.5), 500 mM NaCl, 0.05% Tween 20. HRP was visualized using an ECL kit (Amersham).

Ca2+ flux

B cells were depleted of RBC as described above, washed, and resuspended in loading buffer (HBSS, 1 mM Ca2+, 1 mM Mg2+, 1% FBS). Cells were loaded with 1 μM Indo-1 acetoxymethyl ester (Molecular Probes, Eugene, OR) at 37°C for 30 min. Cells were subsequently stained with PE-conjugated anti-B220 Abs (BD Pharmingen) in loading buffer at room temperature for 20 min, washed, and resuspended in loading buffer at 3 × 106/ml. Cells were prewarmed at 37°C for several minutes before analysis and maintained at 37°C throughout the analysis. Changes in intracellular Ca2+ levels in B cells were measured on a FACStarplus by gating on B220+ cells and measuring Indo-1 fluorescence ratios. Baseline fluorescence ratios were measured for 60 s, at which point cells were stimulated with 20 μg/ml goat anti-mouse IgM F(ab′)2 fragments (Jackson ImmunoResearch Laboratories), and analysis was continued. Data were analyzed with FlowJo software (Tree Star, San Carlos, CA).
ELISA

**Autoantibodies.** Immulon II plates (Dynatech Laboratories, Chantilly, VA) were precoated with Ag: 50 μg/ml calf thymus ssDNA (Sigma-Aldrich, St. Louis, MO), 50 μg/ml calf thymus dsDNA (Sigma-Aldrich), 50 μg/ml calf thymus dsDNA plus 10 μg/ml histones (Boeringher Mannheim, Mannheim, Germany), 10 μg/ml histones, or 3 μg/ml rabbit IgG (Jackson ImmunoResearch Laboratories). After plates were incubated overnight at 4°C with blocking buffer (PBS, 3% BSA, 0.1% gelatin, 3 mM EDTA), 1/100 and 1/400 dilutions of serum were added in duplicate and incubated for 2 h at room temperature. Bound IgM or IgG was detected using alkaline phosphatase-conjugated goat anti-mouse IgM or IgG (Southern Biotechnology Associates) and an alkaline phosphatase substrate kit (Bio-Rad Laboratories). OD405 was read on a Bio-Tek Instruments microplate reader.

**Total IgM and IgG.** Plates were coated with 2 μg/ml goat anti-mouse Ig (Southern Biotechnology Associates, Birmingham, AL) and blocked with 1% BSA in borate-buffered saline (BBS). Serum or Ig standards (mouse IgM and IgG; Sigma-Aldrich) were diluted serially into BBS and added to wells in duplicate. Plates were then incubated with secondary Ab (goat anti-mouse IgM-alkaline phosphatase or goat anti-mouse IgG-alkaline phosphatase; Southern Biotechnology Associates) diluted 1/500 in BBS, 0.05% Tween 20, and 1% BSA and developed with an alkaline phosphatase substrate kit (Bio-Rad). OD405 was read on a Bio-Tek Instruments (Winooski, VT) microplate reader. Total amounts of IgM and IgG were calculated based on the known concentrations of the Ig standards.

**Results**

Btklow transgene restores conventional B cell development in lyn−/−Btk−/− mice

Btk deficiency (Fig. 1A and Ref. 34) and the point mutation in Btk associated with xid (15) each cause a substantial reduction in the number of peripheral B cells in lyn−/− mice. We asked whether this is associated with a developmental block. lyn−/− mice had frequencies of T1, T2, and mature B cells similar to those of wild-type mice as defined by expression of CD21, CD23, and IgM (Fig. 1, B and C), although each of these populations was present in

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**FIGURE 1.** Btklow transgene restores conventional B cell development to lyn−/−Btk−/− mice. A, Splenocytes from 6- to 12-wk-old mice were stained with anti-B220 Abs. The total number of B220+ cells per spleen is indicated. Data are presented as mean ± SEM (wild type (wt), n = 30; lyn−/−, n = 14; lyn−/−Btklow, n = 9; lyn−/− Btk−/−, n = 12). B and C, Splenocytes from 6–12 wk old mice were stained with Abs against B220, CD23, CD21, and IgM. Populations were defined as indicated by the gates in C: marginal zone (MZ), B220+CD23+CD21highIgM−; T1, B220+CD23+CD21 IgM−; T2, B220+CD23+CD21IgMhigh; mature (M), B220+CD23+CD21 IgM−. B, Percentage of B220+ cells having the indicated phenotype. Data are presented as mean ± SD, n = 4–6. C, Representative experiment. The percent of B220+ cells in each gate is indicated. FL, Fluorescence.
mature cells as measured by CD23, CD21, and IgM expression (Fig. 1, B and C) or IgM and IgD expression (34). The relative absence of marginal zone B cells previously described in lyn−/− mice (20) was independent of Btk (Fig. 1, B and C).

These observations make it difficult to conclude whether reduced B cell numbers, impaired B cell development, decreased response to BCR cross-linking, or another mechanism is responsible for the lack of autoantibodies in lyn−/− mice. To address this issue, we used a transgene expressing 25% of endogenous Btk levels (Btklow) in B cells. One allele of this transgene rescues conventional B cell development in lyn−/− mice (48). We predicted that this transgene would have a similar effect on lyn−/− mice. This would allow us to assess the role of Btk in the autoimmune characteristic of lyn−/− mice in the context of normal B cell development. Indeed, the Btklow transgene restored B cell numbers in lyn−/− mice to levels equivalent to those in lyn−/+ animals (Fig. 1A). There was no difference in the frequency of IgMlowIgDhigh, IgMhighIgDhigh, and IgMlowIgDhigh B cells in the spleens of lyn−/− and lyn−/−Btklow mice (data not shown). A relatively normal distribution of cells among the T1, T2, and mature B populations in lyn−/−Btklow mice was also observed as measured by CD23, CD21, and IgM expression, although there was an increase in the frequency of T2 cells compared with lyn−/− or wild-type mice (Fig. 1, B and C). Like lyn−/− mice, lyn−/−Btklow mice had a decreased proportion of marginal zone B cells. Thus, the Btklow transgene will allow us to assess the role of Btk in the autoimmune phenotype of lyn−/− mice in the absence of the substantial impairment in B cell development observed in lyn−/−Btk−/− (Ref. 34 and Fig. 1) and lyn−/−xid (15) animals.

lyn−/−Btklow B cells are hyperresponsive to BCR cross-linking

The correlation between hyperresponsiveness to Ag stimulation and autoimmunity in several mouse mutants (3) suggests that Btk may mediate autoantibody production in lyn−/− mice by regulating BCR signaling thresholds. lyn−/− B cells are hypersensitive to BCR cross-linking as measured by proliferative response, Ca2+ flux, and activation of ERK and Akt (4, 5, 11, 12, 49–52). Btk has been suggested to regulate each of these signaling events (45, 46, 51–56). We thus assessed each of these activation parameters in BCR-stimulated lyn−/−Btklow B cells. Both lyn−/− and lyn−/−Btklow B cells responded vigorously in a [3H]thymidine incorporation assay to low doses (2 μg/ml) of anti-IgM that are poorly mitogenic for wild-type B cells (Fig. 2A). This is consistent with previous studies demonstrating an increased frequency of lyn−/− and lyn−/−Btklow B cells entering the cell cycle upon BCR engagement (34). In contrast, lyn−/−Btk−/− B cells responded poorly as has been described for both lyn−/−Btk−/− and lyn−/−xid mice (15, 34). Under conditions where lyn−/− B cells demonstrated increased BCR-stimulated ERK phosphorylation compared with wild-type B cells, lyn−/−Btklow cells were also hyperresponsive (Fig. 2B). This can be observed as an increase in reactivity with anti-phospho-ERK as well as a greater proportion of total ERK

FIGURE 2. lyn−/−Btklow B cells are hypersensitive to BCR cross-linking: A. Purified splenic B cells from 6- to 12-wk-old mice were stimulated with 2 or 20 μg/ml anti-IgM F(ab')2 fragments for 48 h. Proliferation was measured by [3H]incorporation during the final 8 h of culture. Data are presented as mean ± SEM, n = 3 (left) or 5–8 (right). wt, Wild-type, B, B cells were purified from pools of two or three mice per group and stimulated for 5 min with 0 or 2 μg/ml anti-IgM F(ab')2 fragments. Total cell extracts were blotted to nitrocellulose and blotted sequentially with anti-phospho-ERK (pERK), anti-ERK, anti-phospho-Akt (pAkt), and anti-Akt. Data are representative of three independent experiments. C. Splenocytes from 6- to 12-wk-old mice were loaded with Indo-1 acetoxymethyl ester and stained with PE-conjugated anti-B220. Ca2+ levels (indicated by the ratio of Indo-1 fluorescence intensity) were measured in the B220+ population by flow cytometry. Anti-IgM F(ab')2 fragments were added at the indicated time (arrow). Mean fluorescence ratios were calculated at 10-s intervals, and background fluorescence was subtracted. These results are representative of five independent experiments with wild-type, lyn−/−, and lyn−/−Btklow mice, two of which also included lyn−/−Btk−/− mice.
displaying the retarded mobility characteristic of the phosphorylated, active form. Similarly, enhanced phosphorylation of Akt on Ser473 was observed in lyn−/− and lyn−/−/Btklow B cells upon stimulation with low doses of anti-IgM (Fig. 2B). The increased proliferative response to BCR cross-linking in lyn−/− B cells is also associated with augmented BCR-induced Ca2+ flux. The acute phase, although slightly delayed, has a greater magnitude, and the sustained phase is prolonged (4, 7). Little to no difference was observed among lyn−/−, lyn−/−/Btklow, and lyn−/−/Btkhi B cells in this assay (Fig. 2C). Because increased Ca2+ flux was observed even in lyn−/−/Btk−/− B cells, this response is insufficient to lead to the robust proliferation of lyn−/− B cells. Taken together, these observations demonstrate that lyn−/−/Btklow B cells resemble lyn−/− B cells in their hypersensitivity to BCR cross-linking as measured by multiple parameters.

Although the Btklow transgene rescues the developmental defect in lyn−/−Btk−/− mice, there is an increased frequency of T2 cells in lyn−/−/Btklow mice compared with those in lyn−/− mice (Fig. 1). It has recently been shown that T2 cells are more responsive than mature B cells to BCR cross-linking (57–59). However, we do not believe that this accounts for the hypersensitivity of lyn−/−/Btklow B cells to BCR stimulation. First, 5-bromo-2′-deoxyuridine incorporation assays demonstrate that a similar proportion of lyn−/− and lyn−/−/Btklow B cells undergo S phase in response to anti-IgM stimulation (34). In addition, a similar increase in the frequency of T2 cells (15% of B220+ cells, n = 3) compared with wild-type (9.9% of B220+ cells, n = 4; p = 0.035) does not result in increased proliferative response to BCR cross-linking in Btklow mice with an intact Lyn gene (34, 48). Thus, the hypersensitivity of lyn−/−Btklow B cells is likely due to effects of the Lyn mutation rather than increased numbers of T2 cells.

**Hyperresponsiveness to BCR cross-linking is insufficient for autoantibody production, B-1 cell expansion, and splenomegaly in lyn−/− mice**

lyn−/−Btklow mice therefore have similar numbers of hyperresponsive conventional B cells as lyn−/− mice. If the autoantibodies in lyn−/− mice result solely from increased sensitivity of B cells to BCR cross-linking, then lyn−/−Btklow animals should also develop autoimmunity. However, the spontaneous production of IgM and IgG anti-dsDNA Abs that occurred in the majority of lyn−/− mice was absent in all lyn−/−Btklow mice at 4–6 mo of age (Fig. 3A). These animals remained predominantly anti-dsDNA free until at least 6–8 mo of age (Fig. 3B). Only 1 of 13 lyn−/−Btklow mice produced anti-dsDNA IgM, and none developed anti-dsDNA IgG. This was not because of a general inhibitory effect of the Btk transgene due to its integration site or ectopic expression, given that lyn−/− mice carrying the transgene and the wild-type endogenous Btk gene (lyn−/−/Btkhwb) develop autoantibodies at a frequency and time frame similar to lyn−/− mice (Fig. 3A). The absence of IgG anti-dsDNA in lyn−/−Btklow mice cannot be explained by a general reduction in serum IgG levels (Fig. 3A). The difference in the amount of IgG between lyn−/− and lyn−/−/Btklow
mice is not statistically significant \((p = 0.14)\). However, the elevated total IgM characteristic of \(\text{lym}^{-/-}\) mice was not observed in \(\text{lym}^{-/-}\)Btk\text{low} mice (Fig. 3A). Btk therefore mediates the production of autoantibodies by mechanisms other than regulating BCR signaling thresholds.

The progression of lupus-related autoimmune disease has been suggested to occur in multiple stages, with loss of tolerance to chromatin and ssDNA preceding a more generalized, pathogenic response that includes anti-dsDNA Abs (23). There are thus two checkpoints at which Btk may be regulating autoantibody production in \(\text{lym}^{-/-}\) mice: at the initial loss of tolerance to chromatin; or at the progression to anti-dsDNA reactivity. To address this issue, we assessed the role of Btk in the production of autoantibodies against ssDNA, histone-DNA complexes, and histones (Fig. 3B). Although \(\text{lym}^{-/-}\) mice produced both IgM and IgG against these Ags, none of these autoantibodies was detected in \(\text{lym}^{-/-}\)Btk\text{low} mice. Reduced Btk dosage also impaired the production of rheumatoid factor in \(\text{lym}^{-/-}\) mice. The absence of autoantibodies against multiple Ags, including chromatin and ssDNA, in \(\text{lym}^{-/-}\)Btk\text{low} mice suggests that the effect of reduced Btk dosage likely occurs at the initiation or early in the progression of autoimmune disease.

**Discussion**

The production of autoantibodies in \(\text{lym}^{-/-}\) mice is prevented by loss of Btk function (15, 34). The opposing roles of Btk and Lyn in setting BCR signaling thresholds and the correlation between BCR hyperactivity and autoantibody levels in a number of mutant mouse strains suggest that Btk contributes to autoimmunity at the level of BCR signal transduction. It is likely that Btk-dependent transmission of BCR signals is required for autoantibody production. However, we demonstrate here that the heightened sensitivity to BCR cross-linking observed in \(\text{lym}^{-/-}\) mice is not sufficient for the development of autoimmunity (Table I). Conventional B cells from \(\text{lym}^{-/-}\)Btk\text{low} and \(\text{lym}^{-/-}\) mice develop similarly and are equally hyperresponsive to stimulation of the BCR by several measures: Ca\(^{2+}\) flux; activation of ERK and Akt; and proliferation. However, \(\text{lym}^{-/-}\)Btk\text{low} mice do not exhibit autoimmunity or splenomegaly and have very few B-1 cells. Thus, the role of Btk and Lyn in these processes can be uncoupled from their functions as regulators of BCR signaling thresholds.

It is possible that there are BCR-initiated, Btk-dependent signaling events other than Ca\(^{2+}\) flux, ERK activation, Akt activation, or proliferation that differ between \(\text{lym}^{-/-}\) and \(\text{lym}^{-/-}\)Btk\text{low} B
cells and are required for BCR-mediated B-1 cell development and autoantibody production. It will be interesting to determine whether there are particular alterations in BCR signaling pathways that are specifically associated with autoimmunity, but not B cell hyperactivity, in the lyn−/− model.

An alternative and intriguing consideration is that Btk may mediate autoimmunity via BCR-independent processes. Btk is phosphorylated in response to RP-105, CD38, IL-5, IL-6, and IL-10 and has been shown to be required for the response of B cells to all of these except IL-6 (61–65). Signaling by RP-105 (66) and CD38 (67) is dependent on Lyn, so these molecules are unlikely to contribute to the phenotype of lyn−/− mice. IL-5, IL-6, and IL-10 have each been implicated as regulators of autoantibody production in human SLE and mouse models of lupus, suggesting that they may also play a role in the Btk-dependent autoimmunity in lyn−/− mice. Transgenic mice overexpressing IL-5 develop increased anti-DNA Abs of the IgM class (68). Similarly, in vitro stimulation of B cells from NZB × NZW autoimmune mice with LPS and IL-5 results in production of anti-DNA IgM Abs (69). IL-6 increases secretion of anti-DNA IgG by these B cells (70), whereas anti-IL-6 Ab inhibits the spontaneous production of anti-DNA IgG by SLE B cells in vitro (71) and by NZB × NZW mice in vivo (72). IL-10 accelerates and anti-IL-10 Abs delay the onset of lupus in both NZB × NZW mice (73) and SLE patients (74).

Btk-dependent cytokine production may also play a role in the development of autoantibodies in lyn−/− mice. A massive accumulation of myeloid cells is observed as lyn−/− mice age (12–14, 16). Increased cytokine secretion either directly by these cells or by T cells to which they present Ag may contribute to the autoimmune phenotype. In support of this hypothesis, the splenomegaly resulting from myeloid hyperplasia that is characteristic of lyn−/− animals is severely attenuated in lyn−/−/Btklow (Fig. 5), lyn−/−/Btk−/− (Fig. 5 and 34), and lyn−/−/xid (15) mice. Alternatively, the Btk-dependent myeloid expansion seen in lyn−/− mice could be a secondary consequence rather than a cause of the autoimmunity.

The role of B-1 cells in the autoimmune disease of Lyn-deficient mice is controversial. B-1 cells are clearly the source of autoantibodies in an anti-RBC Ig-transgenic mouse model of severe autoimmune hemolytic anemia (75), and autoimmunity is exacerbated in this system in the absence of Lyn (21). Several studies have described an expansion of B-1 cells in Lyn-deficient mice (12, 14, 15). However, this was not observed in several recent reports (16, 22). In the present study, the frequency of B-1 cells in the peritoneal cavities of young wild-type and lyn−/− mice were similar. One model that incorporates all of these observations is that B-1 cells do contribute to autoimmunity in lyn−/− mice but that the critical function of Lyn is to regulate their activation rather than their numbers. Indeed, whereas normal peripheral B-1 cells die in response to BCR cross-linking, lyn−/− B-1 cells proliferate upon stimulation with anti-IgM (76). Btk, in contrast, would contribute significantly to B-1 cell development and/or maintenance. This scenario is consistent with the correlation between the near-absence of B-1 cells and the lack of autoantibodies in lyn−/−/Btklow, lyn−/−/Btk−/− (34), and lyn−/−/xid (15) mice.

The positive selection of B-1 cells is regulated by BCR signals (2). However, the significant reduction in this cell population in lyn−/−/Btklow mice despite the hyperresponsiveness of conventional B cells to BCR stimulation demonstrates that Btk has other critical roles in this process. IL-5 and IL-10 each signal via Btk (63, 65) and are regulators of both B-1 cell development and activation. Deficiency of IL-5 (77) or its receptor (78) results in reduced B-1 cell numbers, as does treatment with anti-IL-10 Abs (79). Injection of either IL-5 or IL-10 activates autoreactive B-1 cells to secrete autoantibody in the anti-RBC Ig-transgenic mouse model (80). Thus, impaired IL-5 and IL-10 signaling in lyn−/−/Btklow mice could result in both a decrease in the number of B-1 cells and the inability of those few cells that do develop to become activated.

Decreased expression of Lyn has been observed in peripheral B cells of a subset of human SLE patients (81). The uncoupling of Btk-mediated response to BCR cross-linking from autoantibody production in lyn−/− mice suggests that Btk signaling pathways may be an attractive therapeutic target for SLE. Partial inhibition of Btk, its regulators, or its effectors may prevent either the initiating loss of tolerance to chromatin or the production of pathogenic anti-dsDNA autoantibodies (23) while allowing residual B cell function. Interestingly, several naturally occurring loci that suppress the development of SLE have been identified (82). It will be interesting to determine whether these encode components of Btk signaling pathways.

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expression of B cells implicates Bruton tyrosine kinase (btk) as a regulator of CD38
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