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Independent and Opposing Roles For Btk and Lyn in B and Myeloid Signaling Pathways

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Summary

Transphosphorylation by Src family kinases is required for the activation of Bruton’s tyrosine kinase (Btk). Differences in the phenotypes of Btk–/– and lyn–/– mice suggest that these kinases may also have independent or opposing functions. B cell development and function were examined in Btk–/–lyn–/– mice to better understand the functional interaction of Btk and Lyn in vivo. The antigen-independent phase of B lymphopoiesis was normal in Btk–/–lyn–/– mice. However, Btk–/–lyn–/– animals had a more severe immunodeficiency than Btk–/– mice. B cell numbers and response to T cell–dependent antigens were reduced. Btk and Lyn therefore play independent or partially redundant roles in the maintenance and function of peripheral B cells. Autoimmunity, hypersensitivity to B cell receptor (BCR) cross-linking, and splenomegaly caused by myeloerythroid hyperplasia were alleviated by Btk deficiency in lyn–/– mice. A transgene expressing Btk at ~25% of endogenous levels (Btklo) was crossed onto Btk–/– and Btk–/–lyn–/– backgrounds to demonstrate that Btk is limiting for BCR signaling in the presence but not in the absence of Lyn. These observations indicate that the net outcome of Lyn function in vivo is to inhibit Btk-dependent pathways in B and myeloid cells, and that Btklo mice are a useful sensitized system to identify regulatory components of Btk signaling pathways.

Key words: B cell receptor • B cell development • Src family kinases • transgenic mice • immunodeficiency

The development of a diverse repertoire of B cells and the maintenance of self-tolerance depend on signals transduced by the B cell antigen receptor (BCR). The outcome of BCR engagement varies from proliferation and differentiation to deletion depending on the developmental stage of the B cell, concurrent signals, and the degree of BCR cross-linking (for review see reference 1). A complex signaling network translates BCR-mediated signals into the appropriate response given the context in which they are received. One of the initial biochemical consequences of BCR engagement is the sequential activation of a cascade of tyrosine kinases belonging to the Src, Btk/Tec, and Syk/Zap70 families. The phosphorylation of multiple substrates by these kinases leads to signaling events which include stimulation of the Ras/mitogen-activated protein kinase (MAPK) pathway, phosphoinositide hydrolysis, Ca2+ flux, and the activation of PI3-kinase α (for review see reference 2). B cell development is generally blocked at the proB to preB transition in the absence of preB receptor or BCR subunits (3–6), but BCR signaling is less severely affected in mice lacking other molecules downstream of the BCR such as Bruton’s tyrosine kinase (Btk; references 9–11), Lyn (12–14), Fyn (15, 16), PKCβ (17), and Vav (18, 19). This suggests that, although Syk plays a unique role early in B cell development, there may be a significant degree of redundancy among some components of BCR signaling pathways.

Src family kinases, including Lyn, BLK, Fyn, Lck, and Fgr, are activated rapidly upon BCR cross-linking (2).
Among Src family kinases, only mutations in Lyn have been described as affecting BCR signaling (12–16, 20). Intriguingly, Lyn appears to be involved in both the initiation of BCR signals and their subsequent downregulation (14, 20). Anti-IgM-mediated cross-linking of the BCR results in slightly delayed and reduced tyrosine phosphorylation of Igα, Igβ, Syk, and several other substrates in B cells from lyn−/− mice (13, 14). The residual phosphorylation is probably catalyzed by other Src family kinases present in these cells. Despite delayed signal initiation, lyn−/− mice have an immature cell surface phenotype and a shorter life span than do wild-type B cells (14). Serum IgM and IgA levels are increased (12, 13). Aged lyn−/− animals develop autoantibodies and exhibit splenomegaly due to extramedullary hematopoiesis and the expansion of IgM-secreting B lymphoblasts (12–14). The phenotype of lyn−/− mice is strikingly similar to that of motheaten (serum Ig (for review see reference 33). Both BCR, IL-5R, IL-10R, and CD38 are impaired (15, 16, 20). Intriguingly, Lyn appears to be involved in both the initiation of BCR signals and their subsequent downregulation (14, 20). This results from impaired downregulation of BCR signaling via both FcγRIIb-dependent and -independent mechanisms (14).

Mutations in Lyn also affect B cell development. The frequency of peripheral B cells is reduced approximately twofold in lyn−/− mice (12–14, 20). The remaining cells have an immature cell surface phenotype and a shorter life span than do wild-type B cells (14). Serum IgM and IgA levels are increased (12, 13). Aged lyn−/− animals develop autoantibodies and exhibit splenomegaly due to extramedullary hematopoiesis and the expansion of IgM-secreting B lymphoblasts (12–14). The phenotype of lyn−/− mice is strikingly similar to that of motheaten (me) mice (21), which are deficient in the negative regulator of BCR signaling SH2-containing protein tyrosine phosphatase 1 (SHP1). This suggests that other Src kinases cannot compensate for Lyn in the termination of BCR signals.

Several lines of evidence indicate that Btk is downstream of Src family kinases in a BCR signaling pathway. Coexpression of Btk and Lyn in fibroblasts leads to the transphosphorylation of Btk on Y551 and activation of Lyn kinase activity (22, 23). Btk is also phosphorylated on Y551 in response to BCR cross-linking (22, 24). The ability of an activated form of Btk to transform fibroblasts is dependent on both the activity of Src family kinases and the presence of Y551 (25, 26). Mutations of lyn−/− mice prevents Lyn from mediating BCR-induced Ca2+ flux in B cells (27, 28). These combined observations indicate that transphosphorylation by Lyn is critical for Btk function.

Mutations in Btk result in the B cell immunodeficiencies X-linked agammaglobulinemia (XLA) in humans (29, 30) and X-linked immunodeficiency (xid) in mice (31, 32). XLA patients have a block at the pre-B stage of development, resulting in a severe deficit of circulating B cells and serum Ig (for review see reference 33). Both xid and lyn−/− (9–11) mice have a more subtle phenotype (for review see reference 33). They have a 30–50% decrease in the number of peripheral B cells, with the most profound reduction in the mature IgM+IgD+ subset. xid mice have reduced levels of serum IgM and IgG3 and do not respond to type II T cell–independent antigens. They also lack B1 cells. Responses to the engagement of several cell surface receptors including BCR, IL-5R, IL-10R, and CD38 are impaired in the absence of Lyn. Lyn expressing reduced levels of Btk are hypersensitive to anti-IgM (34), suggesting that Lyn is limiting for the transmission of signals from the BCR.

Despite the biochemical evidence that Lyn and Btk operate sequentially in common signaling pathways, the different phenotypes of Btk−/− and lyn−/− mice (low versus high serum IgM, hyporesponsiveness to BCR cross-linking) suggest that these kinases may also have opposing roles in BCR signaling. To clarify this issue, we examined B cell development in mice lacking both Btk and Lyn. If Btk and Lyn oppose each other, Btk deficiency might be expected to rescue the lyn−/− phenotype, analogous to the rescue of the me B cell phenotype by CD45 deficiency (35). If Lyn is the sole upstream activator of Btk, then effects on B cell development should be no more severe in lyn−/− than in lyn−/− mice alone. Increased severity of phenotype would indicate that Btk and Lyn are partially redundant components of one signaling pathway or participants in independent pathways. A combination of these possibilities was observed, indicating that Lyn opposes Btk-mediated signals and plays a positive signaling role independent of or partially redundant with Btk.

Materials and Methods

Mice

Btk−/− (10) and lyn−/− mice (14), each on a mixed C57BL/6 × 129/Sv genetic background, were crossed to generate Btk−/−/lyn−/− F1 progeny. These F1 animals were mated resulting in wild-type (wt), Btk-deficient, Lyn-deficient, and Btk/Lyn-deficient progeny. Genotypes were determined by Southern blot (10) or PCR (14) analysis of tail biopsy DNA as described. For the analysis of limiting Btk dosage, Btk−/−/lyn−/− mice carrying an Ig heavy chain enhancer/promoter-driven Btk transgene expressing ∼25% of endogenous Btk levels in B cells (34). The resulting progeny were on a mixed C57BL/6 × 129/Sv × Balb/c background. The presence of the Btk transgene was determined by Southern blot as previously described (34).

Flow Cytometry

Phenotypic Analysis. Single cell suspensions from spleen, bone marrow, or peritoneal wash were depleted of red blood cells and stained with combinations of the following antibodies (from PharMingen, San Diego, CA, unless otherwise noted): PE-conjugated (PE) anti-B220 (RA2-6B2); FITC-conjugated (FITC) anti-IgM (R6-60.2); anti-IgD (AMS 9.1) PE; anti-IgG2a (217-170) PE; anti-CD5 (53-7.3) FITC; anti-Thy-1.2 (35-2.1) FITC; anti-CD4 (H129.19) PE; anti-CD8 (53-6.7) FITC; and anti-Ter119 (Becton Dickinson) FITC (129/Sv genetic background, were crossed to generate Btk−/−/lyn−/− F1 progeny. These F1 animals were mated resulting in wild-type (wt), Btk-deficient, Lyn-deficient, and Btk/Lyn-deficient progeny. Genotypes were determined by Southern blot (10) or PCR (14) analysis of tail biopsy DNA as described. For the analysis of limiting Btk dosage, Btk−/−/lyn−/− mice carrying an Ig heavy chain enhancer/promoter-driven Btk transgene expressing ∼25% of endogenous Btk levels in B cells (34). The resulting progeny were on a mixed C57BL/6 × 129/Sv × Balb/c background. The presence of the Btk transgene was determined by Southern blot as previously described (34).

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Analysis of In Vivo Bromodeoxyuridine Incorporation. Mice were fed 0.25 mg/ml bromodeoxyuridine (BrdU) and 2.5% glucose in their drinking water continuously for up to 15 d. Single cell suspensions of spleens and peripheral blood were depleted of red blood cells stained with anti-BrdU FITC (Becton Dickinson) and anti-B220 (R A3-6B2) PE (Pharmingen) as previously described (36), and analyzed as above.
Proliferation Assays

BrdU Labeling. Total splenocytes were depleted of red blood cells and plated in RPMI 10% heat-inactivated FCS at 10^5/ml. Where indicated, goat anti-mouse IgM F(ab')2, fragments (Jackson Immunoresearch Labs, West Grove, PA) were added at either 2 or 20 μg/ml. At 24 h, BrdU (Sigma Chemical Co., St. Louis, MO) was added to a final concentration of 10 μM. Cells were harvested at 48 h and FACScan analysis was performed as above.

ELISA

Serum Ig. Plates were coated with 2 μg/ml goat anti-mouse Ig (Southern Biotechnology Associates, Huntsville, AL) and blocked with 1% BSA in borate-buffered saline (BBS). Serum or Ig standards (mouse IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA; Sigma Chemical Co.) were diluted serially into BBS and added to wells in duplicate. Plates were washed, incubated with secondary antibody (goat anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3, or IgA-alkaline phosphatase [ALPH], Southern Biotechnology Associates, Inc., Huntsville, AL) at 2 or 4 μg/ml goat anti-mouse IgM F(ab')2 fragments (Jackson Immunoresearch Labs.). 1 μCi [3H]thymidine (NEN Life Science Products, Boston, MA) was added per well for the final 12-18 h. Cells were harvested and counted on a scintillation counter.

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[14] Hjorthymidine Labeling. B220+ spleen cells were isolated using the Mincams magnetic bead system (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Single cell suspensions were depleted of red blood cells before incubation with magnetic beads. B cell-enriched populations were >90% B220+ by FACScan analysis. B220+ splenic B cells were seeded into 96-well plates at 5 × 10^3/ml in RPMI 10% heat-inactivated FCS. Where indicated, cells were incubated for 60 h with 2 or 20 μg/ml goat anti-mouse IgM F(ab')2 fragments (Jackson Immunoresearch Labs.). 1 μCi [3H]thymidine (NEN Life Science Products) was added per well for the final 12-18 h. Cells were harvested and counted on a scintillation counter.

ELISA

Serum Ig. Plates were coated with 2 μg/ml goat anti-mouse Ig (Southern Biotechnology Associates, Huntsville, AL) and blocked with 1% BSA in borate-buffered saline (BBS). Serum or Ig standards (mouse IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA; Sigma Chemical Co.) were diluted serially into BBS and added to wells in duplicate. Plates were washed, incubated with secondary antibody (goat anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3, or IgA-alkaline phosphatase [ALPH], Southern Biotechnology Associates) diluted 1:500 in BBS/0.05% Tween 20/1% BSA and developed with an ALPH substrate kit (Bio-Rad, Hercules, CA). OD was read on a Vmax kinetic microplate reader (Molecular Devices Corp., Sunnyvale, CA).

Keyhole Limpet Hemocyanin. Mice were immunized intraperitoneally with 100 μg of keyhole limpet hemocyanin (KLH) (Sigma Chemical Co.) in incomplete Freund's adjuvant (GIBCO BRL, Gaithersburg, MD), boosted on day 21 with 50 μg of KLH in PBS, and bled on day 28. ELISAs were performed as above with the following modifications: plates were coated with 2 μg/ml of KLH, and secondary antibodies were goat anti-mouse IgM-ALPH and goat anti-mouse IgG1-ALPH.

2,4,6-Triphenyl-1-Ficoll. Mice were immunized intraperitoneally with 10 μg of 2,4,6-triphenyl (TNP)-Ficoll (a gift of Dr. John Imman, National Institutes of Health, Bethesda, MD) and bled 6 d later. ELISA was performed as above with the following modifications: plates were coated with 25 μg/ml of TNP-Ficoll in PBS, and serum and secondary antibody (goat anti-mouse IgM-ALPH) were diluted into PBS/0.1% BSA, and 0.05% Tween 20.

dsDNA. Unimmunized mice were bled at 16–20 wk of age. Serum antibodies to dsDNA were measured in triplicate by ELISA as previously described (14).

Immunofluorescence

Unimmunized mice were bled at 16–20 wk of age. Serum antibodies to nuclear antigens were measured by immunofluorescence as previously described (14).

Results

Generation of Btk−/−lyn−/− Mice. We bred Btk+/−lyn−− mice to generate wild-type, Btk−/−, lyn−/−, and Btk−/−lyn−− mice in order to examine the interaction of these kinases in B cell development and function. All genotypes occurred in the predicted Mendelian frequencies, indicating that embryonic development occurs normally in the absence of both Btk and Lyn. Btk−/−lyn−/− mice were healthy and fertile. lyn−/− and lyn−− animals were indistinguishable in the assays described below and were therefore used interchangeably. All mice were on a mixed C57B/6 × 129/Sv background. Although the phenotype of Btk deficiency has been described to differ according to genetic background (10, 37, 38), little variation was observed within each group of mice analyzed here.

Reduced Numbers of Peripheral B Cells In Btk−/−lyn−/− Mice. To understand whether Lyn and Btk are components of a common signaling pathway or function independently in B cells, we examined B cell development in the absence of Btk alone, Lyn alone, or both Btk and Lyn. Both the frequency and number of splenic B cells in 8-wk-old Btk−/−lyn−/− mice was reduced two- to fourfold compared to wild-type controls (Fig. 1 A and Table 1). The remaining B cells had an immature IgM+IgD+ phenotype similar to Btk−/− B cells (Fig. 1 B). This decrease was specific to the B lineage. No significant difference in myeloid cell numbers was observed in the spleens of young mice, and T cell numbers were reduced less than twofold compared with wild-type controls (Table 1). The frequency of conventional (B220+CD5−) B cells in the peritoneum was less than in Btk−/−lyn−/− mice compared with single knockouts alone (Fig. 1 D, Table 1).

Either a block in the bone marrow phase of B cell development or reduced half life of peripheral B cells could be responsible for the small number of splenic B cells in Btk−/−lyn−/− mice. Both the B220+IgM− population, consisting of proB and preB cells, and B220+IgM− immature B cells were present in similar frequencies in bone marrow from wild-type, Btk−/−, lyn−/−, and Btk−/−lyn−− mice (Fig. 1 C and Table 1). Therefore, the antigen-independent phase of B cell development proceeds normally in the absence of both Btk and Lyn. B220+IgM− recirculating B cells were three- to fourfold less frequent in both lyn−− (as previously reported in references 12 and 13) and Btk−/−lyn−− mice than in wild-type controls (Fig. 1 C and Table 1). This is consistent with the significant reduction in mature B cell numbers in the periphery.

The turnover rate of the peripheral B cell population was assessed with in vivo BrdU labeling since an early developmental block did not account for the reduced number of splenic B cells in Btk−/−lyn−/− mice. Short-lived B cells in the periphery are replaced by newly generated cells that have incorporated BrdU, whereas long-lived resting cells remain unlabeled. Both splenic and peripheral blood B cells from Btk−/−lyn−− mice turned over faster than wild-type, Btk−/−, or lyn−− B cells (Fig. 2). Greater than 70% of Btk−/−lyn−− B220+ cells were labeled with BrdU after 8 d, compared with ~35% in wild-type mice and 50% in mice lacking either Btk or Lyn alone (Fig. 2). These observations indicate that the reduced number of B cells in mice lacking both...
Impaired B Cell Survival and Function in Btk⁻/⁻ lyn⁻/⁻ Mice

Btk and Lyn is due to poor survival in the periphery rather than decreased production of B cells in the bone marrow.

Serum Ig Levels and Immune Responses Are Decreased in Btk⁻/⁻ lyn⁻/⁻ Mice. To determine whether the reduced number of peripheral B cells in Btk⁻/⁻ lyn⁻/⁻ mice was accompanied by alterations in serum Ig levels, the amount of each Ig isotype present in the serum of 6-8-wk-old wild-type, Btk⁻/⁻, lyn⁻/⁻, and Btk⁻/⁻ lyn⁻/⁻ mice was measured.
Peritoneum

Bone marrow

Spleen

Reduced Numbers of Peripheral B Cells in Btk

Table 1. Reduced Numbers of Peripheral B Cells in Btk<sup>−/−</sup>-Lyn<sup>−/−</sup> Mice

|                    | wt          | Btk<sup>−/−</sup> | Lyn<sup>−/−</sup> | Btk<sup>−/−</sup>-Lyn<sup>−/−</sup> |
|--------------------|-------------|------------------|-----------------|-----------------------------------|
| **Spleen**         |             |                  |                 |                                   |
| Total cells (× 10⁷)| 6.0 ± 1.9   | 5.3 ± 0.98       | 4.7 ± 2.9       | 3.6 ± 1.9                         |
| Percentage of B    | 48 ± 5.0    | 35 ± 8.1         | 27 ± 7.5        | 13 ± 4.6                          |
| B cells (× 10⁷)    | 2.8 ± 0.74  | 1.9 ± 0.68       | 1.2 ± 0.37      | 0.46 ± 0.32                       |
| Percentage of T    | 39 ± 5.2    | 38 ± 2.7         | 44 ± 9.4        | 52 ± 15                           |
| T cells (× 10⁷)    | 2.2 ± 0.45  | 2.0 ± 0.36       | 2.0 ± 0.55      | 1.6 ± 0.44                        |
| Percentage of myeloid cells | 12 ± 2.6 | 18 ± 4.4        | 22 ± 3.7        | 22 ± 4.9                          |
| No. of myeloid cells (× 10⁷) | 0.65 ± 0.25 | 0.93 ± 0.19 | 1.1 ± 0.60 | 0.80 ± 0.48 |

| **Bone marrow**    |             |                  |                 |                                   |
| Total cells (× 10⁷)| 3.8 ± 1.3   | 3.6 ± 0.97       | 3.7 ± 1.5       | 3.7 ± 1.3                         |
| Percentage of B220<sup>+</sup>IgM⁻ | 11 ± 3.0 | 9.9 ± 3.5       | 12 ± 4.6        | 10 ± 3.7                          |
| Percentage of B220<sup>+</sup>IgM⁺ | 5.6 ± 0.79 | 5.4 ± 1.4       | 5.3 ± 2.1       | 4.9 ± 1.6                         |
| Percentage of B220<sup>+</sup>IgG<sub>1</sub> | 3.3 ± 1.6 | 2.5 ± 1.4 | 1.2 ± 0.63 | 0.84 ± 0.62 |

| **Peritoneum**     |             |                  |                 |                                   |
| Percentage of B220<sup>+</sup>CDS<sub>5</sub>⁻ | 41 ± 6.8 | 40 ± 7.9       | 20 ± 5.0        | 13 ± 4.7                          |
| Percentage of B220<sup>+</sup>CDS<sub>5</sub>⁺ | 22 ± 8.4 | 7.3 ± 3.4     | 19 ± 8.5        | 5.8 ± 2.5                          |

Single cell suspensions of spleen, bone marrow, or peritoneal cells from 8-wk-old mice were depleted of red blood cells and stained with the following combinations of antibodies: bone marrow, anti-IgM FITC versus anti-B220 PE; spleen, anti-IgM FITC versus anti-B220 PE, anti-Thy-1.2 FITC versus anti-B220 PE, anti-CD8<sub>1</sub>FITC versus anti-CD<sub>4</sub> PE, and anti-Mac1 FITC versus anti-Gr-1 PE; peritoneum, anti-CD5 FITC versus anti-B220 PE. Live cells were gated based on forward and side scatter. The frequency and absolute number of B (B220<sup>+</sup>), T (Thy-1.2<sup>+</sup> or CD<sub>4</sub><sup>+</sup> and CD<sub>8</sub><sup>+</sup>), and myeloid (Mac1<sup>+</sup> and/or Gr1<sup>+</sup>) cells are indicated for spleen. The frequency of proB and preB cells (B220<sup>−/−</sup>IgM<sup>+</sup>) in bone marrow is shown. The frequency of CD<sub>5</sub><sup>−</sup> and CD<sub>5</sub><sup>+</sup> B cells in the peritoneum was determined for cells in a lymphoid gate. Data are presented as mean ± SD (n = 6).

(Fig. 3). Btk<sup>−/−</sup> mice had a >10-fold reduction in IgM and IgG<sub>3</sub> levels, and a 2–3-fold decrease in IgG<sub>2a</sub> and IgG<sub>2b</sub>. IgM and IgA levels were slightly elevated in lyn<sup>−/−</sup> mice compared with wild-type controls, although the 5–10-fold increase in these isotypes described in lyn<sup>−/−</sup> mice in two previous studies (12, 13) was not observed. This may be due to differences in the age of the animals at the time of analysis, since serum IgM levels in lyn<sup>−/−</sup> mice increase with time (12, 13). Btk<sup>−/−</sup>-Lyn<sup>−/−</sup> mice resembled Btk<sup>−/−</sup>- mice with respect to IgM, IgG<sub>3</sub>, and IgG<sub>2b</sub> levels, and had increased IgA similar to lyn<sup>−/−</sup> animals. The amount of IgG<sub>2b</sub> and IgG<sub>1</sub> was reduced in Btk<sup>−/−</sup>-lyn<sup>−/−</sup> mice compared with all other genotypes, consistent with the low numbers of peripheral B cells in these animals.

The dramatic effect of double Btk and Lyn deficiency on B cell numbers suggested that B cell function may also be more severely inhibited in the absence of both kinases. The ability of Btk<sup>−/−</sup>-lyn<sup>−/−</sup> mice to mount an immune response to the T cell–independent antigen TNP-Ficoll and the T cell–dependent antigen KLH was assessed. Btk<sup>−/−</sup>-lyn<sup>−/−</sup> mice failed to produce antibodies against TNP-Ficoll (Fig. 4 A), as expected, since this response requires Btk. Mice lack-

Figure 2. Increased turnover of Btk<sup>−/−</sup>-lyn<sup>−/−</sup> peripheral B cells. 8–10-wk-old wt, Btk<sup>−/−</sup>, lyn<sup>−/−</sup>, and Btk<sup>−/−</sup>-lyn<sup>−/−</sup> mice were fed BrdU in their drinking water continuously. Single cell suspensions were isolated from the spleen (A) and peripheral blood (B) of mice at the times indicated, depleted of red blood cells, and stained with anti-BrdU FITC and anti-B220 PE. The percentage of B220<sup>+</sup> cells labeled with BrdU is represented. Data were collected in two independent experiments and are presented as mean ± SEM of at least four mice per group.
ing either Btk or Lyn alone have relatively normal secondary responses to T cell-dependent antigens such as KLH (Fig. 4, B and C). Strikingly, anti-KLH IgM was not detectable in Btk−/−lyn−/− mice (Fig. 4 B). IgG1 titers against KLH were measurable but lower on average than in mice of other genotypes (Fig. 4 C). Although the impaired IgG1 response could be attributed to low B cell numbers, the reduction in anti-KLH IgM was significant even when corrected for cell number. This indicates that Btk and Lyn are redundant for the production of IgM against T-dependent antigens.

**Figure 4.** Impaired immune responses in Btk−/−lyn−/− mice. Serum levels of IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA in 6-8-wk-old wt, Btk−/−, lyn−/−, and Btk−/−lyn−/− mice were measured by ELISA. Data are presented as mean ± SEM (n = 8).

**Figure 3.** Serum Ig levels in Btk−/−lyn−/− mice. Serum levels of IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA in 6-8-wk-old wt, Btk−/−, lyn−/−, and Btk−/−lyn−/− mice were measured by ELISA. Data are presented as mean ± SEM (n = 8).

**Figure 5.** Lyn has a net inhibitory effect on Btk-mediated BCR signals. Several aspects of B cell function differ significantly between lyn−/− and Btk−/− mice despite the similar reduction in conventional B cell numbers. Most strikingly, lyn−/− B cells are hyperresponsive to BCR cross-linking (14, 20), whereas Btk−/− cells fail to proliferate in response to anti-IgM (10). A transgenic model, in which varying doses of a Btk transgene driven by the Ig heavy chain enhancer and promoter are expressed on an xid or Btk−/− background, has been used to demonstrate that Btk dosage is limiting for response to BCR cross-linking (34). B cells expressing low levels of transgenic Btk and no endogenous Btk (Btkko) develop normally but have reduced sensitivity to BCR cross-linking (34). This system was used to determine whether the net effect of Lyn is to activate or inhibit Btk function. Low doses of Btk should be less effective in mediating mitogenic response to BCR engagement on a lyn−/− background if Lyn is an essential upstream activator of Btk during signal initiation. Alternatively, the ability of limiting amounts of Btk to transmit BCR signals should be enhanced in the absence of Lyn if the negative role of Lyn predominates.

A Btk transgene expressing ~25% of endogenous Btk levels in splenic B cells (34) was crossed onto both Btk−/− (Btkko) and Btk−/−lyn−/− (Btkko lyn−/−) backgrounds. The frequency and absolute number of B220+ and IgMloIgDhi cells were increased two- to threefold in Btkko spleens relative to Btk−/− spleens in both the presence and absence of Lyn (reference 34 and data not shown), indicating that the transgene restored Btk-dependent signals for maintenance of B cell numbers. Btkko B cells were less sensitive to BCR engagement than were wild-type B cells (reference 34 and Fig. 5 A). In contrast, the BCR-induced proliferative response of Btkko lyn−/− B cells was indistinguishable from that of lyn−/− B cells even at low doses of anti-IgM (Fig. 5 A). Btk−/−lyn−/− B cells failed to proliferate upon anti-IgM stimulation (Fig. 5 A). This was not due to altered T or myeloid cell function, as the same result was obtained with purified B cells (Fig. 5 B) and total splenocytes (Fig. 5 A). Lyn deficiency therefore enhances Btk-dependent signaling by the BCR but cannot bypass a requirement for Btk. These observations suggest that Lyn has a net inhibitory effect on Btk-dependent BCR signaling pathways.

Autoimmunity in lyn−/− mice is dependent on Btk. The development of autoimmunity in aged lyn−/− mice is another feature that distinguishes them from Btk−/− and xid mice (12–14). The xid mutation has been shown to prevent autoantibody production in both NZB×NZW mice (39,
40) and me mice (41). Although six out of six lyn<sup>−/−</sup> mice older than 16 wk developed IgM and IgG antibodies against both dsDNA and nuclear antigens, no Btk<sup>−/−</sup>lyn<sup>−/−</sup> animals of similar age displayed signs of autoimmunity (Table 2). Peritoneal B1 cells are believed to be a major source of anti-self antibodies in autoimmune strains of mice (42, 43). Btk<sup>−/−</sup>lyn<sup>−/−</sup> mice, like Btk<sup>−/−</sup> mice, have a reduced frequency of B<sup>220<sup>−</sup>CD5<sup>−</sup></b> cells in the peritoneum relative to wild-type or lyn<sup>−/−</sup> animals (Fig. 1D, Table 1).

Figure 5. Enhancement of Btk-dependent signaling in the absence of Lyn. (A) Single cell suspensions of spleens from eight to ten week old Btk<sup>−/−</sup>, Btk<sup>−/−</sup>, and wt mice on a lyn<sup>−/−</sup> (left) or lyn<sup>−/−</sup> (right) background were depleted of red blood cells and incubated for 24 h with the indicated concentrations of goat anti-mouse IgM F(ab')<sub>2</sub> fragments. Cells were labeled with BrdU for an additional 24 h and stained with anti-BrdU FITC and anti-B220 PE. The percentage of B220<sup>−</sup> cells labeled with BrdU is indicated. The results are presented as mean ± SEM (n = 3 for transgenic mice, n = 5 for all other groups). (B) B220<sup>−</sup> cells were purified from spleens from 8-wk-old wt, Btk<sup>−/−</sup>, lyn<sup>−/−</sup>, and Btk<sup>−/−</sup>lyn<sup>−/−</sup> mice using the M inimacs magnetic bead system. Cells were incubated in triplicate with the indicated concentrations of goat anti-mouse IgM F(ab')<sub>2</sub> fragments for 48 h and labeled with [3H]thymidine for a further 12–16 h. The results are presented as mean ± SEM (two independent mice per group, triplicate samples per mouse) and are from one of four experiments giving similar results.

Table 2. Autoimmunity in lyn<sup>−/−</sup> Mice Is Btk Dependent

|  | wt | Btk<sup>−/−</sup> | Lyn<sup>−/−</sup> | Btk<sup>−/−</sup>-Lyn<sup>−/−</sup> |
|---|---|---|---|---|
| Antibodies to nuclear antigens* | 1/7 (weak) | 0/5 | 6/6 | 0/7 |
| Antibodies to dsDNA † |  |  |  |  |
| IgM | 0.062 | 0.031 | 0.459 | 0.016 |
| ± 0.018 | ± 0.013 | ± 0.281 | ± 0.006 |
| IgG | 0.017 | 0.004 | 0.417 | 0.005 |
| ± 0.006 | ± 0.002 | ± 0.274 | ± 0.002 |

Mice were 16–20-wk-old at the time of analysis.
* Number of mice positive for antibodies against nuclear antigens/total mice tested by immunofluorescence.
† Antibodies against dsDNA were measured by ELISA assay. OD<sub>490</sub> values are shown as mean ± SEM. Triplicate samples were measured for each mouse, with the number of individual mice per group as follows: wild-type, 7; Btk<sup>−/−</sup>, 5; lyn<sup>−/−</sup>, 6; Btk<sup>−/−</sup>-Lyn<sup>−/−</sup>, 7.
Figure 6. Splenomegaly and extramedullary hematopoiesis in lyn⁻/⁻ mice are uncoupled in the absence of Btk. (A) Spleens from 10–11-mo-old mice are shown. (B) Single cell suspensions from spleens from 10–11-mo-old mice were depleted of red blood cells and stained with anti-Mac1 FITC (middle x-axis) versus anti-Gr-1 PE (middle y-axis) and anti-Ter119 PE (bottom x-axis). The percentage of total cells falling in the indicated regions is shown.
Independent roles for Btk and Lyn in the maintenance of peripheral B cell numbers. A large body of evidence derived from biochemical and tissue culture–based experiments demonstrates that activation of Btk requires Src family kinases (22–28). However, the increased severity of the B cell phenotype in Btk−/−lyn−/− mice compared with lyn−/− mice alone indicates that Btk can still transmit signals in the absence of Lyn. In fact, the ability of limiting doses of Btk to signal is enhanced in lyn−/− B cells. This suggests either that Lyn is redundant with other Src family kinases for the activation of Btk in mature B cells or that Btk can be activated by Src family–independent mechanisms. Although activation of Btk is critically dependent on Src kinases in a fibroblast model (22, 23, 26), additional regulatory mechanisms cannot be ruled out in the B lineage.

Although Lyn may contribute to the activation of Btk in concert with other Src kinases, it also plays a Btk–independent role in the maintenance of the peripheral B cell population. Lyn−/− mice have fewer B cells than did wild-type littermates (12–14). This has been suggested to result from increased negative selection of B cells that are hypersensitive to BCR cross-linking (14). Diminished B cell numbers in Btk−/−lyn−/− mice may be explained by the additive effect of reduced positive selection in the absence of Btk and increased negative selection in the absence of Lyn. However, this is unlikely since lyn−/− B cells do not respond to BCR engagement when they also lack Btk. Lyn is therefore likely to transmit a positive signal for B cell survival that is independent of Btk.

Reduced life span of B cells in the periphery of Btk−/−lyn−/− mice. B cells from both Btk−/− and lyn−/− mice have an increased turnover rate relative to wild-type B cells. The number of long-lived B cells is even further reduced in the absence of both Btk and Lyn. This could be secondary to a block in maturation as the long-lived B cell pool consists predominantly of mature B cells (45). However, this possibility is unlikely as Btk−/− and Btk−/−lyn−/− mice have a similar developmental block at the IgMhiIgDlo to IgMloIgDhi transition. The reduced half-life of Btk−/−lyn−/− B cells could also be a result of impaired positive BCR signaling due to the combined effects of Lyn deficiency on signal initiation and Btk deficiency on signal transmission. The BCR is required for survival of B cells in the periphery (46), and deletion of the cytoplasmic tail of Igα in mice results in a B cell phenotype (4) similar to that of Btk−/−lyn−/− mice. Independent roles for Btk and Lyn in BCR signaling are also supported by the recent demonstration that the Btk/TLc family kinase Itk and the Src family kinase Fyn have independent functions in TCR signaling (47). Alternatively, Btk and Lyn may be redundant for CD40 signaling. Btk−/−lyn−/− mice resemble mice deficient in both Btk and CD40 (48, 49). The impaired response to the T cell–dependent antigens would also be explained by failure to transmit CD40 signals (50–52). Finally, defects in homing of Btk−/−lyn−/− B cells to the proper compartments in secondary lymphoid organs could contribute to their poor survival (53).

The antigen-independent phase of B cell development is normal in Btk−/−lyn−/− mice. Mice lacking the three Src family kinases Lyn, Btk, and Fyn have a block in development at the proB to proB transition (Tarakhovsky, A., personal communication) similar to that observed in Ig heavy chain− (5) or surrogate light chain–deficient (6) mice. These combined results imply that Src family kinases are redundant for the transmission of preB receptor signals. B lymphopoiesis is also blocked at the proB stage in XLA patients (33), suggesting that Btk is an essential substrate of Src family kinases in human preB receptor signaling. In contrast, the antigen-independent phase of B cell development is normal in Btk−/− mice even in the absence of Lyn. The critical target of Src family kinases in murine preB cells is probably Syk rather than Btk since the proB to preB transition is impaired in syk−/− mice (7, 8).

Lyn negatively regulates Btk–dependent signaling pathways. Hypersensitivity to BCR cross-linking in lyn−/− B cells indicates that Lyn plays a critical role in the negative regulation of BCR signaling. Both the failure of Btk−/−lyn−/− B cells to proliferate in response to anti-IgM and the observation that Btk is no longer limiting for response to BCR engagement in the absence of Lyn suggest that Lyn downregulates Btk–dependent signaling pathways. The ability of Btk to promote depletion of intracellular calcium stores in response to BCR cross-linking is prevented by FcγRIIb signaling (27). This inhibition may be mediated by Lyn since FcγRIIb function is partially impaired in lyn−/− B cells (14). The negative regulatory role of Lyn is not limited to Btk–dependent pathways, as BCR–induced activation of the classical mitogen–activated protein kinase (MAPK) pathway does not require Btk (54, 55) but is enhanced in lyn−/− B cells (14).

The transgenic mice expressing low levels of Btk (34) are shown here (Fig. 5 A) to be a useful sensitized system with which to identify negative regulatory components of Btk signaling pathways. Similarly, molecules that contribute positively to Btk signaling could be defined by mutations that further impair BCR signaling in Btk−/− mice. It will be interesting to determine the effect of mutations in the remaining Src family kinases, BCR signal threshold modulators, and other B cell signaling molecules on the transmission of BCR signals by limiting amounts of Btk.

A role for Btk in myeloid expansion. A distinguishing characteristic of older lyn−/− mice is the development of splenomegaly due to extramedullary hematopoiesis (12–14). Surprisingly, although splenomegaly did not occur in old Btk−/−lyn−/− mice, these animals had a similar increase in the frequency of myeloid and erythroid cells as lyn−/− mice. This suggests that the splenomegaly in lyn−/− mice is caused by two separate defects. The first in which the frequency of splenic myeloid and erythroid cells is increased, is independent of Btk. This phase could result from either a shift in the site of hematopoiesis to the spleen or simply “space filling” (56) secondary to a reduction in the number of lymphoid cells. Myeloid and erythroid elements that are
present in lyn−/− spleens then expand in a Btk-dependent manner. Lyn deficiency may render myeloid cells hypersensitive to cytokines, analogous to the reduction of BCR signaling thresholds in B cells. This enhanced response may be attenuated in the absence of Btk. Btk has been implicated as a component of the IL-5 (25, 57, 58), IL-6 (59), and IL-10 (60) cytokine pathways in B cells. However, no alterations in myeloid or erythroid cell development have been reported in xid mice, Btk−− mice, or XLA patients except for some defects in FcεR1 signaling in mast cells (44).

Btk may serve in a general capacity to regulate mitogenic responses and cell survival. These functions would normally be observed only in B cells because of redundant signaling pathways in other lineages. Variation in genetic context or the mutation of other signaling molecules on a Btk−− background may reveal additional roles for Btk in the development and function of hematopoietic cells.

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