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−/− mice. Lnk−/− 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−/− 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−/− 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-3Rβ, IL-3 receptor β 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 NH₂-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 (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⁻/⁻ mice display splenomegaly, hyperplasia of the megakaryocyte 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 pre-hematopoietic progenitors. Consistent with this, we show that Lnk is highly expressed in multipotent cells and pre-hematopoietic progenitors. Consistent with this, we show that Lnk is highly expressed in multipotent cells and pre-hematopoietic progenitors.
and with or without 100 ng/ml IL-7, 50 ng/ml SCF (Peprotech), IL-7 and SCF, 10 μg/ml LPS, and 10 μg/ml anti-IgM for 2–6 d. The cultures were pulsed with 1 μCi/well [3H]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 Cuman, Institut Pasteur, Paris, France). For the proliferation assay of CD43+, 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 μM B-mercaptoethanol, antibiotics, 200 μg/ml transferrin (Sigma-Aldrich), 10 μg/ml insulin (Boehringer), and 1% BSA. Cells were plated in 96-well tissue culture plates at 10^4 (for bone marrow) or 5 x 10^4 cells/well (for spleen) in various concentrations of recombinant murine IL-3 (Peprotech), SCF (Peprotech), or IL-3 and SCF. After 4–6 d in culture, cells were stained with MTT (3-4,5-dimethylthiazol-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 Cells 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 NH2-terminal synthetic peptide (amino acid 72–91) of mouse Lnk. Spleens and testis from wild-type and mutants 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 μg/ml each; Santa Cruz Biotechnology, Inc. and UBI, respectively) or anti-Kit (40) antibodies. Immunoprecipitation and Western blot analysis of IL-3 receptor β chain (IL-3Rβ) 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™ multimag (Bio-Rad Laboratories).

BMMCs In Vitro Proliferation Assay. For in vitro proliferation assays, BMMCs in IMDM complete medium were starved of IL-3 for 12 h; 5 x 10^5 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 μCi/well of [3H]thymidine was added for 12 h. Cells were harvested and [3H]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. Iscove, 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−/− 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 original 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 NH2-terminal Lnk probe (exon 1) or antibodies raised against the NH2-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−/− Mice Exhibit Extramedullary Hematopoiesis. Homozygous Lnk−/− mice were present at the expected Mendelian ratio of 1:2:1 (n = 330) from intercrosses of heterozygous parents. Both Lnk−/− female and male animals were viable and fertile, with normal longevity.

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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−/− mice characterized by splenomegaly (weight, 0.062 ± 0.015 g for +/+ mice compared with 0.195 ± 0.07 g for −/− mice; P < 0.01; Fig. 2 A), fibrosis, and extensive extramedullary hematopoiesis (EMH), in which increased numbers of megakaryocytes (CD41+ cells) and erythrocytes (Ter119+ 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 its characteristic discrete lymphoid follicles (Fig. 2 B), presented dramatic changes in shape (Fig. 2 C), accompanied by an increase in B220+ 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−/− 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−/− mice with the latter two contributing to increased cellularity in this organ and splenomegaly.
**WBC, white blood count; RBC, red blood count.**

**Differential counts (10^6/ml)**

| Parameter            | Wild-type | Lnk-deficient | P valuea |
|----------------------|-----------|---------------|-----------|
| WBC (10^9/ml)        | 7.6 ± 1.5 | 26.6 ± 6.04   | **        |
| RBC (10^6/ml)        | 8.4 ± 0.9 | 7.8 ± 0.7    | NS        |
| Hematocrit (%)       | 48 ± 3.3  | 49 ± 2       | NS        |
| Platelets (10^9/ml)  | 400 ± 107 | 1,903 ± 219  | **        |

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

| Parameter            | Wild-type | Lnk-deficient | P valuea |
|----------------------|-----------|---------------|-----------|
| Peripheral blood     |           |               |           |
| WBC (10^6/ml)        | 7.6 ± 1.5 | 26.6 ± 6.04   | **        |
| RBC (10^6/ml)        | 8.4 ± 0.9 | 7.8 ± 0.7    | NS        |
| Hematocrit (%)       | 48 ± 3.3  | 49 ± 2       | NS        |
| Platelets (10^9/ml)  | 400 ± 107 | 1,903 ± 219  | **        |

**Differential counts (10^6/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.

*Values represent the mean ± SD for 10 animals per determination per genotype.

*Statistical significance was determined using the Student’s t test where *P* ≤ 0.05; **P** ≤ 0.005; NS, not significant.

**Lnk−/− 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−/− 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−/− mice. The percentage of B220+ 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−/− mice (Table II). In addition, the percentage of Ter119+-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+ cells, representing monocyte/granulocyte progenitors, was decreased 15% in the bone marrow, but remained similar in the spleen of Lnk−/− mice. When combined with the increase in cellularity in both the bone marrow and spleen compartments of Lnk−/− 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−/− 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+/+, Lnk+−/−, and Lnk−/− 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−/− 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−/− 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+ | Erythroid Ter119+ | Megakaryocyte CD41+ | Myeloid Gr-1/Mac-1+ |
|----------|-------------|---------------|-------------------|---------------------|--------------------|
|          | 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 |
| Lnk-deficient | 14 ± 1.2 | 6.6 ± 0.71b | 47.5 ± 5.1 | 0.63 ± 0.20 | 4.5 ± 1.3 | 2.3 ± 0.37c | 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 |
| Lnk-deficient | 82 ± 25d | 49 ± 3.1a | 59 ± 3.8 | 4.6 ± 0.98b | 6.0 ± 1.2 | 15 ± 1.4b | 18 ± 1.7 | 2.2 ± 0.51c | 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.

= **P** < 0.001.

= **P** < 0.005.

= **P** < 0.02.

= **P** < 0.05.

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fold) compared with +/+ littermates. Similarly, the number of pre-B lymphoid colony-forming cells in Lnk−/− 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−/− 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).

Moreover, there was a marked difference in the proliferative capacity of Lnk−/− 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−/− mice. These results suggest that the variations in differentiation potential observed are intrinsic to the Lnk−/− progenitor cells, and that these cells have an enhanced responsiveness to cytokines.

Lnk−/− Mice Are Affected in B Cell Maturation and Proliferation. Because there was an increase in the number of B220+ 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+CD43− cells) (Fig. 4 A). Further analysis of the pro-B compartment by four-color FACS staining indicated that Lnk−/− mice have an increase in the percentage of BP.1+HSA+ 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+BP.1+, 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+IgM− and B220+CD43− 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+CD43−IgM− cells and a decreased percentage of more mature, recirculating B220+IgM+ cells were identified in Lnk-deficient mice (Fig. 4 A). In addition, a greater proportion of the IgM+ cells in the bone marrow and spleen of Lnk−/− 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−/− 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+ B cells may arise after the maturation of the expanded pre-B population present in Lnk−/− mice, or may indicate incomplete maturation past the B220+CD43−IgM−, 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−/− compared with 1:11,000 in wild-type mice; unpublished data) in the absence of detectable numbers of IgM− 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
B220−, 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+ 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 demonstrated 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−/− and control mice are able to proliferate above background in low concentrations of IL-7. A slight elevation in background [3H]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 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+CD43+ cells. Numbers indicate percentage of gated cells in the indicated boxes. Plots are representative of three sets of mice of each genotype.
For each experiment, two mice per genotype were used. The results indicate that the B lineage cells derived from wild-type mice maintain growth characteristics that are typical of immunogerminale immature B cells. Differences between wild-type and Lnk-deficient mice are more apparent in reduced levels of IL-7, as IL-7–responsive precursors detected in the spleen more efficiently in Lnk-deficient mice than in wild-type mice. The phenotype observed in reduced levels of IL-7 is not due to the presence of a higher percentage of BP.1 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−/− 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−/− mice maintain growth characteristics that are typical of immunogerminale 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+IgM− 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.

**Figure 5. Functional analysis of Lnk−/− B cells.** (A) Bone marrow sorted pro-B cells from wild-type and Lnk-deficient mice were cultured with the indicated concentrations of IL-7 and proliferation was measured at day 6 by [H]thymidine incorporation. (B and C) Spleenic B cells were cultured with the indicated stimuli and proliferation was measured at day 6 (B) or day 4 (C) by [H]thymidine incorporation. The values are the mean counts per minute (±SD) of triplicate determinations. Significance was determined by Student’s t test: ***: P < 0.001; **: P < 0.005; *: P < 0.02. (B and C) Spleenic B cells were cultured with the indicated stimuli and proliferation was measured at day 6 (B) or day 4 (C) by [H]thymidine incorporation. The values are the mean counts per minute (±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.
**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-3Rβ 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-3Rβ 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
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 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−/− 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−/− mice display a phenotype intermediate between wild-type and mutant mice. These data suggest that Lnk expression and function are tightly regulated

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-3Rβ chain was immunoprecipitated from wild-type and Lnk−/− BMMCs lysates and analyzed with anti-P-Tyr and anti–IL-3Rβ 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−/−. (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-P-Tyr, 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−/−. All data are representative of four independent experiments. Each experiment was done with cells isolated from two mice per genotype.
It was surprising to detect high levels of Lnk in nonhematopoietic tissues such as testis, brain, and muscle because the Lnk-/- 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 differentiated cells. Thus, it will be important to analyze the p85 subunit of PI3K can associate with Lnk in B cells (unpublished data).

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

**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. Radiolabeling 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.
Because of its role in maintaining homeostasis, Link−/− 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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