harmful response in either of two ways. First, in response to orally administered antigen, soluble factors and/or signaling through CD40L and CD40 could favor the generation of antigen-specific CTLs. Indeed, CD40L/CD40-mediated activation of mucosal DC by antigen-specific CD4 T cells has been shown to recruit CD8 T cells into the DC-T cell clusters and to be essential for the subsequent priming of these CD8 T cells (21). Alternatively, since CD8 T cells can express CD40L, mucosal DCs may interact directly with them to promote their activation against specific antigens (22). In this second model, the effect on the CTL response of blocking CD40L, as demonstrated by Hänninen et al. (8), could be explained by a direct effect on CD8 T cells, which might subsequently interact with different DC populations, including those residing in the mucosal compartment.

Recently, several interesting studies have demonstrated a critical and unique role of mucosal DCs in modulating quiescent or aggressive mucosal immune responses that can lead to either mucosally induced tolerance or mucosal immunity (23-25). Manipulation of mucosal DCs could therefore offer novel strategies to induce mucosally induced tolerance without generating active and harmful immune responses, including the generation of autoantigen-specific CTLs. The current work showing that transient blockade of CD40L can dissociate mucosally induced tolerance from the generation of harmful CTLs (Figure 1b) suggests another possible therapeutic strategy that could be applied to autoimmune diseases. However, because the effects of this procedure clearly differ from the effects of complete CD40L deficiency, fundamental questions about the roles of these molecules remain to be addressed before CD40L-inhibitory drugs or antibodies can be considered for clinical applications.

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Enhanced Hematopoiesis by Hematopoietic Progenitor Cells Lacking Intracellular Adaptor Protein, Lnk

Satoshi Takaki, Hatsue Morita, Yoshinari Tezuka, and Kiyoshi Takatsu

Division of Immunology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan

Abstract

Hematopoietic stem cells (HSCs) give rise to variety of hematopoietic cells via pluripotential progenitors and lineage-committed progenitors and are responsible for blood production throughout adult life. Amplification of HSCs or progenitors represents a potentially powerful approach to the treatment of various blood disorders and to applying gene therapy by bone marrow transplantation. Lnk is an adaptor protein regulating the production of B cells. Here we show that Lnk is also expressed in hematopoietic progenitors in bone marrow, and that in the absence of Lnk, the number and the hematopoietic ability of progenitors are significantly increased. Augmented growth signals through c-Kit partly contributed to the enhanced hematopoiesis by $lnk^{-/-}$ cells. Lnk was phosphorylated by and associated with c-Kit, and selectively inhibited c-Kit–mediated proliferation by attenuating phosphorylation of Gab2 and activation of mitogen-activated protein kinase cascade. These observations indicate that Lnk plays critical roles in the expansion and function of early hematopoietic progenitors, and provide useful clues for the amplification of hematopoietic progenitor cells.

Key words: bone marrow transplantation • hematopoietic progenitors • c-Kit • stem cell factor • Gab2

Introduction

All blood cell lineages differentiate from hematopoietic stem cells (HSCs)* which self-renew to produce blood cells throughout the entire lifetime of the organism (1). Recent studies have demonstrated the potential of HSCs or progenitor cells in bone marrow to give rise not only to blood cells, but also to other cell types including hepatocytes, cardiac myocytes, and epithelial cells of the liver, lung, gastrointestinal tract, and skin (2–4). Amplification of HSCs or progenitors represents a potentially powerful approach to the treatment of various blood disorders, to the regeneration of damaged liver, heart, and lung tissues, and may also allow the use of gene therapy by bone marrow transplantation. A modest net gain in HSC numbers has been reported using serum-free cultures containing stem cell factor (SCF), IL-11, and Flt-3 ligand (5). Thrombopoietin has also been shown to promote HSC survival and induce the proliferation of HSCs in the presence of SCF (6, 7). However, culture conditions that allow the amplification of HSCs while retaining their self-renewing abilities have not been established, and the mechanism for the self-renewal of HSCs remains poorly understood.

Lnk is an adaptor protein mainly expressed in lymphocytes (8–11). Lnk forms part of an adaptor protein family, together with APS and Src homology (SH)2-B, whose members share the presence of an NH_2-terminal homologous domain, followed by pleckstrin homology and SH2 domains, and a COOH-terminal conserved tyrosine phosphorylation site (11–13). Lnk regulates B cell production by negatively controlling the expansion of pro-B cells. We have previously reported that mutant mice lacking the *lnk* gene showed enhanced B cell production due to the hypersensitivity of B cell precursors to SCF, a ligand for c-Kit (11). The absence of Lnk confers upon immature bone marrow cells an enhanced ability to support B lymphopoiesis in adoptively transferred host animals, even in a competitive environment such as nonirradiated recombination activating gene (RAG)-$2^{-/-}$ host (11).

The receptor tyrosine kinase c-Kit is a member of a subfamily that includes the platelet-derived growth factor

H. Morita's present address is International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (AIST), 1-1 Higashi, Tsukuba 305-8566, Japan.

Address correspondence to Satoshi Takaki, Division of Immunology, Dept. of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai Minato-ku, Tokyo 108-8639, Japan. Phone: 81-3-5449-5264; Fax: 81-3-5449-5407; E-mail: takakis@ims.u-tokyo.ac.jp

**Abbreviations used in this paper:* CFU-S, CFU in spleen; HSC, hematopoietic stem cell; Lin, lineage marker; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; SCF, stem cell factor; SH, Src homology; RAG, recombination activating gene.

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(PDGF), CSF-1, and Flt3/Flk-2 receptors (14, 15). As with other receptor tyrosine kinases, stimulation of c-Kit by SCF binding leads to dimerization, transphosphorylation, and the recruitment and tyrosine phosphorylation of various cellular proteins, including phosphatidylinositol 3-kinase and Akt that rescue cells from apoptosis, and Gab2 that leads to the activation of mitogen-activated protein kinase (MAPK) pathways (16, 17). Through SCF binding, c-Kit is a key controlling receptor in a number of cell types including HSCs and hematopoietic progenitors, mast cells, melanocytes, and germ cells. The growth and survival of the stem cells for these cell types are severely affected by mutations in the gene loci for c-Kit (Kit^{W}) or SCF (*sl*) (15).

In this study, we show that Lnk is also expressed in hematopoietic progenitors, and that in the absence of Lnk, the number and the hematopoietic ability of progenitors in bone marrow are significantly enhanced. Augmented growth signals through c-Kit partly contribute to the enhanced hematopoiesis by $lnk^{-/-}$ cells. Lnk is phosphorylated by and associates with c-Kit. Lnk selectively inhibits c-Kit–mediated proliferation by inhibiting tyrosine phosphorylation of Gab2 and activation of the MAPK cascade. These observations indicate that Lnk plays critical roles in the expansion and function of early hematopoietic progenitors, and provide useful clues in the understanding of the generation and proliferation of hematopoietic progenitor cells.

Materials and Methods

Mice. $lnk^{-/-}$ mice used in this study were backcrossed with C57BL/6 (B6-Ly5.2) >10 times. $Kit^{W/+}$ or $Kit^{Wv/+}$ mice were purchased from The Jackson Laboratory. Mice congenic for the Ly5 locus (B6-Ly5.1) were bred and maintained at the animal facility of the Institute of Medical Science, University of Tokyo, Tokyo, Japan. All mice were housed and bled in specific pathogen-free conditions.

Flow Cytometry and RT-PCR. Single cell suspensions were prepared, and cells stained using predetermined optimal concentrations of the respective antibodies. The stained cells were then analyzed on a FACScan™ or FACScalibur™ instrument (Becton Dickinson). The following mAbs were used: FITC-conjugated anti–Sca-1 (E13–161.7); PE-conjugated anti–c-Kit (2B8); biotin-conjugated anti–TER-119; biotin-anti-Gr-1 (RB6–8C5); PE- or biotin-anti-CD3ε (145-2C11); FITC-anti-CD8 (53–6.7); PE-anti-CD4 (RM4–5); PE–anti-CD43 (S7) (all purchased from BD PharMingen); FITC- or biotin-anti-Mac-1 (M1/70); FITC-, PE-, or biotin-anti-B220 (RA3–6B2) (from Caltag); and biotin-anti-Ly5.2 (a gift of K. Ikuta, Kyoto University, Japan). PE-conjugated streptoavidin (Ancell), or Tri-Color-conjugated streptoavidin (Caltag) were used for biotin-coupled antibody staining. Bone marrow cells were depleted of lineage-committed cells using a MACS® system (Miltenyi Biotec) after incubation with a cocktail of biotin-conjugated antibodies against various lineage markers (Lins) (B220, CD3, Gr-1, Mac-1, and TER-119) and streptavidin-coupled microbeads. Resulting Lin^{-} cells were stained with PE-anti-c-Kit, and c-Kit^{+} or c-Kit^{-} cells sorted using a FACSVantage™ (Becton Dickinson). Poly$(A)^{+}$ RNA was isolated from purified cells using a Micro-Fast Track kit (Invitrogen), and first strand cDNA templates synthesized by Superscript II reverse transcriptase (GIBCO BRL) using random primers (TaKaRa, Kyoto, Japan). Serial dilutions of cDNA templates were subjected to PCR amplification by using primer-sets encompassing several introns for Lnk (FWD primer: 5′-ATGCCTGACAACCTCTACAC, REV primer: 5′-ATTCACACGTCTGCCTCTCT) or β-actin (FWD primer: 5′-ACACTGTGCCCATCTACGAG, REV primer: 5′-CTAGAAGCACTTGCGGTGCA). Cycling parameters were 1 min at 94°C, 2 min at 64°C and 3 min at 72°C for 34 cycles to detect *lnk* mRNA, or 27 cycles for β-actin. PCR products were separated through 1.0% agarose gels and stained with ethidium bromide.

Colony-forming Unit in Spleen Assay. Nucleated bone marrow cells (10^5) prepared from $lnk^{+/+}$ or $lnk^{-/-}$ mice were injected into lethally irradiated (9.5 Gy) female wild-type mice. The recipient mice were killed 12 d after the injection, and their spleens were removed and fixed in Bouin's solution for macroscopic examination to count colonies.

Competitive Repopulation Assay. Bone marrow cells obtained from $lnk^{-/-}$ or $lnk^{+/+}$ mice (Ly5.2) were depleted of lineage-committed cells using a MACS® system as described above. Resulting Lin^{-} cells (7.0×10^4) were intravenously injected into lethally irradiated (9.5 Gy) recipient mice together with 3.5×10^5 competitor Lin^{-} cells obtained from B6-Ly5.1 mice. For assays using $lnk^{-/-}Kit^{+/+}$ or $lnk^{-/-}Kit^{W/+}$ mice, 1.5×10^5 or 7.5×10^4 bone marrow cells were injected into lethally irradiated recipient mice together with 1.5×10^6 competitor bone marrow cells obtained from B6-Ly5.1 mice. Bone marrow cells, splenocytes, and thymocytes of chimeric animals were isolated and analyzed 10–12 wk after transfer.

Plasmid Construction and Transfection. Full-length *lnk* cDNA (11) was subcloned into the BamHI site of pcDNA3 (Stratagene), an eukaryotic expression vector driven by the cytomegalovirus enhancer and promoter, resulting in pcDNA3-Lnk. Substitutional mutations were introduced into the *lnk* cDNA sequence encoding the COOH-terminal tyrosine residue (Y536) by PCR-based site-directed mutagenesis. The introduced mutation was confirmed by DNA sequencing. The resulting mutated cDNA was subcloned into pcDNA3 (pcDNA3-Y536F). A mouse mast cell line, MC9 was grown in RPMI 1640 medium supplemented with 5% FCS, 50 μM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin and 10 U/ml mIL-3. MC9 cells were transfected by electroporation with pcDNA3-Lnk or pcDNA3-Y536F linearized with *SspI*. 2 d after transfection, cells were seeded into 96-well plates (10^4 cells per well) and selected in medium containing 0.8 mg/ml G418. Transfectants resistant to G418 were expanded and screened for expression of Lnk protein by immunoblotting.

Proliferation Assay and Immunoblotting. Transfectants (10^4) were cultivated in 200 μl of medium in 96-well plates. Cells were stimulated with various concentrations of SCF or IL-3. Cells were pulse-labeled with [^{3}H]thymidine (0.2 μCi per well) during the last 16-h of the 72-h culture period, and incorporated [^{3}H]thymidine measured using a MATRIX 96 Direct Beta Counter (Packard Instrument Co.). Transfectants were washed in Hanks' Balanced Salt solution three times and starved in RPMI 1640 medium containing 2% FCS, 50 μM 2-ME for 4 h. Cells were then collected and stimulated with 1 μg/ml SCF (PeproTech) for the indicated times. After stimulation, cells were collected and lysed on ice in lysis buffer (150 mM NaCl, 50 mM Tris HCl, pH 7.5, 1% NP-40, 10 mM NaF, 1 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and the lysates clarified by centrifugation. Whole

cell lysates or proteins immunoprecipitated using anti-Lnk (11) or anti-Gab2 (Upstate Biotechnology) were resolved by SDS/8%PAGE under reducing conditions, and transferred onto nitrocellulose membranes (Schleicher and Schull). After blocking with 5% skim milk/PBS, blots were probed with antiphosphotyrosine mAb (4G10; Upstate Biotechnology), anti–phospho-Akt, or anti–phospho-Erk1/2 (New England BioLabs), anti–c-Kit (Santa Cruz Biotechnology, Inc.) or anti-Lnk antibodies, and incubated with horseradish peroxidase-conjugated secondary antibodies. Filters were washed in 0.05% Tween 20, Tris-buffered saline, pH 7.5, and developed by enhanced chemiluminescence (NEN Life Science Products).

Results

Increased Number and Enhanced Ability of Hematopoietic Progenitors in lnk$^{-/-}$ Mice. The hyperresponsivity of *lnk*$^{-/-}$ precursor B cells to SCF and the enhanced ability to support B lymphopoiesis in nonirradiated RAG-2$^{-/-}$ host animals (11) prompted us to investigate the function of Lnk in more immature hematopoietic progenitor cells. We first examined whether immature progenitors not yet committed to the B cell lineage expressed *lnk* mRNA. Bone marrow cells that already expressed Lins were depleted and resulting Lin-negative (Lin$^-$) immature cells were further purified by the expression of c-Kit. RT-PCR results demonstrated that *lnk* mRNA was expressed in Lin$^-$ bone marrow cells. Among these Lin$^-$ cells, more *lnk* transcripts were observed in immature c-Kit$^+$ cells than in c-Kit$^-$ cells (Fig. 1 A). Then we compared the distribution of cells expressing c-Kit and Sca-1 within the Lin$^-$ cell population obtained from *lnk*$^{-/-}$ or *lnk*$^{+/+}$ mice. The Lin$^-$c-Kit$^+$Sca-1$^+$ fraction, within which HSCs are found (18), was significantly expanded in *lnk*$^{-/-}$ mice compared with *lnk*$^{+/+}$ mice (Fig. 1 B, Table III). Pluripotential hematopoietic progenitors give rise to CFU in spleen (CFU-S) after 12 d when transplanted into lethally irradiated host. CFU-S d12 in *lnk*$^{-/-}$ bone marrow was significantly increased compared with wild-type (Table I, Fig. 1 C). In addition, CFU-S d12 colonies derived from *lnk*$^{-/-}$ cells were larger in size than those derived from *lnk*$^{+/+}$ cells, suggesting the enhanced proliferating ability of *lnk*$^{-/-}$ hematopoietic progenitors.

The ability of *lnk*$^{-/-}$ progenitors to produce various hematopoietic cell types was examined by competitive repopulation assay. To avoid the contribution of increased B cell precursor populations in *lnk*$^{-/-}$ bone marrow to recipient B cell compartments, Ly5.2$^+$Lin$^-$ cells were purified from *lnk*$^{-/-}$ or *lnk*$^{+/+}$ mice and transferred into irradiated hosts together with a fivefold excess of Ly5.1$^+$ normal Lin$^-$ cells (Fig. 2 A). Reflecting the ratio of testee to competitor cells, only small part of B220^{lo}CD43$^-$ B-lineage cells in bone marrow and B220$^+$ splenocytes were Ly5.2$^+$ in recipient mice repopulated using *lnk*$^{+/+}$ Ly5.2$^+$ progenitors (Fig. 2 B). In contrast, when *lnk*$^{-/-}$ Ly5.2$^+$ progenitors were used, almost all of the B lineage cells were Ly5.2$^+$ despite the original transfer of five times fewer *lnk*$^{-/-}$ progenitors than normal Ly5.1$^+$ competitor cells (Fig. 2 B). This also demonstrates the enhanced ability of *lnk*$^{-/-}$ progenitors to produce B lineage cells compared with normal progenitors. Unexpectedly, results of the competitive repopulation assay showed that the *lnk*$^{-/-}$ progenitors produced not only B lineage cells but also myeloid cells and T cells more efficiently than normal progenitors (Fig. 2 B, Table II). Most of the B220$^-$CD43$^+$ myeloid cells in bone marrow and Mac-1$^+$ splenocytes in mice that received *lnk*$^{-/-}$ progenitors were Ly5.2$^+$. In addition, most CD4$^+$CD8$^+$ thymocytes and CD3$^+$ splenocytes were generated from *lnk*$^{-/-}$

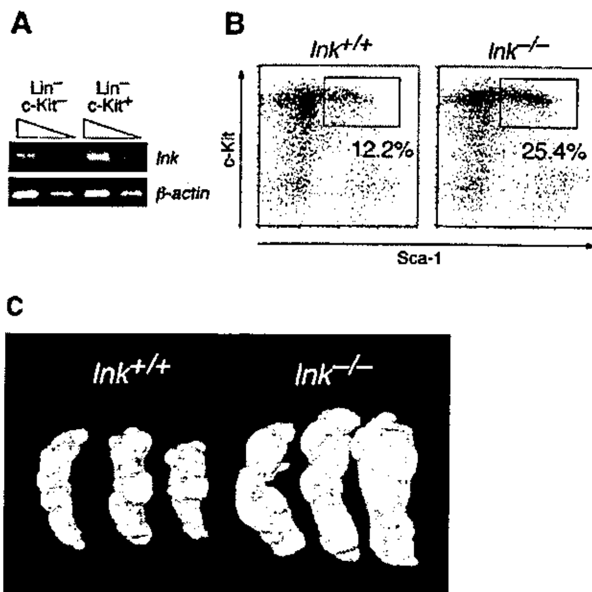

Figure 1. Increased hematopoietic progenitors in *lnk*$^{-/-}$ mice. (A) Expression of *lnk* transcripts in hematopoietic progenitor cells of normal mice. Lin$^-$ bone marrow cells were purified and separated according to c-Kit expression. Serial dilutions (threefold) of cDNA templates were prepared and subjected to RT-PCR analysis using primer sets designed to amplify *lnk* (top) or β-actin (bottom) cDNA fragments. (B) The cell fraction containing HSCs (Lin$^-$Sca-1$^+$c-Kit$^+$) was increased in the bone marrow of *lnk*$^{-/-}$ mice. Sca-1 and c-Kit expression on Lin$^-$ bone marrow cells prepared from *lnk*$^{+/+}$ or *lnk*$^{-/-}$ mice was analyzed by flow cytometry. Representative dual parameter plots are shown. (C) Increased hematopoietic progenitors (CFU-S d12) caused by the *lnk*-deficiency. Bone marrow cells (10^5) were transplanted into lethally irradiated recipient mice. The spleens were removed after 12 d and fixed in Bouin's solution to visualize the colonies.

Table I. *Increased CFU-S d12 in the Bone Marrow of the lnk$^{-/-}$ Mice*

Genotype	CFU-S d12 (per 10^5 cells)
lnk$^{+/+}$	11.5 ± 2.4
lnk$^{-/-}$	20.8 ± 3.3[a]

Bone marrow cells (10^5) were administered intravenously to lethally irradiated recipient mice. The spleens were removed and fixed 12 d after injection, and the number of colones was counted. The values are mean ± SD for quadruplicates.
[a]$P < 0.01$.

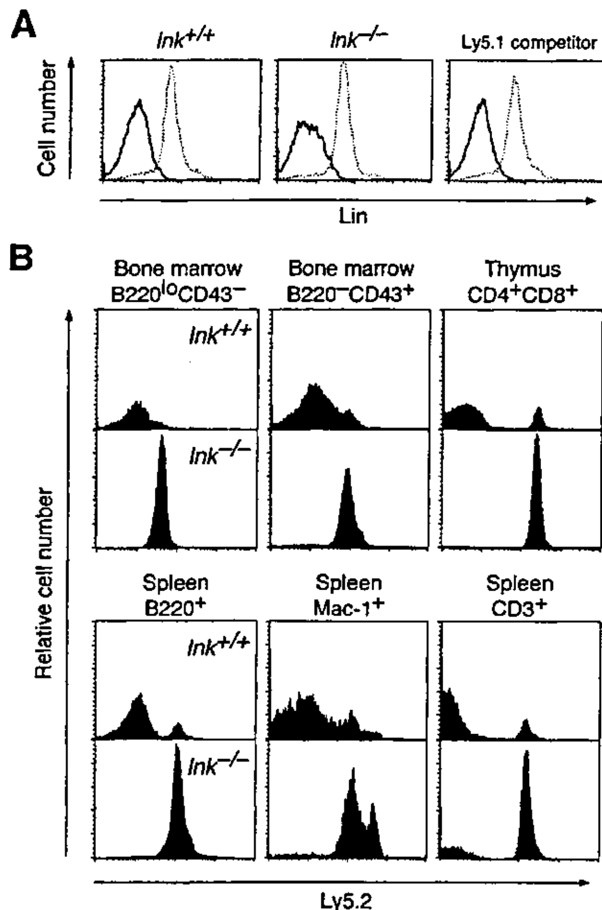

Figure 2. Enhanced repopulating ability of $lnk^{-/-}$ progenitors demonstrated by a competitive repopulation assay. (A) Lin^- bone marrow cells prepared for repopulating assay. Bone marrow cells obtained from $lnk^{-/-}$, $lnk^{+/+}$ mice, or B6-Ly5.1 (competitor) mice were depleted of lineage-committed cells using a MACS® system as described in Materials and Methods. Expression of Lin markers (B220, CD3, Gr-1, Mac-1, and TER-119) on cells before depletion (dotted lines) or after depletion (solid lines) was shown. (B) Lin^- cells obtained from $lnk^{+/+}$ or $lnk^{-/-}$ mice (Ly5.2) were transferred into lethally irradiated (9.5 Gy) recipient mice together with a fivefold excess of Lin^- cells prepared from normal B6-Ly5.1 mice. Ly5.2 expression on $B220^{lo}CD43^-$ B lineage cells in bone marrow, $B220^-CD43^+$ myeloid cells in bone marrow, $CD4^+CD8^+$ thymocytes, $B220^+$ splenic B cells, $Mac\text{-}1^+$ splenocytes, and $CD3^+$ splenic T cells were analyzed 10–12 wk after transfer. Upper profiles show results obtained from animals transferred with $lnk^{+/+}$ cells, and lower profiles show results obtained from animals transferred with $lnk^{-/-}$ cells. Representative results of three independent experiments are shown.

progenitors even in the presence of five times more normal progenitors. Most of the $IL\text{-}2R\beta^+CD3^+$ NKT cells in the spleen and thymus were also derived from $lnk^{-/-}$ progenitors (data not shown). These results indicate that the ability of hematopoietic progenitors to generate various lineages of hematopoietic cells is greatly enhanced by the absence of Lnk.

c-Kit Signaling Is a Major Target Affected by lnk Deficiency. We have previously demonstrated that B cell overproduction observed in $lnk^{-/-}$ mice was due, at least in part to SCF hypersensitivity of the precursors, reflecting enhanced c-Kit signaling in the absence of Lnk (11). To confirm our observations in vivo, we bred $lnk^{-/-}$ mice to $Kit^{W/+}$ mutant mice. The Kit^W mutation consists of a deletion in the c-Kit protooncogene that results in reduced cell surface expression of c-Kit protein in $Kit^{W/+}$ heterozygote mice (19). $Kit^{W/Wv}$ mice are commonly used as viable mutant mice carrying nonfunctional c-Kit. However, the fraction of B lineage cells in the bone marrow of these mice varied from animal to animal, presumably due to malnutrition accompanied with severe anemia. Therefore, we compared $Kit^{+/+}$ with $Kit^{W/+}$ mutant mice in a $lnk^{-/-}$ background. $Kit^{W/+}$ mice ($lnk^{+/+}Kit^{W/+}$) showed no abnormalities in B cell development compared with normal ($lnk^{+/+}Kit^{+/+}$) mice (Table III). Total bone marrow preparations from $lnk^{-/-}Kit^{+/+}$ mice contained higher cell numbers than from normal mice, with both $B220^{lo}IgM^-$ and $B220^{lo}IgM^+$ fractions significantly increased. Reduction of c-Kit expression by the introduction of the Kit^W mutation on a $lnk^{-/-}$ background partially but significantly normalized B cell overproduction in $lnk^{-/-}Kit^{W/+}$ mice (Table III). This confirms that the main reason for B cell overproduction in $lnk^{-/-}$ mice is enhanced signaling through the c-Kit tyrosine kinase receptor.

The fraction of $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ progenitors in $lnk^{-/-}Kit^{W/+}$ bone marrow was then examined. Expression levels of c-Kit were reduced in $lnk^{-/-}Kit^{W/+}$ mice compared with $lnk^{-/-}Kit^{+/+}$ mice (Fig. 3 A). Importantly, the increased $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ fraction in $lnk^{-/-}Kit^{+/+}$ mice was partly but significantly reduced in $lnk^{-/-}Kit^{W/+}$ mice (Fig. 3 A, Table III). This suggests that the increase in numbers of hematopoietic progenitors in $lnk^{-/-}$ mice also results from enhanced c-Kit signaling. Despite the partial normalization of B cell overproduction and increased $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ progenitors, the competitive repopulation assay demonstrated an enhanced ability of hematopoietic progenitors from $lnk^{-/-}Kit^{W/+}$ mice comparable to that from $lnk^{-/-}Kit^{+/+}$ mice. Both $lnk^{-/-}Kit^{W/+}$ and $lnk^{-/-}Kit^{+/+}$ progenitors ($Ly5.2^+$) generated most of the B and myeloid lineage cells and thymocytes in the presence of 10-fold more competitor cells ($Ly5.1^+$) (Fig. 3 B, Table IV). Even in the presence of 20-fold more competitor cells, $lnk^{-/-}Kit^{W/+}$ as well as $lnk^{-/-}Kit^{+/+}$ progenitors continued to generate the majority of B, myeloid and T lineage cells (Fig. 3 B, Table IV). These results strongly suggest that the enhanced hematopoietic ability of $lnk^{-/-}$ progenitors involves as yet unidentified mechanism(s), in addition to the enhanced c-Kit signaling that lead to the increase of $Lin^-ca\text{-}1^+c\text{-}Kit^+$ progenitors.

Mechanisms for the Selective Inhibition of c-Kit Signaling by Lnk. The mechanism by which Lnk regulates signaling through c-Kit was studied using the SCF-dependent mast cell line MC9, which endogenously expresses low levels of Lnk (Fig. 4 A). MC9 transfectants that expressed Lnk at moderate (Lnk#1) or high (Lnk#2) levels were established and responsiveness to SCF examined. APS, a member of the Lnk adaptor family, is phosphorylated at a tyrosine residue at the COOH-terminal end and forms a binding site for c-Cbl (13), which is involved in the degradation of var-

Table II. *Repopulating Ability of Hematopoietic Progenitors in Irradiated Hosts Is Enhanced by the Absence of Lnk*

Experiment	Tested donor	Percent chimerism (Ly5.2$^+$ cells, %)		
		Bone marrow B220^{lo}CD43$^-$	Bone marrow B220$^-$CD43$^+$	Thymus CD4$^+$CD8$^+$
Exp. 1	Ly5.1 alone	2.3	7.4	0.1
	lnk$^{+/+}$	10.9	28.9	0.1
	lnk$^{-/-}$	87.1	48.9	99.8
	lnk$^{-/-}$	85.1	78.1	87.5
Exp. 2	*lnk*$^{+/+}$	16.6	23.9	17.2
	lnk$^{+/+}$	18.0	16.9	16.1
	lnk$^{-/-}$	97.5	93.9	99.2
	lnk$^{-/-}$	97.6	95.8	99.4
Exp. 3	Ly5.1 alone	5.4	9.9	2.2
	lnk$^{+/+}$	33.3	42.3	30.4
	lnk$^{-/-}$	94.8	96.3	99.7
	lnk$^{-/-}$	95.3	95.2	98.2

Lin$^-$ bone marrow cells obtained from *lnk*$^{+/+}$ or *lnk*$^{-/-}$ mice (Ly 5.2) were transferred into lethally irradiated (9.5 Gy) recipient mice together with a fivefold excess of Lin$^-$ cells prepared from normal B6-Ly5.1 mice. Bone marrow cells and thymocytes were isolated 10–12 wk after transfer, and percentage of Ly5.2$^+$ cells in the indicated cell fractions were determined.

ious proteins (20). The phosphorylation of the c-Cbl binding site is required for the negative regulatory functions of APS in cytokine receptor signaling, such as through PDGF-R and erythropoietin-R (21, 22). Therefore, we also generated transfectants that expressed a mutant Lnk (Y536F) that carried a substitution at the COOH-terminal tyrosine residue expected to be phosphorylated by analogy with APS. Lnk#2 and Y536F expressed c-Kit at amounts comparable with control transfectants (Neo), and c-Kit was similarly downregulated in all three transfectants upon stimulation with SCF (Fig. 4 B). Even when the transfectants were stimulated with low amounts of SCF, no differences between transfectants were detected (data not shown). This indicates that early signals are efficiently transmitted, and that the c-Kit degradation or internalization is not affected in the presence of excess amounts of Lnk. Interestingly, SCF-induced proliferative responses were impaired in a dose–response manner by Lnk expression (Fig. 4 C). However, IL-3–induced proliferation remained intact. Y536F also showed impaired proliferation upon stimulation by SCF but not by IL-3. Apoptosis of MC9 cells induced by the depletion of SCF and IL-3 was not affected by overexpression of Lnk (data not shown). Therefore, Lnk selectively inhibits growth signaling mediated through c-Kit and the putative c-Cbl binding site of Lnk is not required for the negative regulatory effect on c-Kit.

We next analyzed the tyrosine phosphorylation of cellular proteins. SCF stimulation rapidly induced tyrosine phosphorylation of various cellular proteins, including c-Kit.

Table III. *B Cell Overproduction and Increased in Lin$^-$Sca-1$^+$c-Kit$^+$ Hematopoietic Progenitors in lnk$^{-/-}$ Mice Is Partly Normalized by Reducing Expression of c-Kit*

Genotype	Cell number	B220^{lo}IgM$^-$	B220^{lo}IgM$^+$		Lin$^-$Sca-1$^+$c-Kit$^+$		
	(10^7 cells/femur)	*(%)*	*(%)*		*(%)*	*(% in Lin$^-$ cells)*	
lnk$^{+/+}$*Kit*$^{+/+}$	1.65 ± 0.16	19.5 ± 5.1	8.9 ± 3.6	(*n* = 12)	0.45 ± 0.07	16.2 ± 2.2	(*n* = 5)
lnk$^{+/+}$*Kit*$^{W/+}$	1.71 ± 0.19	18.2 ± 4.6	8.0 ± 3.1	(*n* = 10)	0.38 ± 0.06	14.9 ± 1.7	(*n* = 4)
lnk$^{-/-}$*Kit*$^{+/+}$	2.03 ± 0.37^a	3.6 ± 4.8^a	14.1 ± 2.1^a	(*n* = 11)	0.70 ± 0.06^a	26.1 ± 2.1^a	(*n* = 5)
lnk$^{-/-}$*Kit*$^{W/+}$	1.91 ± 0.28	28.9 ± 3.9^b	11.8 ± 1.7^b	(*n* = 8)	0.56 ± 0.13	21.3 ± 1.4^b	(*n* = 5)

Bone marrow cells obtained from 8–10-wk-old mice were analyzed, and mean cell number (±SD), mean percentage (±SD) of the indicated cell fractions and the number of mice analyzed are shown.

^{a}$P < 0.05$ compared to *lnk*$^{+/+}$*Kit*$^{+/+}$.

^{b}$P < 0.05$ compared to *lnk*$^{-/-}$*Kit*$^{+/+}$.

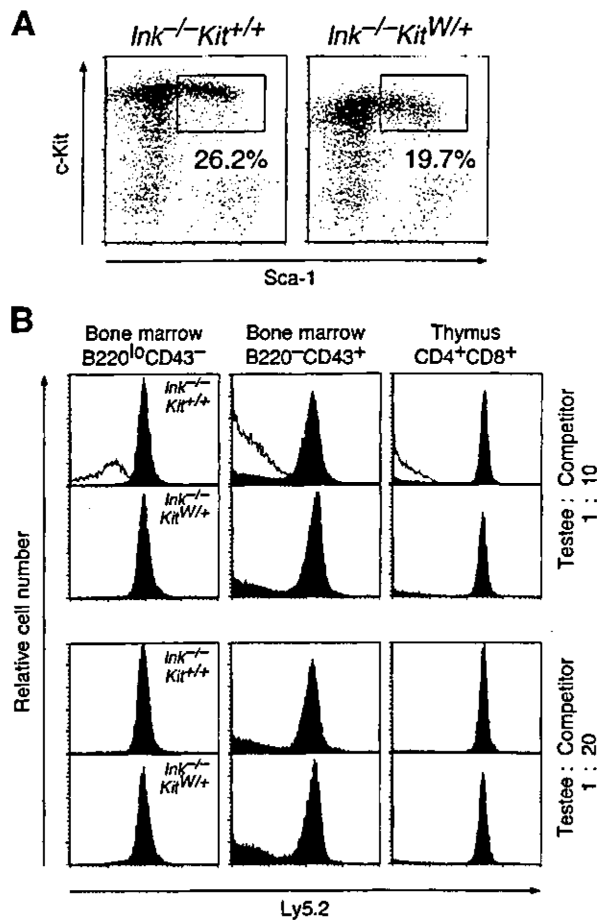

Figure 3. Increased number but not enhanced ability of *lnk*$^{-/-}$ hematopoietic progenitors is partly normalized by reducing expression of c-Kit. (A) Expression of Sca-1 and c-Kit on Lin^{-} bone marrow cells of *lnk*$^{-/-}$*Kit*$^{+/+}$ or *lnk*$^{-/-}$*Kit*$^{W/+}$ mice. Shown are representative dual parameter plots analyzed by flow cytometry. (B) Comparable repopulating ability of progenitors obtained from *lnk*$^{-/-}$*Kit*$^{+/+}$ or *lnk*$^{-/-}$*Kit*$^{W/+}$ mice. Bone marrow cells obtained from *lnk*$^{-/-}$*Kit*$^{+/+}$ or *lnk*$^{-/-}$*Kit*$^{W/+}$ mice (Ly5.2) were transferred into lethally irradiated (9.5 Gy) recipient mice together with 10 times (top) or 20 times (bottom) more competitor cells prepared from normal B6-Ly5.1 mice. Ly5.2 expression on B220^{lo} CD43^{-} B lineage cells in bone marrow, B220^{-}CD43^{+} myeloid cells in bone marrow, and CD4^{+}CD8^{+} thymocytes of chimeric animals were analyzed 10–12 wk after transfer. Upper profiles show results obtained from animals transferred with *lnk*$^{-/-}$*Kit*$^{+/+}$ cells, and lower profiles show results obtained from animals transferred with *lnk*$^{-/-}$*Kit*$^{W/+}$ cells. Thin lines in top panels show profiles of mice transferred with Ly5.1 competitor cells alone. Representative results of two independent experiments are shown.

Table IV. *Comparable Repopulating Ability of HSCs Obtained from lnk*$^{-/-}$*Kit*$^{+/+}$ *or lnk*$^{-/-}$*Kit*$^{W/+}$ *Mice*

			Percent chimerism (Ly5.21 cells, %)		
Experiment	Tested donor	Testee: Competitor	Bone marrow B220^{lo}CD43^{-}	Bone marrow B220^{lo}CD43^{+}	Thymus CD4^{+}CD8^{+}
Exp. 1	Ly5.1 alone		4.7	5.1	0.3
	lnk$^{-/-}$*Kit*$^{+/+}$	1:10	98.1	76.6	94.8
	lnk$^{-/-}$*Kit*$^{W/+}$	1:10	98.4	73.1	77.5
	lnk$^{-/-}$*Kit*$^{+/+}$	1:20	98.5	71.1	96.1
	lnk$^{-/-}$*Kit*$^{W/+}$	1:20	94.4	64.4	24.6
Exp. 2	Ly5.1 alone		4.4	3.1	0.1
	lnk$^{-/-}$*Kit*$^{+/+}$	1:10	81.2	65.8	81.2
	lnk$^{-/-}$*Kit*$^{W/+}$	1:10	87.6	77.1	76.9
	lnk$^{-/-}$*Kit*$^{+/+}$	1:20	86.1	67.3	97.0
	lnk$^{-/-}$*Kit*$^{W/+}$	1:20	87.2	74.5	86.8

Bone marrow cells obtained from *lnk*$^{-/-}$ *Kit*$^{+/+}$ or *lnk*$^{-/-}$*Kit*$^{W/+}$ mice (Ly5.2) were transferred into lethally irradiated (9.5 Gy) recipient mice together with 10-fold or 20-fold excess of competitor bone marrow cells prepared from normal B6-Ly5.1 mice. Bone marrow cells and thymocytes were isolated 10–12 wk after transfer, and percentage of Ly5.2^{+} cells in the indicated cell fractions was determined.

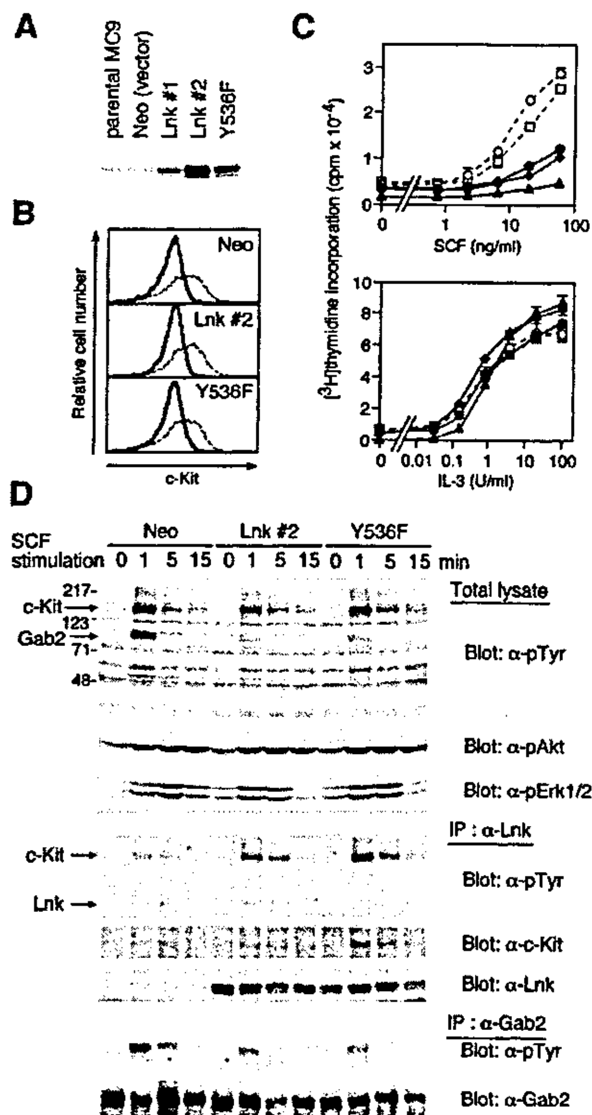

Figure 4. Lnk specifically inhibits c-Kit–mediated growth signaling in MC9 cells responsive to SCF or IL-3. (A) MC9 cells were transfected with expression plasmid without insert (Neo), plasmid encoding Lnk (Lnk#1, Lnk#2), or mutant Lnk (Y536F) that lacks the COOH-terminal tyrosine phosphorylation site. Lnk protein expression levels were analyzed by immunoblot. (B) Downregulation of c-Kit induced by SCF. Expression levels of c-Kit on unstimulated MC9 transfectants (broken lines) or cells stimulated with 100 ng/ml SCF for 30 min (solid lines) were analyzed by flow cytometry. Representative results of multiple experiments are shown. (C) Proliferative responses of parental MC9 (○), Neo (□), Lnk#1 (●), Lnk#2 (▲), and Y536F (◆) upon stimulation with various concentrations of SCF (top) or IL-3 (bottom). Values shown are mean ± SD of triplicate determinations. Representative results of three independent experiments are shown. (D) Phosphorylation of cellular proteins induced by SCF stimulation of MC9 transfectants. Transfectants were starved for 4 h and stimulated with 1 μg/ml of SCF for the indicated times. Whole cell lysates (top), anti-Lnk immunoprecipitants (middle), and anti-Gab2 immunoprecipitants (bottom) were separated through SDS/PAGE gels and transferred onto nitrocellulose membranes. Resultant membranes were probed with the indicated antibodies and visualized by ECL. Representative results of multiple experiments are shown.

The pattern of protein tyrosine phosphorylation was comparable between the three transfectants except for the attenuation of phosphorylation of a 90–100-kD protein in Lnk#2 and Y536F cells (Fig. 4 D). c-kit itself was efficiently phosphorylated, and we observed neither attenuated nor augmented c-Kit phosphorylation. Akt was also similarly phosphorylated among the three transfectants (Fig. 4 D). Interestingly, MAPK activation as detected by its phosphorylation was perturbed in Lnk#2 and Y536F, correlating with the attenuated phosphorylation of the 90–100-kD protein. Immunoprecipitation experiments demonstrated that Lnk was phosphorylated by and associated with c-Kit. This association was dependent on phosphorylation of c-Kit, but not of Lnk, as the mutant Lnk Y536F was no longer phosphorylated upon SCF stimulation but still efficiently bound c-Kit. Finally, the 90–100 kD phosphoprotein was identified as Gab2, which is thought to be a substrate of c-Kit (17). Anti-Gab2 reacted with and immunoprecipitated the 90–100 kD phosphoprotein (Fig. 4 D). These results demonstrate that Lnk is a substrate of c-Kit, specifically interacting with phosphorylated c-Kit, and that Lnk negatively regulates c-Kit-mediated signaling for cell growth, at least in part, by attenuating Gab2 phosphorylation and the subsequent activation of the MAPK pathway.

Discussion

We demonstrated that hematopoietic progenitors were increased in the bone marrow of $lnk^{-/-}$ mice as a consequence of enhanced c-Kit signaling, and that $lnk^{-/-}$ hematopoietic progenitors exhibited a greatly increased capacity to repopulate and produce various blood cell lineages after transplantation into lethally irradiated host animals. While it is likely that the increased progenitor fraction partly accounts for the enhanced hematopoiesis by $lnk^{-/-}$ progenitors, the enhanced hematopoiesis by $lnk^{-/-}Kit^{W/+}$ progenitors was comparable to that by $lnk^{-/-}Kit^{+/+}$ progenitors in competitive repopulation assays. This suggests the existence of Lnk-dependent but c-Kit–independent signaling pathways that lead to the augmented ability of hematopoietic progenitors to repopulate host bone marrow and undergo hematopoiesis. Flt3/Flk-2 may be involved in such a pathway as it has been shown to support proliferation and differentiation of hematopoietic progenitor cells, while disruption of the Flt3/Flk-2 perturbed the production of various blood cell lineages (23). However, injection of anti-Flt3/Flk-2 antibodies into $lnk^{-/-}$ mice failed to normalize the B cell overproduction in $lnk^{-/-}$ mice (unpublished data), indicating that Flt3/Flk-2 signaling is not likely to be a target affected by the *lnk*-deficiency. Alternatively, the fact that the increased reconstituting activity of $lnk^{-/-}$ progenitors was not affected by the reduction of c-Kit expression would indicate difference in the threshold of c-Kit–dependent signals in early hematopoietic progenitors or HSCs, late-stage progenitors and B cell precursors. Since c-Kit is not absent but only reduced in $Kit^{W/+}$ mice, c-Kit signaling with a relatively low threshold in early progenitors or HSCs

might fully function in the condition where c-Kit–dependent growth signaling in the majority of $Lin^-c\text{-}Kit^+Sca\text{-}1^+$ progenitors and B cell precursors were affected.

We found that Lnk is also expressed in hematopoietic progenitors and that the ability to generate various lineages of blood cells was significantly enhanced in irradiated host animals in the absence of Lnk. In $lnk^{-/-}$ mice, however, the B lineage is affected to a greater degree while other lineages of cells are kept intact. The high expression of Lnk in B lineage cells (9) presumably accounts for the selective accumulation of B cells in $lnk^{-/-}$ mice despite the importance of c-Kit in directing the development of a wide-range of blood cells. As Lnk expression is maintained at a high level in the B cell lineage in normal mice, B cell lineage is severely affected in the absence of Lnk. Although other lineage of $lnk^{-/-}$ cells may also have some growth advantage compared with corresponding wild-type cells, the advantage is likely to be masked by a massively enhanced growth ability of B lineage cells in $lnk^{-/-}$ bone marrow.

Biochemical analysis using c-Kit$^+$ MC9 cells demonstrated that Lnk was tyrosine-phosphorylated by c-Kit and interacted with phosphorylated c-Kit. Lnk specifically inhibited c-Kit–mediated signaling for cell growth, at least in part, by attenuating Gab2 phosphorylation and the subsequent activation of MAPK pathway. Although the residue of Y536 of Lnk was the main target of c-Kit, tyrosine phosphorylation of Y536 was not required for the negative regulatory effect of Lnk on c-Kit. Moreover, SCF-induced downregulation of c-Kit was not affected by Lnk overexpression. These are in contrast to APS that inhibits Janus kinase (JAK)2 or PDGF-R–mediated signaling in combination with c-Cbl. Phosphorylation of the COOH-terminal tyrosine is essential for c-Cbl binding and subsequent inhibitory effects of APS (21, 22). The inhibitory function of Lnk on c-Kit is also in contrast to the positive regulatory roles of SH2-B and APS in signaling via receptors for various cytokines and growth factors (24–26), while both SH2-B and APS share significant similarities in structure with Lnk.

Phosphorylation of cellular proteins induced by c-Kit stimulation was hardly detectable in primary progenitor cells prepared freshly from normal or $lnk^{-/-}$ bone marrow cells. Even if we used primary pro-B cells obtained by a long-term bone marrow culture, c-Kit–dependent phosphorylation was hardly detectable. Given the ubiquitous expression of MAPK pathway components and Gab2 (17, 27), however, the observation obtained using mast cell lines overexpressing Lnk should reflect the function of Lnk in hematopoietic progenitors exposed to low concentration of SCF in the bone marrow microenvironment. The transgenic mice overexpressing Lnk in B cell precursors resulted in the reduction of B lineage cells (unpublished data), presenting a good contrast to the phenotype of $lnk^{-/-}$ mice and a reasonable synonym with the inefficient SCF-dependent growth of MC9 cells overexpressing Lnk. Recently, it has been reported that $Gab2^{-/-}$ mice are viable and show normal steady-state hematopoiesis except reduced mast cells in skins and peritoneal cavity (28). Gab2 is expressed widely like its related protein Gab1 (17, 27), and both Gab1 and Gab2 are involved in MAPK activation in response to several growth factors and cytokines (17). Bone marrow–derived mast cells, however, express only Gab2 but not Gab1 (28). Although Lnk overexpression resulted in attenuated phosphorylation of Gab2 in mast cell line MC9, Lnk might also act on and regulate Gab1 in other lineages of cells. Whether Lnk regulates Gab1 as well as Gab2 and whether Gab1 and Gab2 function redundantly in hematopoietic progenitors remain to be elucidated.

The expression of c-Kit has been one of best markers for HSCs commonly used in various purification protocols to enrich HSCs (18, 29–32). The definitive hematopoiesis is severely perturbed by mutations in the gene loci for c-Kit (*Kit^W*) or SCF (*sl*) (15). In addition, administration of anti–c-Kit antibodies abrogates development of hematopoietic cells (29, 30). These indicate the importance of c-Kit expressed on HSCs. Recent report, however, has demonstrated the existence of c-Kit$^-$ HSC fraction (33). Although c-Kit$^-$ HSCs that are dormant/quiescent population do not radioprotect or form CFU-S, they show delayed or slow reconstitution kinetics when cotransplanted with radioprotective bone marrow cells. c-Kit$^-$ HSCs can give rise to c-Kit$^+$ HSCs not in primary recipients but in secondary recipients when transplanted repeatedly into irradiated hosts (33). One of possible dysregulations caused by the *lnk*-deficiency resulting in the enhanced hematopoiesis might be a rapid and augmented transition from c-Kit$^-$ HSCs into c-Kit$^+$ HSCs in irradiated hosts that is independent on c-Kit signaling.

Defined characterization of $lnk^{-/-}$ HSCs by the evaluation of the hematopoietic ability of single HSC and the repopulation to various tissues other than blood cells will be of great interest. HSCs transferred into host animals need to migrate into bone marrow, find a marrow niche best suited for their self-renewal, then proliferate and differentiate. Studying the effect of the *lnk*-deficiency on HSCs and clarifying the processes affected by the *lnk*-deficiency would provide useful information to reveal HSCs function in vivo. In addition, identification of Lnk-dependent signaling pathways and target genes that exhibit altered expression levels in the absence of Lnk would provide useful clues in the understanding of the self-renewal abilities of HSCs or the expansion of progenitor cells. Inhibiting Lnk function by a mutant form of Lnk, inhibition of Lnk-dependent signaling pathways, or forced expression of Lnk-target genes may eventually allow us to control the expansion and generation of hematopoietic progenitor cells from HSCs.

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Pathogenesis of Murine Experimental Allergic Rhinitis: A Study of Local and Systemic Consequences of IL-5 Deficiency[1]

Hiroko Saito,* Koichiro Matsumoto,* Avram E. Denburg,* Lynn Crawford,* Russ Ellis,* Mark D. Inman,* Roma Sehmi,* Kiyoshi Takatsu,† Klaus I. Matthaei,‡ and Judah A. Denburg[2]*

Recent studies have demonstrated an important role for IL-5-dependent bone marrow eosinophil progenitors in allergic inflammation. However, studies using anti-IL-5 mAbs in human asthmatics have failed to suppress lower airway hyperresponsiveness despite suppression of eosinophilia; therefore, it is critical to examine the role of IL-5 and bone marrow responses in the pathogenesis of allergic airway disease. To do this, we studied the effects of IL-5 deficiency (IL-5$^{-/-}$) on bone marrow function as well as clinical and local events, using an established experimental murine model of allergic rhinitis. Age-matched IL-5$^{+/+}$ and IL-5$^{-/-}$ BALB/c mice were sensitized to OVA followed by 2 wk of daily OVA intranasal challenge. IL-5$^{-/-}$ OVA-sensitized mice had significantly higher nasal mucosal CD4^{+} cells and basophilic cell counts as well as nasal symptoms and histamine hyperresponsiveness than the nonsensitized group; however, there was no eosinophilia in either nasal mucosa or bone marrow; significantly lower numbers of eosinophil/basophil CFU and maturing CFU eosinophils in the presence of recombinant mouse IL-5 in vitro; and significantly lower expression of IL-5Rα on bone marrow CD34^{+}CD45^{+} progenitor cells in IL-5$^{-/-}$ mice. These findings suggest that IL-5 is required for normal bone marrow eosinophilopoiesis, in response to specific Ag sensitization, during the development of experimental allergic rhinitis. However, the results also suggest that suppression of the IL-5-eosinophil pathway in this model of allergic rhinitis may not completely suppress clinical symptoms or nasal histamine hyperresponsiveness, because of the existence of other cytokine-progenitor pathways that may induce and maintain the presence of other inflammatory cell populations. *The Journal of Immunology,* **2002, 168: 3017–3023.**

Although the role of IL-5 in the differentiation, proliferation, and migration of eosinophils in allergic inflammation has been well documented (1–9), it remains unclear how critical IL-5 is to the development of clinical disease. Indeed, recent studies using anti-IL-5 mAbs in vivo in human asthmatic subjects have failed to confirm that IL-5 is both necessary and sufficient to cause lower airway hyperresponsiveness, even though it appears responsible for the development of blood and tissue eosinophilia (10–12). In animal models involving IL-5 deficiency and/or overexpression, there are only a few studies in which both pathological and clinical variables have been evaluated (13–19).

Recent reports (3, 4, 20–37) have demonstrated an important role for the bone marrow as a source of eosinophils and other allergic inflammatory cells such as basophils, mast cells, or lymphocytes in upper or lower airway allergic inflammation. Bone marrow CD34^{+}CD45^{+} progenitor cells are increased and phenotypically altered to preferentially differentiate—in response to allergen and IL-5—into eosinophils and basophils during the development of airway allergic disorders (14, 38–42). These progenitors migrate to airway tissues, providing a source for local expansion of eosinophils and basophils (5, 39, 40). Because it is possible that the failure of anti-IL-5 Abs to suppress asthmatic responses occurs because treatment is insufficient to control systemic progenitor responses, it becomes critical to carefully examine IL-5-dependent bone marrow responses in allergic airway disease, including whether or not IL-5 deficiency per se has any effect on this pathogenetic mechanism and the concomitant development of clinical disease. To do this, we have used a recently developed murine experimental allergic rhinitis model (14) to critically examine the pathological and clinical roles of IL-5.

Materials and Methods

Animals and OVA sensitization

Age-matched (8- to 10-wk-old, N_{14}.BALB/c, female and male, crossed to the BALB/c from the C57BL/6 strain for 14 generations) IL-5$^{+/+}$ and IL-5$^{-/-}$ mice were placed into one of two groups: 1) OVA/OVA group, which was given OVA sensitization followed by 2 wk of OVA intranasal daily challenge (IL-5$^{+/+}$ and IL-5$^{-/-}$ mice, $n = 10$ each), and 2) sham/sham group, which was given normal saline instead of OVA in the same schedule (IL-5$^{+/+}$ and IL-5$^{-/-}$ mice, $n = 10$ each). Under pathogen-free conditions, mice in the OVA/OVA group were sensitized using OVA Ag as follows. A total of 40 μg/kg OVA (Sigma-Aldrich, St. Louis, MO) diluted by sterile normal saline with aluminum hydroxide gel (alum adjuvant, 40 mg/kg) were administered to unanesthetized animals four times by i.p. injection on days 1, 5, 14, and 21. This was followed by daily challenge with OVA diluted by sterile normal saline intranasally (20 μl of 25 mg/ml OVA per mouse) from day 22 to 35 (Fig. 1).

Clinical symptoms and specimens

Nasal symptoms were evaluated for each mouse in each group at the time points of days 28 and 35 by counting the number of sneezes and nasal

*Asthma Research Group, Division of Clinical Immunology and Allergy, Department of Medicine, McMaster University, Hamilton, Ontario, Canada; †Institute for Medical Sciences, Tokyo University School of Medicine, Tokyo, Japan; and ‡John Curtin School of Medical Research, Canberra, Australia

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[2] Address correspondence and reprint requests to Dr. Judah A. Denburg, Asthma Research Group, Division of Clinical Immunology and Allergy, Department of Medicine, McMaster University, HSC-3V46, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada. E-mail address: denburg@mcmaster.ca

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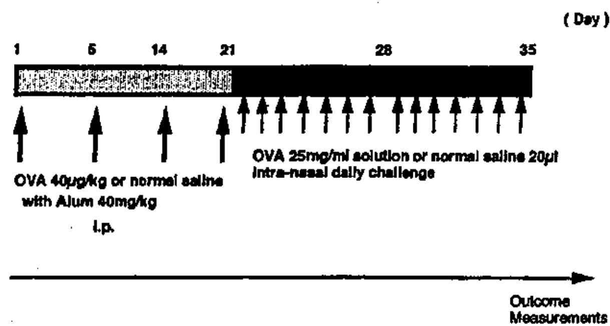

FIGURE 1. Protocol for OVA sensitization and subsequent OVA intranasal challenge. Sham/sham mice were treated with diluent both during sensitization and challenge instead of OVA. In contrast, OVA/OVA mice were given daily OVA challenge intranasally from day 22 to 35 after OVA i.p. sensitization from day 1 to 21.

itching motions (nasal rubbing) for 10 min after OVA intranasal provocation. Nasal histamine responsiveness (NHR)[3] was also measured by determining the concentration of histamine which caused sneezing and itching and was expressed as the limiting concentration of histamine (log_{10} picograms per milliliter) as previously described (14). The mice in each group were euthanized by deep anesthesia using a solution that contained ketamine hydrochloride (Ketalean; Bimeda-MTC, Cambridge, Ontario, Canada) and Xylazine (Rompun; Bayer, Toronto, Ontario, Canada) diluted in normal saline, at 24-h postintranasal provocation. Nasal mucosa and bone marrow tissues were taken and immediately processed.

All procedures were performed in accordance with the ethical guidelines in the Guide to the Care and Use of Experimental Animals of the Canadian Council on Animal Care and approved by the Animal Ethics Committee of McMaster University (Hamilton, Ontario, Canada).

Tissue preparation

Nasal mucosal tissues were treated by the following methods (previously described in Ref. 43). Briefly, specimens were cut into 3- to 4-mm^3 pieces and fixed overnight in cold acetone containing protease inhibitors at −20°C before processing in glycolmethacrylate resin. The embedded tissues were cut into 4-μm thin sections using a microtome for ultra-thin sections (Ultra Cut; Leica Microsystems, Wetzlar, Germany) and recruited to the immunostaining procedure. Bone marrow cells were obtained from sternal or femoral bone marrow, suspended in McCoy's 3+ culture medium, which was made from modified McCoy's 5A medium and 15% FCS (Life Technologies, Grand Island, NY) with 1% penicillin-streptomycin and 0.35% 2-ME, as previously described (28). Total bone marrow cells, as well as mononuclear cells that were separated by density gradient centrifugation over LymphPrep (Nycomed, Oslo, Norway) for 25 min at 2000 rpm in room temperature, were diluted to a concentration of 5 × 10^5/ml with PBS, and cytocentrifuge slides were prepared (Cytospin 3; Thermo Shandon, Sewickly, PA) on Silane-coated glass slides. Another set of mononuclear cells, which were isolated as described from femoral bone marrow, were incubated in plastic flasks for 2 h at 37°C and 5% CO_2 to remove adherent cells and then prepared for methylcellulose culture or immunofluorescence staining followed by flow cytometry analysis.

Immunostaining

For cell differential counts of bone marrow cells on cytocentrifuge slides, Diff-Quick stain (Dade Behring, Dudingen, Switzerland) was performed. To identify eosinophils and basophilic cells in the nasal mucosa, slides were fixed in acetone-methanol at room temperature for 10 min then stained in Diff-Quick; slides were then embedded in Permount (Fisher Scientific, Fair Lawn, NJ). To identify CD4$^+$ lymphocytes in nasal mucosa and CD34$^+$ cells in bone marrow cytocentrifuge preparations, a streptavidin-biotin complex immunostaining system method was used, using various anti-murine mAbs: anti-murine CD4 mAb (L3T4, rat IgG2a, κ; BD PharMingen, Mississauga, Ontario, Canada) and anti-murine CD34 mAb (rat IgG2a, κ; BD PharMingen) as previously described, with some modification (43). Briefly, glycolmethacrylate resin-embedded tissue slides and acetone-fixed cytocentrifuge slides were pretreated to inhibit endogenous peroxidase with a solution of 0.1% sodium azide and 0.3% hydrogen peroxide for 30 min, washed with TBS, then treated with TBS containing 1% Carnation skim milk (Nestle, Don Mills, Ontario, Canada) and 10% FBS (Life Technologies) for 30 min followed by incubation with saturated BSA (Sigma-Aldrich) in sterile water for 20 min and 10% normal goat serum in TBS to block nonspecific reactivity, followed by the application for 2 h of each mAb. Bound Abs were then labeled with biotinylated second-stage Abs appropriate to the isotype of each primary Ab (DAKO, Missisauga, Ontario, Canada) for 2 h, and detected using a streptavidin-biotin peroxidase detection system (StreptABComplex/HRP; DAKO). Aminoethylcarbazole (Sigma-Aldrich) was then applied as a chromogen and the sections were counterstained with Mayer's hematoxylin (Sigma-Aldrich) to contrast with the red positive staining by aminoethylcarbazole. Negative control sections were similarly treated with the same isotype Ig Ab (DAKO) instead of the primary Ab.

Evaluation and quantitation of staining

In the lamina propria of the nasal mucosa, total numbers of cells expressing positive immunoreactivity for cellular surface markers or any of the intracellular cytokines were enumerated. The area of the nasal tissue was measured, excluding glands, using an eye piece with a grid: 10 high-power fields were randomly evaluated after the stained slides were coded by a person unconnected with the study and blinded from the investigator until all evaluations were complete. The cell count results were expressed as the number of cells per square millimeter of lamina propria. For bone marrow pathological evaluation, differential cell counts were performed of cytospin preparations of bone marrow cells after Diff-Quick stain, eosinophilic cells and basophilic cells on each slide were enumerated by light microscopy, 1000 bone marrow cells were counted, and the result was expressed as a percentage in total sternal bone marrow cells. CD34$^+$ cells on bone marrow were also counted by light microscopy: 1000 mononuclear bone marrow cells were counted and the result was expressed as the percentage of positive cells in total mononuclear cells.

Bone marrow methylcellulose cultures

Nonadherent mononuclear cells (NAMNC) were cultured in 35 × 10-mm tissue culture dishes (Falcon Plastics; BD Biosciences Labware, Franklin Lakes, NJ) in culture medium, which was made up of 0.9% methylcellulose (Dow Chemical, Midland, Michigan), 20% FCS and Iscove's Dulbecco's medium (with 1% penicillin-streptomycin, 0.35% 2-ME, and 0.1% BSA) and the following recombinant mouse (rm) cytokines (R&D Systems, Minneapolis, MN): rmIL-5 (0.5, 1, 5, 10 ng/ml) with 1 × 10^5 NAMNC, rmIL-3 (5 ng/ml) with 5 × 10^4 NAMNC, or rmGM-CSF (5 ng/ml) with 2.5 × 10^4 NAMNC. With each batch of growth factor, dose response experiments were performed. After 6 days, colonies of >40 cells were counted using inverse microscopy and eosinophil/basophil (Eo/Baso)-CFU were classified using morphological and histological criteria (tight, compact, round refractile cell aggregates). To identify the differentiated cells from colonies as Eo/Baso-CFU, sample cells in each 10-day culture were evaluated, 3 ml of PBS was added to the sample in each culture dish, and then the sample was centrifuged at 1200 rpm for 10 min at 4°C. After the sample was resuspended in 1 ml of PBS, cytocentrifuge slides were created on Silane-coated glass slides and stained with Diff-Quick.

Immunofluorescence staining and flow cytometry analysis

Samples of 1 × 10^6 NAMNC derived from femoral bone marrow tissues at the time point of 24-h postintranasal provocation on day 35 were suspended in 100 μl of washing buffer, which contained PBS with 0.02% sodium azide, 0.02% EDTA, and 1% BSA, then incubated with anti-mouse CD32/CD16 Ab (Fc Block; BD PharMingen) for 15 min at 4°C to reduce FcγII/IIIR-mediated Ab binding, which could contribute to background. This was followed by staining with saturating amounts of biotin-conjugated anti-murine IL-5Rα Ab (T21, rat IgG2a, κ; provided by Dr. K. Takatsu, Tokyo University, Tokyo, Japan) (44) or biotin-conjugated isotype-matched negative control for 10 min, then FITC anti-murine CD34 Ab (rat IgG2a, κ; BD PharMingen) or isotype-matched negative control, and CyChrome anti-murine CD45 Ab (rat IgG2b, κ; BD PharMingen) or isotype-matched negative control at each saturating concentration; samples were then incubated for 35 min at 4°C in the dark. The cells were washed with 3 ml of washing buffer, then incubated with streptavidin-conjugated PE (BD PharMingen) to label biotinized IL-5Rα-positive cells for 30 min at 4°C in the dark after incubation with anti-mouse CD32/CD16 Ab for 15 min at 4°C in the dark to reduce binding by streptavidin-PE, which could contribute to background. The cells were washed with 3 ml washing buffer twice, then fixed in 500 μl of PBS plus 1% paraformaldehyde and kept at 4°C in the dark until analysis 24 h later. For analysis, FACScan (BD Biosciences, San Jose, CA) was used as a flow cytometer with flow cytometry

[3] Abbreviations used in this paper: NHR, nasal histamine responsiveness; NAMNC, nonadherent mononuclear cell; rm, recombinant mouse; Eo/Baso, eosinophil/basophil.

analysis software (CellQuest from BD Biosciences and FlowJo from Tree Star, San Carlos, CA). To measure $CD34^+CD45^+$ progenitor cells a gating strategy was used as previously described (41, 42, 45), and IL-5Rα-positive $CD34^+CD45^+$ progenitor cells were enumerated as a percentage of total $CD34^+CD45^+$ progenitor cells in the marrow.

Statistics

For all cell counts of stained slides, slides were read randomly and in blinded fashion. The Mann-Whitney U test and ANOVA followed by Student's Neuman-Keuls test were used for comparison of data between groups.

Results

Clinical symptoms

OVA-sensitized IL-$5^{+/+}$ mice developed significant nasal symptoms of sneezing and nasal itching (rubs) during 2 wk of daily intranasal OVA challenge, as was previously reported (14). IL-$5^{-/-}$ mice in the OVA/OVA group had significant nasal symptoms only at day 35, the number of sneezes and rubs being significantly higher compared with the sham/sham group. IL-$5^{+/+}$ mice developed sneezing significantly more quickly than IL-$5^{-/-}$: means ± SE days after the first day of sensitization were 25.3 ± 0.3 for IL-$5^{+/+}$ mice and 31.2 ± 0.7 for IL-$5^{-/-}$ mice ($p < 0.0001$) (Fig. 2). In the comparison between OVA-sensitized IL-$5^{+/+}$ and IL-$5^{-/-}$ mice, there were significant differences in each symptom at day 28 (sneezing, $p < 0.0001$; itching, $p < 0.0001$) and in the OVA/OVA group (sneezing, $p < 0.02$; itching, $p < 0.05$).

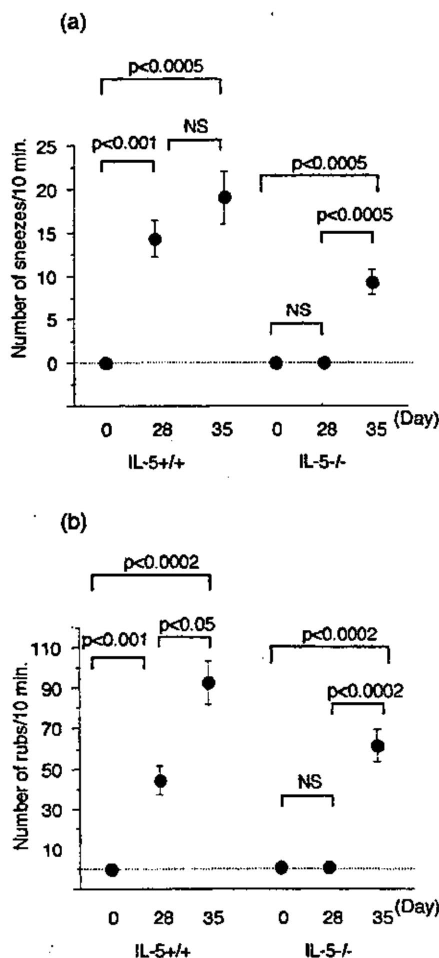

FIGURE 2. Nasal symptoms in IL-$5^{+/+}$ and IL-$5^{-/-}$ mice. Clinical allergic nasal symptoms at days 0 (sham/sham group), 28, and 35 (OVA/OVA group) in OVA sensitization protocol. *a*, Sneezes. *b*, Nasal rubs. Each value represents mean ± SE.

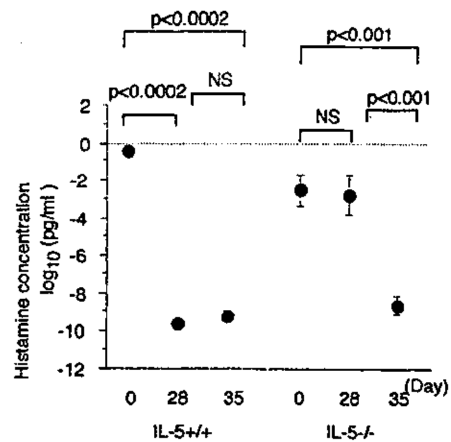

FIGURE 3. NHR in IL-$5^{+/+}$ and IL-$5^{-/-}$ mice. NHR is represented by the limiting concentration of histamine that caused sneezing and itching at days 0 (sham/sham group), 28, and 35 (OVA/OVA group) in OVA sensitization protocol.

Nasal histamine responsiveness

NHR correlated strongly with clinical symptoms (with sneezing, $r = 0.691$ and $p < 0.0001$; with itching, $r = 0.811$ and $p < 0.0001$) (Fig. 3). NHR in OVA/OVA IL-$5^{+/+}$ or IL-$5^{-/-}$ mice was significantly higher than in sham/sham mice; however, in this case, too, IL-$5^{-/-}$ mice developed NHR, manifested by nasal symptoms, later than IL-$5^{+/+}$ mice.

Pathological changes in airway tissue

In IL-$5^{+/+}$ mice, the numbers of allergic inflammatory cells in the nasal mucosa were increased in OVA/OVA mice (at 24-h postintranasal provocation on day 35) compared with sham/sham mice, including eosinophils ($p < 0.01$) and $CD4^+$ cells ($p < 0.05$) but not basophilic cells (NS). In OVA/OVA IL-$5^{-/-}$ mice, at 24-h postintranasal provocation on day 35, significantly higher numbers of $CD4^+$ lymphocytes ($p < 0.02$) and increased basophilic cell counts were observed compared with sham/sham IL-$5^{-/-}$ mice, without eosinophilia in the nasal mucosa, as shown in Table I.

Bone marrow analysis

In comparisons between the two groups, a significant increase in sternal bone marrow eosinophil counts was observed in OVA/OVA IL-$5^{+/+}$ mice compared with sham/sham IL-$5^{+/+}$ mice; no

Table I. *Pathological changes in OVA-sensitized mice*[a]

	Sham/Sham Group	OVA/OVA Group
Eosinophils		
IL-$5^{+/+}$	0.0 ± 0.0	469.9 ± 145.9[b,c]
IL-$5^{-/-}$	0.0 ± 0.0	0.0 ± 0.0
$CD4^+$ cells		
IL-$5^{+/+}$	0.8 ± 0.3[b]	34.6 ± 12.0[d]
IL-$5^{-/-}$	8.9 ± 5.2	86.3 ± 39.4[d]
Basophilic cells		
IL-$5^{+/+}$	10.6 ± 5.0	23.1 ± 6.4
IL-$5^{-/-}$	10.9 ± 5.8	26.0 ± 5.9

[a] The number of eosinophils, $CD4^+$ cells, and basophilic cells in nonsensitized and OVA-sensitized murine nasal tissue. Shown are mean ± SE of cell numbers per square millimeter.
[b] Value of $p < 0.01$ compared to IL-$5^{-/-}$.
[c] Value of $p < 0.01$ compared to sham/sham group.
[d] Value of $p < 0.05$ compared to sham/sham group.

differences were seen between the IL-$5^{-/-}$ mice in the two groups (Fig. 4). Also, a significantly higher percentage of eosinophils in the bone marrow was detected in IL-$5^{+/+}$ mice than in IL-$5^{-/-}$ mice in the OVA/OVA group ($p < 0.01$); however, there was no significant difference between IL-$5^{+/+}$ mice and IL-$5^{-/-}$ mice in the sham/sham group. Basophilic cells and $CD34^{+}$ cells also increased in the bone marrow in the OVA/OVA mice of both IL-$5^{+/+}$ and IL-$5^{-/-}$ strains when compared with sham/sham mice. There was no significant difference in the number of basophilic cells or $CD34^{+}$ cells between mice of either strain in the OVA/OVA group.

Eo/Baso-CFU analysis

In 6-day methylcellulose assays, the number of Eo/Baso-CFU that grew from NAMNC derived from murine bone marrow increased significantly in the presence of rmIL-5, rmIL-3, or rmGM-CSF in OVA/OVA, compared with sham/sham, IL-$5^{+/+}$ mice (Fig. 5). In IL-$5^{-/-}$ mice, the number of Eo/Baso-CFU was higher in the OVA/OVA group than in the sham/sham group in the presence of rmIL-3 or rmGM-CSF in vitro; however, in the presence of rmIL-5 in vitro, the number of Eo/Baso-CFU did not increase significantly in the OVA/OVA group. Furthermore, in the comparison between IL-$5^{+/+}$ and IL-$5^{-/-}$ mice, there were significantly higher numbers of Eo/Baso-CFU grown in the presence of rmIL-5 in OVA/OVA IL-$5^{+/+}$ mice ($p < 0.02$), whereas in the presence of rmIL-3 or rmGM-CSF there were no significant differences between IL-$5^{+/+}$ and IL-$5^{-/-}$ mice.

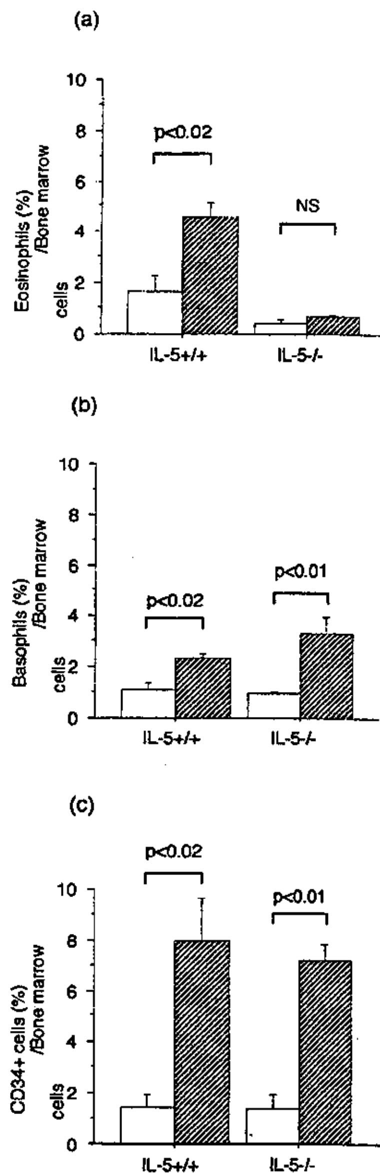

FIGURE 4. Bone marrow changes in murine allergic rhinitis. The percentages of eosinophils (*A*), basophilic cells (*B*), and $CD34^{+}$ cells (*C*) in the bone marrow. Hatched bar, result from the OVA/OVA group; open bar, result from the sham/sham group.

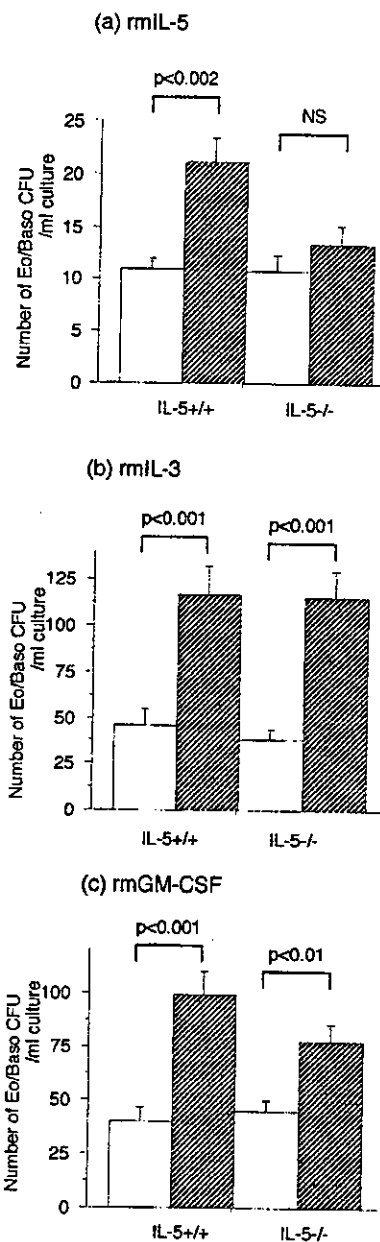

FIGURE 5. Eo/Baso-CFU in 6-day methylcellulose cultures of murine bone marrow. Eo/Baso-CFU in the presence of 5 ng/ml each rmIL-5 (*A*), rmIL-3 (*B*), and rmGM-CSF (*C*). Hatched bar, result from the OVA/OVA group; open bar, result from the sham/sham group.

IL-5Rα expression on $CD34^{+}CD45^{+}$ progenitor cells in murine bone marrow

As shown in Table II, there were significant differences in IL-5Rα expression on $CD34^{+}CD45^{+}$ progenitor cells between IL-$5^{+/+}$

Table II. *IL-5Rα expression on murine bone marrow CD34⁺CD45⁺ progenitor cells*[a]

	Sham/Sham Group[b]	OVA/OVA Group[c]
IL-$5^{+/+}$	4.14 ± 0.64	11.97 ± 2.77[d]
IL-$5^{-/-}$	2.01 ± 0.55	2.48 ± 0.30

[a] Each value is shown as a percentage of IL-5Rα$^+$CD34$^+$CD45$^+$ cells in bone marrow CD34$^+$CD45$^+$ progenitor cells.
[b] Values of $p < 0.05$.
[c] Values of $p < 0.001$.
[d] Values of $p < 0.02$ compared to sham/sham group.

and IL-$5^{-/-}$ mice, in both sham/sham and OVA/OVA groups, with IL-$5^{+/+}$ mice showing higher IL-5Rα expression. Looking at IL-$5^{+/+}$ mice across groups, the OVA/OVA group had significantly higher IL-5Rα expression on CD34$^+$CD45$^+$ cells.

Eosinophil differentiation in 10-day methylcellulose bone marrow culture

As shown in Table III, sham/sham IL-$5^{-/-}$ mice had a significantly higher ratio of immature eosinophilic cells in 10-day cultures compared with sham/sham IL-$5^{+/+}$ mice; along these lines, OVA/OVA IL-$5^{-/-}$ mice had significantly lower ratios of mature eosinophils compared with OVA/OVA IL-$5^{+/+}$ mice.

Discussion

Eosinophilia has been studied as an important phenomenon in allergic disorders. There have been many studies of IL-5 as an important factor in eosinophilic inflammation, because this cytokine controls the differentiation, proliferation, and migration of eosinophils. However, other cytokines, such as GM-CSF, IL-4, and IL-13, or chemokines, such as eotaxin, also influence the inflammatory process (2, 9, 31, 46–53). Whether eosinophils take part in the pathogenesis of allergic disease or behave as bystanders has been the subject of much discussion recently (10–12, 54). Views that cast doubt on the pathogenetic role of eosinophils have come from mainly clinical studies in which data have been gathered after a relatively short period of sensitization and Ag challenge, and in which protocols varied widely, making comparisons of the results difficult. In this study, for the first time, the roles of IL-5 and eosinophils in upper airway allergic inflammation were studied over a relatively protracted period.

In our model, significant nasal symptoms were surprisingly observed in IL-5-deficient animals, although these were delayed compared with wild-type mice. For a better understanding of the pathological basis for this clinical result, we measured NHR and inflammatory changes in the nasal mucosa. NHR was, like nasal symptoms, significantly higher in OVA/OVA compared with sham/sham IL-$5^{-/-}$ mice, but again this was significantly delayed compared with wild-type mice. The pathological changes observed in OVA/OVA IL-$5^{+/+}$ mice were the same as previously reported (14): the numbers of eosinophils, basophilic cells, and CD4$^+$ lymphocytes were increased, while IL-5-deficient mice showed no eosinophilia in either sham/sham or OVA/OVA groups and the numbers of other cellular populations, basophilic cells, and CD4$^+$ lymphocytes were elevated and showed no significant differences when comparing OVA/OVA IL-$5^{+/+}$ to IL-$5^{-/-}$ mice. These results demonstrate that eosinophils are not the only cells responsible for the development of NHR in allergic rhinitis; rather, nasal mucosal mast cells and basophils might be just as important in the expression of both NHR and clinical symptoms. Also, the possibility of differences between human and murine biology, including Eo/Baso function, should be considered. Although some papers have discussed comparisons of murine and human cell functions (55–58), this is an area in which further work is needed to explore the contribution of these and other types of cells, such as macrophages, lymphocytes, neutrophils, and various epithelial cells, to allergic responses.

Previous reports from our group and others (3, 4, 20–37, 59) have shown that a systemic up-regulation of the bone marrow may play a pivotal role in the development and maintenance of not only lower, but also upper, airway allergic inflammation, as exemplified by asthma and allergic rhinitis, respectively. In the present study, we performed detailed examination of bone marrow cellular and molecular events, as well as function, in response to allergen sensitization and challenge in our model of experimental rhinitis. While marrow eosinophils, basophilic cells, and CD34$^+$ cells were increased after Ag sensitization and nasal Ag challenges in wild-type mice, there was no increase of eosinophils in the marrow of IL-5-deficient mice after challenge. Nonetheless, all other inflammatory cell types were increased to the same extent as in wild-type mice, accompanied by the same nasal mucosal pathologic changes. Thus, though IL-$5^{-/-}$ deficiency resulted in suppression of Ag-dependent eosinophil progenitor differentiation (i.e., reduced bone marrow Eo/Baso-CFU), the actual number of progenitor cells was not lower at baseline than in wild-type mice. This defective functional response of eosinophil progenitors was attended by reduced IL-5Rα expression on CD34$^+$CD45$^+$ bone marrow cells in IL-5 mice both before and after Ag sensitization and challenge, with defective up-regulation following Ag sensitization.

The functional consequences of reduced IL-5Rα expression on CD34$^+$CD45$^+$ bone marrow cells in IL-$5^{-/-}$ mice were seen in 6-day methylcellulose assays. While the number of marrow Eo/Baso-CFU increased significantly in vitro in the presence of rmIL-5, rmIL-3, or rmGM-CSF in OVA/OVA IL-$5^{+/+}$ animals, it was only in the presence of rmIL-3 or rmGM-CSF, but not IL-5 in vitro, that Eo/Baso-CFU increased in the OVA/OVA IL-$5^{-/-}$ mice. Related to this, in sham/sham IL-$5^{-/-}$ mice there was a significantly higher ratio of immature eosinophilic cells in colonies enumerated at day 10 compared with significantly higher ratios of

Table III. *Eosinophil differentiation from murine bone marrow-derived NAMNC in 10-day methylcellulose semisolid culture assay in the presence of rmIL-5 (5 ng/ml)*[a]

	Sham/Sham Group		OVA/OVA Group	
	IL-$5^{+/+}$	IL-$5^{-/-}$	IL-$5^{+/+}$	IL-$5^{-/-}$
Mature eosinophils	22.6 ± 3.3	25.7 ± 1.4	53.1 ± 3.9[b]	11.5 ± 2.4
Immature eosinophilic cells	26.0 ± 3.0[c]	46.6 ± 4.6	15.6 ± 2.3	21.5 ± 5.7
Other cell types	51.4 ± 3.9[c]	27.7 ± 4.4	31.3 ± 4.2[c]	67.0 ± 8.0

[a] The number of mature eosinophils, immature eosinophilic cells, and other cell types. Shown are mean ± SE of the percentage of total cells.
[b] Value of $p < 0.002$ compared to IL-$5^{-/-}$.
[c] Value of $p < 0.01$ compared to IL-$5^{-/-}$.

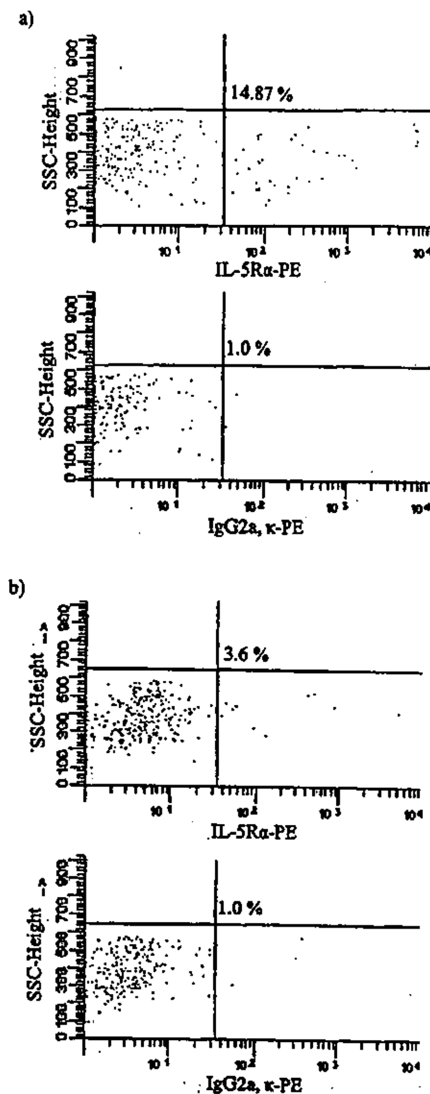

FIGURE 6. Enumeration of bone marrow-derived CD34$^+$CD45$^+$ progenitor cells that express IL-5Rα. Samples of bone marrow-derived NAMNC from OVA/OVA group mice were stained with CD34FITC/CD45CyChrome and either PE-linked IL-5Rα or isotype control Ab. CD34$^+$CD45$^+$ progenitor cell populations that were analyzed by using a gating strategy were back-scattered onto a dot plot of IL-5Rα vs side scatter (SSC). Quadrant statistics (i.e., data in *lower right corner*) are presented as the percentage of total CD34$^+$CD45$^+$ progenitor cells that demonstrated positive staining with anti-IL-5Rα or rat IgG2a,κ isotype-matched control Ab. *A*, Result from a mouse that is in the IL-5$^{+/+}$ OVA/OVA group. *B*, Result from a mouse that is in the IL-5$^{-/-}$ OVA/OVA group.

mature to immature eosinophils in OVA/OVA IL-5$^{+/+}$ mice. This poor response to IL-5 in vitro of both eosinophil colony numbers and eosinophil maturation within colonies is presumably a direct consequence of defective expression of eosinophil progenitor IL-5Rα in response to Ag sensitization and nasal challenge in IL-5-deficient mice. One can speculate that IL-5 is required in vivo for the proper induction—as we have observed in human asthmatics (25)—of marrow progenitor, as well as mature eosinophil, expression of IL-5Rα; this is in agreement with Tavernier's recent demonstration that IL-5 can induce the expression of IL-5R on maturing eosinophils in vitro (60).

Because the numbers of Eo/Baso-CFU responsive to IL-3 or GM-CSF were not suppressed in IL-5-deficient mice, it appears that these latter cytokines can replace IL-5 functionally, giving rise to small numbers of eosinophils and, more importantly, to normal numbers of basophilic cells, which can probably account for the persistence of clinical symptomatology and nasal hyperresponsiveness, albeit delayed. Lantz et al. (61) have shown IL-3 dependency of basophilic responses in mice; whether upper airway inflammation and bone marrow responses are defective in IL-3-deficient mice remains to be investigated. To clarify the role of basophilic cells in the systemic and local pathogenesis of allergic rhinitis, investigations of IL-3 deficiency and/or IL-3 expression in this model would be an important future direction.

In conclusion, IL-5 is required for the development of tissue and marrow eosinophilia, the formation of Eo/Baso-CFU, and the early development of symptoms, but not for other components of the inflammatory response in murine experimental allergic rhinitis. Our findings confirm that IL-5 is required for normal bone marrow eosinophilopoiesis, in response to specific Ag sensitization during the development of experimental allergic rhinitis. However, the results also point out that the suppression of the IL-5-eosinophil pathway in the pathogenesis of allergic rhinitis (and also, by inference, asthma) may not fully suppress clinical symptoms or airway hyperresponsiveness due to the possible existence of other cytokine-progenitor pathways that may induce and maintain the presence of other inflammatory cell populations.

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Impaired Lymphopoiesis and Altered B Cell Subpopulations in Mice Overexpressing Lnk Adaptor Protein

Satoshi Takaki,[2]* Yoshinari Tezuka,* Karsten Sauer,[3]† Chiyomi Kubo,* Sang-Mo Kwon,* Erin Armstead,† Kazuki Nakao,‡ Motoya Katsuki,§ Roger M. Perlmutter,¶ and Kiyoshi Takatsu*

Lnk is an adaptor protein expressed primarily in lymphocytes and hemopoietic precursor cells. Marked expansion of B lineage cells occurs in *lnk*$^{-/-}$ mice, indicating that Lnk regulates B cell production by negatively controlling pro-B cell expansion. In addition, *lnk*$^{-/-}$ hemopoietic precursors have an advantage in repopulating the hemopoietic system of irradiated host animals. In this study, we show that Lnk overexpression results in impaired expansion of lymphoid precursor cells and altered mature B cell subpopulations. The representation of both B lineage and T lineage cells was reduced in transgenic mice overexpressing Lnk under the control of a lymphocyte-specific expression vector. Whereas the overall number of B and T cells was correlated with Lnk protein expression levels, marginal zone B cells in spleen and B1 cells in the peritoneal cavity were relatively resistant to Lnk overexpression. The C-terminal tyrosine residue, conserved among Lnk family adaptor proteins, was dispensable for the negative regulatory roles of Lnk in lymphocyte development. Our results illuminate the novel negative regulatory mechanism mediated by the Lnk adaptor protein in controlling lymphocyte production and function. *The Journal of Immunology,* **2003, 170: 703–710.**

B cells are continuously generated from hemopoietic progenitors in the fetal liver and in adult bone marrow (BM).[4] Multiple sequentially developing B cell precursor populations can be characterized based on the expression of various surface markers (1). Immature B cells generated in the BM emigrate into the peripheral immune system and give rise to a heterogeneous peripheral B cell population, consisting of recirculating cells located in follicles in the spleen and lymph nodes, and nonrecirculating cells mainly enriched in the splenic marginal zone (MZ). The majority of cells are the follicular (FO) B cells, with marginal zone (MZ) B cells representing 5–10% of the splenic B cells in an adult mouse (2, 3). There exists another self-renewing B cell subset, B1 cells, which predominates in the peritoneal and pleural cavities (4). MZ B cells and B1 cells produce natural Abs and provide a first line of defense against Ags. FO B cells are involved in thymus-dependent (TD) Ab responses, in which memory and plasma cells are generated (5, 6).

Lymphocyte differentiation is a series of finely regulated processes whereby the coordinate regulation of cell proliferation, differentiation, and death directs the development of functional cells. Through these processes, lymphocytes reactive against self-Ags are eliminated from the developing repertoire, and sufficient numbers of functional lymphocytes are produced to guarantee the prompt and effective elaboration of immune responses. Cell-to-cell contact and soluble growth factors play important roles in the regulation of developmental processes. Self-Ags presented on stromal cells trigger Ag receptors, signals from which help to determine the fate of lymphoid precursors (7). Various growth factors, such as stem cell factor (SCF) and IL-7, assist in regulating lymphoid precursor expansion by binding to c-Kit and the IL-7R, respectively. (8)

Binding of extracellular ligands to these polypeptide receptors initiates a cascade of events through the activation of intracellular protein kinases (9, 10). The phosphorylation events catalyzed by these kinases both modulate the catalytic activity of effector enzymes and mediate protein-protein interactions that juxtapose critical signal transduction elements. Although the details of how signaling molecules are activated or recruited to receptors remain incompletely elucidated, studies in recent years have defined an array of adaptor proteins that integrate and regulate multiple signaling events (11–13). Adaptor proteins lack kinase, phosphatase, or transcriptional domains, and instead consist of multiple binding sites mediating protein-protein or protein-lipid interactions, such as Src homology (SH) 2, SH3, or pleckstrin homology (PH) domains.

Lnk is an adaptor protein expressed mainly in lymphocytes (14, 15). Together with adaptor molecule containing PH and SH2 domains (APS) and SH2-B, Lnk is part of an adaptor protein family, whose members share the presence of a homologous N-terminal domain with putative proline-rich protein interaction motifs, followed by PH and SH2 domains, and a conserved C-terminal tyrosine phosphorylation site (16–18). SH2 domains of the Lnk family proteins, whose binding specificity remains unknown, are

*Division of Immunology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan; †Department of Immunology and Rheumatology, Merck Research Laboratories, Rahway, NJ 07065; ‡Animal Resources and Genetic Engineering, RIKEN Center for Developmental Biology, Kobe, Japan; §National Institute for Basic Biology, Aichi, Japan; and ¶Amgen, Thousand Oaks, CA 91320

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[2] Address correspondence and reprint requests to Dr. Satoshi Takaki, Division of Immunology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan. E-mail address: takakis@ims.u-tokyo.ac.jp

[3] Current address: Genomics Institute, Novartis Research Foundation, San Diego, CA 92121.

[4] Abbreviations used in this paper: BM, bone marrow; MZ, marginal zone; FO, follicular; TD, thymus-dependent; SCF, stem cell factor; SH, Src homology; PH, pleckstrin homology; KLH, keyhole limpet hemocyanin; TNP, trinitrophenyl; APS, adaptor molecule containing PH and SH2 domains; *hGH*, human growth hormone; HSA, heat stable Ag.

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approximately the same size and share over 90% similarity. Lnk regulates B cell production by negatively controlling pro-B cell expansion. Mutant mice lacking the *lnk* gene show enhanced B cell production (16, 19). This B cell overproduction is due to the hypersensitivity of B cell precursors to SCF, a c-Kit ligand (16). The absence of Lnk confers upon immature BM cells an enhanced ability to support B lymphopoiesis in adoptively transferred host animals, even in a competitive environment, such as the nonirradiated $RAG2^{-/-}$ host (16). In addition, the numbers of hemopoietic progenitors in the bone marrow increase in *lnk*-deficient mice (20). Competitive repopulation assays in irradiated host animals demonstrate that the ability of hemopoietic progenitors to generate various lineages of hemopoietic cells is greatly enhanced by the absence of Lnk.

In this study, we used a transgenic approach to define critical aspects of Lnk function in more detail. Lymphocyte production was impaired in a dose-dependent manner upon overexpression of Lnk in lymphoid cells. In addition to its importance in lymphopoiesis at the early developmental stages, Lnk also plays a role in peripheral maturing B cells. In transgenic mouse spleens, skewed B cell subpopulations and abnormalities in B cell morphology and cell cycle status were observed. Our results illuminate the novel negative regulatory mechanism mediated by the Lnk adaptor protein in controlling lymphocyte production and function.

Materials and Methods

Mice

All mice were bred and maintained at the animal facility of the Institute of Medical Science (University of Tokyo, Tokyo, Japan) under specific pathogen-free conditions. The *NcoI-EcoRI* cDNA fragment encompassing the entire coding region of the mouse Lnk cDNA was subcloned into the *BamHI* site of pcDNA3 (Stratagene, La Jolla, CA), a eukaryotic expression vector driven by the CMV enhancer and promoter, resulting in pcDNA3-Lnk as previously described (20). The *BamHI* fragment containing the Lnk cDNA was subcloned into the *BamHI* cloning site of the p1026x vector that consists of the murine *lck* proximal promoter, Ig intronic H chain enhancer Eμ, and a human growth hormone (*hGH*) gene cassette (21). A substitutional mutation at the C-terminal tyrosine residue to phenylalanine (Y536F) was introduced into the Lnk cDNA by PCR-based site-directed mutagenesis, and confirmed by DNA sequencing (20). The resulting mutated cDNA was also inserted into the p1026x vector. The *lnk* transgenes, purified as *NotI* fragments, were injected into C57BL/6J mouse zygote pronuclei as previously described (22). Transgenic founders were detected by hybridization of genomic tail DNA with a *hGH* probe or PCR, and stable mouse lines were generated by backcrossing founders with C57BL/6J mice.

Western blotting

Single cell suspensions were prepared from lymphoid organs of 6- to 8-wk-old mice. Cells were lysed with lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 10 mM NaF, 1 mM Na_3VO_4, 2 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin), and the lysates were clarified by centrifugation. Total lysates derived from 4×10^6, 2×10^6, or 1×10^6 thymocytes were separated on 8% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were probed with anti-Lnk-C-terminal Abs (15). Bound Abs were detected using HRP-conjugated secondary Abs via chemiluminescence.

Flow cytometry

Single cell suspensions were prepared from lymphoid organs of 6- to 8-wk-old mice, and cells were stained using predetermined optimal concentrations of the respective Abs. The stained cells were then analyzed on a FACScan or FACSCalibur instrument (BD Biosciences, San Jose, CA). The following mAbs were used: PE-conjugated anti-CD43 (S7), biotin-conjugated anti-BP-1/Ly-51, FITC-coupled anti-heat stable Ag (anti-HSA, J11d), FITC-anti-CD8 (53-6.7), FITC- or PE-anti-CD4 (RM4-5), biotin-anti-CD25 (PC61), PE-anti-CD44 (IM7), biotin-anti-CD23 (B3B4), FITC-anti-CD21/CD35 (7G6), PE- or biotin-anti-CD3ε (145-2C11), biotin-anti-TER-119, biotin-anti-Gr-1 (RB6-8C5), (all purchased from BD PharMingen, San Diego, CA); FITC-, PE-, or biotin-anti-B220 (RA3-6B2), PE-anti-IgM $F(ab')_2$, FITC-anti-Mac-1 (M1/70), (obtained from Caltag Laboratories, Burlingame, CA), and biotin-anti-IgD (CS15, a gift from Dr. K. Miyake, University of Tokyo). PE-streptavidin (Ancell, Bayport, MN), Tri-color-conjugated streptavidin (Caltag Laboratories) or allophycocyanin-conjugated streptavidin (BD PharMingen) were used for biotin-coupled Ab staining. In some staining, 2 μg/ml 7-amino-actinomycin D (Sigma-Aldrich) were used to gate out dead cells. For DNA staining, splenocytes were stained with FITC-anti-CD21/CD35 and PE-anti-IgM $F(ab')_2$ and $CD21^-IgM^+$ T1 or $CD21^{high}IgM^{high}$ T2 and MZ B cells were purified using FACSVantage (BD Biosciences). Cells were then fixed in ethanol and stained in 20 μg/ml propidium iodide, 0.5 mg/ml RNase H, and 0.2% Tween 20 at room temperature for 60 min. Stained cells were then analyzed on a FACSCalibur instrument.

Serology

Serum concentrations of each Ig isotype were determined by isotype-specific ELISA as described previously (23). To examine the Ab production against TI-2 Ags, mice were i.p. injected with 100 μg of trinitrophenyl (TNP)-Ficoll in saline and were bled 10 days after the injection. To examine the response against TD Ags, mice were immunized i.p. with 100 μg of keyhole limpet hemocyanin (KLH) in a 1:1 homogenate with CFA (Difco, Detroit, MI), and were bled on day 12. Serum serial dilutions were analyzed for TNP- or KLH-specific Ig isotypes by ELISA using dinitrophenyl-coupled BSA (cross-reacts with anti-TNP Abs) or KLH as the capture reagent.

Proliferation assay

Splenic B cells were purified using a MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) after incubation with biotin-conjugated anti-CD43 and streptavidin-coupled microbeads. Resulting purified B cells (1×10^5) were cultivated in 200 μl of medium in 96-well plates. Cells were stimulated with various concentrations of anti-IgM $F(ab')_2$ (The Jackson Laboratory, Bar Harbor, ME), anti-CD40 mAb (BD PharMingen), IL-4 (PeproTech, London, U.K.), or LPS. Cells were pulse-labeled with [^{3}H]thymidine (0.2 μCi per well) during the last 16 h of the 72-h culture period, and incorporated [^{3}H]thymidine was measured using a Matrix 96 direct beta counter (Packard Instrument, Meriden, CT).

Results

Generation of transgenic mice overexpressing Lnk at various levels

In mice lacking the *lnk* gene, an accumulation of B lineage cells caused by overproduction of pro-B cells was observed (16). To characterize further the importance of Lnk, and to reveal its roles in lymphopoiesis and lymphocyte function, we generated transgenic mice that overexpress Lnk under the control of the *lck* proximal promoter in combination with the Eμ enhancer (Fig. 1*A*). The promoter drives expression of the inserted cDNA in T and B lineage cells from their early developmental stages (21). Seven lines of transgenic mice were obtained from independent founders, and five lines that overexpress Lnk at various levels were further analyzed. Lnk protein expression levels in thymocytes from each transgenic line were measured by immunoblotting a 2-fold serial dilution of the lysates using anti-Lnk Ab in combination with densitometric quantification (Fig. 1*B*). Transgene expression in peripheral T or B cells was well correlated with that in thymocytes in low or medium expressers (data not shown). Severe reduction of peripheral B cells and altered distribution of mature B cell fractions in a high expressing line (see below) made it difficult to directly measure the Lnk protein expression in B cells. In contrast, T cell development assessed on the basis of CD4 and CD8 expression was unaffected in the transgenic lines (see below). Thus, expression levels in the thymus were used to compare the Lnk levels in each transgenic line. The Lnk no. 4 line expressed Lnk at the highest level, 23-fold over endogenous Lnk protein levels in normal C57BL/6 thymocytes. The Lnk no. 99 line expressed Lnk at the lowest level, 2.5-fold greater than the endogenous level.

Perturbed B lymphopoiesis in BM by Lnk overexpression

B cell development in each transgenic mouse line was analyzed. The highest expresser, Lnk no. 4, showed severe reduction of B lineage cells as shown in Fig. 2. The BM contained very few