

The Adaptor Protein BLNK Is Required for B Cell Antigen Receptor-induced Activation of Nuclear Factor- κ B and Cell Cycle Entry and Survival of B Lymphocytes*

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B lymphocytes lacking the adaptor protein B cell linker (BLNK) do not proliferate in response to B cell antigen receptor (BCR) engagement. We demonstrate here that BCR-activated BLNK^{-/-} B cells fail to enter the cell cycle, and this is due to their inability to induce the expression of the cell cycle regulatory proteins such as cyclin D2 and cyclin-dependent kinase 4. BCR-stimulated BLNK^{-/-} B cells also do not up-regulate the cell survival protein Bcl-x_L, which may be necessary for the cells to complete the cell cycle. In addition, BLNK^{-/-} B cells exhibit a high rate of spontaneous apoptosis in culture. Examination of the various BCR-activated signaling pathways in mouse BLNK^{-/-} B cells reveals the intact activation of Akt and mitogen-activated protein kinases but the impaired activation of nuclear factor (NF)- κ B that is known to regulate genes involved in cell proliferation and survival. The inability to activate NF- κ B in BCR-stimulated BLNK^{-/-} B cells is due to a failure to induce the degradation of the inhibitory κ B protein. In all these aspects, BLNK^{-/-} B cells resemble *xid* B cells that have a mutation in Bruton's tyrosine kinase (Btk). Recently, phospholipase C (PLC)- γ 2 has also been demonstrated to be essential for NF- κ B activation. Since BLNK has been shown separately to interact with both Btk and PLC- γ 2, our finding of normal Btk but impaired PLC- γ 2 activation in BCR-stimulated BLNK^{-/-} B cells strongly suggests that BLNK orchestrates the formation of a Btk-PLC- γ 2 signaling axis that regulates NF- κ B activation. Taken together, the NF- κ B activation defect may be sufficient to explain the similar defects in BCR-induced B cell proliferation and T cell-independent immune responses in BLNK^{-/-}, Btk^{-/-}, and PLC- γ 2^{-/-} mice.

generation and activation of B lymphocytes. Its signaling can lead to various distinct cellular responses including receptor editing, anergy, and cell death of immature B cells and activation, proliferation, and differentiation of mature B lymphocytes (1). In molecular terms, the engagement of the BCR is known to activate cytoplasmic protein tyrosine kinases (PTKs) of the Syk, Src, and Tec families such as Syk, Lyn, Blk, and Bruton's tyrosine kinase (Btk). The activation of these PTKs generally leads to calcium fluxes, the phosphorylation of other signaling molecules, and, ultimately, to the induction of transcription factors that result in new gene expression (2). However, little is known about how these downstream signaling events are being coordinated with the activation of the BCR-proximal PTKs. In particular, it is still not clear how the biochemical events initiated by BCR signaling could be translated into distinct cellular responses.

Recently, adaptor proteins have been shown to interface PTK activation with selective downstream molecules and could therefore channel BCR signaling to elicit distinct cellular outcomes (3). One such adaptor molecule in B-lymphocyte is B cell linker (BLNK) (4), otherwise known as SLP-65 (5) or BASH (6). BLNK is a Src homology 2 and 3 domain-containing protein that bears homology to another adaptor molecule called SLP-76 that is found in T cells (7). BLNK is phosphorylated upon BCR stimulation (5) and couples Syk activation to Grb2, Vav, Nck, and phospholipase C (PLC)- γ 2 that is intimately associated with intracellular Ca²⁺ mobilization (8). In addition, BLNK has been shown to play a role in the activation of all three major classes of mitogen-activated protein kinases (MAPKs), namely the extracellular signal-regulated kinase (ERK), the c-Jun NH₂-terminal kinase (JNK), and p38 MAPK in the DT40 chicken B cell line (8). BLNK also interacts with Btk through the Src homology 2 domain of the latter (9, 10). Btk is a key molecule in the BCR signaling and is known to regulate the survival (11) and cell cycle status (12, 13) of activated B lymphocytes. Mutation in Btk causes X-linked immunodeficiency (*xid*) in mice and X-linked agammaglobulinemia in humans (14–17).

We and others have previously generated mice lacking BLNK (18–21). BLNK^{-/-} mice have a severe but incomplete block in B cell development and lack specifically CD5⁺ B cells due to a defect in the generation and maintenance of this B cell subset (22). In addition, they do not respond to T cell-independent antigen (18, 19). BLNK^{-/-} B cells also do not proliferate and up-regulate the expression of activation markers upon BCR cross-linking (18, 19, 21). Overall, the phenotype of BLNK^{-/-} mice closely resembles that of *xid* or Btk^{-/-} mice (23, 24).

Given the similarities in the phenotypes of Btk^{-/-} and BLNK^{-/-} mice and the demonstrated interaction between Btk and BLNK (9, 10), we now examine the BCR-initiated molecu-

The B cell antigen receptor (BCR)¹ plays a pivotal role in the

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¹ The abbreviations used are: BCR, B cell antigen receptor; BLNK, B cell linker protein; BrdUrd, 5'-bromo-2'-deoxyuridine; Btk, Bruton's tyrosine kinase; cdk, cyclin-dependent kinase; ERK, extracellular signal-regulated kinase; IgM, immunoglobulin M; I κ B, inhibitory κ B; JNK, c-Jun NH₂-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor κ B; PI, propidium iodide; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PTK, protein-tyrosine kinase; *xid*, X-linked immunodeficiency; PBS, phosphate-buffered saline; BSA, bovine serum albumin; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) labeling reagent; FACS, fluorescence-activated cell sorting.

lar events leading to cell cycle entry and survival in BLNK^{-/-} B cells. In this report, we demonstrate that BCR-activated BLNK^{-/-} B cells fail to enter the cell cycle; this is due to their inability to express the cell cycle regulatory and cell survival proteins. Finally, we examine the BCR-activated signal transduction pathways that may be perturbed in mouse primary B lymphocytes lacking BLNK and show that these mutant cells specifically do not activate the NF- κ B signaling pathway that have been implicated in the regulation of genes essential for B cell proliferation and survival.

EXPERIMENTAL PROCEDURES

Mice—The generation of BLNK^{-/-} mice has been described previously (18). All mice were used at 6–12 weeks of age and in accordance with institutional guidelines.

Preparation of Cells and Extracts—Single cell suspensions were prepared from spleens of wild-type and BLNK^{-/-} mice and depleted of erythrocytes by treatment with red blood cell lysis solution (0.15 M NH₄Cl, 0.1 mM Na₂EDTA, pH 7.2). Primary B lymphocytes were isolated by negative selection of splenic cell samples using anti-CD43 monoclonal antibody-coupled MACS beads (Miltenyi, Germany). The purity of the B cells recovered was ~90%, as assessed by FACS analyses using anti-B220 and anti-IgM antibodies.

Purified primary B cells were stimulated with LPS (Sigma), PMA (Sigma), or goat anti-mouse IgM F(ab')₂ fragment (Jackson ImmunoResearch Laboratories). For the NF- κ B assays, cells were cultured initially at 5 × 10⁶ cells/200 μ l in Opti-MEM I reduced serum medium containing HEPES buffer, 2400 mg/liter sodium bicarbonate and L-glutamine (Life Technologies, Inc.) to reduce the background levels of NF- κ B activity. For the I κ B α assays, cells were pre-treated with 50 μ M cycloheximide (Sigma) for 30 min and subsequently stimulated in the continued presence of the drug.

For the preparation of nuclear extracts, cells were lysed for 20 min on ice in a hypotonic buffer (5 mM Tris-Cl, pH 7.5, 5 mM KCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5 μ g/ml leupeptin, and 5 μ g/ml aprotinin). The nuclear extracts were spun through Microcon centrifugal filter devices (Millipore) to concentrate and desalt the samples. A Bio-Rad DC Protein Assay was employed to determine the amount of nuclear proteins present using known concentrations of bovine serum albumin (BSA) as standards. For the preparation of total cell lysate, cells were lysed for 20 min on ice in a phosphotyrosine lysis buffer (1% Nonidet P-40, 10 mM Tris-Cl, pH 8, 150 mM NaCl, 1 mM EDTA, 0.2 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml aprotinin).

Cell Proliferation Assay—A colorimetric MTT assay (Roche, Germany) was used according to the manufacturer's instructions to measure cell proliferation *in vitro*. Briefly, 5 × 10⁵ B cells were stimulated with varying concentrations of F(ab')₂ goat anti-mouse IgM (Jackson ImmunoResearch Laboratories) antibody or LPS (Sigma) for 48 h in a 96-well tissue culture plate. Subsequently, the cells were incubated with the MTT labeling reagent for 4 h and with the second step reagent overnight. Cell proliferation was quantified using an enzyme-linked immunosorbent assay reader at 570 nm wavelength.

Cell Cycle and Cell Death Analyses—For cell cycle analysis, wild-type and BLNK^{-/-} B cells that were not treated or were stimulated with anti-IgM (20 μ g/ml) or LPS (25 μ g/ml) were cultured in the presence of 40 μ M 5'-bromo-2'-deoxyuridine (BrdUrd; Sigma) at 4 × 10⁶ cells/well in a 48-well tissue culture plate. After 48 h of incubation, cells were harvested and washed once with 1% BSA in PBS. The cell pellet was subsequently resuspended in 200 μ l of PBS and fixed overnight in 1 ml of 70% ethanol at -20 °C. After centrifugation, the cells were incubated with 1 ml of 2 N HCl/Triton X-100 at room temperature for 30 min to denature the DNA. The cells were further washed with 0.5% Tween 20/BSA/PBS. Anti-BrdUrd antibody (Becton Dickinson) was added to the cells for 30 min at room temperature. Finally, propidium iodide (PI; Sigma) was added at 5 μ g/ml to the fixed cells, and the analyses were carried out on a FACScan using CellQuest software (Becton Dickinson).

For the analysis of cell death, *ex vivo* cells and cells that were non-treated or stimulated with anti-IgM or LPS overnight were harvested, washed once in PBS, and fixed overnight in ethanol at -20 °C. Subsequently, the cells were washed once in PBS, resuspended in 100 μ l of 100 μ g/ml ribonuclease A (Roche Molecular Biochemicals), and incubated for 5 min at room temperature. Finally, propidium iodide was added at 50 μ g/ml to the cells prior to their analyses on a FACScan.

Electrophoretic Mobility Shift Assays—Nuclear extract (10 μ g) isolated from B cells was incubated with an [α -³²P]ATP end-labeled, dou-

ble-stranded oligonucleotide probe that contains two tandemly positioned NF- κ B-binding sites, 5'-CATGGCCTGGGAAAGTCCCCTCAAC-T-3' and 5'-CATGAGTTGAGGGGACTTTCCAGGC-3'. The reaction was performed in 20 μ l of binding buffer (60 mM Hepes, pH 7.9, 20 mM Tris-HCl, pH 7.9, 300 mM KCl, 150 mM NaCl, 25 mM MgCl₂, 25 mM dithiothreitol, 62.5% glycerol, and 1 μ g of poly(dI-dC)) for 30 min on ice. After incubation, the samples were resolved in a 5% native gel for 2 h. The gel was dried for 1 h and exposed to an autoradiography film. Densitometry analysis was carried out using a Bio-Rad imaging densitometer and multi-analysis software (Bio-Rad).

Western Blot Analyses—After stimulation with anti-IgM, LPS, or PMA, the purified B cells were lysed on ice for 15 min in a 1% Nonidet P-40 buffer (10 mM Tris-Cl, pH 8, 150 mM NaCl, 1 mM EDTA, 0.2 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml aprotinin). Proteins corresponding to 5 × 10⁶ cells or 5 μ g of nuclear extracts were electrophoresed in 9–12% SDS-polyacrylamide gels and transferred onto immunoblot polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h at room temperature and incubated separately with the various antibodies that recognized the different molecules being studied. Protein bands were visualized using horseradish peroxidase-coupled secondary antibodies and the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The following antibodies were used in this study: anti-cyclin D2 (M-20, Santa Cruz), anti-cdk4 (SC-22, Santa Cruz), anti-Bcl-x_L (clone 44, Transduction Laboratories), anti-bcl-2 (Ab-4, Oncogene), anti-tubulin (Ab-4, NeoMarkers), anti-NF- κ B p50 (H-119, Santa Cruz), anti-NF- κ B p65 (C-20, Santa Cruz), anti-I κ B α (SC-371, Santa Cruz), anti-p38 MAPK (SC-721, Santa Cruz), anti-phospho-p38 MAPK (catalog no. 9211, Cell Signaling Technology (CST)), anti-JNK (catalog no. 9252, CST), anti-phospho-SAPK/JNK (catalog no. 9251, CST), anti-ERK2 (SC-154, Santa Cruz), anti-phospho-ERK (SC-7383, Santa Cruz), anti-Akt (catalog no. 9272, CST), and anti-phospho-Akt (catalog no. 9271, CST). All antibodies were used according to the manufacturers' instructions.

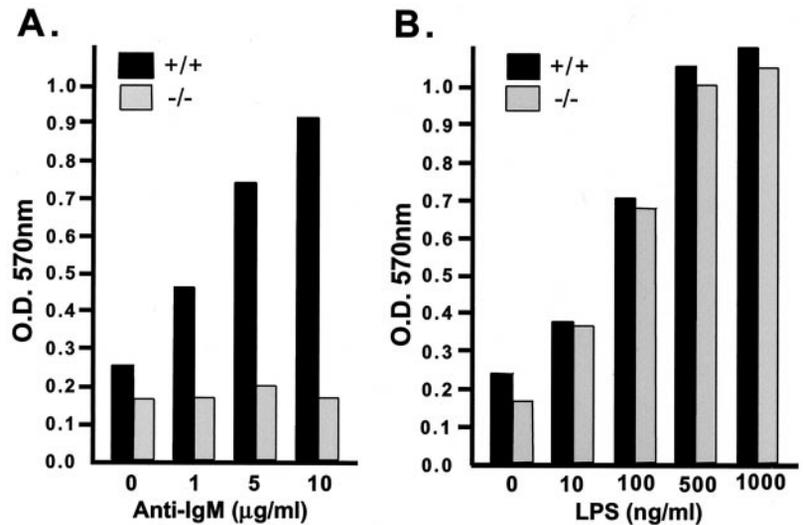
Immunoprecipitations of Btk and PLC- γ 2—For immunoprecipitation studies, anti-Btk (catalog no. 65251A, PharMingen) or anti-PLC- γ 2 (SC-407, Santa Cruz) antibodies were coupled to Protein A/G Plus-Agarose (SC-2003, Santa Cruz) at 4 °C overnight. The beads were washed twice in lysis buffer and incubated with pre-cleared total cell lysates of untreated or goat anti-mouse IgM F(ab')₂ (10 μ g/ml) stimulated B cells for 1 h at 4 °C. Subsequently, the beads were boiled in loading buffer for 3 min, and the released proteins were resolved in a 7% SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes. The membranes were first probed with horseradish peroxidase coupled anti-phosphotyrosine antibody (P11625, Transduction Laboratories) and subsequently with the immunoprecipitating antibodies.

RESULTS

Anti-IgM-stimulated BLNK^{-/-} B Cells Fail to Enter the Cell Cycle—We have shown previously that anti-IgM stimulation that induces BCR signaling does not lead to the proliferation of BLNK^{-/-} B cells (18). This is re-capitulated and shown in Fig. 1A, where BLNK^{-/-} B cells are non-responsive to increasing amount of anti-IgM stimulation. In comparison and as expected, wild-type B cells undergo cellular proliferation in a manner proportional to the extent of their BCR cross-linking. The inability of BLNK^{-/-} B cells to proliferate upon BCR engagement is specific to this stimulus as lipopolysaccharides (LPS), a potent mitogen, induces similar levels of proliferation in both the wild-type and BLNK^{-/-} B cells (Fig. 1B). This phenomenon whereby BLNK^{-/-} B cells respond to LPS but not anti-IgM stimulations was observed regardless of the age of the mice in which the cells were isolated (data not shown).

Anti-IgM stimulation causes B cell to enter the cell cycle (25). The failure of BLNK^{-/-} B cells to proliferate upon BCR engagement would suggest that these cells are defective in their entry and/or completion of their cell cycle. To distinguish between these possibilities, we performed cell cycle analysis on anti-IgM-treated wild-type and BLNK^{-/-} B cells. As described previously (26), the combined use of BrdUrd incorporation and PI staining of total cellular DNA content allows one to discriminate cells in the various stages of the cell cycle. In contrast to

FIG. 1. Defective proliferation of BLNK^{-/-} B cells in response to anti-IgM but not LPS stimulation. Purified splenic wild-type and BLNK^{-/-} B cells were stimulated for 48 h with increasing concentrations of goat anti-mouse IgM F(ab')₂ fragment (A) or LPS (B). Cell proliferation was quantified in an MTT colorimetric assay. Figure shown is representative of five independent experiments and reproducible with B cells obtained from mice of different ages. O.D., optical density.



cells in the resting G₀/G₁ phase, cells in the S phase of the cell cycle are actively synthesizing DNA and will incorporate BrdUrd. They will also exhibit a higher level of PI staining due to their increased cellular DNA content. As seen in Fig. 2, analysis of anti-IgM-stimulated wild-type B cells clearly reveals a population of cells in the S phase of the cell cycle. In contrast, such S phase cycling cells are absent in anti-IgM-treated samples of BLNK^{-/-} B cells, suggesting that the mutant cells are arrested at the G₀/G₁ phase. Again as control, BLNK^{-/-} B cells are able to enter the cell cycle upon treatment with LPS, and they show a pattern of BrdUrd incorporation indistinguishable from that of LPS-treated wild-type B cells (Fig. 2). This is consistent with the proliferation data shown in Fig. 1B, which indicate the ability of BLNK^{-/-} B cells to respond to LPS stimulation. Thus, the data presented in Figs. 1 and 2 together show that the failure of BLNK^{-/-} B cells to proliferate in response to anti-IgM stimulation is due to their inability to enter the cell cycle.

Impaired Induction of Cell Cycle Regulatory Proteins in Anti-IgM-stimulated BLNK^{-/-} B Cells—The entry of cells into the cell cycle is regulated by the activity of specific proteins such as the cyclins and the cyclin-dependent kinases (cdks) (27). The D-type cyclins and their kinase partners, cdk4 and cdk6, are the earliest cell cycle regulatory protein complexes to be expressed when cells leave quiescence and enter the cell cycle (28). Normal proliferating B cells express cyclins D2 and D3 but not D1 (25). Cyclin D2 and its kinase partner, cdk4, are up-regulated from mid to late-G1 phase of the cell cycle and are readily detected in cycling B cells (29). To determine if cyclin D2 and cdk4 can be induced in BLNK^{-/-} B cells, we treated the wild-type and mutant cells with anti-IgM antibodies for various times. As shown in Fig. 3, cyclin D2 is maximally induced in wild-type B cells 24 h after BCR cross-linking and its expression can be detected even after 48 h of treatment. Similarly, cdk4, which is expressed at basal level in normal resting B cells, is also up-regulated upon anti-IgM stimulation. In contrast, both cyclin D2 and cdk4 are not expressed or up-regulated in BLNK^{-/-} B cells regardless of the duration of their BCR cross-linking.

As control, we examined the induction of cell cycle regulatory proteins in BLNK^{-/-} B cells after LPS treatment. As expected, LPS induces the up-regulation of cyclin D2 and cdk4 in wild-type B cells (Fig. 3). LPS is also able to induce the expression of cyclin D2 and cdk4 in BLNK^{-/-} B cells, consistent with the fact that LPS-treated mutant B cells do proliferate (Fig. 1), incorporate BrdUrd (Fig. 2), and enter the cell cycle. This suggests that the failure to express the cell cycle regulatory proteins in

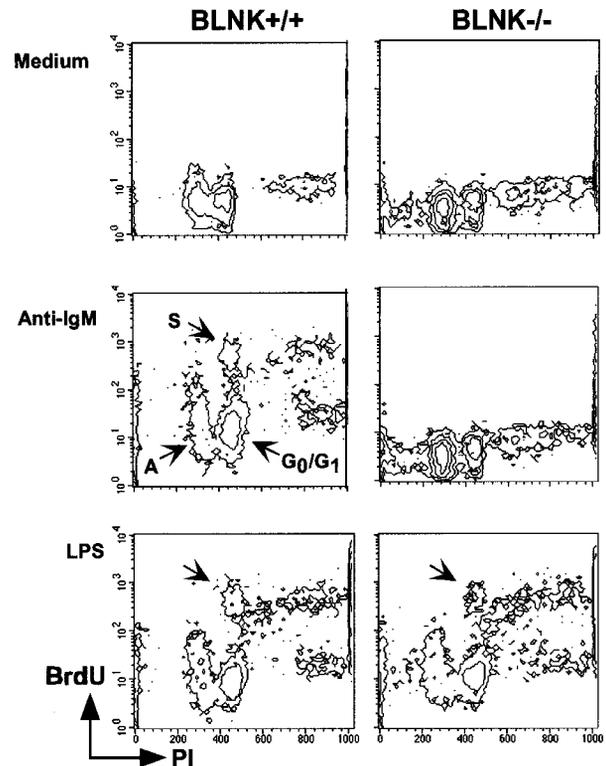


FIG. 2. Anti-IgM-stimulated BLNK^{-/-} B cells failed to enter the cell cycle. Purified splenic wild-type and BLNK^{-/-} B cells were cultured in the presence of 40 μ M BrdUrd (BrdU) for 48 h without stimulus or treated with 20 μ g/ml goat anti-mouse IgM F(ab')₂ fragment or 25 μ g/ml LPS. BrdUrd incorporation and PI staining of total cellular DNA content are used to reveal cells at various stages of the cell cycle. Arrows indicate apoptotic cells (A) or cells in the G₀/G₁ and S phase of the cell cycle.

BLNK^{-/-} B cells is specific to anti-IgM stimulation. Thus, the failure of anti-IgM-stimulated BLNK^{-/-} B cells to proliferate may be due to the inability of these cells to transduce the signal for the induction of specific regulatory proteins that are critical for the entry into cell cycle.

BLNK^{-/-} B Cells Do Not Express Bcl-x_L upon Anti-IgM Stimulation—In addition to the induction of cell cycle regulatory proteins, the cross-linking of the BCR on normal B cells also leads to the expression of the cell survival protein, Bcl-x_L (30, 31). The induction of Bcl-x_L has been correlated with the ability of activated B cells to undergo cellular proliferation (30) and may be required for them to complete the cell cycle (11, 13).

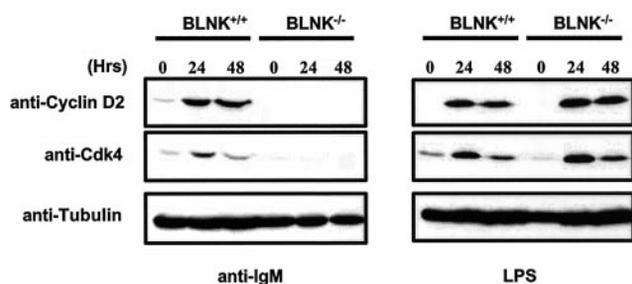


FIG. 3. Lack of induction of cell cycle regulatory proteins in anti-IgM-stimulated BLNK^{-/-} B cells. Purified splenic wild-type and BLNK^{-/-} B cells were treated with goat anti-mouse IgM F(ab)₂ fragment or LPS for various times and examined for the expression of cell cycle regulatory proteins in Western blot analyses. The anti-tubulin blot served as a control for the loading of whole cell lysate. Figure shown is representative of three separate analyses.

As shown in Fig. 4A, this protein is expressed in anti-IgM-treated wild-type or BLNK^{+/-} B cells within 24 h of stimulation and its continued expression can be detected up to 72 h of culture (data not shown). In contrast, Bcl-x_L is absent in BLNK^{-/-} B cells treated with anti-IgM for the same time duration (Fig. 4A and data not shown). Again as control, Bcl-x_L expression can be detected within 24 h in both LPS-treated wild-type and BLNK^{-/-} B cells (Fig. 4B). Thus, the data indicate that anti-IgM-treated BLNK^{-/-} B cells may also abort the cell cycle due to their inability to express the cell survival Bcl-x_L protein upon activation.

Normal B lymphocytes also express the anti-apoptotic protein, Bcl-2 (32). To examine if BLNK deficiency affects the expression of Bcl-2, as is the case for Bcl-x_L above, we examined the expression level of this protein in normal and BLNK^{-/-} B cells before and after various stimulations. As shown in Fig. 5 (A and B), Bcl-2 is expressed at equivalent level in wild-type and BLNK^{-/-} B cells *ex vivo* and its expression level remains relatively unchanged upon stimulation with anti-IgM antibodies or LPS. Thus, a BLNK deficiency specifically affects the expression of Bcl-x_L but not that of Bcl-2 in B lymphocytes.

BLNK^{-/-} B Cells Exhibit a High Rate of Spontaneous Apoptosis in Culture—In the course of the cell cycle analysis as shown in Fig. 2, it was noticed that BLNK^{-/-} B cells cultured for 48 h in medium alone or in the presence of anti-IgM antibodies show a higher degree of apoptosis compared with similarly treated wild-type B cells. To determine if BLNK^{-/-} B cells indeed exhibit a higher propensity to undergo spontaneous apoptosis *in vitro*, we examined these cells after overnight culture, either non-stimulated or treated with the various stimuli. Apoptotic cells can be distinguished in FACS analyses by their reduced cellular DNA content, as revealed by PI staining, and by their smaller size, as shown by a reduction in the forward and side scatter profiles. As shown in Fig. 6, BLNK^{-/-} B cells are twice as likely (50–65%) to undergo spontaneous apoptosis in medium after overnight culture as compared with their normal counterparts (33–38%). Anti-IgM treatment can reduce the fraction of dying wild-type (20–23%) but not BLNK^{-/-} (55–63%) B cells. This can be explained by the ability of wild-type but not BLNK^{-/-} B cells to express Bcl-x_L upon activation (see Fig. 4). Finally, LPS treatment substantially reduced the fraction of dying cells in both the wild-type and BLNK^{-/-} B cell samples, although the population of apoptotic cells is still higher in the latter compared with the former. This is consistent with the fact that LPS can induce the expression of Bcl-x_L in both the wild-type and BLNK^{-/-} B cells (see Fig. 4).

Normal Activation of MAPKs and Akt in Mouse BLNK^{-/-} B Cells—The various analyses above describe the cellular defects of BLNK^{-/-} B cells in response to BCR signaling. Given the fact that BLNK is an adaptor protein involved in signal trans-

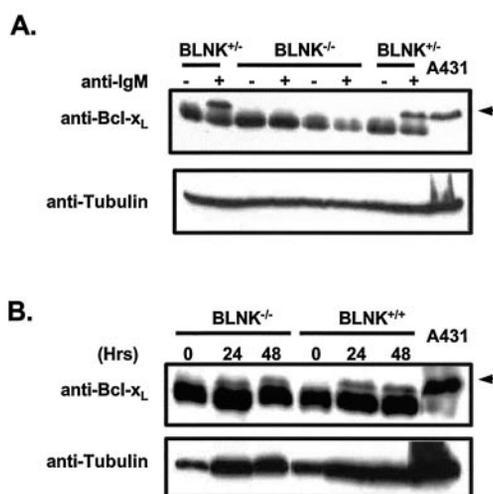


FIG. 4. Absence of Bcl-x_L expression in anti-IgM-stimulated BLNK^{-/-} B cells. Western blot analysis of Bcl-x_L expression in wild-type, BLNK^{+/-} and BLNK^{-/-} B cells stimulated with 10 μ g/ml goat anti-mouse IgM F(ab)₂ fragment for 48 h (A) or 10 μ g/ml LPS for 24–48 h (B). The A431 cell line provides the specificity control for the anti-Bcl-x_L antibody. The anti-tubulin blot serves as a control for the loading of whole cell lysate.

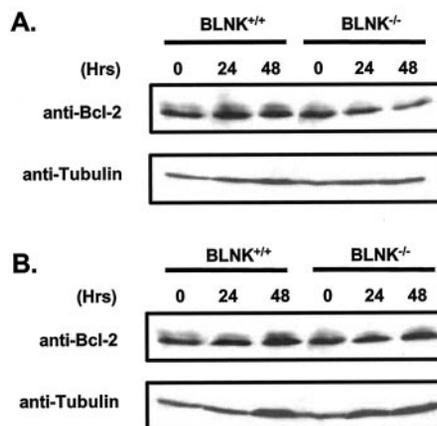


FIG. 5. The expression of Bcl-2 is not altered in wild-type and BLNK^{-/-} B cells treated with various stimuli. Western blot analysis of Bcl-2 expression in wild-type and BLNK^{-/-} B cells stimulated for 24–48 h with either 10 μ g/ml goat anti-mouse IgM F(ab)₂ fragment (A) or 10 μ g/ml LPS (B). The anti-tubulin blot serves as a control for the loading of whole cell lysate.

duction, it would be of interest to correlate the above cellular defects with disruption of distinct signaling pathways. BCR engagement is known to activate the three different classes of MAPKs: ERK, JNK, and p38 MAPK, that have been shown to regulate proliferation, survival, and differentiation in various cellular systems (33). In the DT40 chicken B cell line, BLNK is required for the activation of both JNK and p38 MAPK and for the sustained activation of ERK in response to BCR signaling (8). We therefore re-examined if the activation of MAPKs was similarly affected in mouse primary B cells lacking BLNK and if these signaling defects (if any) could plausibly explain the cellular defects that we have observed above.

As shown in Fig. 7A, the phosphorylation of the p42 and p44 forms of ERK occurs within 30 s and is sustained for as long as 10 min (data not shown) after BCR cross-linking in wild-type B cells. ERK phosphorylation can also be detected with the same kinetics after BCR stimulation in BLNK^{-/-} B cells, suggesting that the activation of ERK is normal in these mutant B cells. Similarly, Western blot analyses of whole cell lysates derived from non-treated and anti-IgM-stimulated wild-type and

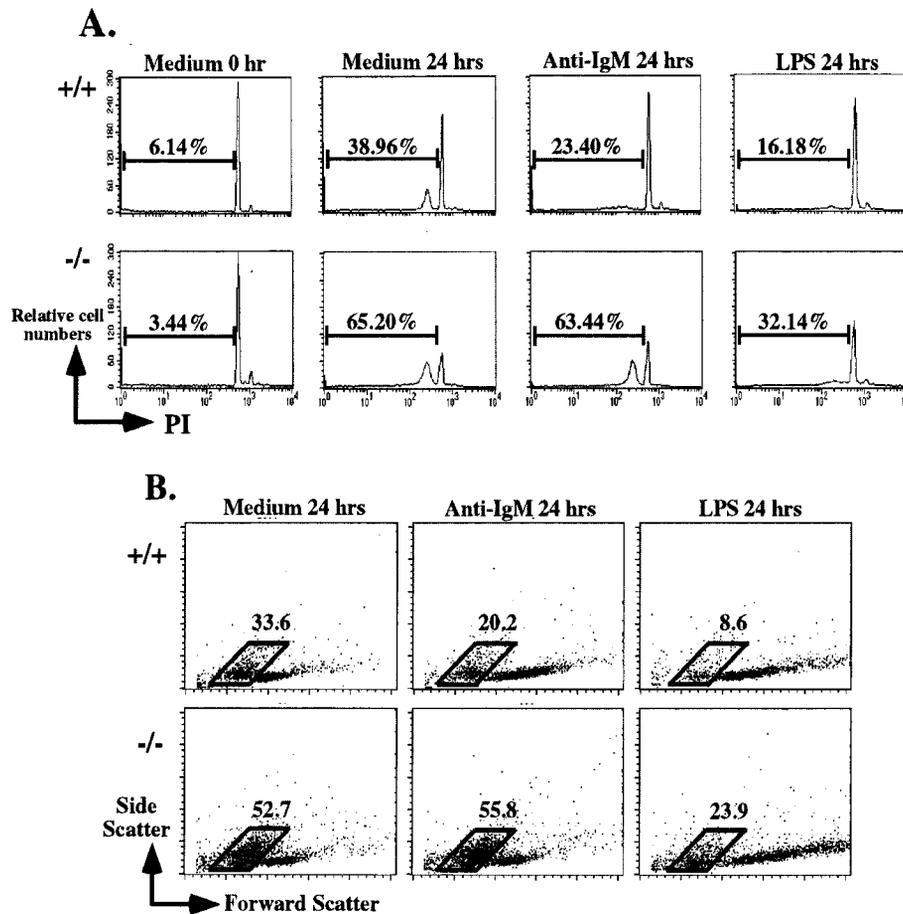


FIG. 6. **High rate of spontaneous apoptosis of BLNK^{-/-} B cells in culture.** Purified splenic wild-type and BLNK^{-/-} B cells were cultured overnight in media alone or with 20 μ g/ml goat anti-mouse IgM F(ab)₂ fragment or 25 μ g/ml LPS. **A**, apoptotic cells with sub-G₀ amount of DNA as revealed by PI staining in FACS analyses were marked and expressed as a percentage of total cells analyzed. **B**, apoptotic cells as revealed by their reduced forward and side scatter profiles in FACS analyses were gated and expressed as a percentage of total cells examined.

BLNK^{-/-} B cells reveals that JNK (Fig. 7B) and p38 MAPK (Fig. 7C) are also phosphorylated with the same kinetics in both the samples tested. The intact activation of the three classes of MAPKs in BLNK^{-/-} mouse primary B cells is in contrast to the impaired activation of these kinases in the DT40 chicken B cell line (8).

Other than the MAPKs, BCR engagement also activates the Akt signaling pathway that is known to regulate cell survival (34). As such, we examine if this pathway is compromised in BLNK^{-/-} B cells. As shown in Fig. 7D, the kinetics of Akt activation is again similar in both the wild-type and BLNK^{-/-} B cells stimulated by anti-IgM antibodies. Thus, the overall data suggest that BLNK plays no role in transducing the BCR signal that activates the three different classes of MAPKs and Akt in mouse primary B lymphocytes and that these signaling pathways are not likely responsible for the cellular defects described earlier.

BLNK Is Required for the Activation of NF- κ B in Response to BCR Engagement—The engagement of the antigen receptor on B lymphocyte also activates the transcription factor NF- κ B that is known to regulate genes involved in cell proliferation and survival (35). Several groups have shown that the predominant form of NF- κ B in B cells is largely the p50-c-Rel heterodimer (36); in particular, c-Rel was shown to be essential for B cell proliferation after BCR engagement (35, 36).

To investigate the role of BLNK in BCR-induced NF- κ B activation, nuclear extracts were prepared from non-treated and anti-IgM-stimulated wild-type and BLNK^{-/-} mouse primary B cells. As shown in the EMSA in Fig. 8A, BCR stimula-

tion of wild-type B cells leads to a marked increase in nuclear NF- κ B activity (lanes 1 and 2) as evidenced by the binding of a radiolabeled probe that contains two consensus NF- κ B binding sequences. In contrast, there was no increase in BCR-induced NF- κ B activity above the background levels in the nuclear extract obtained from BLNK^{-/-} B cells (lanes 4 and 5). As control, the treatment of wild-type and BLNK^{-/-} B cells with phorbol ester (PMA) leads to a corresponding increase in nuclear NF- κ B activity in both the samples tested (compare lanes 1 and 3 and lanes 4 and 6). This suggests that BLNK^{-/-} B cells can activate NF- κ B in response to other stimulus but not to anti-IgM stimulation. Thus, the data indicate that BLNK is specifically required for BCR-induced activation of NF- κ B.

The immediate events regulating NF- κ B activation have been elucidated. In non-stimulated cells, NF- κ B/Rel factors are sequestered in the cytoplasm in complexes with the I κ B family of proteins (35, 36). Upon treatment of cells with specific stimulus, the I κ B kinases are activated and this results in the serine/threonine phosphorylation of I κ B proteins and their subsequent degradation. The NF- κ B/Rel proteins are released from the inhibitory complexes and localized to the nucleus to effect gene transcription (35, 36).

The lack of nuclear NF- κ B binding activity in BCR-stimulated BLNK^{-/-} B cells could therefore be due to reduced NF- κ B proteins in the nucleus. To determine if this is indeed the case, we examined the amount of c-Rel and the p50 subunit in the nucleus of mutant cells. As seen in the Western blot analysis in Fig. 8B, PMA or anti-IgM stimulation led to an accumulation of c-Rel in the nucleus of wild-type B cells (lanes 2 and 3). How-

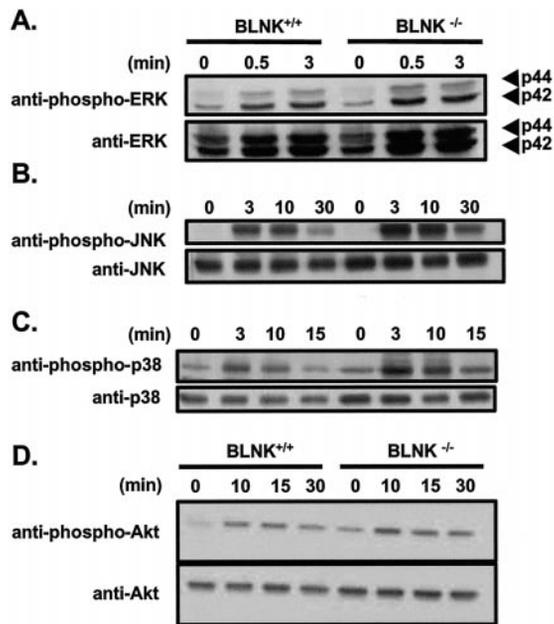


FIG. 7. Normal activation of MAPKs and Akt in BCR-stimulated BLNK^{-/-} B cells. Wild-type and BLNK^{-/-} B cells were treated with anti-IgM antibodies and the expression and activation of ERK (A), JNK (B), p38 MAPK (C), and Akt (D) were examined in Western blot analyses. The whole cell lysates were first probed with an antibody that recognizes the phosphorylated form and subsequently with an antibody that binds the non-phosphorylated form of the protein that is being studied.

ever, in contrast, the accumulation of nuclear c-Rel was effected only with PMA but not with anti-IgM treatment of BLNK^{-/-} B cells (lanes 5 and 6). These data are consistent with the NF- κ B binding assay shown in Fig. 8A. The reduced level of nuclear c-Rel in the BCR-induced BLNK^{-/-} B cell sample was not due to variation in the integrity of the nuclear extracts, as control Western blot analysis revealed the presence of similar amount of nuclear transcription factor IID in all the samples tested (Fig. 8B, middle panel). Examination of the p50 subunit also reveals that it is not translocated into the nucleus of BLNK^{-/-} B cells after anti-IgM stimulation (Fig. 8B, lower panel) similar to the situation found with c-Rel.

It is conceivable that BLNK^{-/-} B cells may produce reduced amount of total c-Rel/p50 proteins and this in turn affects the amount found in the nucleus. To explore this possibility, we examine the amount of c-Rel in the whole cell lysates of wild-type and BLNK^{-/-} B cells. Western blot analysis shown in Fig. 8C reveals that untreated, PMA-stimulated, or anti-IgM-stimulated BLNK^{-/-} B cells produced amounts of c-Rel equivalent to that produced by similarly treated wild-type B cells. Thus, the data suggest that BLNK^{-/-} B cells have specific impairment in the nuclear translocation of NF- κ B factors in response to BCR engagement.

As mentioned above, NF- κ B/Rel transcription factors are sequestered in the cytoplasm by I κ B factors (35, 36). The failure of NF- κ B/Rel transcription factors to translocate into the nucleus of BCR-stimulated BLNK^{-/-} B cells could be due to impairment in the degradation of the I κ B proteins. To test this possibility, we examine the degradation of I κ B α in these cells in response to the different stimuli. Both the wild-type and BLNK^{-/-} splenic B cells were cultured in the presence of cycloheximide to prevent the *de novo* synthesis of I κ B α . As seen in Fig. 8D and consistent with the data presented earlier, I κ B α was degraded in wild-type B cells stimulated with either PMA or anti-IgM antibodies (lanes 2 and 3). In comparison, the degradation of I κ B α was normal in BLNK^{-/-} B cells treated

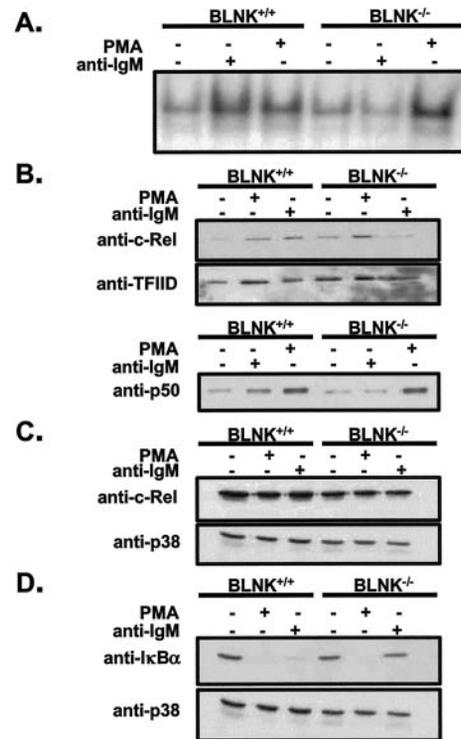


FIG. 8. Impaired NF- κ B activation in BCR-stimulated BLNK^{-/-} B cells. A, lack of NF- κ B binding activity in the nuclear extract of BLNK^{-/-} B cells. EMSA analysis of nuclear NF- κ B activity in wild-type (lanes 1–3) and BLNK^{-/-} (lanes 4–6) splenic B cells that were either non-treated or stimulated with PMA (2 μ g/ml) or anti-IgM antibodies (50 μ g/ml) for 3 h. B, impaired nuclear translocation of c-Rel and the p50 subunit in BCR-stimulated BLNK^{-/-} B cells. Western blot analysis of nuclear extracts (5 μ g) obtained from wild-type and BLNK^{-/-} B cells that were non-treated or stimulated with PMA or anti-IgM antibodies. The blot was first probed with anti-c-Rel antibody and subsequently re-probed with anti-transcription factor IID (anti-TFIIID) antibody to control for the amount and integrity of the nuclear extracts used. In a separate blot, the amount of nuclear p50 (bottom panel) was examined. C, equivalent amount of cytoplasmic c-Rel is found in wild-type and BLNK^{-/-} B cells. Western blot analysis of whole cell lysates (5 \times 10⁶ cells) obtained from wild-type and BLNK^{-/-} B cells that were treated as above. The blot was initially probed with the anti-c-Rel antibody and re-probed with the anti-p38 MAPK antibody as control. D, degradation of I κ B α is impaired in BCR-stimulated BLNK^{-/-} B cells. Splenic wild-type and BLNK^{-/-} B cells were pre-cultured and later stimulated with PMA or anti-IgM antibodies for 3 h in the continuous presence of cycloheximide. Anti-I κ B α antibody was used to determine for the presence of the I κ B α proteins, whereas anti-p38 MAPK antibody was used as control for the equivalent loading of whole cell lysates.

with PMA (lane 5) but impaired in the mutant cells stimulated with anti-IgM antibodies (lane 6). This suggests that the failure of NF- κ B to translocate to the nucleus in BCR-stimulated BLNK^{-/-} B cells is due to a specific impairment in the degradation of I κ B factors.

Taken together, the data indicate that BLNK is required for BCR-induced NF- κ B activation in B cells and this occurs via a mechanism that involves the degradation of the I κ B subunits and the subsequent nuclear translocation of the NF- κ B/Rel transcription factors.

The Activation of Bruton's Tyrosine Kinase Is Normal, but That of PLC- γ 2 Is Impaired in BCR-stimulated BLNK^{-/-} B Cells—Recently Btk has been shown to be essential for the BCR-induced activation of NF- κ B in B cells (37, 38), and this involves a similar mechanism that requires the degradation of I κ B subunits. BLNK has been shown to associate with Btk (9, 10). Hence, it is possible that the inactivation of BLNK may affect the expression and/or activation of Btk, which in turn leads to the impairment in NF- κ B activation in BLNK^{-/-} B

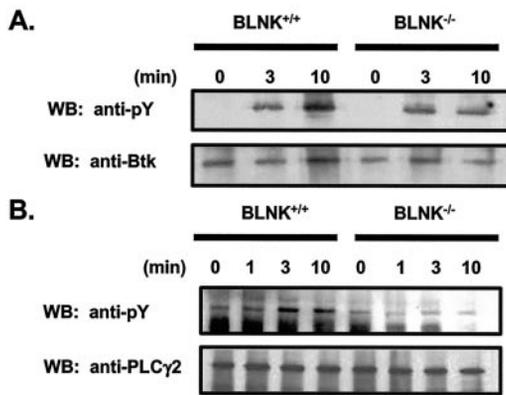


FIG. 9. Expression and activation of Btk and PLC- γ 2 in anti-IgM-stimulated BLNK^{-/-} B cells. Btk (A) and PLC- γ 2 (B) were immunoprecipitated from whole cell lysates of wild-type and BLNK^{-/-} B cells that were treated with anti-IgM antibodies for various times and probed with anti-phosphotyrosine (pY) and the immunoprecipitating antibodies in Western blot (WB) analyses. The figures shown are representative of five separate experiments.

cells. We therefore immunoprecipitated Btk from lysates of non-treated or anti-IgM-stimulated wild-type and mutant B cells to explore this possibility. As shown in Fig. 9A (lower panel), Btk is expressed at equivalent levels in both the wild-type and BLNK^{-/-} B cells that were either non-treated or stimulated with anti-IgM antibodies. In addition, Btk is also activated with the same kinetics in both the wild-type and mutant B cells, as revealed by the anti-phosphotyrosine antibody that reveals the phosphorylation and activation status of Btk (Fig. 9A, upper panel). Thus, the inability of BLNK^{-/-} B cells to activate NF- κ B in response to BCR stimulation is not due to a defect in the expression or activation of Btk *per se*.

While the current work was in progress, Petro and Khan (39) showed that the enzyme PLC- γ 2 that catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol is also essential for the BCR-induced activation of NF- κ B. We thus immunoprecipitated PLC- γ 2 from BLNK^{-/-} B cells that were stimulated with anti-IgM antibodies for various times to determine if the enzyme is activated normally in these cells. As shown in Fig. 9B, PLC- γ 2 is fully activated within 3 min, and its phosphorylation persisted for at least 10 min after BCR engagement in wild-type B cells. In contrast, PLC- γ 2 remains non-phosphorylated and therefore not activated after BCR stimulation for the same time duration examined in BLNK^{-/-} B cells. Thus, the defect in NF- κ B activation in BLNK^{-/-} B cells can be correlated to a defect in PLC- γ 2 activation in response to BCR engagement.

DISCUSSION

BLNK^{-/-} B cells do not proliferate upon BCR stimulation (Fig. 1). Our data presented here indicate that this defect is due to the failure of mutant B cells to enter the cell cycle in response to this stimulus. In contrast to wild-type B cells, BLNK^{-/-} B cells fail to incorporate BrdUrd and are arrested prior to the S phase of the cell cycle (Fig. 2). Indeed, this specific impairment is correlated at the molecular level to the inability of anti-IgM activated BLNK^{-/-} B cells to express the cell cycle regulatory proteins such as cyclin D2 and cdk4 (Fig. 3) that are necessary for the progression of cell cycle beyond the G₀/G₁ phase (29). Anti-IgM treatment of BLNK^{-/-} B cells also fails to induce the expression of the cell survival protein Bcl-x_L (Fig. 4) that has been postulated to be critical for the viability of proliferating B cells (11, 13, 30). Thus, BLNK^{-/-} B cells may have a compound defect in not being able to sustain cell survival for the progression of the cell cycle after anti-IgM stimulation. Taken together, the data strongly suggest that BLNK^{-/-} B

cells fail to enter and abort the cell cycle upon BCR engagement.

The defect in cell cycle progression and cell proliferation may explain why, at the physiological level, BLNK^{-/-} mice failed to mount an effective humoral immune response to T cell-independent antigen that involved extensive BCR cross-linking (18, 19). Antigen-specific B cells are rare and undergo clonal expansion when activated. It is likely that antigen-activated B cells are not clonally expanded in BLNK^{-/-} mutant mice due to their inability to undergo the cell cycle and to their higher propensity to undergo apoptosis. Interestingly, BLNK^{-/-} mice are able to mount a normal T cell-dependent immune response (18, 19). This would suggest that T cell help in the form of co-stimulation or secreted cytokines might overcome the cell cycle defect associated with the BCR signaling resulting from a BLNK deficiency.

Of the major signaling pathways that are known to regulate cell proliferation and/or survival such as those of Akt (34), MAPKs (33) and NF- κ B (35, 36), we show here that only the latter is impaired in BCR-activated BLNK^{-/-} B cells (see Figs. 7 and 8). The finding that Bcl-x_L, which is regulated by NF- κ B (40), is not induced in BLNK^{-/-} B cells in response to BCR stimulation is consistent with this signaling defect.

Previous analyses of BLNK^{-/-} mice (18–21) and our current biochemical study of BLNK^{-/-} B cells suggest that the defects resulting from a BLNK deficiency mirror more closely to those resulting from a lack of Btk as in *xid* mice and Btk^{-/-} B cells (23, 24). BLNK^{-/-} and *xid* B cells are both unable to up-regulate the cell cycle regulatory proteins and enter the cell cycle upon BCR engagement (12), and they both show a propensity to undergo a higher rate of spontaneous apoptosis in culture (11, 12). In addition, BCR-stimulated Btk^{-/-} B cells also fail to activate the NF- κ B signaling pathway (37, 38). We demonstrate here that the expression and activation of Btk is normal in BLNK^{-/-} B cells (Fig. 9). Hence, the phenotypes of BLNK^{-/-} mice and cells are not likely due to defective expression and activation of Btk *per se*. Recently, PLC- γ 2 was also shown to be essential for the activation of NF- κ B in response to BCR engagement (39). Our demonstration that this enzyme is not activated in BCR-stimulated BLNK^{-/-} B cells suggests that this is the likely defect that impairs NF- κ B activation in these mutant cells.

Taken together, a detailed model for the activation of NF- κ B in response to BCR stimulation is now emerging (see Fig. 10). Engagement of the BCR activates the immediate downstream tyrosine kinases Syk and Btk (2). It is known that both Syk and Btk are required for the full phosphorylation and activation of PLC- γ 2 (41). BLNK is the adaptor molecule that couples Syk to PLC- γ 2 (4). Since BLNK has also been shown separately to associate with Btk (9, 10), it is likely to couple Btk to PLC- γ 2 as well, although this has not been directly proven. Our data of normal Btk but impaired PLC- γ 2 activation in BCR-stimulated BLNK^{-/-} B cells are consistent with this model of tri-molecular interaction. Thus, BLNK emerges as the key adaptor molecule that couples Syk and Btk, either in concert or sequentially to activate PLC- γ 2, which in turn activates NF- κ B that regulates genes involved in proliferation and survival such as *bcl-x*. These signaling molecules Syk, Btk, BLNK, and PLC- γ 2 together form a “signalosome” (42). Inactivation of BLNK (this report) or Btk or PLC- γ 2 will disrupt this signalosome and lead to a common NF- κ B signaling defect. This may provide an explanation for the similar B cell defects found in BLNK^{-/-} (18–21), Btk^{-/-} (23, 24), and PLC- γ 2^{-/-} (43, 44) mice. By extrapolation, one would then expect that PLC- γ 2^{-/-} B cells would also not up-regulate cell cycle regulatory and cell survival molecules upon BCR engagement, and this awaits further

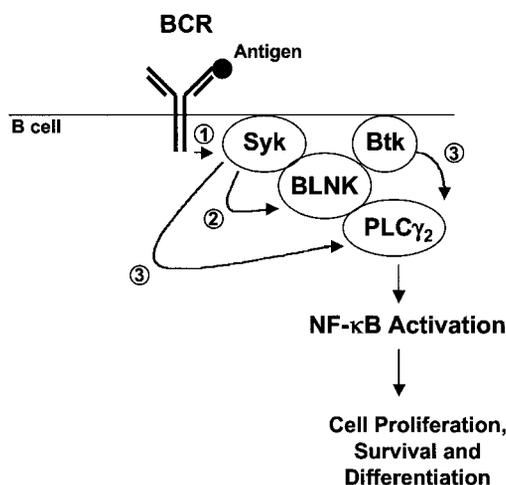


FIG. 10. **A model for the BCR-induced activation of NF- κ B.** Engagement of the BCR activates Syk and Btk (1). Syk phosphorylates BLNK (2). BLNK couples Syk and Btk to PLC- γ 2 (3), and this results in its activation and to the eventual activation of NF- κ B that regulates genes involved in cell survival and proliferation such as *bcl-x*.

confirmation. Finally, it is worthy to note that B cells deficient in the various components of NF- κ B also exhibit proliferation defects (35, 36, 45, 46), again consistent with the model that we presented here.

Our current analyses indicate that BLNK^{-/-} B cells are able to proliferate upon LPS stimulation. This contrasts with previous reports that have stated otherwise (19, 21). The discrepancy in the response to LPS may be due to the relative sensitivity of the various assays used to examine cellular proliferation. It may be that the MTT assay used in our current study is more easily saturated compared with the thymidine incorporation assay used by others (19, 21) and thus failed to measure the reduction in the LPS-induced proliferation of BLNK^{-/-} B cells compared with the wild-type B cells. However, it is noted that, in one of the previous published reports (19), BLNK^{-/-} B cells did respond to some extent to LPS stimulation and thus did not contradict our current data qualitatively. Indeed, the various experiments outlined in this paper that examine the incorporation of BrdUrd (Fig. 2), the induction of cell cycle regulatory proteins (Fig. 3), and cell survival protein, Bcl-x_L (Fig. 4), are all consistent with the fact that BLNK^{-/-} B cells are able to respond to LPS stimulation and proliferate. Finally, examination of LPS-stimulated BLNK^{-/-} B cells (Fig. 6) indicates that they are bigger in size (as reflected by the forward scatter profile) compared with anti-IgM-stimulated or non-treated BLNK^{-/-} B cells, again consistent with the notion that BLNK^{-/-} B cells are activated by LPS. LPS signals through CD14 and the Toll-like receptors (47), and, although it activates NF- κ B (48), it appears to do so via a pathway that does not require BLNK. Incidentally, it was reported that PLC- γ 2^{-/-} B cells, which have defects similar to those of BLNK^{-/-} B cells, also proliferate normally in response to LPS stimulation (43).

Much of the recent work in BCR signaling had been done using the chicken DT40 B cell line. It was shown in this system that the BCR-induced activation of ERK and JNK was impaired in the absence of Syk and Btk (49). Given that BLNK interacts with both Syk and Btk (4, 9, 10), it would be reasonable to assume that some of the MAPKs might also be affected by a BLNK deficiency. Indeed, it was shown recently that the BCR-induced activation of ERK, JNK, and p38 MAPK was also perturbed in the DT40 cell line lacking BLNK (8). However, our data presented in this paper seem to contradict the latter observation. We show here that the BCR-induced activation of

all three classes of MAPKs remain intact in mouse primary B cells lacking BLNK (see Fig. 7). A likely explanation in the discrepancy is the cellular context of the systems used, namely the DT40 chicken B cell line used by others (4, 8–10) versus the mouse primary B lymphocytes used in this report. Such a discrepancy has indeed been observed in several instances previously. First of all, it was shown that the BCR-induced Ca²⁺ response was reduced in human B cell lines and mouse primary B lymphocytes lacking Btk (50) but totally abolished in the DT40 chicken B cells (41). Similarly, DT40 chicken B cells lacking BLNK do not flux Ca²⁺ in response to BCR cross-linking (8), whereas mouse B cells lacking BLNK do although the response was again reduced (19). Thus, there may exist real signaling differences in mouse primary B lymphocytes and the chicken DT40 cell line. Alternatively, it remains possible that the DT40 chicken B cell line is in a different state of maturation compared with the splenic B cells that we used in this paper. It is interesting to note that mouse primary B cells lacking BLNK (19) or Btk (50) still retain the ability to flux Ca²⁺ to some extent, although the activation of PLC- γ 2 is impaired in these mutant cells. On the same note, mouse primary B cells lacking PLC- γ 2 (43) also retain the ability to flux Ca²⁺ in response to BCR engagement, although the magnitude is again much reduced.

Finally, it has been suggested that BCR specificity and hence signaling may play a role in the development of CD5⁺ B cells (51). Mice deficient in BLNK (18–21), Btk (23, 24), or PLC- γ 2 (43, 44), which are molecules involved in BCR signaling, all lack CD5⁺ B cells. Recently, it was shown that cyclin D2 expression is also essential for CD5⁺ B cell development (52). CD5⁺ B cells are thought to undergo self-renewal, a process that is most likely induced through the recognition of an antigen by their BCR (51). Our current data and those previously published (37, 38) indicate that BLNK and Btk are part of a signalosome that transduces the BCR signal that leads to the expression of cyclin D2 and the entry into cell cycle. Thus, in the absence of BLNK or Btk, the BCR signal that leads to the expression of cell cycle regulatory molecules is impaired and this will result in the subsequent defect in BCR-induced cellular proliferation that may affect the self-renewal of a population of CD5⁺ B cells.

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