

# Cytokine Signaling and Hematopoietic Homeostasis Are Disrupted in *Lnk*-deficient Mice

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## Abstract

The adaptor protein Lnk, and the closely related proteins APS and SH2B, form a subfamily of SH2 domain-containing proteins implicated in growth factor, cytokine, and immunoreceptor signaling. To elucidate the physiological function of Lnk, we derived *Lnk*-deficient mice. *Lnk*<sup>-/-</sup> mice are viable, but display marked changes in the hematopoietic compartment, including splenomegaly and abnormal lymphoid and myeloid homeostasis. The *in vitro* proliferative capacity and absolute numbers of hematopoietic progenitors from *Lnk*<sup>-/-</sup> mice are greatly increased, in part due to hypersensitivity to several cytokines. Moreover, an increased synergy between stem cell factor and either interleukin (IL)-3 or IL-7 was observed in *Lnk*<sup>-/-</sup> cells. Furthermore, *Lnk* inactivation causes abnormal modulation of IL-3 and stem cell factor-mediated signaling pathways. Consistent with these results, we also show that Lnk is highly expressed in multipotent cells and committed precursors in the erythroid, megakaryocyte, and myeloid lineages. These data implicate Lnk as playing an important role in hematopoiesis and in the regulation of growth factor and cytokine receptor-mediated signaling.

Key words: knockout • hematopoiesis • cell proliferation • growth factors • signal transduction

## Introduction

The growth, differentiation, and function of immune and hematopoietic cells are controlled by the coordinated actions of multiple cytokines (1). Members of the cytokine receptor superfamily function through their association with one or more members of the Janus (JAK)\* family of cytoplasmic tyrosine kinases (2, 3). Cytokines induce oligomerization of their receptors, followed by transphosphorylation and activation of the JAK kinases. The activated

JAKs phosphorylate tyrosine residues in the receptor and subsequently downstream substrates, such as the signal transducers and activators of transcription (STAT) proteins (4). Once recruited to the receptor complex, STAT proteins are themselves phosphorylated on tyrosine, and dimerize and translocate into the nucleus, where they activate the transcription of genes mediating cytokine-induced responses. The JAK/STAT pathway is tightly regulated, and deregulated JAK or STAT activity has been implicated in several hematopoietic disorders, autoimmunity, inflammatory diseases, and in cell transformation (5–8).

Cytokines also activate other signaling cascades, such as the Ras/mitogen-activated protein kinase (MAPK) and the phosphoinositide 3-kinase (PI3K)/Akt pathways (9–11). These have been implicated in the proliferation, survival, and differentiation of several cell types in the hematopoietic system (12–14). Indeed, deregulation of these pathways has been implicated in the pathogenesis of hematopoietic diseases (15–18).

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\*Abbreviations used in this paper: BCR, B cell receptor; BMMC, bone marrow-derived mast cell; EMH, extramedullary hematopoiesis; EPO, erythropoietin; ES, embryonic stem; FACS, flow cytometric analysis; IL-3R $\beta$ , IL-3 receptor  $\beta$  chain; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; pBCR, pre-B cell receptor; PH, pleckstrin domain; PI3K, phosphoinositide 3-kinase; SCF, stem cell factor; SH2, src homology 2; STAT, signal transducer and activator of transcription.

Lnk was originally identified as a 38-kD adaptor protein expressed mainly in hematopoietic tissues (19, 20). However, it was later found that the Lnk protein is much larger than initially reported (21, 22, and this work). The full-length mouse Lnk protein contains an NH<sub>2</sub>-terminal proline rich region, a pleckstrin homology (PH) domain, a Src homology 2 (SH2) domain, and potential tyrosine phosphorylation sites. Lnk, and the closely related proteins APS and SH2-B, form a subfamily of SH2-containing proteins with potential adaptor functions (23, 24). Members of the Lnk family are expressed in a wide variety of tissues, and therefore, may be involved in diverse signaling pathways. Indeed, APS and SH2-B have been shown to be phosphorylated after stimulation of growth factor (23, 25–30), cytokine (23–33), and immunoreceptors (21, 23, 24). SH2-B $\beta$  (an isoform of SH2-B) and APS are substrates of the Trk family of neurotrophin receptors, and positive mediators of nerve growth factor signaling in PC12 cells (27). SH2-B is also an activator of JAK2 in response to growth hormone (34), whereas APS appears to be a negative regulator of JAK2 after erythropoietin (EPO) stimulation (32) and inhibits platelet-derived growth factor-induced mitogenesis (35). These observations suggest that these adaptor proteins are regulators of growth factor and cytokine receptor-mediated pathways.

Despite substantial data implicating this new family of adaptor proteins in signaling, their physiological roles in hematopoietic cell signaling have not been defined. However, Takaki et al. (22) have demonstrated that *Lnk*-deficient mice display defects in B lymphopoiesis. We have also generated *Lnk*-deficient mice and have also observed an effect on B cell production and function. In addition, we demonstrate here that *Lnk*<sup>-/-</sup> mice display splenomegaly, hyperplasia of the megakaryocytic lineages, and increased numbers of both erythroid cells in the spleen and hematopoietic progenitors. Consistent with this, we show that Lnk is highly expressed in multipotent cells and precursors of hematopoietic lineages. Moreover, *Lnk*<sup>-/-</sup> cells show an increased sensitivity to several cytokines and altered activation of the Ras/MAPK pathway in response to IL-3 and stem cell factor (SCF). These results demonstrate that Lnk regulates proliferation of several hematopoietic cell lineages and suggest that it is an important component of growth factor and cytokine signal transduction in hematopoietic cells.

## Materials and Methods

**Generation of *Lnk*-deficient Mice.** A 129/Sv mouse genomic library (Stratagene) was screened with a rat *Lnk* cDNA probe. Exon/intron boundaries were determined by restriction enzyme mapping, DNA sequencing, and PCR. To generate the targeting construct, a 5-kb *Sac*I fragment containing 5' *Lnk* genomic DNA and a 2-kb *Sac*I fragment containing 3' sequence were cloned into pPNT. The targeting construct was linearized with *Not*I and electroporated into R1 embryonic stem (ES) cells, and transfectants were selected as described previously (36, 37). Aggregation chimeras were generated with targeted ES cells, and chimeric

males were mated with 129/Sv females and germline transmission of the mutated allele was verified by PCR analysis of tail DNA from F1 mice. Heterozygous offspring were intercrossed to generate homozygous mutants. No differences in phenotype were observed between *Lnk* mutant mice from different genetic backgrounds (129/Sv or C57BL/6).

Poly(A)<sup>+</sup> RNA from E13.5 embryos from each genotype was purified once on an oligo-(dT) column. 2  $\mu$ g of poly(A)<sup>+</sup> RNA was fractionated in a 1% agarose gel containing 2.2 M formaldehyde, and transferred to a Hybond-N membrane (Amersham Pharmacia Biotech). Hybridization was performed in Church buffer (500 mM phosphate buffer, pH 6.8, and 7% SDS) at 65°C overnight. A 500-bp NH<sub>2</sub>-terminal *Lnk* cDNA fragment was used as a probe. The membrane was washed in 0.1 $\times$  SSC, 0.1% SDS at 65°C. The Northern blot was stripped by boiling for 5 min in 0.1% SDS, 0.1 $\times$  SSC, and 0.05% sodium pyrophosphate. RNA loading was evaluated by hybridization with a 1.4-kb glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

**Immunohistochemistry and Peripheral Blood Analysis.** Mouse tissues were fixed in 10% buffered formalin, paraffin embedded, sectioned, and stained with hematoxylin and eosin. For immunohistochemical analysis, spleens were embedded in OCT compound (Tissue-Tek Sakura), snap frozen in dry ice, and stored at -70°C. Cryostat sections (8–10  $\mu$ m thick) were dried overnight and fixed with acetone. Slides were rehydrated and preincubated for 1 h in staining buffer (1 $\times$  PBS and 0.1% NP-40) containing 5% heat-inactivated rat serum. Sections were incubated with FITC- or phycoerythrin (PE)-conjugated primary antibodies (BD PharMingen) in a humidified chamber for 1 h at room temperature. After three washes in staining buffer and one wash in 1 $\times$  PBS, the slides were mounted in *p*-phenylenediamine (Sigma-Aldrich) in PBS and glycerol (50% vol/vol) to retard photobleaching. The antibodies used for immunohistochemistry were PE-anti-B220, PE-anti-Ter119, and FITC-anti-CD41. Visual data were acquired with a fluorescent microscope (Leica).

Peripheral blood analysis was performed on a blood analyzer (Coulter electronics). Peripheral blood smears were stained with Wright-Giemsa (Fisher) and scored microscopically based on morphology.

**Flow Cytometric Analysis.** Bone marrow and spleen cells obtained from wild-type or mutant mice were made into single cell suspensions in staining buffer (cold PBS supplemented with 2% FBS). The cells were stained on ice for 30 min with FITC, PE, APC, or biotin-conjugated mAbs. The stained cells were washed twice in staining buffer and analyzed using a FACScan<sup>TM</sup> flow cytometer and CellQuest<sup>TM</sup> software (Becton Dickinson). The following mAbs were used: PE, APC, or biotin-anti-B220, PE-anti-Ter119, PE-anti-Mac-1, PE-anti-CD43, PE-anti-CD22, PE-anti-CD23, FITC-anti-CD41, FITC-anti-Gr-1, FITC-anti-BP.1, and biotin-anti-HSA; all were purchased from BD PharMingen. FITC-anti-IgM is the clone 33.60 (38). Biotin-stained cells were labeled with Quantum red (Sigma-Aldrich).

**B Cell Proliferation Assays.** To obtain enriched B cell populations, splenocytes from wild-type and mutant mice were treated with ACK (0.155 M ammonium chloride, 0.1 mM disodium EDTA, and 0.01 M potassium bicarbonate) for red cell lysis followed by T cell depletion. Cells were incubated with anti-Thy1.2 antibody (clone H013.4.9), anti-CD4 (clone RL172.4H), and anti-CD8 (clone 3.168) supernatants and low tox guinea pig complement (Cedarlane Laboratories). The resulting enriched splenic B cells were cultured at 10<sup>5</sup> cells/well in flat-bottom 96-well plates in OPTI-MEM (GIBCO BRL) medium + 5% FBS

and with or without 100 ng/ml IL-7, 50 ng/ml SCF (Pepro-Tech), IL-7 and SCF, 10  $\mu$ g/ml LPS, and 10  $\mu$ g/ml anti-IgM for 2–6 d. The cultures were pulsed with 1  $\mu$ Ci/well [ $^3$ H]thymidine for 6 h and harvested. Incorporated radioactivity was determined by a Betaplate Counter (Packard Instrument Co.). IL-7 was obtained from the supernatant of the stably transfected J558 line (Dr. A Cumano, Institute Pasteur, Paris, France). For the proliferation assay of CD43<sup>+</sup> pro-B cells, bone marrow cells were enriched and prepared as described previously (39).

**In Vitro Bone Marrow and Spleen Cultures.** For liquid cultures, single cell suspensions of bone marrow and spleen cells were prepared in IMDM (GIBCO BRL) supplemented with 15% heat-inactivated FBS, 50  $\mu$ M B-mercaptoethanol, antibiotics, 200  $\mu$ g/ml transferrin (Sigma-Aldrich), 10  $\mu$ g/ml insulin (Boehringer), and 1% BSA. Cells were plated in 96-well tissue culture plates at  $10^4$  (for bone marrow) or  $5 \times 10^4$  cells/well (for spleen) in various concentrations of recombinant murine IL-3 (Pepro-Tech), SCF (PeproTech), or IL-3 and SCF. After 4–6 d in culture, cells were stained with MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) as suggested by the manufacturer (Boehringer).

**In Vitro Hematopoietic Progenitor Colony Assays.** Single cell suspensions were prepared from bone marrow aspirates or spleen under sterile conditions. Triplicate samples of  $10^4$  or  $10^5$  cells (for CFU-GEMM) were plated in methylcellulose (MethoCult M3434) containing cytokines, including erythropoietin (Stem Cell Technologies Inc.), and incubated at 37°C, 5% CO<sub>2</sub>, 100% humidity. Progenitor colony-forming units were counted at 2 d (CFU-E), 8 d (BFU-E), and 10 d (CFU-GM, CFU-GEMM) after benzidine staining. Megakaryocyte progenitors ( $2 \times 10^4$  cells) were assayed in collagen-based media (MegaCult-C; Stem Cell Technologies Inc.). Colonies were stained for acetylcholinesterase activity and counted after 6 d incubation. Bone marrow pre-B cell progenitors ( $5 \times 10^4$  cells) were detected by culture in methylcellulose media (MethoCult M3231; Stem Cell Technologies Inc.) supplemented with IL-7 (10 ng/ml; BD PharMingen) or IL-7 and SCF (50 ng/ml; PeproTech). Colonies were scored microscopically after 7 d.

**Immunoprecipitation and Western Blot Analysis.** Polyclonal rabbit antisera was raised against an NH<sub>2</sub>-terminal synthetic peptide (amino acid 72–91) of mouse Lnk. Spleens and testis from wild-type, heterozygous, and homozygous mice were homogenized and lysed in ice-cold lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 10% glycerol, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 1 mM PMSF, 10  $\mu$ g/ml aprotinin, and 10  $\mu$ g/ml leupeptin). Immunoprecipitations were done with anti-Lnk-NH<sub>2</sub>-terminal antibodies and separated on a 8–12% SDS-PAGE gradient gel. Western blot analysis was performed with anti-Lnk-NH<sub>2</sub>-terminal antibodies, followed by incubation with HRP-conjugated protein A (Bio-Rad Laboratories) and detection via ECL reagents (Pierce Chemical Co.).

**Preparation of Bone Marrow-derived Mast Cells and In Vitro Cultures.** Primary cultures of IL-3-dependent bone marrow-derived mast cells (BMMCs) from 6–8-wk-old wild-type and mutant mice were prepared and maintained as described previously (40). For MAPK analysis, BMMCs were plated in starving media (IMDM: 0.5% FBS and 1% BSA, pH 7) for 16 h and stimulated with 100 ng/ml SCF (PeproTech) or 100 ng/ml IL-3 (BD PharMingen) for the indicated times at 37°C. Cells were then washed once with ice-cold PBS containing 1 mM Na<sub>3</sub>VO<sub>4</sub>, and lysed in ice-cold lysis buffer for 30 min on ice. Lysates were cleared by centrifugation at 13,000 rpm for 10 min, and supernatants containing the whole cell lysates were analyzed on SDS-

PAGE gels. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) and immunoblotted with monoclonal antiphosphotyrosine 4G10 (UBI), phospho-specific Akt, or phospho-specific ERK1/2 MAPK (both from New England Biolabs, Inc.) antibodies. To verify equivalent loading and to confirm the identity of phosphorylated MAPK and Kit receptor, membranes were stripped as recommended (ECL; Amersham Pharmacia Biotech) and then blotted with anti-ERK1 and ERK2 (0.5  $\mu$ g/ml each; Santa Cruz Biotechnology, Inc. and UBI, respectively) or anti-Kit (40) antibodies. Immunoprecipitation and Western blot analysis of IL-3 receptor  $\beta$  chain (IL-3R $\beta$ ) were done as described previously (41). Immunoblots were visualized with enhanced chemiluminescence detection reagents (ECL; Amersham Pharmacia Biotech). Densitometric scanning was performed with a high resolution Fluor-S Max<sup>TM</sup> multimager (Bio-Rad Laboratories).

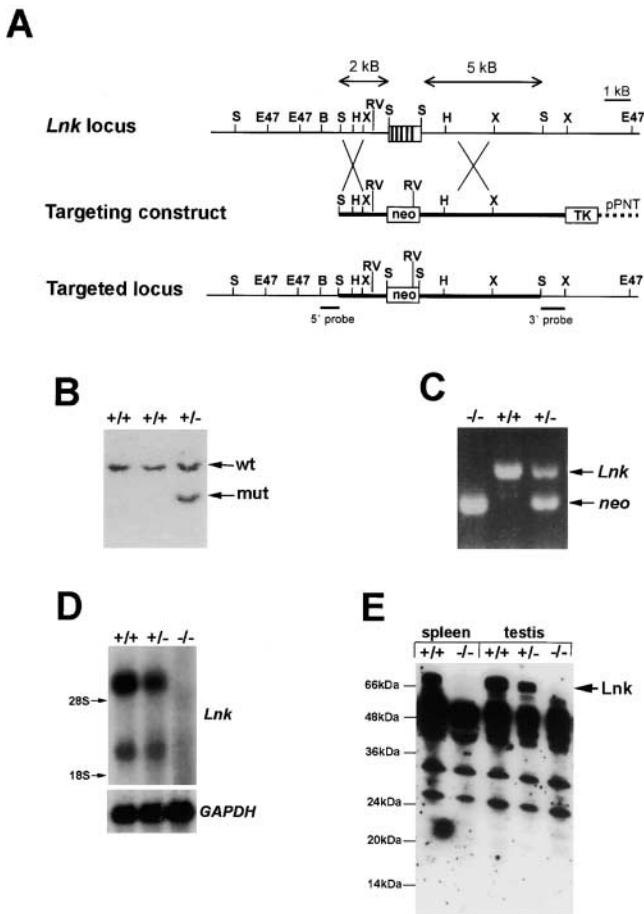
**BMMC In Vitro Proliferation Assay.** For in vitro proliferation assays, BMMCs in IMDM complete medium were starved of IL-3 for 12 h;  $5 \times 10^4$  cells were seeded in triplicate in 96-well plates and stimulated with the indicated concentrations of SCF or IL-3 for 28 h. After 20 h, 1  $\mu$ Ci/well of [ $^3$ H]thymidine was added for 12 h. Cells were harvested and [ $^3$ H]thymidine incorporation was measured by a Betaplate counter (Packard Instrument Co.).

**Expression Analysis.** A Slot blot containing amplified cDNAs from various hematopoietic cells ranging from pentapotent precursors to terminally mature cells in erythroid, myeloid, and lymphoid lineages, or different organs (a gift from Norman N. Iacove, Ontario Cancer Institute) was hybridized as described previously (42). A 350-bp fragment containing the terminal 3' untranslated (close to the polyadenylation site) sequence of *Lnk* was used as a probe. L32, a constitutively expressed “housekeeping” transcript encoding a ribosomal subunit protein, was used as a probe to confirm equal loading of cDNAs samples.

## Results

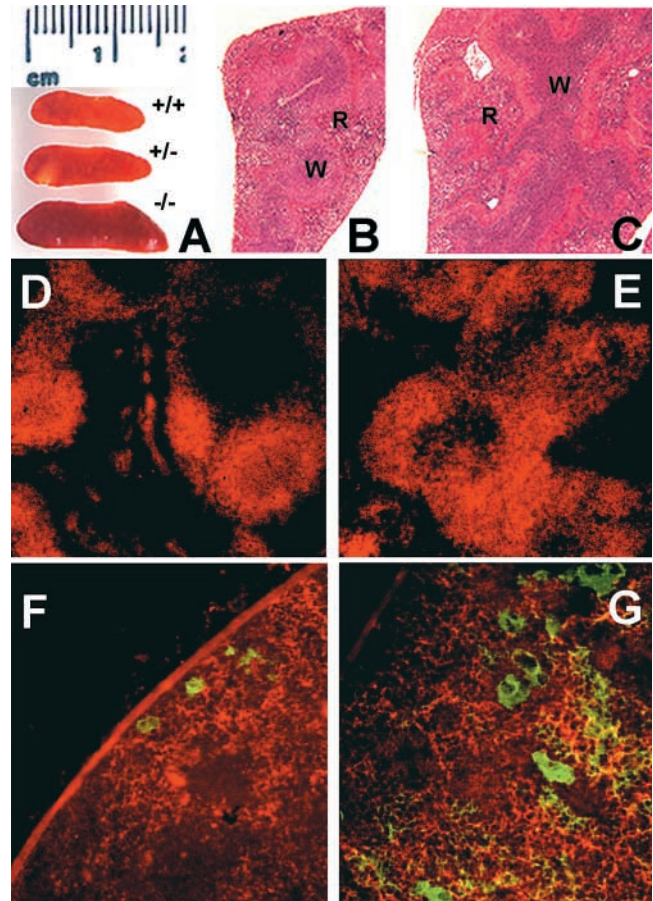
**Disruption of the *Lnk* Gene and Generation of *Lnk*<sup>-/-</sup> Mice.** To address the biological function of *Lnk* in vivo, we generated mice carrying a germline mutation in *Lnk* by homologous recombination in RI mouse ES cells. A targeting vector was designed to replace a 1.6-kb DNA fragment containing exons II–VII of *Lnk* with a PGK-neomycin cassette (Fig. 1 A). The deleted exons encoded the originally reported coding sequence of Lnk (19). Successful homologous recombination and germline transmission was confirmed by Southern blot and PCR analysis of ES cells and tail-derived genomic DNA (from offspring of heterozygous parents), respectively (Fig. 1, B and C). Northern and Western blot analyses of different tissue extracts derived from mice of each genotype were performed with a 500-bp NH<sub>2</sub>-terminal Lnk probe (exon 1) or antibodies raised against the NH<sub>2</sub>-terminal region of the Lnk protein, respectively (Fig. 1, D and E). This analysis confirmed the absence of Lnk-specific protein products in the *Lnk*-deficient mice.

***Lnk*<sup>-/-</sup> Mice Exhibit Extramedullary Hematopoiesis.** Homozygous *Lnk*<sup>-/-</sup> mice were present at the expected Mendelian ratio of 1:2:1 ( $n = 330$ ) from intercrosses of heterozygous parents. Both *Lnk*<sup>-/-</sup> female and male animals were viable and fertile, with normal longevity.



**Figure 1.** Targeted disruption of the *Lnk* locus. (A) Structure of the *Lnk* locus (top), targeting construct (middle), and predicted *Lnk* mutant allele (bottom) is shown; 1 kb = 0.5 cm. (B) Southern blot analysis of EcoRV-digested ES cell-derived genomic DNA with a 3' external probe. The wild-type (wt) fragment and the mutant (mut) allele are indicated. (C) PCR analysis of tail-derived genomic DNA from F2 mice resulting from a cross between two *Lnk*<sup>+/-</sup> mice with both *Lnk* and *neo*-specific primers. (D) Northern blot analysis of *Lnk* expression. 2  $\mu$ g poly(A)<sup>+</sup>RNA from E13.5 embryos was hybridized with an NH<sub>2</sub>-terminal *Lnk* cDNA (top) or *GAPDH* probe (bottom). (E) Immunoprecipitation and Western blot analysis of spleen and testis extracts from wild-type (+/+), heterozygous (+/-), and homozygous (-/-) *Lnk* mice. The *Lnk* antibody used was raised against amino acid 72–91 of the *Lnk* protein. H, HindIII; X, XbaI; S, SacI; E47, EcoR47; B, BamHI; RV, EcoRV; neo, neomycin resistance cassette; TK, thymidine kinase.

We performed a histological analysis of neonatal and adult mice to identify tissue abnormalities in *Lnk*-deficient animals. This identified a severe splenic disorder in *Lnk*<sup>-/-</sup> mice characterized by splenomegaly (weight, 0.062  $\pm$  0.015 g for +/+ mice compared with 0.195  $\pm$  0.07 g for -/- mice;  $P < 0.01$ ; Fig. 2 A), fibrosis, and extensive extramedullary hematopoiesis (EMH), in which increased numbers of megakaryocytes (CD41<sup>+</sup> cells) and erythrocytes (Ter119<sup>+</sup> cells) were apparent in the splenic red pulp (Fig. 2, C, F, and G). This increased EMH in the spleen of mutant mice was apparent from birth (unpublished data). In general, the architecture of the spleen was considerably changed in the *Lnk*-deficient mice. The white pulp, with



**Figure 2.** Primary splenomegaly and extramedullary hematopoiesis in *Lnk*<sup>-/-</sup> mice. (A) Gross anatomy of spleens from *Lnk* wild-type (top), +/+ (middle), and -/- (bottom) mice. (B and C) Low power magnification (10 $\times$ ) of hematoxylin and eosin staining of splenic sections from wild-type (B) and *Lnk*<sup>-/-</sup> (C) mice showing enlargement of the white (W) and red (R) pulp. (D–G) Cryosections of spleens from wild-type (D and F) or *Lnk*-deficient mice (E and G) were stained with anti-B220 (red, D and E), anti-Ter119 (red, F and G), and anti-CD41 (green, F and G)-conjugated antibodies. Original magnification: 50 $\times$  (D and E), 200 $\times$  (F and G).

its characteristic discrete lymphoid follicles (Fig. 2 B), presented dramatic changes in shape (Fig. 2 C), accompanied by an increase in B220<sup>+</sup> lymphocytes (Fig. 2, D and E). The bone marrow histology of *Lnk* mutant mice revealed an increase in the number of megakaryocytes and a slight decrease in erythroid cell number (unpublished data). Lymph nodes were often enlarged in *Lnk*<sup>-/-</sup> mice and showed increased numbers of B cells (unpublished data). No morphological differences between wild-type and *Lnk*-deficient animals were observed in any other tissue, including thymus, liver, kidney, and lungs. For all parameters analyzed, the *Lnk* heterozygous mice showed an intermediate phenotype between the wild-type and homozygous mutant mice (Fig. 2 A and unpublished data). Together, these results revealed hyperplasia of the megakaryocytic, erythroid, and B cell lineages in the spleen of *Lnk*<sup>-/-</sup> mice with the latter two contributing to increased cellularity in this organ and splenomegaly.

**Table I.** Hematological Parameters of Wild-type and *Lnk*-deficient Mice

Parameter	+ / + <sup>a</sup>	- / -	<i>P</i> value <sup>b</sup>
Peripheral blood			
WBC (10 <sup>6</sup> /ml)	7.6 ± 1.5	26.6 ± 6.04	**
RBC (10 <sup>6</sup> /ml)	8.4 ± 0.9	7.8 ± 0.7	NS
Hematocrit (%)	48 ± 3.3	49 ± 2	NS
Platelets (10 <sup>6</sup> /ml)	400 ± 107	1,903 ± 219	**
Differential counts (10 <sup>6</sup> /ml)			
Lymphocytes	6.44 ± 1.39	22.12 ± 5.55	**
Monocytes	0.130 ± 0.05	0.420 ± 0.102	**
Neutrophils	1.04 ± 0.51	3.91 ± 1.15	**
Eosinophils	0.106 ± 0.07	0.394 ± 0.22	*

WBC, white blood count; RBC, red blood count.

<sup>a</sup>Values represent the mean ± SD for 10 animals per determination per genotype.

<sup>b</sup>Statistical significance was determined using the Student's *t* test where \*, *P* ≤ 0.05; \*\*, *P* ≤ 0.005; NS, not significant.

*Lnk*<sup>-/-</sup> Mice Exhibit Defects in Hematopoietic Homeostasis. Based on these observations, we examined specific hematological parameters in the mutant mice. No significant differences in peripheral red blood cell counts or in hematocrits were observed between wild-type and *Lnk*<sup>-/-</sup> mice. However, the white blood cell counts and circulating platelet levels in *Lnk* mutants were markedly increased as compared with wild-type mice. Differential counts of peripheral blood smears revealed a substantial elevation in the numbers of circulating lymphocytes, monocytes, and neutrophils (Table I).

Flow cytometric analysis (FACS) of bone marrow and spleen cells was then performed to further characterize the

hematopoietic perturbations in the *Lnk*<sup>-/-</sup> mice. The percentage of B220<sup>+</sup> lymphocytes was increased in the bone marrow and in the spleen (8–13% above +/+ littermates) of *Lnk*-deficient mice (Table II). Similarly, the percentage of cells positive for CD41, expressed on megakaryocytes, was elevated in the bone marrow (twofold) and more dramatically in the spleen (threefold) of *Lnk*<sup>-/-</sup> mice (Table II). In addition, the percentage of Ter-119–positive erythroid cells in *Lnk*-deficient mice was decreased 36% in the bone marrow but increased twofold in the spleen of *Lnk* mutant mice (Table II), which is consistent with the elevated splenic erythropoiesis observed previously (Fig. 2 A). Finally, the percentage of Gr-1/Mac-1<sup>+</sup> cells, representing monocyte/granulocyte progenitors, was decreased 15% in the bone marrow, but remained similar in the spleen of *Lnk*<sup>-/-</sup> mice (Table II). When combined with the increase in cellularity in both the bone marrow and spleen compartments of *Lnk*<sup>-/-</sup> mice, these FACS data reveal a significant elevation of both B lymphoid and megakaryocyte populations, as well as enhanced erythroid numbers in the spleen (Table II). The FACS data are consistent with the changes observed in the peripheral blood counts and with the histological findings in bone marrow and spleen of *Lnk*-deficient mice.

*Lnk*<sup>-/-</sup> Mice Have Altered Hematopoietic Progenitor Numbers. To determine if the perturbations in the hematopoietic organs and peripheral blood arise at the progenitor level, bone marrow and spleen cells from *Lnk*<sup>+/+</sup>, *Lnk*<sup>+/-</sup>, and *Lnk*<sup>-/-</sup> mice were grown in vitro in semi-solid media with various growth factors to quantitate multilineage (CFU-GEMM), pre-B, megakaryocytic (CFU-Meg), and erythroid (BFU-E and CFU-E) progenitor cells (Fig. 3 A). The absolute numbers of clonogenic progenitors observed with *Lnk*<sup>+/-</sup> cells were intermediate between wild-type and mutant levels (unpublished data). The number of multilineage colony-forming cells (CFU-GEMM) was significantly increased in *Lnk*<sup>-/-</sup> bone marrow (2.5-fold) and spleen (8-

**Table II.** Cell Population in Wild-type and *Lnk*-deficient mice (in millions)

	Total cells	B cells B220 <sup>+</sup>		Erythroid Ter119 <sup>+</sup>		Megakaryocyte CD41 <sup>+</sup>		Myeloid Gr-1/Mac-1 <sup>+</sup>		
		No.	%	No.	%	No.	%	No.	%	
Bone marrow										
Wild type	13 ± 3.6	4.3 ± 0.59	34.1 ± 4.6	0.90 ± 0.20	7.0 ± 1.5	1.1 ± 0.10	8.5 ± 0.81	6.1 ± 1.1	48 ± 8.7	
<i>Lnk</i> -deficient	14 ± 1.2	6.6 ± 0.71 <sup>b</sup>	47.5 ± 5.1	0.63 ± 0.20	4.5 ± 1.3	2.3 ± 0.37 <sup>c</sup>	17 ± 2.6	5.0 ± 0.66	33 ± 4.7	
Spleen										
Wild type	63 ± 15.4	32.1 ± 5.4	51 ± 8.6	2.2 ± 0.98	3.5 ± 1.6	4.0 ± 0.11	6.0 ± 0.17	1.5 ± 0.30	12 ± 2.3	
<i>Lnk</i> -deficient	82 ± 25 <sup>d</sup>	49 ± 3.1 <sup>a</sup>	59 ± 3.8	4.6 ± 0.98 <sup>b</sup>	6.0 ± 1.2	15 ± 1.4 <sup>b</sup>	18 ± 1.7	2.2 ± 0.51 <sup>c</sup>	13 ± 3.2	

The phenotype of each cell population was determined by flow cytometry. The numbers and percentage of each group of cells were determined for each mouse for at least three to five mice per genotype, and the mean and SD were calculated.

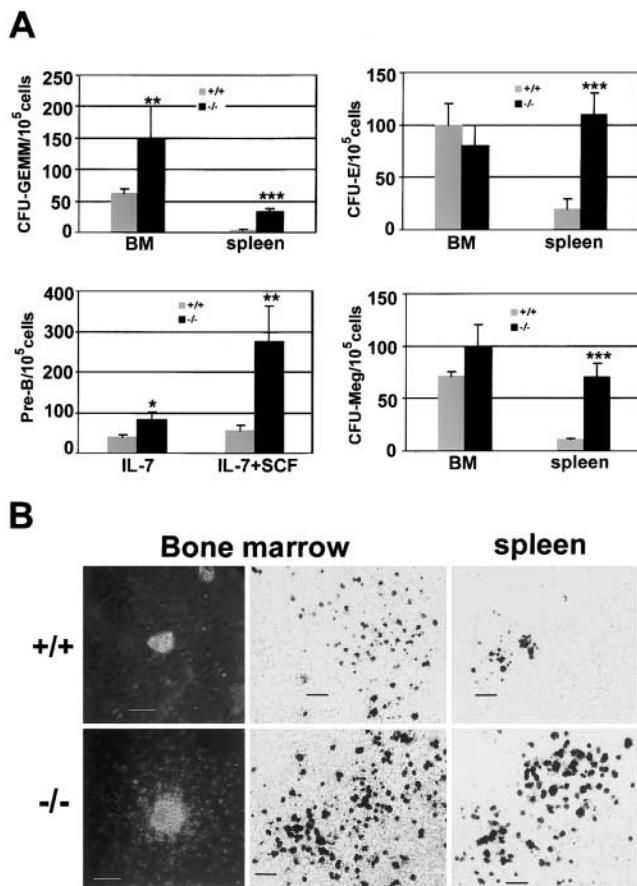
<sup>a</sup>*P* < 0.001.

<sup>b</sup>*P* < 0.005.

<sup>c</sup>*P* < 0.02.

<sup>d</sup>*P* < 0.05.

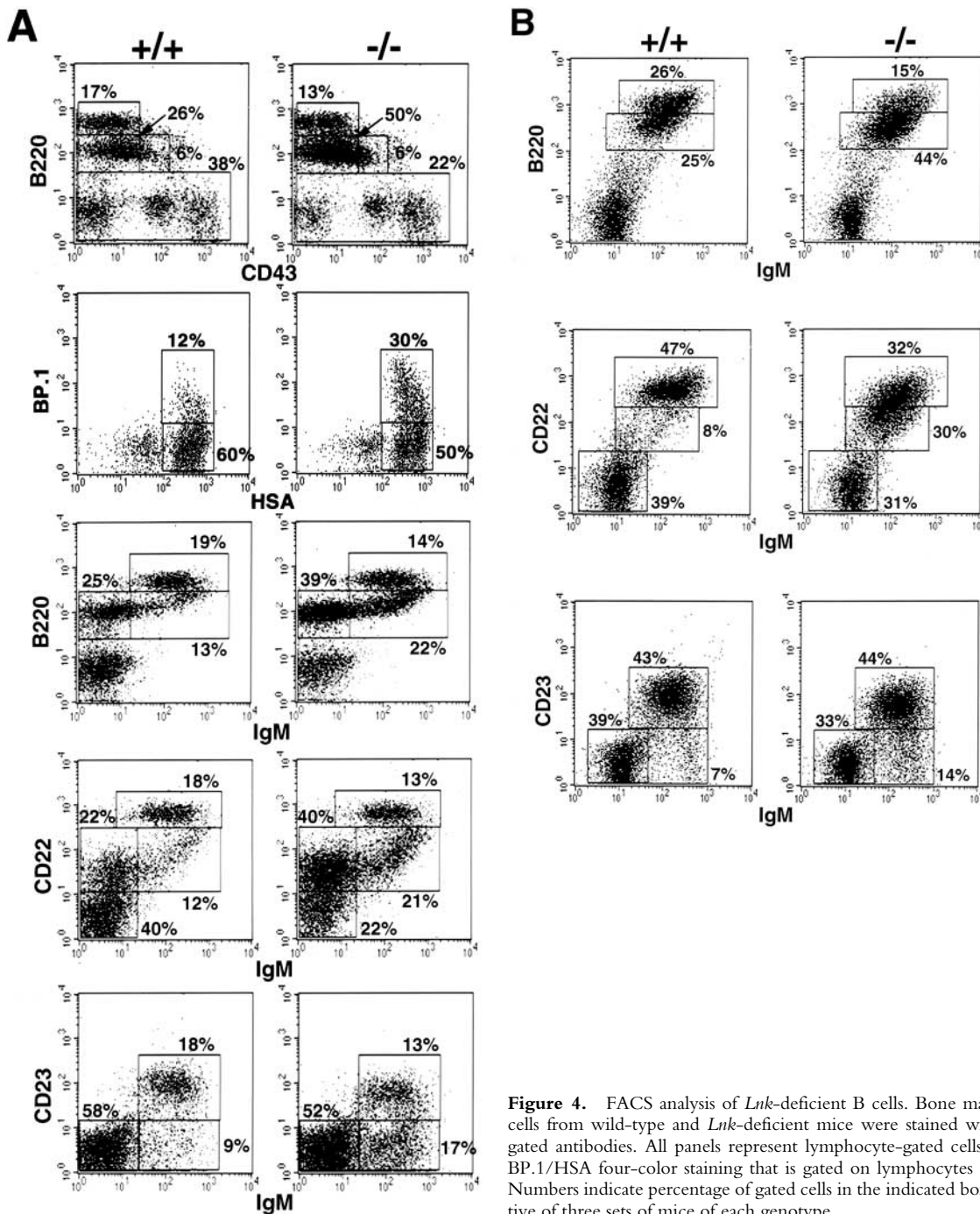
fold) compared with +/+ littermates. Similarly, the number of pre-B lymphoid colony-forming cells in *Lnk*<sup>-/-</sup> bone marrow was increased two- and sevenfold relative to wild-type mice in the presence of IL-7 or IL-7 and SCF, respectively (Fig. 3 A). Although only slight differences were observed in the number of precursors of small erythroid 2-d colonies (CFU-E) and CFU-megakaryocyte (CFU-Meg) in *Lnk*<sup>-/-</sup> bone marrow, increased numbers of these colonies were seen in cells derived from the spleen. A similar result was observed with precursors of large erythroid-only 6–8-d colonies (BFU-E) and granulocyte-macrophage (CFU-GM) progenitor numbers (unpublished data).



**Figure 3.** In vitro proliferative capacity of early hematopoietic progenitors in bone marrow and spleen cells from wild-type and *Lnk* mutant mice. (A) In vitro colony-forming ability of bone marrow and spleen hematopoietic progenitors from wild-type and *Lnk*-deficient mice. The mean and SD of the number of colonies/10<sup>5</sup> cells are shown from assays using three mice of each genotype in triplicate. Statistical significance was determined using the Student's *t* test: \*, *P* ≤ 0.05; \*\*, *P* < 0.02; \*\*\*, *P* < 0.01. (B) Typical appearance of pre-B (left) in IL-7 + SCF and CFU-Meg (center and right) colonies derived from wild-type and *Lnk*-deficient cells after 7 d of incubation in methylcellulose media containing optimal concentrations of appropriate recombinant growth factors (Materials and Methods). CFU-GEMM, colony-forming units of granulocyte, erythroid, macrophage, and megakaryocyte cells; CFU-E, precursors of small erythroid 2-d colonies; CFU-Meg, colony-forming units of megakaryocyte cells; BM, bone marrow. Scale bar: pre-B colonies, 20 μm; CFU-Meg colonies, 200 μm.

Moreover, there was a marked difference in the proliferative capacity of *Lnk*<sup>-/-</sup> progenitors. The pre-B lymphoid and CFU-Meg colonies from bone marrow and spleen of *Lnk*-deficient mice were typically three to five times larger than the colonies derived from wild-type mice (Fig. 3 B). We observed a similar effect on the colony size of CFU-GEMM from *Lnk*<sup>-/-</sup> mice. These results suggest that the variations in differentiation potential observed are intrinsic to the *Lnk*<sup>-/-</sup> progenitor cells, and that these cells have an enhanced responsiveness to cytokines.

*Lnk*<sup>-/-</sup> Mice Are Affected in B Cell Maturation and Proliferation. Because there was an increase in the number of B220<sup>+</sup> cells in the *Lnk* mutant mice, we examined which B cell populations were affected by the lack of the Lnk protein. FACS analysis of bone marrow-derived B cells revealed that wild-type and *Lnk* mutant mice display similar percentages of pro-B (B220<sup>+</sup>CD43<sup>+</sup>) cells (Fig. 4 A). Further analysis of the pro-B compartment by four-color FACS staining indicated that *Lnk*<sup>-/-</sup> mice have an increase in the percentage of BP.1<sup>+</sup>HSA<sup>+</sup> cells relative to wild-type mice (Fig. 4 A). This population corresponds to the IL-7-responsive, Fraction C stage of B cell development (43). The increase in Fraction C cells is accompanied by a slight decrease in the percentage of HSA<sup>+</sup>BP.1<sup>-</sup>, Fraction B cells (Fig. 4 A). A more profound increase is observed in the pre-B cell population in *Lnk*-deficient animals, which can be visualized by the B220<sup>+</sup>IgM<sup>-</sup> and B220<sup>+</sup>CD43<sup>-</sup> populations shown in Fig. 4 A. Later stages of development were also altered in the absence of *Lnk* expression. An accumulation of the immature population of B220<sup>lo</sup>CD43<sup>-</sup>IgM<sup>+</sup> cells and a decreased percentage of more mature, recirculating B220<sup>hi</sup>IgM<sup>+</sup> cells were identified in *Lnk*-deficient mice (Fig. 4 A). In addition, a greater proportion of the IgM<sup>+</sup> cells in the bone marrow and spleen of *Lnk*<sup>-/-</sup> mice expressed reduced levels of CD22 and CD23, which is consistent with the observed accumulation of cells at the immature B cell stage of development (Fig. 4, A and B). The differences in the proportion of B cell precursors in wild-type and *Lnk*-deficient animals are more pronounced when the increase in *Lnk*<sup>-/-</sup> bone marrow cellularity is taken into account. This analysis also reveals that the absolute number of recirculating cells is similar to that found in control mice (1.5-fold increase, compared with 3–4-fold increase in pro-, pre-, and immature B cells; unpublished data). Elevated numbers of immature IgM<sup>+</sup> B cells may arise after the maturation of the expanded pre-B population present in *Lnk*<sup>-/-</sup> mice, or may indicate incomplete maturation past the B220<sup>lo</sup>CD43<sup>-</sup>IgM<sup>+</sup>, Fraction E cell stage in the absence of *Lnk* expression. Support for the hypothesis of incomplete maturation of the B cell lineage was found in the spleen, where the presence of a high frequency of IL-7-responsive cells was observed (1:100 in *Lnk*<sup>-/-</sup> compared with 1:11,000 in wild-type mice; unpublished data) in the absence of detectable numbers of IgM<sup>-</sup> B cell precursors (Fig. 4 B). Together, these data indicate that an increase in B lineage cells is evident as early as the IL-7-responsive, Fraction C stage of development, and that an accumulation of cells at the

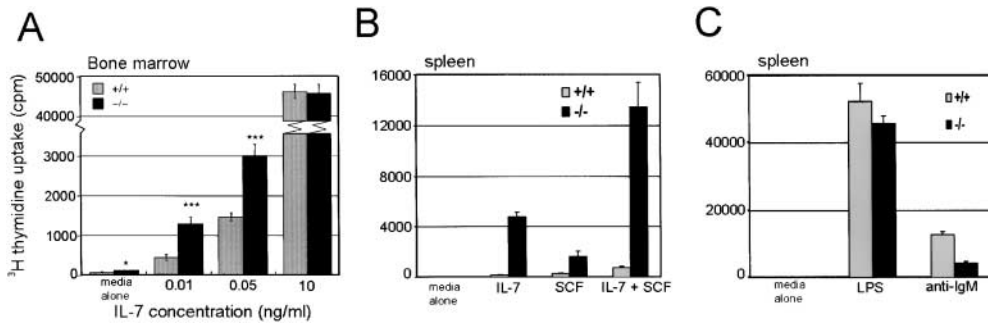


**Figure 4.** FACS analysis of *Lnk*-deficient B cells. Bone marrow (A) or splenic (B) cells from wild-type and *Lnk*-deficient mice were stained with the indicated conjugated antibodies. All panels represent lymphocyte-gated cells, with the exception of BP.1/HSA four-color staining that is gated on lymphocytes and B220<sup>+</sup>CD43<sup>+</sup> cells. Numbers indicate percentage of gated cells in the indicated boxes. Plots are representative of three sets of mice of each genotype.

B220<sup>lo</sup>, immature B cell stage occurs in the bone marrow and spleen in *Lnk*-deficient mice.

To further assess the maturation state of B cell precursors in the *Lnk*-deficient mice, we analyzed the ability of bone marrow and splenic B cells to proliferate in response to various stimuli. CD43<sup>+</sup> pro-B cells were sorted from the bone marrow and grown in a titration of IL-7. These culture conditions permit the expansion of IL-7-responsive pro-B cells, as well as their maturation to the pre-B cell receptor (pBCR)-expressing pre-B stage. Recent reports have dem-

onstrated that a signal downstream of the pBCR is required for proliferation in picogram concentrations of IL-7 (39). As shown in Fig. 5 A, cells from both the *Lnk*<sup>-/-</sup> and control mice are able to proliferate above background in low concentrations of IL-7. A slight elevation in background [<sup>3</sup>H]thymidine incorporation by *Lnk*-deficient cells is observed in the absence of IL-7. However, these cells exhibit a more significant twofold elevation in proliferation when stimulated with picogram per milliliter concentrations of IL-7. FACS staining revealed that cells from both geno-



**Figure 5.** Functional analysis of *Lnk*<sup>-/-</sup> B cells. (A) Bone marrow sorted pro-B cells from wild-type and *Lnk*-deficient mice were cultured with the indicated concentration of IL-7 and proliferation was measured at day 4 by [<sup>3</sup>H]thymidine incorporation. The values are the mean counts per minute ( $\pm$ SD) of triplicate determinations. Significance was determined by Student's *t* test: \*\*\*, *P* < 0.001; \*\*, *P* < 0.005; \*, *P* < 0.02. (B and C) Splenic B

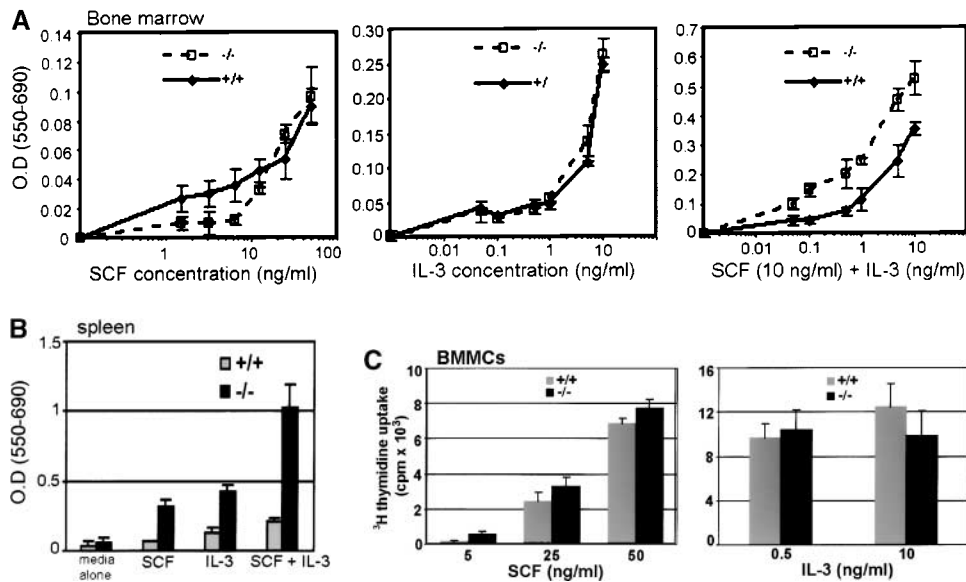
cells were cultured with the indicated stimuli and proliferation was measured at day 6 (B) or day 4 (C) by [<sup>3</sup>H]thymidine incorporation. The values are the mean counts per minute ( $\pm$ SD) of triplicate determinations. All graphs are representative of independent experiments repeated three to five times. For each experiment, two mice per genotype were used.

types matured to express CD2 in high and low concentrations of IL-7 (unpublished data). The absence of a significant increase in the proliferation of mutant precursors in response to nanogram concentrations of IL-7 suggests that the phenotype observed in reduced levels of IL-7 is not due to the presence of a higher percentage of BP.1<sup>+</sup> cells in the sorted population. Together, these results indicate that *Lnk* expression may not be required to transmit pBCR signals at this stage in development, and suggest that the pre-B cells have not fully matured and lost the ability to respond to IL-7 (44). Similarly, splenic B cells from *Lnk*-deficient mice showed a significant increase in proliferation after IL-7 stimulation (Fig. 5 B). This is consistent with the elevated frequency of IL-7-responsive precursors detected in the mutant spleen, and is indicative of a very immature phenotype extending to the periphery. In addition, the proliferation of *Lnk*-deficient splenic B cells is further enhanced by concurrent stimulation with IL-7 and SCF (Fig. 5 B).

Splenic B cells were also assayed for proliferation after BCR stimulation. Although *Lnk*-deficient cells maintain the ability to proliferate in response to the mitogen LPS, the response of splenic *Lnk*<sup>-/-</sup> B cells to anti-IgM was partially reduced at any of the time points examined (Fig. 5 C and unpublished data). Cells at the immature B cell stage are unable to mount a proliferative response after BCR stimulation (45). This is consistent with the elevated numbers of immature B cells observed in the spleen (Fig. 4 B). Alternatively, *Lnk* may be required by the BCR to transmit a signal leading to proliferation. Further experiments are underway to examine these issues. Together, these results indicate that the B lineage cells derived from *Lnk*<sup>-/-</sup> mice maintain growth characteristics that are typical of immature populations. The B cell phenotype that we observed is similar to the one identified by Takaki et al. (22). However, in contrast with this group, we do not detect a striking difference between wild-type and *Lnk*-deficient mice in the mature B220<sup>hi</sup>IgM<sup>+</sup> population recirculating in the bone marrow from the periphery. Therefore, in our hands, the absence of the *Lnk* protein does not prevent the recirculation of mature B cells into the bone marrow compartment, but does lead to an accumulation of phenotypically immature B cells.

*Lnk*<sup>-/-</sup> Cells Exhibit Increased Proliferative Responses to Different Cytokines. The results of the clonogenic and B cell proliferation assays suggest that *Lnk* may play an important role in regulating myeloid and B cell progenitor proliferation. SCF, in combination with other growth factors, such as IL-3, stimulates the proliferation of most hematopoietic progenitors and mature cells (46). Therefore, we examined the growth response of other hematopoietic cells derived from wild-type and *Lnk*<sup>-/-</sup> mice to various mitogenic stimuli. We first analyzed the response of primary bone marrow and splenic cells from wild-type and *Lnk*<sup>-/-</sup> mice to various concentrations of SCF, IL-3, or a combination of both factors. No difference in proliferation was detected between wild-type and *Lnk*<sup>-/-</sup> bone marrow cells with SCF or IL-3 alone (Fig. 6 A, left and middle). The addition of SCF to bone marrow cultures stimulated with IL-3 induced an increased expansion of *Lnk*<sup>-/-</sup> cells that was not seen in wild-type cells (Fig. 6 A, right). A similar result was observed when scoring the number of progenitor colonies formed in the presence of SCF and different concentrations of IL-3 from *Lnk*<sup>-/-</sup> mice (unpublished data). However, primary *Lnk*<sup>-/-</sup> splenocytes exhibited an enhanced sensitivity to either SCF or IL-3 alone relative to wild-type cells (Fig. 6 B). This hypersensitive proliferation of *Lnk*<sup>-/-</sup> cells was more pronounced in cultures stimulated with both SCF and IL-3 (Fig. 6 B). To pursue the effects of *Lnk* disruption in mitogenic signals, we examined the growth properties of wild-type and *Lnk*<sup>-/-</sup> BMDCs in response to SCF and IL-3. These cells provide a model system to study the effects of these cytokines on cell proliferation and survival in a homogenous population. The mitogenic effects of SCF and IL-3 were characterized by determining dose-dependent DNA synthesis on SCF or IL-3 stimulation. Inactivation of *Lnk* resulted in increased incorporation rates of [<sup>3</sup>H]thymidine upon treatment of BMDCs with SCF (Fig. 6 C). However, no consistent difference was found between wild-type and *Lnk*-deficient BMDCs stimulated with IL-3 (Fig. 6 C). These observations suggest that *Lnk* negatively regulates the proliferative response to growth factors in hematopoietic cells of different origin and demonstrate that SCF alone or in synergy with IL-3 can induce a hypersensitive proliferative response in vitro in *Lnk*<sup>-/-</sup>-derived cells.





**Figure 6.** Effect of *Lnk* disruption on cell proliferation in hematopoietic cells. Total bone marrow (A) or splenic cells (B) from wild-type and *Lnk*<sup>-/-</sup> mice were cultured with the indicated growth factors and proliferation was measured at day 4 (A) and day 6 (B) by an MTT assay. The values represent the mean ( $\pm$ SD) of triplicate determinations. (C) BMMCs from wild-type and *Lnk*<sup>-/-</sup> mice were cultured with the indicated concentrations of SCF or IL-3 for 28 h and proliferation was measured as [<sup>3</sup>H]thymidine incorporation. The values are the mean counts per minute ( $\pm$ SD) of triplicate determinations. All graphs are representative of two independent experiments, each done with two mice per genotype.

*Lnk*<sup>-/-</sup> Cells Show Defects in SCF and IL-3 Signaling. To assess the role of *Lnk* in the SCF and IL-3 pathways, we used BMMC from wild-type and *Lnk*-deficient mice. These cells express both Kit, the receptor for SCF, and IL-3R chains, and their responses to IL-3 and SCF have been well characterized (47, 48). Cells from both *Lnk*-deficient and wild-type mice were starved for 12 h and then treated for 10 min with IL-3. Both types of cells showed a similar activation of the IL-3R $\beta$  chain after IL-3 stimulation as judged by antiphosphotyrosine blotting (Fig. 7 A). We next examined the kinetics and extent of MAPK and Akt activation on IL-3 stimulation of BMMCs. Cells were starved and stimulated at different times with IL-3. No differences in the activation of Akt were detected between wild-type and *Lnk*<sup>-/-</sup> BMMCs (Fig. 7 A). However, the analysis of MAPK activation showed that the kinetics of ERK1/2 were altered, such that the peak activation of ERK1/2 was slightly delayed in *Lnk*<sup>-/-</sup> BMMCs (Fig. 7, A and B). We next analyzed the response of BMMCs to SCF stimulation. Cells from wild-type and *Lnk* mutant mice showed a similar activation of Kit and phosphorylation of whole cell lysates (Fig. 7 C). To determine if a difference in the extent of MAPK activation might be responsible for the increased DNA synthesis rates observed in mutant cells (Fig. 6 C), we analyzed the duration of MAPK activation upon SCF stimulation. BMMCs from *Lnk*<sup>-/-</sup> mice displayed a higher level of phospho-ERK1/2 (1.5–4-fold), which decreased with a slower kinetics compared with wild-type BMMCs cells (Fig. 7, C and D). Together, these results suggest that *Lnk* negatively regulates signaling pathways downstream of both the IL-3R $\beta$  and Kit receptors that are implicated in cell proliferation.

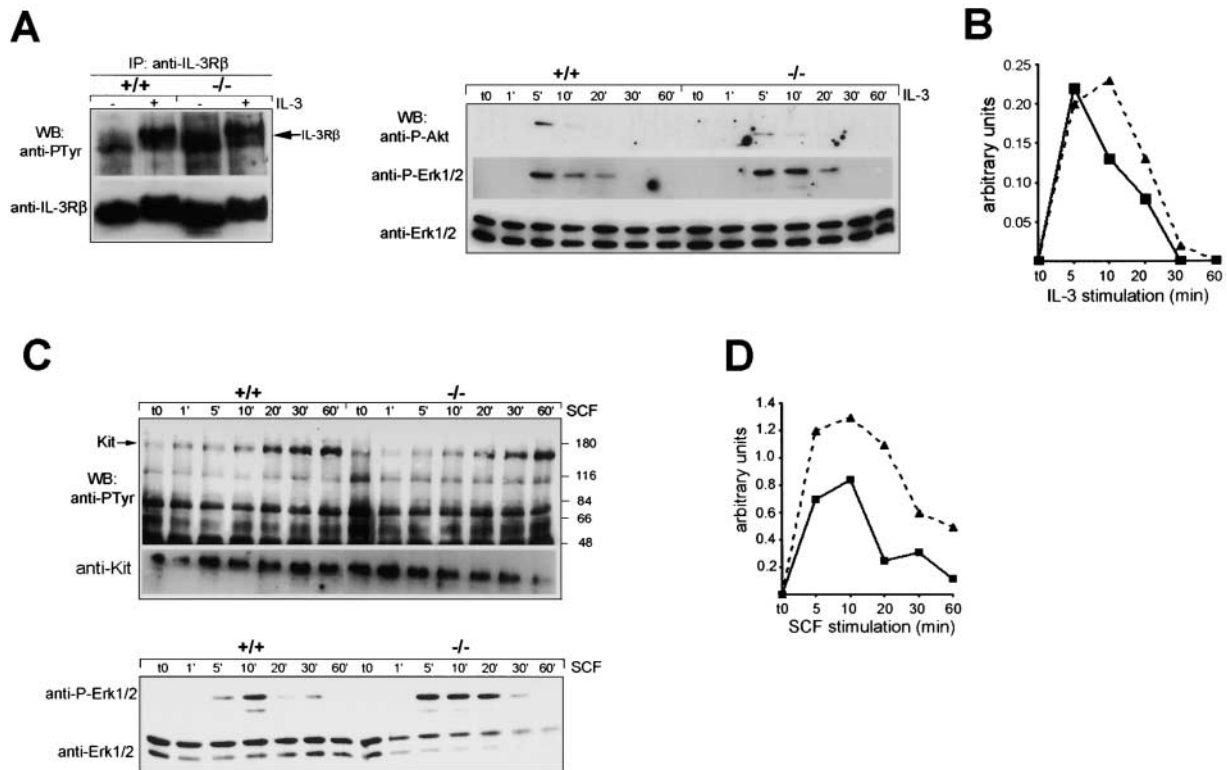
*Lnk* Expression Is Regulated during Hematopoiesis. *Lnk* is reportedly expressed throughout thymocyte development and at high levels in B220<sup>+</sup> splenocytes (20). However, the increased number of clonogenic progenitors observed in *Lnk*<sup>-/-</sup> mice suggests that *Lnk* expression may be regulated

during hematopoietic differentiation. To assess *Lnk* expression during hematopoiesis, a hierarchy blot was hybridized with a radiolabeled *Lnk* cDNA probe including the terminal 3' untranslated sequence (Fig. 8 A) and densitometric analysis was performed. Hybridization of the *Lnk* probe was strong in tripotent and unipotent precursors cells of megakaryocyte and myeloid lineages, as well as in mature megakaryocytes and macrophages, moderate in pentapotent and unipotent precursors cells of the erythroid lineage (BFU-E and CFU-E), and low in tetrapotent cells and other terminally mature cells (erythrocytes, neutrophils, mast, B and T cells).

To further characterize *Lnk*, we analyzed its expression in different organs. *Lnk* was highly expressed in some non-hematopoietic organs examined such as testis, brain, and muscle (Fig. 8 B). Interestingly, although *Lnk* expression is high in the bone marrow and lymph nodes, only low levels of *Lnk* RNA were detected in the spleen (Fig. 8 B), partially due to *Lnk* being weakly expressed in the mature cells of which the spleen is primarily composed. All slot blots were hybridized with a probe for *L32*, a constitutively expressed housekeeping transcript, to verify equal loading of all cDNAs (Fig. 8 C). The specificity of the *Lnk* probe was also confirmed by hybridization of a Northern blot. The same *Lnk* transcripts detected in wild-type and heterozygous cells with an NH<sub>2</sub>-terminal *Lnk* cDNA probe (Fig. 1 D) were observed with this 3' *Lnk* probe (Fig. 8 C). These data demonstrate that *Lnk* is regulated in the hematopoietic compartment, and is most abundantly expressed in hematopoietic precursors.

## Discussion

*Lnk*-deficient mice display an abnormal accumulation of erythroid cells, megakaryocytes, and B lymphocytes in the different hematopoietic compartments, indicating a defect in lymphoid and myeloid homeostasis. One of the most

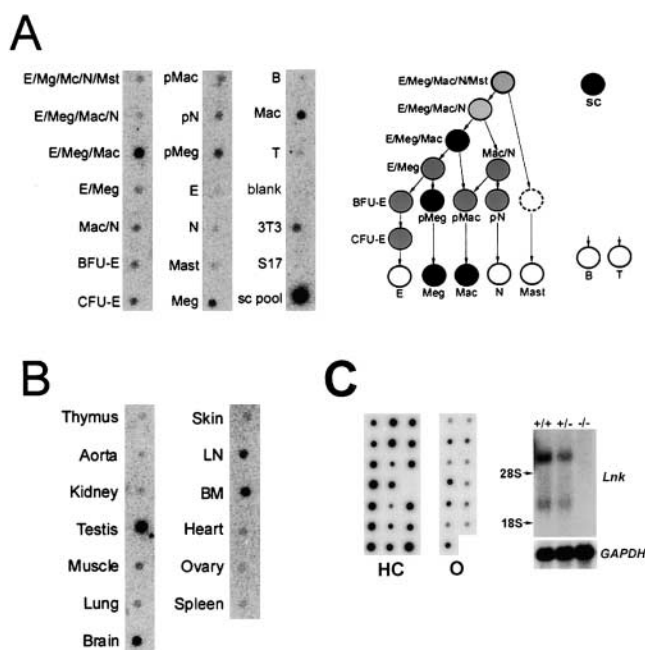


**Figure 7.** Analysis of IL-3 and SCF signaling pathways in *Lnk*-deficient BMMCs. (A and B) BMMCs from wild-type (+/+) and *Lnk* mutant (-/-) mice were stimulated with 100 ng/ml IL-3 for 10 min or for the indicated times. IL-3R $\beta$  chain was immunoprecipitated from wild-type and *Lnk*<sup>-/-</sup> BMMCs lysates and analyzed with anti-Ptyr and anti-IL-3R $\beta$  antibodies. Whole-cell lysates were subjected to Western blot analysis with anti-phospho-Akt and anti-phospho-ERK1/2 antibodies. Anti-ERK1/2 antibodies were used to show equal loading. Normalized densitometric analysis of ERK phosphorylation is shown in B. (solid line) Wild-type; (dashed line) *Lnk*<sup>-/-</sup>. (C and D) Total BMMCs lysates were stimulated with 100 ng/ml SCF for the indicated times and then subjected to Western blot analysis with anti-Ptyr, anti-Kit, and anti-phospho-ERK1/2 antibodies. Anti-ERK1/2 antibodies were used to show similar loading. Normalized densitometric analysis of ERK phosphorylation is shown in D. (solid line) Wild-type; (dashed line) *Lnk*<sup>-/-</sup>. All data are representative of four independent experiments. Each experiment was done with cells isolated from two mice per genotype.

striking features of *Lnk*-deficient mice is a profound splenomegaly with excessive fibrosis and EMH. Morphologically, the abnormal expansion of B lymphocytes and erythrocytes causes considerable change in the architecture of the spleen, whereas the accumulation of a large number of megakaryocytes may be responsible for the extensive fibrosis observed. This phenotype resembles human myeloproliferative disorders that are characterized by abnormal proliferation of more than one hematopoietic cell lineage (49): the myeloid lineage in chronic myelogenous leukemia (CML); the erythrocytic and megakaryocytic lineages in polycythemia vera; and megakaryocytic cells in essential thrombocythemia and primary myelofibrosis. With the exception of CML, the molecular mechanisms responsible for the clonal proliferation in these disorders are unknown. Our results indicate that ablation of *Lnk* expression causes a dysregulation of hematopoiesis reminiscent of these diseases. *Lnk*-deficient mice exhibit amplification of both the erythroid lineage (specifically in spleen) and the megakaryocytic lineage, enhanced sensitivity to several cytokines, and abnormal responses to IL-3 and SCF. Interestingly, transgenic mice expressing activated forms of the IL-3R $\beta$  chain display a chronic myeloproliferative disorder and an

acute leukemia-like syndrome (50, 51). Moreover, it has been shown that the synergistic action of cytokines, such as SCF, IL-3, IL-7, and EPO, provides a growth advantage to multipotent progenitors and increases the frequency of myeloid, lymphoid, and erythroid progenitor cells (45, 52, 53). Indeed, preliminary analysis of erythroid progenitor colony growth in the presence of IL-3 and different concentrations of EPO, showed a hypersensitive pattern of growth in *Lnk*<sup>-/-</sup> cells (unpublished data). Therefore, it is possible that in the *Lnk* mutant mice, multiple cytokines are able to stimulate or continuously sensitize multipotent progenitors via synergistic combinations of growth factors, such as SCF, IL-3, and other cytokines.

One important question deals with the regulation of *Lnk* expression. This is particularly interesting because (1) *Lnk* expression is first detected in 11.5-d embryos, at a stage in which definitive hematopoiesis within the fetal liver is established (unpublished data; reference 54); (2) its expression is higher in hematopoietic precursors than in mature cells; (3) the *Lnk* mutant phenotype is already visible in newborn mice; and (4) *Lnk*<sup>+/-</sup> mice display a phenotype intermediate between wild-type and mutant mice. These data suggest that *Lnk* expression and function are tightly regulated



**Figure 8.** *Lnk* expression during hematopoiesis. (A and B) A slot blot containing either independent amplified cDNA samples from hematopoietic cells ranging from pentapotent precursors to terminally maturing cells in erythroid, myeloid, and lymphoid lineages (A) or different organs (B) was hybridized with a 3' radiolabeled probe for *Lnk*. Radioactivity was quantified with a Phosphorimager (Bio-Rad Laboratories), and the results from A are shown in a hierarchy tree scheme: (black circles) high expression; (gray circles) moderate expression; (white circles) low expression. The ghosted circle represents a committed mast cell precursor stage, not actually sampled. E, erythroid; Meg, megakaryocyte; Mac or Mc, monocyte/macrophage; N, neutrophil; Mast or Mst, mast cell; B, B cells; T, T cells; BFU-E, precursors of large erythroid-only 6–8-d colonies; CFU-E, precursors of small erythroid 2-d colonies; p, precursors of 7-d colonies; pentapotent (E/Meg/Mac/N/Mst), tetrapotent (E/Meg/Mac/N), tripotent (E/Meg/Mac), and bipotent (E/Meg) precursor cells; 3T3, NIH3T3 fibroblasts; S17, stromal cell line; sc, stem cell; BM, bone marrow; LN, lymph node. (C) The same slot blots used in A and B were hybridized with the *L32* probe to confirm equal loading. HC, hematopoietic cells; O, organs, same order as shown in A and B, respectively. A similar Northern blot as the one used in Fig. 1 D was hybridized with the 3' *Lnk* probe used in A and B to confirm specificity.

during hematopoiesis. It is possible that the high levels of *Lnk* expression at later stages of hematopoiesis are important for attenuating cell proliferation and inducing the terminal stages of cell differentiation. This possibility is consistent with the phenotype of the *Lnk*-deficient mice, particularly the predominance of more immature cells in peripheral sites. In this regard, it was surprising to observe IL-7-responsive cells in the spleen of *Lnk*<sup>-/-</sup> animals. It is also possible that hematopoietic progenitors that migrate out of the bone marrow retain a proliferative capacity and cytokine sensitivity that would normally be controlled through the induction of *Lnk* expression. The increase in progenitor cell numbers from the *Lnk* mutant mice was associated with an increase in the number of cells within each colony. This increase could be due to the capacity of these cells to undergo more rounds of proliferation or to survive for longer. Our data with *Lnk*-deficient cells suggest that

*Lnk* mainly exerts an effect on proliferation. In any case, it is possible that *Lnk* expression is increased before differentiation, thereby limiting colony size. Furthermore, it is evident that terminal differentiation can occur in *Lnk* mutant cells, demonstrating that *Lnk* is not essential for this function, but rather serves a regulatory role.

The phenotypes observed in *Lnk*-deficient mice exhibit several similarities with the phenotypes of mice deficient for other molecules involved in the regulation of hematopoietic signaling such as p62<sup>dok</sup>, SHIP, SHP-1, and SOCS3 (17, 55–57). For example, BMMCs derived from SHP-1<sup>-/-</sup> mice exhibit an augmented and prolonged activation of ERK-1 in response to SCF compared with that observed in *Lnk*<sup>-/-</sup> mice (58). These parallels suggest that *Lnk* may also function as a negative regulator of tyrosine kinase signaling in hematopoietic cells. The mechanism by which the *Lnk* family members influence growth factor and cytokine signaling is hypothesized to be through their ability to positively or negatively regulate the JAK/STAT and/or the Ras/MAPK pathways, based on overexpression studies of SH2-B and APS (27, 32, 34, 35). In this regard, we have not detected an effect of *Lnk* ablation on the activation of JAK2 in BMMCs stimulated with IL-3 (unpublished data). However, we have not yet examined the activation of the JAK/STAT pathway in other cytokine-stimulated progenitors. Experiments are in progress to analyze this pathway in more detail. Our results with *Lnk*<sup>-/-</sup> cells demonstrate that *Lnk* can modulate the kinetics of activation of Ras/MAPK, correlating with altered proliferation. Furthermore, *Lnk* appears important in modulating the cellular response to distinct growth factors (IL-3, IL-7, or SCF). A recent report has demonstrated that MAPK activation is required for the pBCR to signal proliferation of pre-B cells in low concentrations of IL-7 (39). It is possible that the elevated proliferation of *Lnk*<sup>-/-</sup> pre-B cells observed in these conditions (Fig. 5 A) is due to an inability to properly regulate ERK activation in the absence of *Lnk* expression. Identification of *Lnk*-interacting proteins may provide insight into how *Lnk* achieves its negative effect on cellular signaling, and preliminary data indicate that the p85 subunit of PI3K can associate with *Lnk* in B cells (unpublished data).

It was surprising to detect high levels of *Lnk* in nonhematopoietic tissues such as testis, brain, and muscle because the *Lnk*<sup>-/-</sup> phenotype appears to exclusively affect the hematopoietic system. This is likely explained by the fact that SH2B and APS are highly expressed in nonhematopoietic tissues and therefore could compensate for the absence of *Lnk* in other organs. APS is also highly expressed in the hematopoietic system, but mainly present in mature B cell lines (23, 31, 32). This suggests that APS functions in mature cells where *Lnk* expression is very low and therefore could explain the absence of a *Lnk* phenotype in terminally mature cells. Thus, it will be important to analyze the phenotypes of mice genetically deficient in other *Lnk* family members, separately or in combination, to establish the biological specificity of these adaptor proteins.

In conclusion, our results indicate that *Lnk* is an important regulator of cytokine signaling during hematopoiesis.

Because of its role in maintaining homeostasis, *Lnk*<sup>-/-</sup> mice provide a powerful model in which to pursue Lnk function in hematopoietic progenitor and mature cells. Future studies with cells derived from these mice should contribute to the understanding of the molecular mechanisms by which signaling thresholds control hematopoietic homeostasis.

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