Table 2. Effects of simvastatin on trabecular bone volume and bone formation rates. Simvastatin was given in doses of 5 to 50 mg/kg/day by oral gavage for 35 days to (i) 3-month-old virgin female rats (experiment 1), (ii) 3-month-old virgin female rats that had been ovariectomized within 7 days after the start of treatment (experiment 2), and (iii) 3-month-old virgin female rats that had been ovariectomized 2 months before treatment (experiment 3). In each experiment, the rats were weight matched and divided into treatment groups of 10. The rats were lightly anesthetized with isofluorane before ovariectomy. Animals were pair fed throughout the experimental period and body weights were determined weekly. Values in parentheses are percent change from vehicle-treated controls. BV/TV, bone volume/tissue volume; Ocl, osteoclasts; BFR, bone formation rate; OVX/veh, ovariectomized rats treated with vehicle; hPTH, human PTH; ND, not determined.

Treatment	Trabecular bone volume (% BV/TV)	BFR ($\mu m^3/\mu m^2$/day)	No. of Ocl/mm^2 of bone surface
	Experiment 1		
Control	13.4 ± 1.4		13.7 ± 1.2
Simvastatin (10 mg/kg/day)	18.6 ± 1.4* (+39)	ND	11.6 ± 1.4 (−15)
hFGF-1 (100 μg/kg/day)	21.4 ± 1.7* (+60)	ND	7.5 ± 1.3* (−45)
	Experiment 2		
OVX/veh	6.9 ± .87	0.6 ± 0.1	8 ± 0.2
Simvastatin (1 mg/kg/day)	8.6 ± .41 (+25)	ND	ND
Simvastatin (10 mg/kg/day)	13.4 ± 2* (+94)	1.2 ± .11 (100*)	7 ± 0.3 (−12.5)
	Experiment 3		
OVX/veh	4.6 ± 0.58	0.151 ± 0.01	1.2 ± 0.1
Simvastatin (5 mg/kg/day)	9 ± 0.8* (+96)	0.196 ± .021* (30)	0.9 ± 0.1 (−25)
Simvastatin (10 mg/kg/day)	8.6 ± 0.9* (+87)	0.229 ± .034* (52)	0.78 ± .06* (−33)
hPTH (80 μg/kg/day)	20 ± 1.9* (+348)	0.228 ± .025* (51)	0.84 ± 0.15 (−30)

*Significantly greater than control ($P < 0.01$).

with this process may lead to osteoclast apoptosis and cessation of bone resorption (*18, 20*). We cannot exclude the possibility that the statins both inhibit bone resorption and promote bone growth, and we did observe a concomitant decrease in osteoclast numbers (Table 2). However, this effect appeared minor in comparison to the effect on new bone formation and osteoblast maturation.

The statins used in our studies and currently on the market are not ideal for use as systemic bone-activation agents. These statins were selected for their capacity to lower serum cholesterol, which requires targeting to HMG Co-A reductase in hepatic cells. Thus, the concentration of statin in other tissues is much lower than in the liver. The most efficacious statins would be those that distribute themselves to the bone or bone marrow. A preliminary retrospective analysis of older women taking lipid-lowering agents suggests that statin use is accompanied by greater hip bone mineral density and lower risk of hip fractures (relative risk = 0.30) (*21*); however, the sample size (598 statin users) was too small to yield definitive information.

The most powerful anabolic agents for bone are the peptide growth factors intrinsic to the tissue. For example, systemically administered FGF-1 restores trabecular microarchitecture and increases bone volume (*15*). However, all of the peptide growth factors have disadvantages—they can be mitogenic to other bone cells and nonselective in their effects. In addition, the FGFs cause hypotension, which limits their potential use in elderly patients (*22*).

Our results suggest that statins, which are orally bioavailable and have been safely administered to patients for more than a decade, may merit further investigation as potential anabolic agents for bone. When the doses are extrapolated from humans to rats with respect to lipid lowering, the statins' effects on bone occur at doses similar to the lipid-lowering doses used in humans.

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Requirement for B Cell Linker Protein (BLNK) in B Cell Development

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Linker proteins function as molecular scaffolds to localize enzymes with substrates. In B cells, B cell linker protein (BLNK) links the B cell receptor (BCR)–activated Syk kinase to the phosphoinositide and mitogen-activated kinase pathways. To examine the in vivo role of BLNK, mice deficient in *BLNK* were generated. B cell development in *BLNK*$^{-/-}$ mice was blocked at the transition from B220$^+$CD43$^+$ progenitor B to B220$^+$CD43$^-$ precursor B cells. Only a small percentage of immunoglobulin M^{++} (IgM^{++}), but not mature IgM^{lo}IgD^{hi}, B cells were detected in the periphery. Hence, BLNK is an essential component of BCR signaling pathways and is required to promote B cell development.

Engagement of the BCR activates distinct families of cytoplasmic protein tyrosine kinases (PTKs) to phosphorylate enzymes that are required for the generation of second messengers (*1*). In turn, the coordinate generation of second messengers is important for normal B cell function because disruption of selected signaling pathways is associated with B cell anergy (*2*). Linker or adapter molecules play integral roles in linking the BCR-activated PTKs with these enzymes. One such linker molecule, BLNK (also known as SLP-65, BASH, and BCA), is phos-

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phorylated by Syk after BCR activation and interacts with enzymes, including phospholipase C–γ, Bruton's tyrosine kinase, and Vav (a guanine nucleotide exchanger for the Rho-GTPases), as well as the Grb2 and Nck linker proteins (*3–5*). An essential role for BLNK in BCR activation was demonstrated in a chicken $BLNK^{-/-}$ DT40 cell line that cannot increase the intracellular calcium concentration ($[Ca^{2+}]_i$) or efficiently activate the Erk-, JNK-, and p38-mediated signaling pathways (*6*).

To better define the expression pattern of BLNK, we developed an intracellular fluorescence-activated cell sorting (FACS) staining assay for BLNK. Consistent with earlier reports (*3, 5*), BLNK expression was detected in peripheral B, but not T, lymphocytes (*7*) (Fig. 1A). Analysis of bone marrow–derived cells showed the highest BLNK expression in early development, with progressively lower expression during B cell maturation (*7, 8*) (Fig. 1B). Hence, BLNK is expressed throughout B cell ontogeny and suggests a potential role for BLNK in B cell development, maturation, or function.

To investigate the in vivo role of BLNK, we undertook a gene-targeting approach to generate and analyze $BLNK^{-/-}$ mutant mice. Because BLNK is a substrate of Syk and $syk^{-/-}$ mice hemorrhage extensively in utero and die during the perinatal period (*3, 9*), we were concerned that $BLNK^{-/-}$ mice might suffer a similar fate. In addition, gene targeting of the BLNK homolog, *SLP-76*, results in mice that die from hemorrhage caused by a defect in collagen-induced platelet aggregation (*10*). To circumvent the embryonic lethality that may be encountered in germ line knockout mice, we also used the $RAG2^{-/-}$ blastocyst complementation system to assay for BLNK function in lymphocytes (*11*). The data presented here represent analyses from both approaches.

Disruption of *BLNK* was accomplished by a targeted mutation of exon 1, which encodes amino acids 1 through 60, including the initiation codon (*12*) (Fig. 2A). For $RAG2^{-/-}$ blastocyst complementation, the $BLNK^{+}$ allele in the $BLNK^{+/-}$ embryonic stem (ES) clone was further targeted with a puromycin selection cassette to generate $BLNK^{-/-}$ ES cells (*13*) (Fig. 2B). To generate germ line mutant mice, $BLNK^{+/-}$ ES cells were injected into B6 blastocysts to yield chimerae that were then crossed with wild-type B6 mice to generate $BLNK^{+/-}$ germ line mutants. The genotypes of the mature offspring from such crosses occurred at the expected Mendelian ratios and the $BLNK^{-/-}$ mutation did not incur any embryonic or perinatal lethality (*14*). Furthermore, mature $BLNK^{-/-}$ mice were healthy under specific pathogen-free conditions and did not display any evidence of gross hemorrhaging (*14*). To evaluate the developmental potential of $BLNK^{-/-}$ ES cells, we also injected these cells into $RAG2^{-/-}$ blastocysts to generate chimerae ($R2{:}BLNK^{-/-}$) for direct analysis (*15*).

To assess the effect of the mutation on BLNK expression, we used the Ly 9.1 surface marker to distinguish between bone marrow cells derived from the $RAG\ 2^{-/-}$ blastocyst ($Ly\ 9.1^{-}$) and the $BLNK^{-/-}$ ES cells ($Ly\ 9.1^{+}$) (*16*). Whereas the $B220^{+}Ly\ 9.1^{+}$ cells from wild-type 129 mice and $B220^{+}Ly9.1^{-}$ cells from $RAG2^{-/-}$ mice expressed BLNK (Fig. 2D, left two bottom panels), no BLNK was detected in the $B220^{+}Ly9.1^{+}$ cells isolated from the $R2{:}BLNK^{-/-}$ chimerae, as assessed by intracellular staining (Fig. 2D, bottom right panel). Similar to the results from the $R2{:}BLNK^{-/-}$ chimerae, no BLNK protein was detected in cell lysates of total bone marrow from germline $BLNK^{-/-}$ mice (*17*) (Fig. 2E).

The effects of BLNK deficiency on lymphocyte development in vivo was examined by analyzing cells isolated from primary and secondary lymphoid organs. Consistent with the absence of BLNK expression in T cells, T cell number, development, and function were normal in both $BLNK^{-/-}$ germ line and $R2{:}BLNK^{-/-}$ chimeric mice (*14, 18–20*). In contrast, an ~65% reduction in splenocyte number was found in $BLNK^{-/-}$ mice as compared to $BLNK^{+/+\ \text{or}\ +/-}$ mice (*18*). Because the development and function of peripheral T cells were normal (*14, 19*), we further investigated the nature of this defect by analyzing the B cell compartment in primary and secondary lymphoid organs. Although the numbers of cells recovered from the bone marrow of $BLNK^{+/+}$ and $BLNK^{-/-}$ mice were similar (*18*), bone marrow cells from $BLNK^{-/-}$ germ line and $R2{:}BLNK^{-/-}$ chimeric mice displayed a profound block in B cell devel-

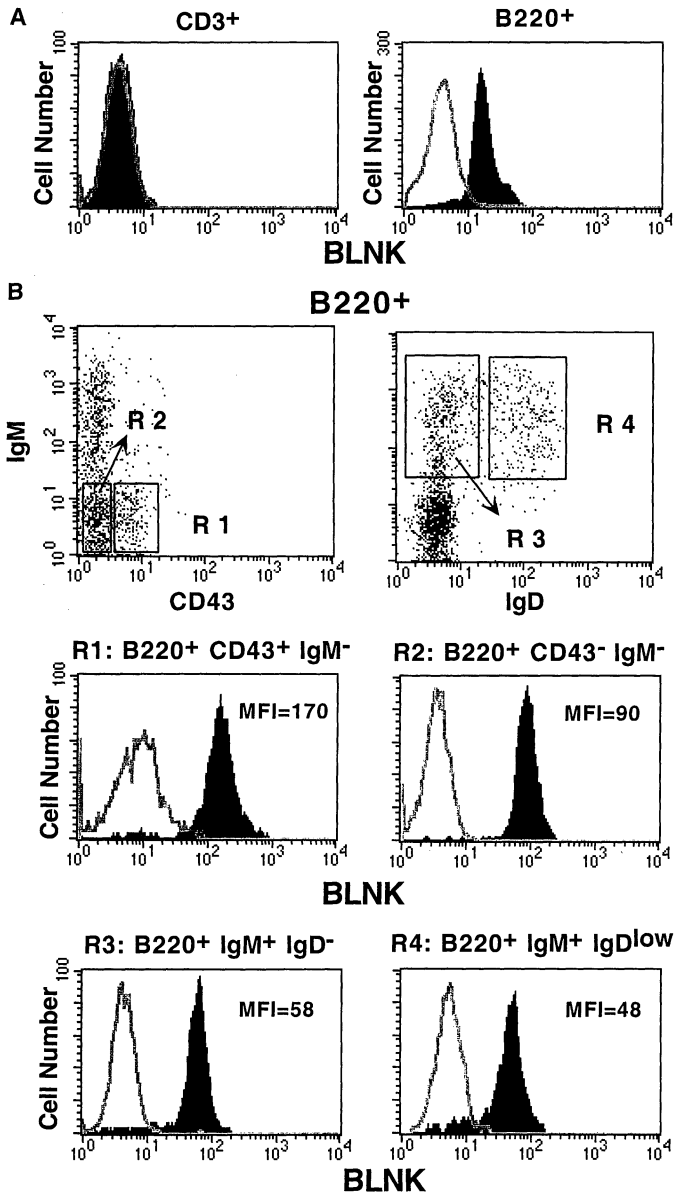


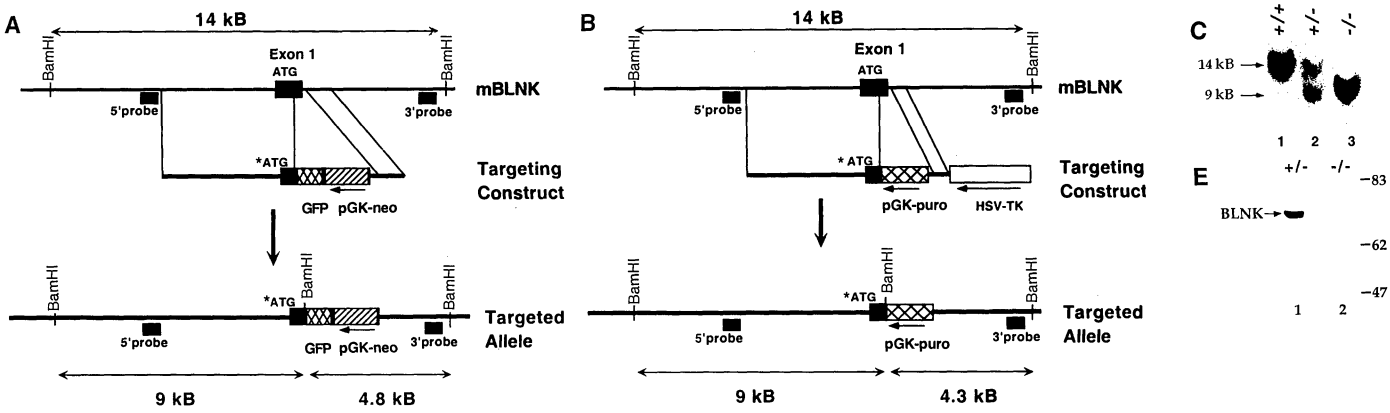
Fig. 1. Expression of BLNK in lymphocyte development. (**A**) BLNK is expressed in murine B, but not T, cells. $CD3^{+}$ (left panel) or $B220^{+}$ (right panel) splenocytes isolated from C57BL/6 mice were analyzed by intracellular staining with an antiserum to BLNK (shadowed areas) or preimmune serum (solid line) (*7*). (**B**) BLNK expression during murine B cell development. Bone marrow–derived cells isolated from C57BL/6 mice were analyzed with four-color FACS analysis (*8*). Cells stained for B220, IgM, and CD43 (left panel) or for B220, IgM, and IgD (right panel) were analyzed as described above. Each developmental subset— $B220^{+}CD43^{+}IgM^{-}$ (pro-B cells; R1), $B220^{+}CD43^{-}IgM^{-}$ (pre-B cells; R2), $B220^{+}IgM^{+}IgD^{-}$ (immature B cells; R3), and $B220^{+}IgM^{+}IgD^{lo}$ (mature B cells; R4)—was analyzed for BLNK expression (*7*).

opment. *BLNK*$^{-/-}$ mice accumulated B220^{+}CD43^{+} progenitor B cells (pro-B cells) (*21*) (Fig. 3A). Consistent with the presence of pro-B cells, the levels of V_H to DJ_H recombination were comparable in *BLNK*$^{+/+}$ and *BLNK*$^{-/-}$ bone marrow–derived cells (*14*). *BLNK*$^{-/-}$ mice had CD43^{+} pro-B cells but failed to develop B220^{hi}CD43^{-} B cells, although a small percentage of B220^{+}CD43^{-} B cells was present (10.0 ± 8.7% for *BLNK*$^{-/-}$ versus 44.4 ± 14% for *BLNK*$^{+/-\ or\ +/+}$, $P < 0.001$, $n = 11$) (*20*) (Fig. 3A). Because the transition from the CD43^{+} to CD43^{-} stage is normally associated with a decrease in cell size, as measured by the forward scatter value (*8*), the B220^{+}CD43^{-} B cells isolated from *BLNK*$^{-/-}$ mice remained large, in contrast to the smaller B220^{+}CD43^{-} B cells from *BLNK*$^{+/-}$ mice (*14*). In addition, the *BLNK*$^{-/-}$ bone marrow (B lineage) cells failed to progress efficiently from the immature B220^{lo}IgM^{lo} (immunoglobulin M, IgM) stage to transitional B220^{lo}IgM^{hi} or mature B220^{hi}IgM^{+} stages (1.3% ± 0.9% in *BLNK*$^{-/-}$ mice for the latter two stages versus 13.1 ± 5 in *BLNK*$^{+/+\ or\ +/-}$ mice, $P < 0.001$, $n = 10$) (*20*) (Fig. 3A). The small percentage of IgM^{lo} bone marrow B cells that develop in *BLNK*$^{-/-}$ mice express a mature surface BCR because many are also Igκ^{+} (*14*).

Analysis of splenocytes revealed a substantial decrease in the numbers of IgM^{+} peripheral B cells (2.4 ± 2.6% for *BLNK*$^{-/-}$ versus 30.7 ± 6.2% for *BLNK*$^{+/+\ or\ +/-}$, $P < 0.001$, $n = 10$) (*20*) (Fig. 3B). As in the bone marrow, the few *BLNK*$^{-/-}$ IgM^{+} B cells found in the spleen were also larger in size than IgM^{hi} *BLNK*$^{+/+}$ B cells (*14*). Concomitant with the profound decrease in peripheral B cells in the spleen, IgM^{+} B cells were also reduced in the lymph node (Fig. 3C). Hence, the absence of BLNK results in a developmental block that leads to reduced numbers of IgM^{+} cells in the periphery. Older *BLNK*$^{-/-}$ mice (8 to 13 weeks old) showed increased numbers of B220^{+}IgM^{+} B cells [(1.4 ± 1.2) × 10^{6} B220^{+}IgM^{+} cells, $n = 8$] as compared to younger *BLNK*$^{-/-}$ mice [3 to 6 weeks old; (0.58 ± 0.31) × 10^{6}, $P < 0.001$, $n = 9$]. In spite of this accumulation, these older *BLNK*$^{-/-}$ mice still have more than 10 times fewer B220^{+}IgM^{+} B cells than their age-matched *BLNK*$^{+/+\ or\ +/-}$ counterparts [(19 ± 6.7) × 10^{6} B220^{+}IgM^{+} cells in older *BLNK*$^{+/+\ or\ +/-}$ mice, $n = 6$, versus (1.4 ± 1.2) × 10^{6} B220^{+}IgM^{+} cells in older *BLNK*$^{-/-}$ mice, $P < 0.001$, $n = 8$] (*20*).

Analysis for mature B cells revealed a marked reduction of B220^{hi}IgM^{+} cells (<1%) in the bone marrow of young and old *BLNK*$^{-/-}$ mice (Fig. 3A) (*14*). Mature IgM^{lo}IgD^{hi} cells were similarly reduced (<1%) in the periphery of young and old *BLNK*$^{-/-}$ mice (Fig. 4, A and B). Staining with CD21 revealed the presence of CD21^{+}IgM^{hi} T2 transitional B cells and a reduction of CD21^{+}IgM^{lo} mature B cells (<1%) in *BLNK*$^{-/-}$ mice (*22*) (Fig. 4B). Consistent with the decrease in mature B cells, serum Ig in older *BLNK*$^{-/-}$ mice was significantly reduced as compared to the amount in wild-type mice (*23*) (Fig. 4C).

The *BLNK*$^{-/-}$ B cells that accumulated in the periphery of older mice further revealed a maturation defect in these cells. In contrast to *BLNK*$^{+/+}$ mice, in which transitional B220^{+}IgM^{++}IgD^{+} B cells develop into mature B220^{hi}IgM^{lo}IgD^{hi} B cells, and in contrast to the B cells that accumulate in the λ*5*$^{-/-}$ mice (*24*), *BLNK*$^{-/-}$ splenic B cells are larger in size and express higher mem-



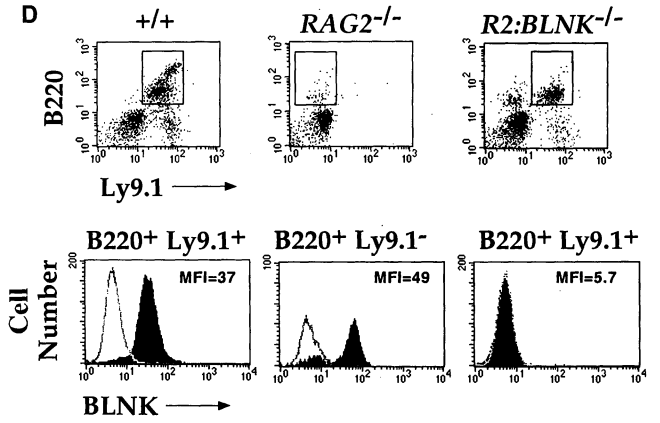


Fig. 2. Generation of *BLNK*$^{-/-}$ mice. (**A**) Targeting of *BLNK*. The genomic structure surrounding exon 1 of *BLNK* (top), the targeting construct (middle), and the targeted allele (bottom) are depicted (*12*). Exon 1 includes amino acids 1 through 60 of the BLNK coding region. The correctly integrated construct converts the 14-kb wild type into 9- and 4.8-kb fragments when detected with the 5′ and 3′ probes, respectively. A GFP cDNA was also inserted into the targeting construct. However, GFP fluorescence was not detected in *BLNK*$^{+/-}$ splenocytes or bone marrow–derived cells, which was likely caused by transcriptional silencing of GFP by the PGK-neo cassette (*12*). (**B**) Targeting of the second *BLNK* allele. The genomic structure surrounding exon 1 of *BLNK* (top), the targeting construct (middle), and the targeted allele (bottom) are depicted (*13*). The correctly integrated construct converts the remaining 14-kb wild type into 9- and 4.3-kb fragments when detected with the 5′ and 3′ probes, respectively. (**C**) Southern (DNA) blot analysis of *BLNK*$^{+/+}$, *BLNK*$^{+/-}$, and *BLNK*$^{-/-}$ mice. Bam HI–digested tail DNA was separated by electrophoresis and hybridized with the 5′ probe to detect the wild-type and mutant fragments (*13*). Blotting with the 3′ probe also revealed the predicted mutant 4.8-kb fragment in *BLNK*$^{+/-}$ and *BLNK*$^{-/-}$ mice (*14*). (**D**) Absence of BLNK protein in *RAG2*$^{-/-}$ chimeric mice. B220^{+} bone marrow cells from 129 wild-type (Ly9.1^{+}; left panels), *RAG2*$^{-/-}$ (Ly9.1^{-}; middle panels), or *R2:BLNK*$^{-/-}$ chimeric (Ly9.1^{+}; right panels) mice were analyzed by intracellular staining for BLNK as described in Fig. 1A (*7*). (**E**) Absence of BLNK protein in *BLNK*$^{-/-}$ bone marrow–derived cells. Bone marrow–derived cells from germ line *BLNK*$^{+/-}$ (lane 1) and *BLNK*$^{-/-}$ (lane 2) littermates were immunoblotted with an antiserum to BLNK (*17*). Equal loading of cell lysates was confirmed by immunoblotting with an antiserum to actin (Sigma) (*14*).

brane IgM (Fig. 4B) (*14*). These IgM^{hi} cells may represent B cells that have matured through the pro- to precursor B cell (pre-B cell) transition but are blocked in IgM signaling and, therefore accumulate as large IgM^{hi} cells. Alternatively, these cells may result from a selection bias in which B cells can bypass the absence of BLNK by increasing membrane Ig expression and decreasing the signaling threshold. As *CD45*$^{-/-}$ immature B cells expressing a transgenic BCR can be rescued from death by chronic exposure to antigen (*25*), heightened BCR signaling may bypass the requirement for CD45. Similarly, BLNK deficiency may abolish development and result in the death of most B cells, except those that express very large amounts of IgM, which partially compensates for the signaling defect incurred by BLNK deficiency. The IgM^{++} *BLNK*$^{-/-}$ B cells could increase free cytoplasmic calcium after BCR cross-linking,

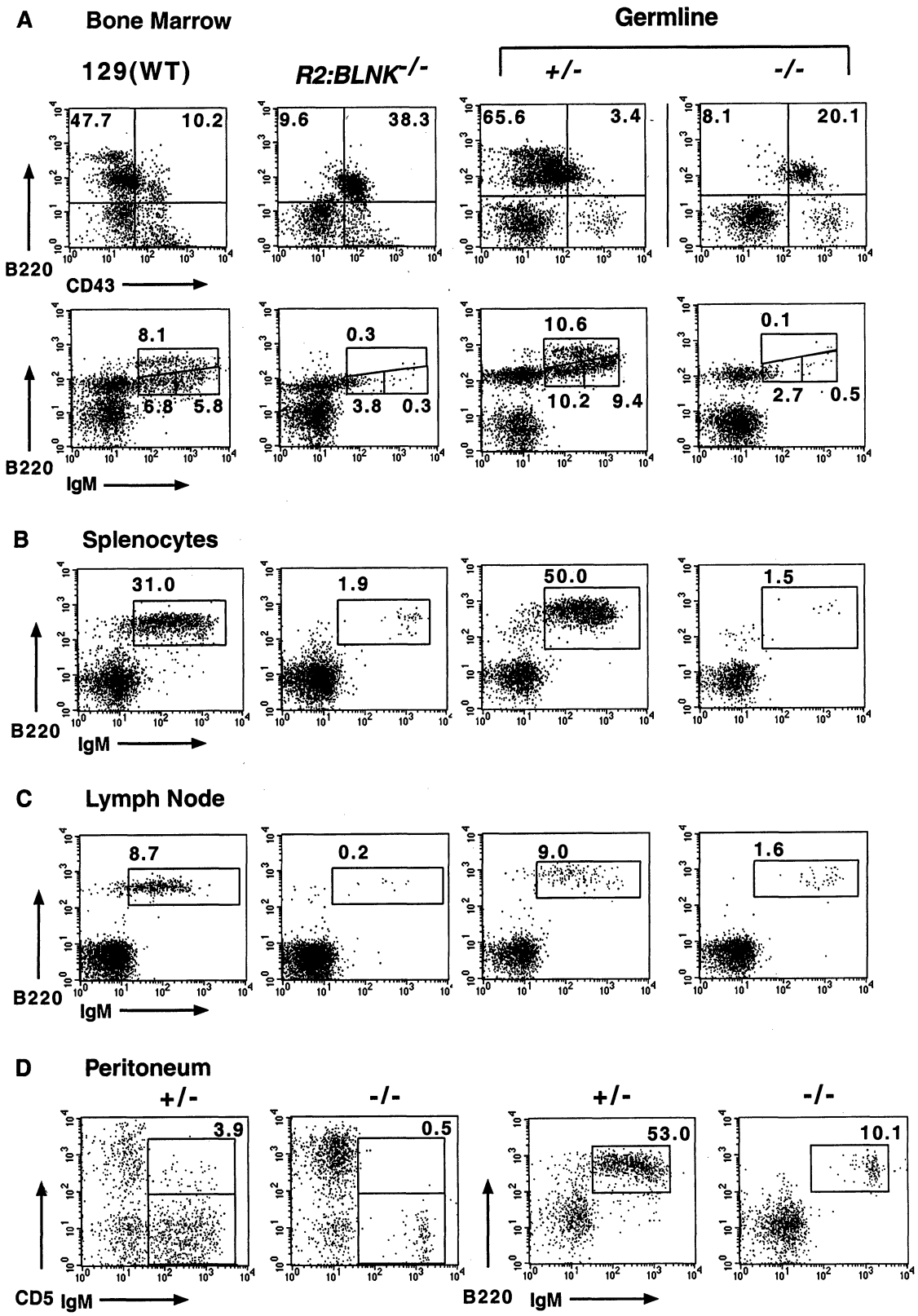


Fig. 3. B cell development in *BLNK*$^{-/-}$ mice. Cells isolated from bone marrow (**A**), spleen (**B**), and lymph nodes (**C**) of 3- to 5-week-old animals were stained with the antibodies indicated in each figure and analyzed by FACS analysis (*21*). Data from both *R2:BLNK*$^{-/-}$ chimeric [left two panels for (A) through (C)] and germ line [right two panels for (A) through (C)] mice are shown. In the *RAG2*$^{-/-}$ blastocyst complementation assay, 129 wild-type and *RAG2*$^{-/-}$ age-matched mice were analyzed in parallel as controls (*14*). No differences were detected between *BLNK*$^{+/+}$ and *BLNK*$^{+/-}$ mice (*14*). The percentages of gated cells are indicated. These analyses were representative of a minimum of five pairs each of *RAG2*$^{-/-}$ chimeric and germ line animals. Experiments from both approaches produced similar results. (**D**) Peritoneal cells isolated from 6- to 13-week-old mice were stained with the antibodies indicated and analyzed by FACS analysis (*21*). Cell recoveries were comparable in yield from *BLNK*$^{+/-}$ and *BLNK*$^{-/-}$ mice [4.5×10^6 for *BLNK*$^{+/-}$ and $(3.2 \pm 0.6) \times 10^6$ for *BLNK*$^{-/-}$ mice, $n = 5$].

albeit less efficiently than wild-type cells, despite expressing 10- to 50-fold more membrane IgM than $BLNK^{+/+}$ B cells (*26*) (Fig. 4D). In addition, $BLNK^{-/-}$ B cells also up-regulate CD69 and CD86 cell surface activation markers after BCR cross-linking (*27*) (Fig. 4E). Hence, these large IgM^{++} $BLNK^{-/-}$ B cells are capable of some BCR-mediated signaling functions.

We also analyzed the development of a distinct subset of B cells known as B-1a cells. These cells are distinguished from conventional B-2 cells by their expression of CD5 and their capacity for self-renewal (*28*). Whereas $BLNK^{+/+}$ and $BLNK^{+/-}$ mice had comparable numbers of peritoneal cells, $BLNK^{-/-}$ mice had a substantial decrease in the $CD5^{+}IgM^{+}$ B-1a B cell population (<1%) in the peritoneum in young and old mice (Fig. 3D) (*14*). In addition, $CD5^{-}CD11b^{+}IgM^{+}$ B-1b B cells were also absent (<0.5%) in the peritoneum and the spleen of $BLNK^{-/-}$ mice (*14, 29*). Hence, BLNK is also required for development of B-1 cells.

Because signals from both the pre-B and IgM BCRs are required for normal B cell development (*1, 30*), these studies showed the critical role of BLNK in the development of IgM^{+} cells. Similar to $syk^{-/-}$ mice (*9*), the absence of BLNK also compromises pre-BCR function to affect the development of $B220^{+}CD43^{-}$ B cells that, in turn, limit their differentiation into $B220^{hi}IgM^{+}$ B cells in the bone marrow. As a result, few B cells are present in the periphery. However, whereas the IgM^{+} B cells that develop in $syk^{-/-}$ mice express little membrane IgM (*9*), the B cells that accumulate in $BLNK^{-/-}$ mice express large amounts of membrane IgM (Fig. 4B). This difference suggests that additional substrates of Syk might exist to partially transduce pre-BCR signals in the absence of BLNK. In accordance with this, the IgM^{++} B cells that accumulate in the periphery of older $BLNK^{-/-}$ mice can generate second messengers after BCR activation. Additional studies aimed at comparing $syk^{-/-}$ and $BLNK^{-/-}$ mice will be required to assess this possibility.

Finally, the developmental block at the pro- to pre-B cell transition observed in a BLNK-deficient patient is similar, though not identical, to the phenotype observed in $BLNK^{-/-}$ mice (*31*). Although IgM^{hi} B cells accumulate in the periphery of $BLNK^{-/-}$ mice, no peripheral B cells were detected in this adult patient. Similar discordance in phenotypes has been observed in immunodeficiencies involving Btk and λ5 in which the human phenotype appears to be more severe than the murine phenotype (*32*). These differences may reflect a greater dependence on pre-BCR function in human B cell development, a species-specific difference in the regulation of signaling molecules that dictate activation thresholds, or both. Such species-specific differences have been observed in T cell development in which Syk is more highly expressed in developing human $CD4^{+}$ T cells than in murine $CD4^{+}$ T cells and may provide a mechanism to explain the phenotypic differences observed between ZAP-70–deficient mice and humans (*33*). Additional investigation is required to determine whether species-specif-

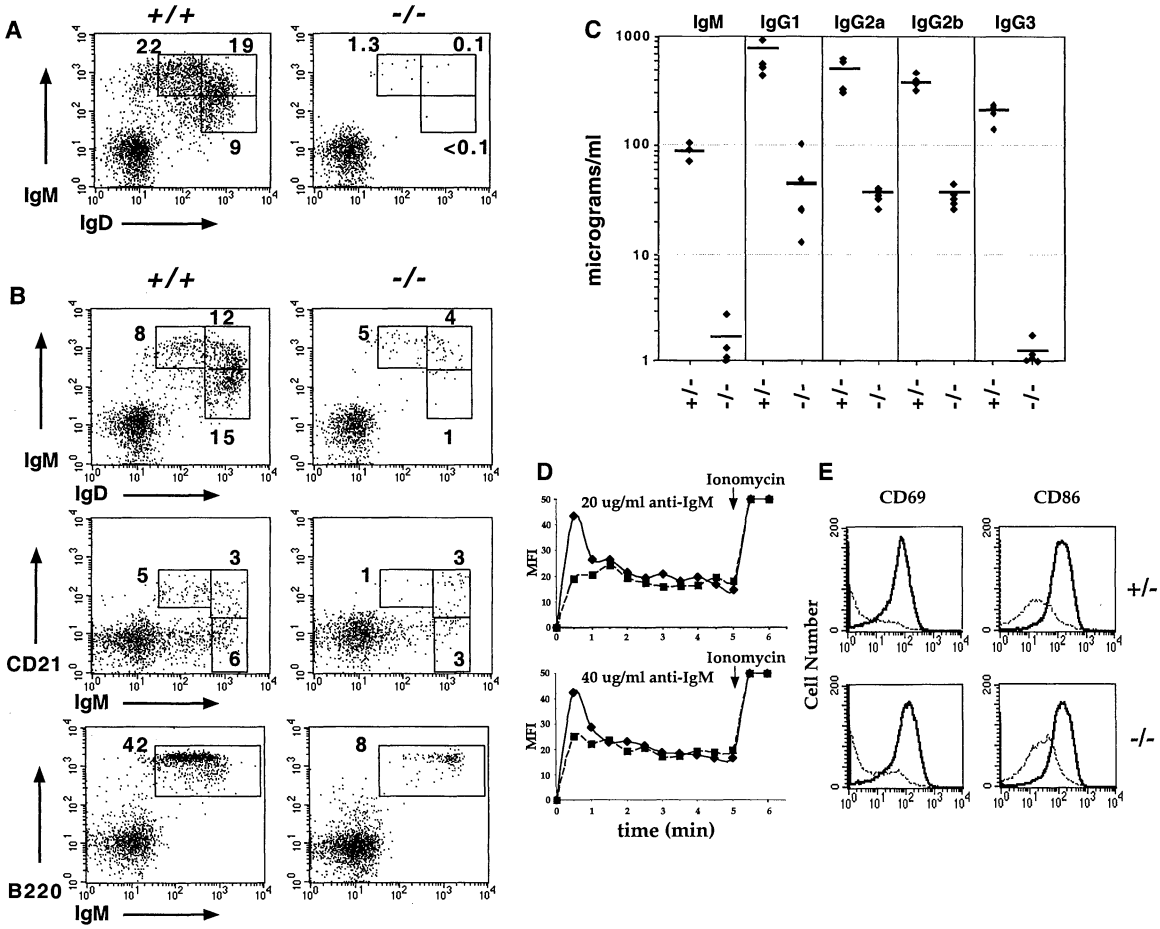


Fig. 4. Decreased maturation of B cell development and function in $BLNK^{-/-}$ mice. (**A**) Splenocytes isolated from 3- to 5-week-old $BLNK^{+/+}$ or $BLNK^{-/-}$ mice were analyzed by FACS staining for IgM and IgD (*21*). (**B**) Splenocytes from older $BLNK^{+/+}$ or $BLNK^{-/-}$ mice (8 to 13 weeks old) were stained with the antibodies indicated in each figure and analyzed by FACS analysis (*21*). The percentages of gated cells are indicated. $BLNK^{-/-}$ spleens were reduced in cell number by ~70% as compared to $BLNK^{+/+}$ spleens (*14, 18*) (see text for absolute numbers of cells recovered). (**C**) Serum immunoglobulin levels of 8- to 13-week-old wild-type, germ line $BLNK^{-/-}$ and *R2:*$BLNK^{-/-}$ mice were determined by ELISA (*23*). Each diamond represents the value derived from an individual mouse. The black bar denotes the mean of each group. (**D**) $B220^{+}$ B cells from older $BLNK^{+/+}$ (solid line) and $BLNK^{-/-}$ (dotted line) mice were analyzed by FACS analysis for increases in $[Ca^{2+}]_i$ after BCR cross-linking with antibody to IgM $F(ab')_2$ fragments (20 and 40 μg/ml) (*26*). Antibody to IgM $F(ab')_2$ was added at time 0 at the indicated concentrations. Ionomycin was added at 5 min to ensure proper loading of cells with Fluo-4. Diamond, $BLNK^{+/+}$ splenocytes; square, $BLNK^{-/-}$ splenocytes. (**E**) $B220^{+}$ B cells from older $BLNK^{+/+}$ (top) and $BLNK^{-/-}$ (bottom) mice were analyzed by FACS analysis for up-regulation of CD69 and CD86 expression after BCR cross-linking (*27*). The dotted line represents cells treated with media. The solid line represents cells treated with antibody to IgM $F(ab')_2$ (10 μg/ml).

ic differences in the regulation of BLNK or other regulators of B cell development may account for the differences observed between human and murine BLNK deficiencies. However, the present studies in a human and in mice demonstrate a central role for BLNK in relaying signals in the pre-BCR and BCR signaling pathways.

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7. Cells (4×10^6) were prepared with the CytoFix/CytoPerm Kit (PharMingen) according to manufacturer's recommendations. After permeabilization, cells were stained with 2 μg of an antiserum to BLNK (*3*) in buffer containing 50% fetal calf serum (FCS) at 4°C for 30 min. Cells were washed twice with 1 ml of wash buffer and stained with 1 μg of fluorescein isothiocyanate–conjugated goat anti-rabbit IgG (heavy and light chains) (Jackson Research Labs) for 30 min at 4°C. Cells were washed twice with 1 ml of wash buffer, resuspended in 0.4 ml of cold phosphate-buffered saline, and prepared for FACS analysis.
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12. The targeting construct, containing a PGK-neomycin (neo) selection cassette flanked by loxP sites, was provided by T. Ley (Washington University, St. Louis, MO). A 4.5-kb fragment of *BLNK* upstream of the initiation codon and a 1.3-kb fragment downstream of exon 1 were subcloned into the targeting construct. In addition, the initiation codon of BLNK was mutated to ATC, and a green fluorescent protein (GFP) cDNA with its own Kozak and initiation codon was inserted upstream of the short arm. GFP fluorescence was not detected in $BLNK^{+/-}$ splenocytes or bone marrow–derived cells; this may be due to transcriptional silencing of GFP by the PGK-neo cassette. The targeting construct was linearized and electroporated into 129/SVJ ES cells at 250 mV and 500 μF with a Gene Pulser II (Bio-Rad, Hercules, CA). Cells were selected in neomycin (0.2 mg/ml) and clones were expanded after 7 days of selection. Fifteen hundred neomycin-resistant clones were screened, of which three represented correct recombinants.
13. DNA was harvested from mouse tails and digested with Bam HI. A 500–base pair (bp) fragment 6 kb upstream of exon 1 was used as the 5′ probe, and a downstream 300-bp Xba I–Bam HI fragment served as the 3′ probe.
14. R. Pappu and A. Chan, unpublished results.
15. To generate $BLNK^{-/-}$ ES cells, a $BLNK^{+/-}$ ES cell was targeted with a vector containing a puromycin selection cassette. This second vector contained the same targeting arms as the first one. In addition, the vector also contained a herpes simplex virus–thymidine kinase (HSV-TK) gene for negative selection. $BLNK^{+/-}$ ES cells were transfected with this construct under the identical conditions described above (*12*) and were selected in media containing puromycin (1 μg/ml), gancyclovir (2 μM), and neomycin (0.2 mg/ml). Clones were screened and analyzed as described above (*13*). The targeting frequency was ~0.5% (*14*). To ensure that the $BLNK^{-/-}$ ES clone was not contaminated with $BLNK^{+/-}$ ES cells, individual clones were subcloned for one round before injection into blastocysts.
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17. Bone marrow–derived cells (5×10^6) were lysed in 10 mM tris (pH 8.0), 150 mM NaCl, and 1% NP-40 buffer containing protease and phosphatase inhibitors (*3*). Cell debris was clarified at 14,000*g* for 10 min at 4°C. The supernatant fraction was then analyzed by SDS–polyacrylamide gel electrophoresis and immunoblotting with antisera to BLNK (*3*) or actin (*14*). The antiserum to BLNK was raised against amino acids 1 through 204 and recognizes amino acids 61 through 204.
18. Cell recoveries from the thymus were (161 ± 51) $\times$ 10^6 ($n = 7$) for $BLNK^{+/+ \text{ or } +/-}$ mice and (152 ± 78) $\times$ 10^6 ($n = 7$) for $BLNK^{-/-}$ mice ($P = 0.334$) (*20*). Recoveries from the bone marrow from two femurs per mouse were (15.3 ± 6.5) $\times$ 10^6 ($n = 12$) for $BLNK^{+/+ \text{ or } +/-}$ mice and (14.1 ± 6.4) $\times$ 10^6 ($n = 11$) for $BLNK^{-/-}$ mice ($P = 0.397$) (*20*). Splenocyte numbers were (64.6 ± 32) $\times$ 10^6 ($n = 10$) for $BLNK^{+/+ \text{ or } +/-}$ mice and (24.0 ± 12) $\times$ 10^6 ($n = 10$) for $BLNK^{-/-}$ mice ($P < 0.001$) (*20*). Cell recoveries from four lymph nodes per mouse were (4.4 ± 2.1) $\times$ 10^6 ($n = 9$) for $BLNK^{+/+ \text{ or } +/-}$ mice and (4.7 ± 2.2) $\times$ 10^6 ($n = 11$) for $BLNK^{-/-}$ mice ($P = 0.388$) (*20*). No statistically significant differences were observed between $BLNK^{+/+}$ and $BLNK^{+/-}$ mice.
19. Supplementary information is available at *Science* Online at www.sciencemag.org/feature/data/1045303.shl
20. Statistical analysis is presented as mean $\pm$ SD; *n* represents sample size and *P* values are derived from comparisons of independent sample tests.
21. For each FACS analysis, 1×10^6 cells were examined. All antibodies were purchased from PharMingen. Data were collected with a FACS Calibur (Becton Dickinson, San Jose, CA) and analyzed with Cell Quest Analysis software. All data were collected from live cells within the lymphocyte gate as defined by forward and side scatter values.
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26. Splenocytes (2×10^7) were incubated at room temperature for 20 min in Dulbecco's modified Eagle's medium (DMEM), 10% FCS, and 3.3 μM Fluo-4 (Molecular Dynamics). Cells were diluted 10-fold with DMEM and 10% FCS and were incubated for an additional 20 min at room temperature. Cells were washed twice with media, resuspended at 2×10^7 cells/ml and, stained with antibodies to B220 (PharMingen) according to the manufacturer's recommendations. Changes in $[Ca^{2+}]_i$ were measured on $B220^+$ B cells every 30 s by FACS analysis.
27. 10^6 cells isolated from the spleen were cultured overnight at 37°C in media alone or in media containing antibody to $F(ab')_2$ (10 μg/ml, Jackson Labs). The expression of CD69 and CD86 was analyzed by FACS analysis gated on $B220^+$ B cells.
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An Essential Role for BLNK in Human B Cell Development

Yoshiyuki Minegishi,[1] Jurg Rohrer,[1] Elaine Coustan-Smith,[2] Howard M. Lederman,[3] Rajita Pappu,[4] Dario Campana,[2,5] Andrew C. Chan,[4] Mary Ellen Conley[1,5]*

The signal transduction events that control the progenitor B cell (pro-B cell) to precursor B cell (pre-B cell) transition have not been well delineated. In evaluating patients with absent B cells, a male with a homozygous splice defect in the cytoplasmic adapter protein BLNK (B cell linker protein) was identified. Although this patient had normal numbers of pro-B cells, he had no pre-B cells or mature B cells, indicating that BLNK plays a critical role in orchestrating the pro-B cell to pre-B cell transition. The immune system and overall growth and development were otherwise normal in this patient, suggesting that BLNK function is highly specific.

Cross-linking of the B cell antigen receptor (BCR) results in rapid phosphorylation of the adapter protein BLNK [also called SLP-65 (Src homology 2 domain–containing leukocyte protein of 65 kD) and BASH (B cell adapter containing Src homology 2 domain)], a hematopoietic-specific cytoplasmic protein with homology to SLP-76 (*1, 2*). Once BLNK is phosphorylated by Syk, it serves as a scaffold to assemble the downstream targets of antigen activation, including Grb2, Vav, Nck, and phospholipase C–γ (PLCγ). Hence, BLNK is positioned to coordinate a number of signaling pathways activated by the BCR. Studies in a

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