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Absence of an Essential Role for Thymic Stromal Lymphopoietin Receptor in Murine B-Cell Development

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The murine cytokine thymic stromal lymphopoietin (TSLP) supports the development of B220$^+$ IgM$^+$ immature B cells and induces thymocyte proliferation in vitro. Human TSLP, by contrast, activates CD11c$^+$ dendritic cells, but not B or T cells. Recent studies have demonstrated that the receptor for TSLP consists of a heterodimer of the interleukin 7 (IL-7) α chain and a novel protein that resembles the hematopoietic cytokine receptor common γ chain. We examined signal transduction by the γ-like chains using chimeric receptor proteins. The cytoplasmic domain of the human, but not of the murine, γ-like chain, activates Jak2 and Stat5 and supports the proliferation of hematopoietic cell lines. In order to assess the role of the murine γ-like chain in vivo, we generated γ-like chain-deficient mice. Receptor-deficient mice are unresponsive to TSLP but exhibit no obvious phenotypic defects. In particular, hematopoietic cell development appeared normal. B-cell development, including the IgM$^+$ compartment, was unaffected by loss of the TSLP pathway, as were T lymphopoiesis and lymphocyte proliferation in vitro. Cytokine receptors that utilize the common γ chain signal through the lymphocyte-specific kinase Jak3. Mice deficient in Jak3 exhibit a SCID phenotype but harbor a residual B220$^+$ splenic lymphocyte population. We demonstrate here that this residual lymphocyte population is lost in mice lacking both the γ-like chain and Jak3.

The proliferation and differentiation of mammalian hematopoietic cells are regulated by the coordinated action of a large group of structurally related cytokines. Hematopoietic cytokines induce proliferation and differentiation of their target cells by signaling through cognate receptor proteins belonging to the cytokine receptor superfamily. Members of the cytokine receptor family can consist of a single polypeptide chain that homodimerizes upon receptor engagement, two distinct polypeptide chains that heterodimerize upon cytokine binding, or three separate polypeptide chains that multimerize. Cytokine receptors utilize members of the Jak family of tyrosine kinases to couple to intracellular signaling pathways. Upon receptor engagement, Jak kinases are activated, enabling them to phosphorylate both the cytoplasmic tails of their cognate receptors and a variety of intracellular substrates. Among their targets are members of the Stat family of transcription factors (7, 9).

Thymic stromal lymphopoietin (TSLP) was originally isolated from a mouse thymic stromal cell line and was found to support development of B220$^+$ IgM$^+$ immature B cells in vitro and induce thymocyte proliferation in vitro (3, 12, 20, 23). Recent studies have demonstrated that the receptor for TSLP (TSLP-R) consists of a heterodimer of the interleukin 7 (IL-7) α chain and a recently identified novel murine cytokine receptor subunit (4, 12, 15, 16). Because of its similarity to the common γ chain, the novel subunit has been referred to as the common γ-like chain (2, 4, 15, 16). The human forms of TSLP and TSLP-R have proved to be remarkably divergent from their murine orthologues in both sequence and function (19, 21, 27, 33), with the main role of human TSLP identified so far being the activation of CD11c$^+$ dendritic cells (5, 24). In contrast to human TSLP, an in vivo role for murine TSLP has not been established. In IL-7-deficient mice, B-cell development is arrested at a point later than in mice lacking the IL-7 receptor α chain (18, 29), suggesting that TSLP might support B lymphopoiesis. In this report, we provide evidence that murine TSLP provides no essential support for B-cell lymphopoiesis in vivo.

MATERIALS AND METHODS

Isolation of human and murine *TSLP-R* genes. A partial cDNA clone of the human γ-like gene was identified by digital cloning based on the WSXWS consensus sequence in the extracellular domain of hematopoietic cytokine receptors. The full-length human cDNA clone was then isolated by 5′ rapid amplification of cDNA ends and used to screen the database for homologues. An expressed sequence tag (EST) corresponding to murine *TSLP-R* (GenBank accession no. AA008678) was identified and used as a probe to screen a randomly primed mouse spleen library (Stratagene). One full-length murine γ-like receptor was isolated from screening 10^6 individual clones by standard techniques.

Construction of chimeric receptors. Cytoplasmic regions of the human and mouse TSLP receptors were cloned from cDNA templates by PCR and fused to a truncated murine erythropoietin receptor (EpoR) cDNA carried in expression vector pRK5 (human chimeric) or pcDNA3 (Invitrogen; mouse chimeric) at the *Bgl*II site containing I^{281} and overlapping W^{282} of the EpoR. TSLP-R cytoplasmic domains extend from R^{256} to the terminal L^{371} (human) or from L^{265} to the terminal L^{361} (mouse). A tagged version of the human chimeric receptor was also

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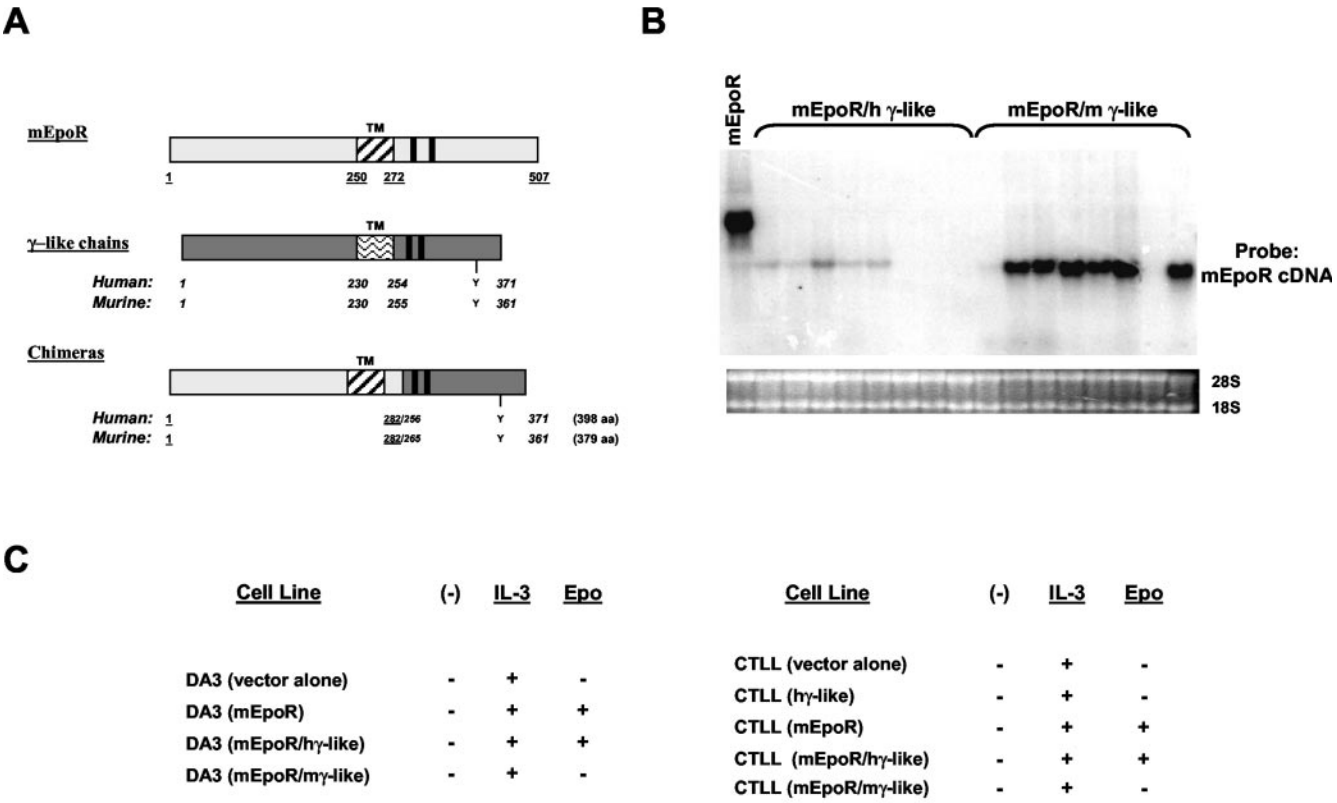


FIG. 1. Chimeric receptors. (A) The cytoplasmic domain of the human or murine γ-like chain was fused in frame to the extracellular and transmembrane portions of the murine EpoR (mEpoR) as indicated. The chimeric receptors contain 10 amino acids (aa) of the EpoR cytoplasmic domain. The box 1 and box 2 regions (black boxes) and the single cytoplasmic domain tyrosine (Y) are shown. The numbers are amino acid residue positions. TM, transmembrane domains. (B) Northern analysis of individual DA3 clones expressing either the EpoR–human γ-like chimeric receptor (EpoR/h γ-like) or the EpoR–murine γ-like chimeric receptor (EpoR/m γ-like). Expression levels of EpoR–human γ-like receptors were uniformly lower in all clones examined. rRNA loading controls are shown in the bottom blot. (C) Mass cultures of transfected DA3 or CTLL cells were cultured in medium without a cytokine (−) or in medium containing the indicated cytokine. The ability (+) or inability (−) to proliferate in long-term cultures is indicated.

made by using a cDNA to which the eight amino acids of the FLAG sequence had been fused onto the C-terminal leucine residue. The presence of the tag had no effect on cell growth or Jak activation (see below). All junctions were confirmed by dideoxynucleotide sequencing.

Cell lines and transfections. Cell lines were cultured in RPMI 1640 medium (Mediatech) containing 4 mM glutamine, 2 g of sodium bicarbonate per liter, 50 U of penicillin per ml, 50 μg of streptomycin per ml, 10% fetal calf serum (FCS) (HyClone), and murine IL-3 (R&D, 0.9 ng/ml) (DA3) or human IL-2 (Chiron, 500 ng/ml) (CTLL). Viability was assessed by trypan blue exclusion. DA3 is a murine IL-3-dependent myeloid line (8); CTLL is a murine IL-2-dependent T-cell line (1). Neither expresses the endogenous EpoR (13, 31, 22). Cell were cotransfected by electroporation with 30 μg of linearized plasmids (pRK5mEpoR [containing the murine erythropoietin cDNA], pRK5hγ-like [containing the human TSLP-R cDNA], pRK5mEpoR/hγ-like with or without the C-terminal FLAG tag, pcDNA3 [Invitrogen] alone, or pcDNA3mEpoR/mγ-like) and 3 μg of linearized pGKhygro, which contains the *Escherichia coli* hygromycin B phosphotransferase gene. The transfected cells were subjected to selection with 1 mg of hygromycin B (Roche) per ml. Established mass cultures were cultured in medium without cytokine, with IL-3 (DA3) or IL-2 (CTLL), or with human Epo (Amgen, 3 U/ml), all with hygromycin, and tested for sustained growth for 3 weeks (DA3) or 6 weeks (CTLL). Both lines containing the human chimeric receptor grew well in Epo. Clones of DA3 chimeric receptor transfectants were produced by serial dilution of mass cultures growing in IL-3. Northern analysis was performed with a full-length murine EpoR cDNA (kindly supplied by Paul Ney).

Generation of TSLP-R-deficient mice. Overlapping genomic clones containing the *TSLP-R* locus were isolated from an embryonic day 14 (E14) embryonic stem cell genomic library by screening with [α-^{32}P]dCTP-labeled EST (GenBank accession no. AA008678) as a probe. A restriction enzyme map of the *TSLP-R* locus was determined using specific genomic clones and murine genomic DNA. A 15-kb fragment of *TSLP-R* genomic sequence containing all eight coding exons was subcloned into pBluescript. A fragment containing exons 3 and 4 was replaced with a cDNA encoding the neomycin resistance gene driven by the thymidine kinase promoter oriented in the direction opposite that of *TSLP-R* transcription. A cDNA encoding the diphtheria toxin gene to be used in negative selection was then placed 3′ of the *TSLP-R* homology region to complete the targeting construct. *TSLP-R*$^{-/-}$ mice were generated essentially as previously described (28). Briefly, 129/SvJ E14 embryonic stem cells were electroporated with 20 μg of linearized targeting vector and selected with 350 μg of Geneticin (Invitrogen) per ml. One thousand G418-resistant clones were isolated, expanded, and analyzed by Southern analysis for correct integration of the mutation, by probing with a *Bam*HI genomic fragment as illustrated below (see Fig. 3). Three clones heterozygous for the induced mutation were injected into C57BL/6 blastocysts, which were then implanted into pseudopregnant females. Male chimeras from one clone successfully passed the induced mutation through the germ line. Heterozygote agouti mice were interbred to produce wild-type and mutant littermates on a mixed 129/SvJ/C57BL/6 background. Genotype was determined by PCR using the following primers: for the wild-type allele, AAC AAATGATTAGGGACGGTACTTC and ACAGGAAATATCGCGGGCA GGGT (150 bp); for the knockout allele, AACAAATGATTAGGGACGGTA CTTC and ATGGCGATGCCTGCTTGCCGAATA (350 bp). Animals were housed under specific-pathogen-free conditions under institutional guidelines.

DNA and RNA analyses. Genomic DNA was isolated by lysing cells in buffer containing 100 mM Tris (pH 8.0), 5 mM EDTA, 0.2% sodium dodecyl sulfate, 200 mM NaCl, and 250 μg of proteinase K per ml. The cells were incubated for 12 h at 55°C, and DNA was extracted with phenol/chloroform and precipitated with ethanol. After restriction enzyme digestion, DNA was separated by agarose gel electrophoresis and transferred to positively charged nylon (Amersham) for

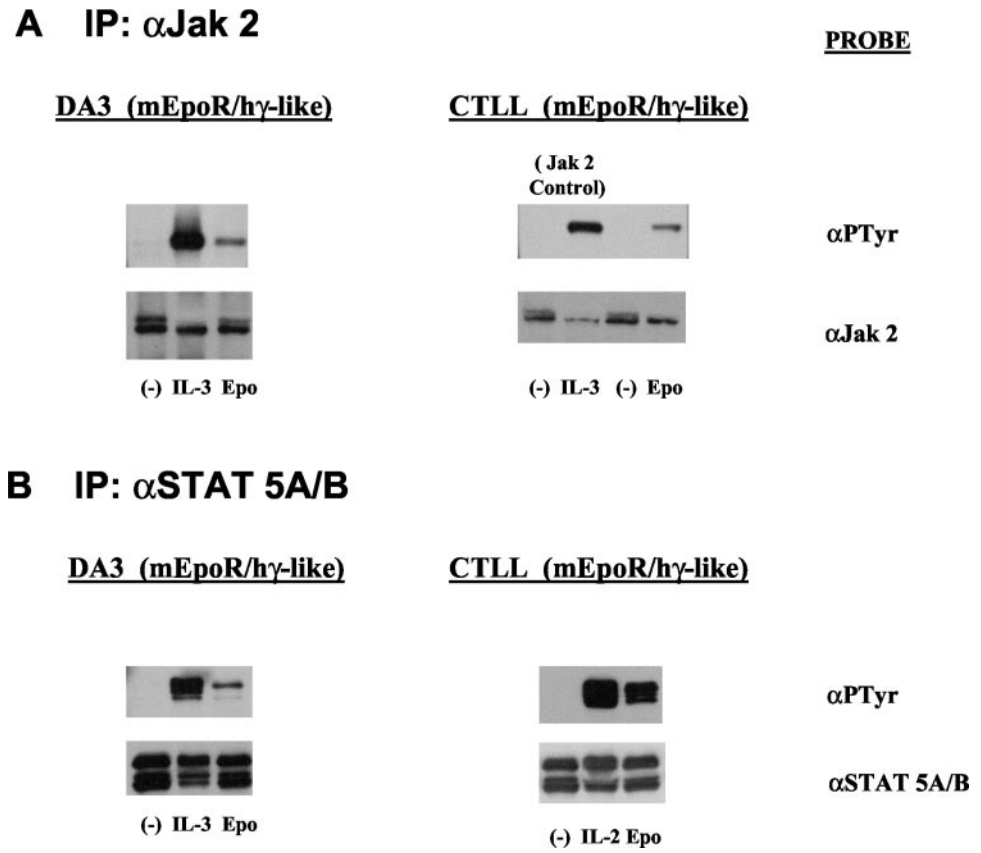


FIG. 2. Activation of Jak2 and Stat5 in transfected cell lines in response to Epo stimulation. DA3 and CTLL clones expressing the murine EpoR–human γ-like chimeric receptor (mEpoR/hγ-like) were used. Transfected cells were starved overnight and treated with the indicated stimuli or not treated (−). Lysates were prepared and subjected to immunoprecipitation (IP) with the anti-Jak2 antibody (αJak 2) and antibody against Stat5a and Stat5b (αSTAT 5A/B) shown. The activation of Jak2 (A) and Stat5 (B) was assessed by probing with antibodies to phosphotyrosine (αPTyr). Blots were stripped and reprobed with the indicated antibodies to assess protein levels.

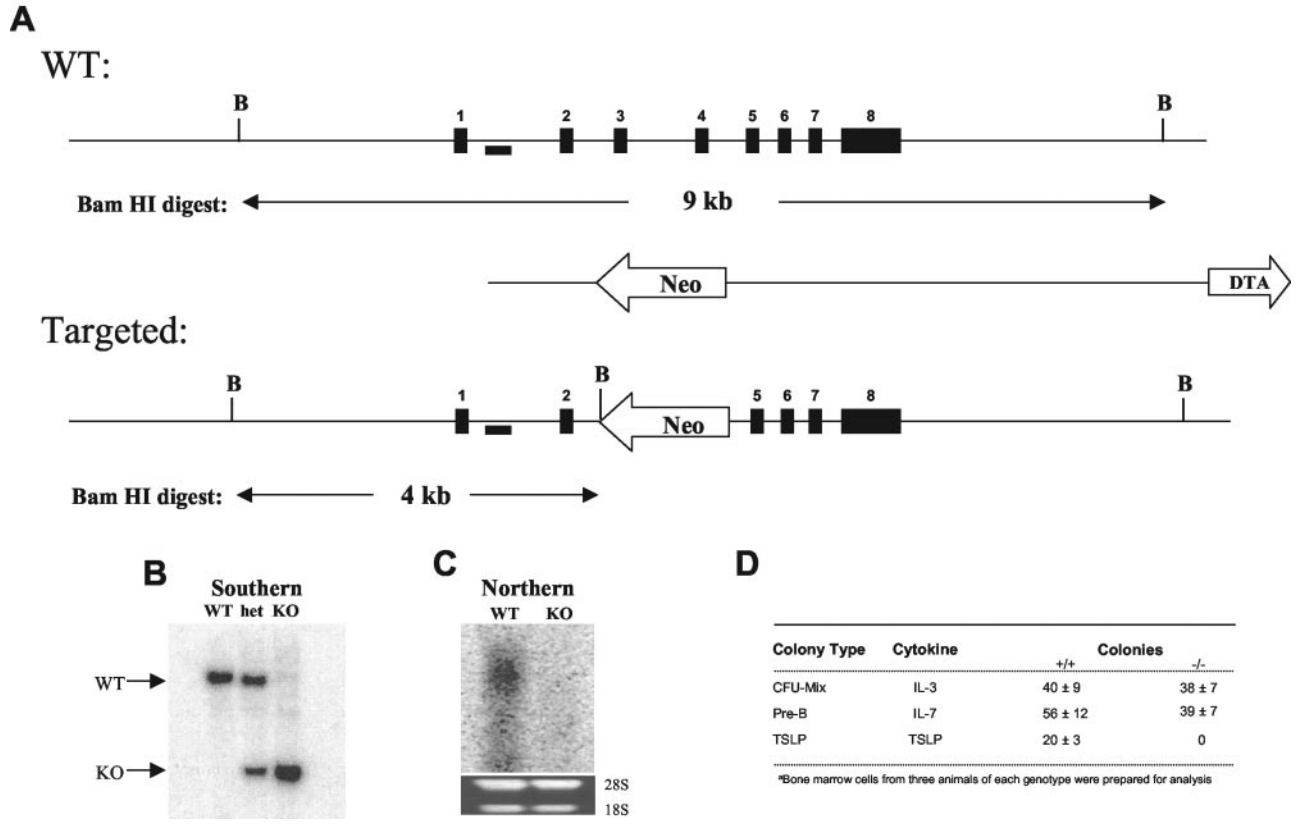
hybridization with labeled probes (Rediprime; Amersham) by standard techniques. RNA was prepared by extracting cells or tissue with Trizol (Invitrogen), separated by formaldehyde gel electrophoresis, and analyzed as described above.

Immunoprecipitations and Western blotting. Cells were washed three times in medium without serum, grown in medium without added cytokines overnight, and then cultured for 10 min (for Jak proteins) or 30 min (for Stat proteins) without cytokine or with IL-3 (9 ng/ml) or Epo (10 U/ml). Treated cells were centrifuged, lysed at 5×10^7 cells/ml in cold lysis buffer (50 mM Tris [pH 8.0], 0.1 mM EDTA, 0.1 mM sodium orthovanadate, 50 mM NaF, 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 1 mM dithiothreitol, 3 μg of aprotinin per ml, 2 μg of pepstatin A per ml, 1 μg of leupeptin per ml, 0.4 mM phenylmethylsulfonyl fluoride), vortexed, rotated at 4°C for 60 to 90 min, and centrifuged, with supernatants stored at −70°C. Aliquots (200 μl) were brought to 1 ml with immunoprecipitation buffer (10 mM Tris [pH 7.6], 5.0 mM EDTA, 0.1 mM sodium orthovanadate, 50 mM NaF, 50 mM NaCl, 30 mM sodium pyrophosphate, 1 mM dithiothreitol, 3 μg of aprotinin per ml, 2 μg of pepstatin A per ml, 1 μg of leupeptin per ml, 1.0 mM phenylmethylsulfonyl fluoride), to which preimmune serum or antiserum to Jak1, Jak2, Jak3, Tyk2, or Stat1 to Stat6, was added, followed by protein A-Sepharose CL-4B (Pharmacia). Beads were washed with an equal volume of 2× loading buffer (0.25 M Tris [pH 6.8], 20% glycerol, 0.142 M β-mercaptoethanol, 10% sodium dodecyl sulfate, bromophenol blue), boiled, and centrifuged, and the supernatants were loaded on 7.5% polyacrylamide gels for electrophoresis. Proteins were transferred to nylon filters by electroblotting and probed with antiphosphotyrosine antibody diluted in 20 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween 20, and 3% bovine serum albumin, followed by anti-Fc horseradish peroxidase-conjugated antibody (Amersham). Bands were visualized by using an ECL kit (Amersham). Filters were stripped and reprobed in the same manner with the immunoprecipitating antibodies for expression controls.

FACS analysis and cell purification. Whole spleens, thymi, or lymph nodes were dissected from littermates and forced through 70-μm-pore-size nylon mesh in phosphate-buffered saline containing 2% FCS. Bone marrow was obtained by flushing the femurs with the same buffer. Red blood cells were lysed by the addition of buffer (pH 7.2) containing 150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA, debris was removed by straining, and cells were labeled with fluorescein-conjugated antibodies. Routine fluorescence-activated cell sorting (FACS) analysis was performed with a FACSCalibur (Becton Dickinson). For proliferation assays, B and T cells were purified to >98% purity by labeling with fluorescein isothiocyanate-conjugated Thy1.2 antibody (Pharmingen) and phycoerythrin-conjugated B220 antibody (Pharmingen), and sorting with a MoFlo cell sorter (Cytomation). Alternatively, to obtain T cells (>90% pure) for proliferation assays, negative selection (utilizing a cocktail of phycoerythrin-conjugated anti-B220, anti-Gr-1, anti-CD11b, and anti-Ter119 antibodies) with an AutoMACs (Miltenyi Biotech) was employed.

Blood cell counts and colony assays. To analyze peripheral blood, samples were taken from the orbital sinus using heparinized microhematocrit capillary tubes (Fisher). Twenty microliters was analyzed by a MASCOT Hemavet 3700R counter (CDC Technologies), scoring for white blood cells and red blood cells. Hematocrits were calculated by a Hemavet counter based on red cell counts and mean corpuscular volume. To assay bone marrow progenitor colony-forming ability, bone marrow was flushed as described above, cells were plated, and colonies were scored as previously described (32).

Proliferation assays. B cells or T cells (10^5) were placed in individual wells of a round-bottom 96-well plate in RPMI 1640 medium containing 10% FCS, 10 mM HEPES (pH 7.0), 2 mM glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol, 0.1 mM nonessential amino acids, and 10 μg of gentamicin per ml. B-cell mitogens were at the following concentrations: immunoglobulin M (IgM), 10 μg/ml; IL-4, 100 ng/ml; anti-CD40, 1 μg/ml; lipopolysaccharide, 10 μg/ml. For



Colony Type	Cytokine	Colonies +/+	Colonies -/-
CFU-Mix	IL-3	40 ± 9	38 ± 7
Pre-B	IL-7	56 ± 12	39 ± 7
TSLP	TSLP	20 ± 3	0

[a]Bone marrow cells from three animals of each genotype were prepared for analysis

FIG. 3. γ-like chain targeting. (A) Structure of the γ-like chain targeting construct. The numbered black boxes represent exons 1 to 8. *Bam*HI (B) sites are indicated, as is the location of the probe utilized in Southern analysis to distinguish wild-type (WT) and targeted alleles (black bar). The position and direction of the neomycin resistance cassette (Neo) and diphtheria toxin cassette (DTA) (30) are shown. (B) Southern blot of *Bam*HI-digested genomic DNA from +/+ (wild type), +/− (heterozygote), and −/− (knockout [KO]) mice. Bands generated by the wild-type (WT), heterozygote (het), and targeted alleles are indicated. (C) Northern analysis of RNA isolated from +/+ (WT) or γ-like chain-deficient (KO) thymi. The blot was probed with a γ-like chain cDNA probe as indicated in Materials and Methods. The bottom blot shows the 28S and 18S RNAs as loading controls. (D) In vitro colony formation of bone marrow hematopoietic progenitors from wild-type (+/+) or γ-like chain-deficient (−/−) littermates. CFU-Mix, mixture of colonies.

T-cell proliferation assays, anti-CD3ε (catalog no. 145-2C11; Pharmingen) diluted in phosphate-buffered saline at various concentrations was used to coat the bottom of each well by incubation for 2 h at 37°C. Cells were also treated with phorbol myristate acetate (10 ng/ml) and ionomycin (125 ng/ml). Cells were cultured for 48 h, labeled with 1 μCi of [^{3}H]thymidine per well for 14 h and harvested with a TOMTECH Harvester 96 MachIII. Each assay was conducted in triplicate, and the data presented are representative of three individual experiments.

Immune response. Specific concentrations of IgM, IgG1, IgG2a, IgG2b, and IgG3 in serum were measured by coating Nunc Maxisorp 96-well plates (Thomas Scientific, Swedesboro, N.J.) with isotype-specific goat anti-mouse antibodies (Southern Biotechnology Associates, Birmingham, Ala.). Bound immunoglobulin from test sera was detected using alkaline phosphatase-conjugated goat anti-mouse isotype-specific antibodies (Southern Biotechnology), followed by alkaline phosphatase substrate (*p*-nitrophenyl phosphate; Sigma). The absorbance of the reaction mixture at a wavelength of 405 nm was quantitated using an enzyme-linked immunosorbent assay plate reader (Bio-Rad model 3550; Bio-Rad Laboratories). To assess hapten-specific responses after immunization, mice were immunized intraperitoneally with keyhole limpet hemocyanin-conjugated tri-nitrophenyl phosphate (100 μg/mouse) in complete Freund adjuvant at week zero and with tri-nitrophenyl phosphate-KLH in incomplete Freund adjuvant at weeks six and twelve. Serum immunoglobulin levels were determined as described previously (11).

RESULTS

The human γ-like chain, but not the murine γ-like chain, supports proliferation. In a screen to identify cytokine receptor-related genes, we identified a human cDNA (GenBank accession no. AF338733) with distant homology to the common γ chain. Other researchers independently identified the murine homologue of the human γ-like chain as a subunit of the TSLP-R4, 12, 15, 16). We utilized a murine EST (GenBank accession no. AA008678) representing a partial cDNA to screen a murine spleen cDNA library and isolate the full-length murine homologue (GenBank accession no. NM016715). To explore the function of the γ-like proteins, chimeric receptors in which the extracellular and transmembrane domains of the human and murine γ-like chains were replaced by those of the EpoR were generated (Fig. 1A). The chimeric receptors were transfected into IL-3-dependent DA3 myeloid cells or IL-2-dependent CTLL cells. Both DA3 cells and CTLL cells expressing the EpoR–human γ-like chimeric receptor proliferated in response to Epo (Fig. 1C). However, the EpoR–murine γ-like chimeric receptor was unable support proliferation, despite being more highly expressed in all individual clones tested (Fig. 1B). This suggests that the human and murine receptors function differently with respect to the activation of downstream signaling pathways that lead to cell proliferation.

The Jak-Stat pathway is a well-characterized signaling path-

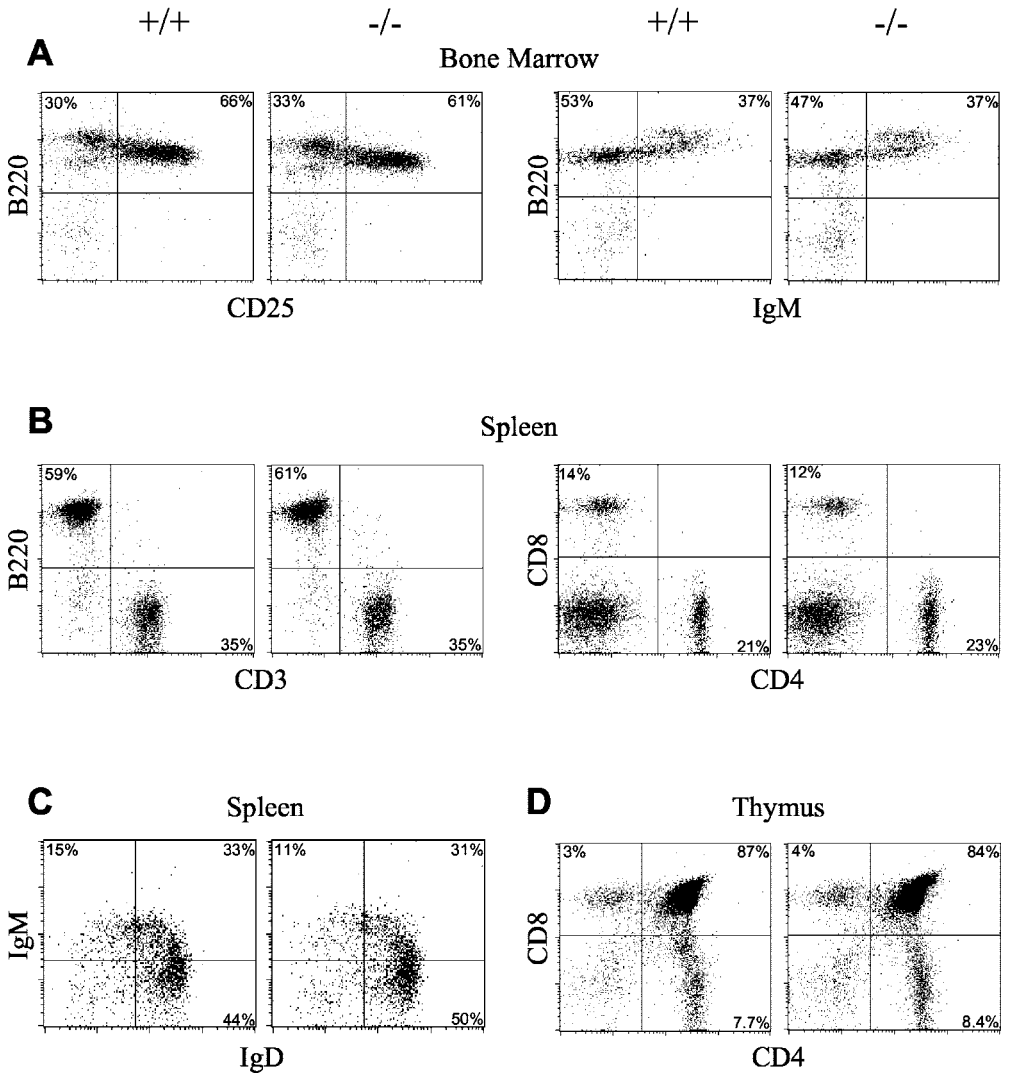


FIG. 4. Flow cytometric analysis of *TSLP-R*$^{+/+}$ (+/+) and *TSLP-R*$^{-/-}$ (−/−) lymphocytes. Calculated percentages are displayed within each quadrant. Representative FACS analyses of at least six mice of each genotype are shown. (A) To assess B-lymphocyte development, bone marrow lymphocytes from wild-type and TSLP-R-deficient mice were stained with fluorochrome-conjugated antibodies as indicated. (B) Splenocytes from wild-type and TSLP-R-deficient mice were stained with anti-B220 and anti-CD3 antibodies (left). Alternatively, splenocytes were stained with anti-CD4 and anti-CD8 antibodies. The CD4/CD8 profile of splenic T cells is displayed (right). (C) Splenocytes from *TSLP-R*$^{+/+}$ (+/+) or *TSLP-R*$^{-/-}$ (−/−) mice were stained with anti-IgD, anti-IgM, and anti-B220 antibodies. The IgM/IgD profile of splenocytes gated on B220^{+} cells is displayed. (D) Thymocytes from *TSLP-R*$^{+/+}$ or *TSLP-R*$^{-/-}$ mice were stained with anti-CD4 and anti-CD8 antibodies. The CD4/CD8 profile of gated thymocytes is displayed.

way that is activated by many cytokine receptors. The murine TSLP-R (mTSLP-R) has been reported to activate Stat5, although no Jak kinase has been shown to be involved in Stat5 activation by mTSLP-R (12, 10, 4). To assess whether the human γ-like chain utilized a Jak kinase, we examined Jak activation in DA3 and CTLL cells expressing the chimeric EpoR–human γ-like chain receptor. Epo strongly induced the tyrosine phosphorylation and activation of Jak2 but not of Jak3 in cells expressing the chimeric receptor (Fig. 2A and data not shown). Jak1 and Tyk2 were expressed below detectable levels in these cell lines. We also detected tyrosine phosphorylation and activation of Stat5a and Stat5b upon Epo stimulation (Fig. 2B). Stat1, Stat3, and Stat6 were not tyrosine phosphorylated upon Epo stimulation (data not shown), while Stat2 and Stat4 were not detected.

Derivation of γ-like chain-deficient mice. To explore the in vivo function of the murine γ-like gene, γ-like receptor-deficient mice were generated by gene targeting (Fig. 3A). A targeting construct that replaced the third and fourth exons of the murine receptor with a neomycin resistance gene cassette was developed. Embryonic stem clones containing properly integrated constructs were injected into mouse blastocysts. Chimeric mice were bred to obtain germ line transmission of the disrupted allele, and heterozygous mice were bred to obtain γ-like receptor-deficient mice. Transmission of the mutated allele was confirmed by Southern analysis (Fig. 3B). No

TABLE 1. Lymphocyte populations from wild-type and *TSLP-R*$^{-/-}$ mice[a]

Tissue and parameter	Mice	
	Wild-type	*TSLP-R*$^{-/-}$
Bone marrow (n = 8)		
No. of cells (10^6)	31.2 ± 4.9	31.4 ± 5.0
% B220^{+b}	80.1 ± 10	79.4 ± 6.7
% CD43^{+c}	5.3 ± 2.1	5.9 ± 3.1
% CD25^{+} IgM^{-c}	42.0 ± 2.8	38.6 ± 6.8
% CD25^{+} IgM^{+c}	14.3 ± 3.6	14.5 ± 3.8
% CD25^{-} IgM^{+c}	29.7 ± 3.7	31.1 ± 7.5
Spleen (n = 8)		
No. of cells (10^6)	65.4 ± 16.7	65.0 ± 11.0
% B220^{+b}	56.2 ± 4.3	65.9 ± 4.7
% IgM^{+} IgD^{-c}	25.9 ± 4.6	26.8 ± 2.7
% IgM^{+} IgD^{+c}	11.0 ± 1.3	14.0 ± 4.7
% IgM^{-} IgD^{+c}	48.1 ± 4.4	46.5 ± 2.7
% Thy 1.2^{+b}	40.3 ± 4.6	30.8 ± 4.5
% CD4^{+} CD8^{-d}	66.4 ± 2.1	68.0 ± 1.8
% CD4^{-} CD8^{+d}	29.5 ± 2.6	25.5 ± 1.5
Thymus (n = 8)		
No. of cells (10^6)	182 ± 60	189 ± 30
% CD4^{+} CD8^{-}	9.6 ± 0.8	8.2 ± 1.1
% CD4^{+} CD8^{+}	86.3 ± 1.6	87.9 ± 2.0
% CD4^{-} CD8^{+}	2.5 ± 0.5	2.5 ± 0.8

[a] Single-cell suspensions of cells prepared from the indicated tissues were analyzed by FACS with fluorochrome-conjugated antibodies
[b] Determined as a percentage of gated lymphocytes.
[c] Determined as a percentage of B220^{+} gated lymphocytes.
[d] Determined as a percentage of Thy1.2^{+} gated lymphocytes.

full-length or truncated γ-like transcripts were detected in homozygous mutant mice, demonstrating that the disrupted allele resulted in a null mutation (Fig. 3C). Although antibodies were not available to determine whether a partial receptor protein product was generated, a bone marrow colony assay was employed to determine γ-like receptor functionality in homozygous mutant mice. TSLP failed to induce colonies from bone marrow progenitor cells (Fig. 3D), confirming that the induced mutation of the γ-like chain gene resulted in functional disruption of the TSLP pathway.

Characterization of *TSLP-R*$^{-/-}$ mice. Interbreeding of *TSLP-R*$^{+/-}$ mice produced litters with the expected Mendelian ratio of *TSLP-R*$^{+/+}$, *TSLP-R*$^{+/-}$, and *TSLP-R*$^{-/-}$ mice (1:2:1). At a gross level, TSLP-R-deficient mice were indistinguishable from their wild-type and heterozygote littermates. They developed normally and did not succumb to any unusual pathological condition, and their life spans were comparable to those of wild-type mice. Histological examination of various organs revealed no differences between wild-type and TSLP-R-deficient mice. In particular, the architectures of the thymus, spleen, and lymph nodes were indistinguishable from those of wild-type mice. Because of the reported involvement of TSLP in hematopoiesis, various hematological parameters were examined. There were no significant differences between wild-type and mutant mice in the number of red cells, hemoglobin levels, or hematocrits. Similarly, there were no significant effects on the number and morphology of peripheral blood leukocytes, including neutrophils, eosinophils, basophils, monocytes, and lymphocytes. The recovery of bone marrow cells was normal, and in colony assays of bone marrow hematopoietic progenitors, there were no significant alterations in the frequency and morphology of colonies in response to a variety of cytokines (data not shown).

To assess whether loss of TSLP-R function affected the development of murine lymphocytes, we analyzed subpopulations of B cells and T cells by flow cytometry. Receptor-deficient mice displayed similar ratios of bone marrow pro-B cells (IgM^{-}, B220^{low}, CD43^{+}, and CD25^{-}), pre-B cells (IgM^{-}, B220^{+}, CD43^{-}, and CD25^{+}) or mature B cells (IgM^{+}, B220^{+}, CD43^{-}, and CD25^{-}) in the bone marrow B-lymphocyte populations (6) (Fig. 4A and Table 1). Splenic lymphocyte populations were also examined. In particular, the ratios of splenic B220^{+} B cells to Thy1.2^{+} T cells were comparable in wild-type and knockout mice (Fig. 4B, left), although γ-like receptor-deficient mice tended to display a mild increase in the levels of B220^{+} B lymphocytes relative to wild-type mice, with a concomitant mild decrease in the levels of Thy1.2^{+} T lymphocytes (Table 1). The CD4^{+}/CD8^{+} ratios of splenic T cells (Fig. 4B, right) and IgM^{+}/IgD^{+} ratios of splenic B cells (Fig. 4C) were also similar in wild-type and receptor-deficient mice. Finally,

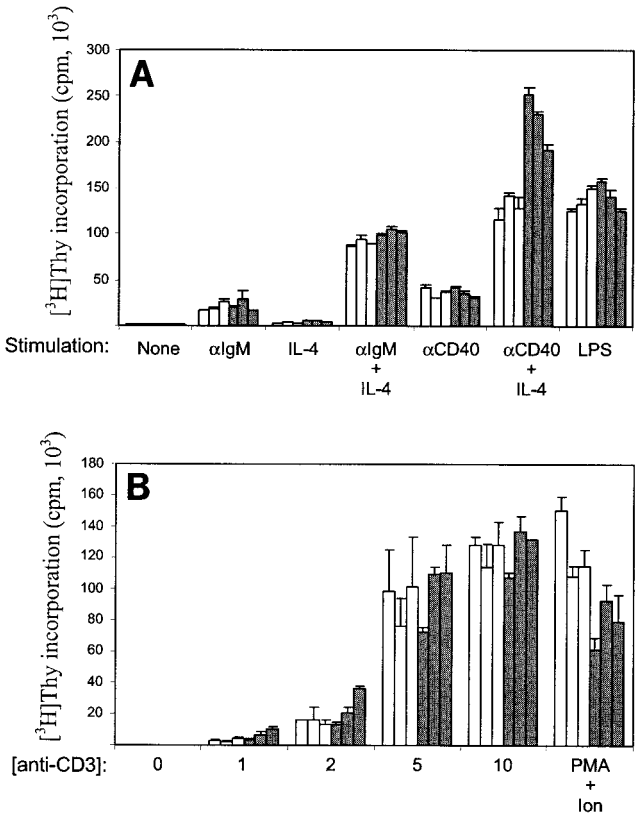


FIG. 5. Lymphocyte proliferation assays. (A) FACS-purified splenic B cells were cultured in the presence of the indicated stimuli (αIgM, anti-IgM; LPS, lipopolysaccharide) for 48 h. Cell proliferation was measured by adding 1 μCi of [^{3}H]thymidine ([^{3}H]Thy) to the cultures. The means ± standard deviations (error bars) for three samples are shown. The data are from one representative experiment of three experiments. (B) FACS-purified splenic T cells were cultured in the presence of the indicated stimuli for 48 h. The cells were stimulated with the indicated concentrations (in micrograms per milliliter) of anti-CD3 antibody or with phorbol myristate acetate and ionomycin (PMA + Ion). Cell proliferation was measured as in panel A.

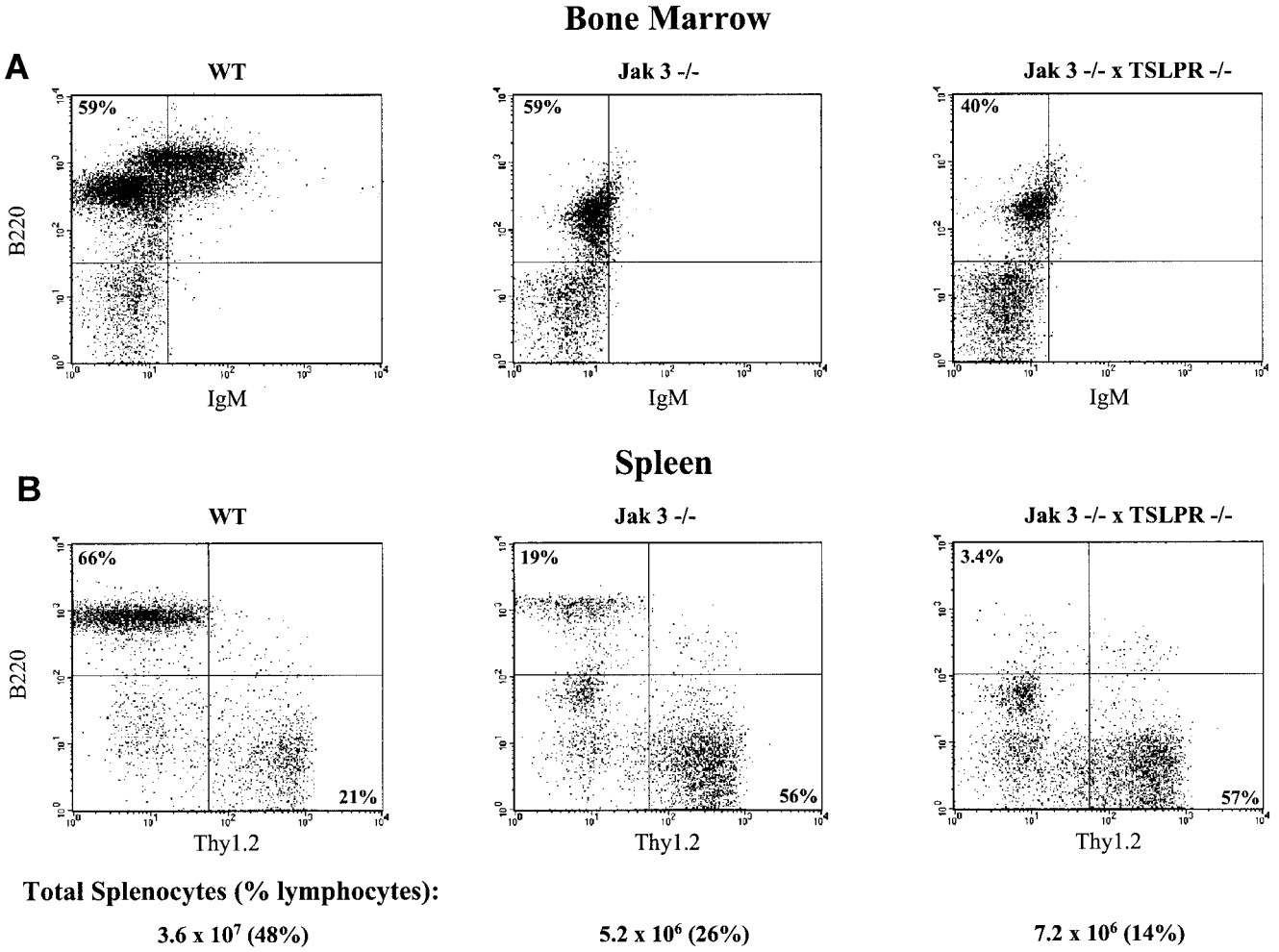


FIG. 6. Flow cytometric analysis of wild-type (WT), *Jak3*$^{-/-}$, and *Jak3*$^{-/-}$ × *TSLP-R*$^{-/-}$ lymphocytes. Calculated percentages are displayed within each quadrant. Representative FACS analyses of at least four mice of each genotype are shown. (A) To assess B-lymphocyte development, bone marrow lymphocytes from wild-type (WT), *Jak3*$^{-/-}$, and *Jak3*$^{-/-}$ × *TSLP-R*$^{-/-}$ mice were stained with fluorochrome-conjugated antibodies as indicated. (B) Splenocytes from wild-type (WT), *Jak3*$^{-/-}$, and *Jak3*$^{-/-}$ × *TSLP-R*$^{-/-}$ mice were stained with antibodies as indicated. Total numbers of splenocytes and percentages of gated lymphocytes for each sample are indicated below plots.

flow cytometric analysis of developing thymocytes revealed no difference in the numbers and proportions of double-negative ($CD4^-$ $CD8^-$), double-positive ($CD4^+$ $CD8^+$), and single-positive ($CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$) thymocytes (Fig. 4D), suggesting that T-cell development was unaffected by TSLP-R deficiency. Splenic natural killer cells ($CD3^-$ $NK1.1^+$) were also unaffected in the knockout, as were splenic granulocytes and monocytes ($CD11b^+$ $CD11c^+$) (data not shown).

The proliferative ability of peripheral lymphocytes isolated from wild-type or TSLP-R-deficient mice was also assessed. B cells were purified by FACS and cultured in the presence of various mitogenic agents. Figure 5A demonstrates the absence of a significant difference between the ability of wild-type and TSLP-R-deficient B cells to respond to different stimuli. Comparable results were obtained with purified peripheral T cells (Fig. 5B). Serum immunoglobulin levels in unimmunized mice were comparable in wild-type and *TSLP-R* knockout mice, as were hapten-specific immunoglobulin levels in immunized mice.

TSLP supports development of Jak3-independent $B220^+$ cells in vivo. Mice deficient in IL-7 or the IL-7 receptor exhibit a profound defect in B lymphopoiesis (18, 29). A similar defect is observed in mice lacking Jak3, although *Jak3*$^{-/-}$ mice exhibit a residual splenic $B220^+$ lymphoid population (14, 26, 17). To test whether this latter population was TSLP-R dependent, we crossed *TSLP-R*$^{-/-}$ mice with mice deficient in Jak3. Introduction of the TSLP-R mutation onto a *Jak3*$^{-/-}$ background had no measurable effect on the distribution of B lymphocytes in the bone marrow or on the total numbers of B and T cells remaining in these mice. Indeed, the only difference detected in double-knockout mice was a consistent loss of the residual $B220^+$ lymphocyte population found in *Jak3*$^{-/-}$ spleens (Fig. 6).

DISCUSSION

One important approach to defining gene function is to determine the consequences of eliminating the gene in the general context of mouse development. As illustrated here, we were able to develop a strain of mice in which the γ-like subunit of the mTSLP-R contained an internal deletion that resulted in a null phenotype. Characterization of these mice

has failed to identify any phenotypic characteristics that are different from those of wild-type mice.

Early studies demonstrated that cells growing in fetal liver and bone marrow cultures in response to TSLP display B-lineage, but not myeloid, markers, and that TSLP promotes the development of IgM^+ immature B cells from pre-B cells (3, 12, 20, 23). It was also shown that TSLP costimulates proliferation of thymocytes in vitro (3, 23). In neonatal mice, TSLP injection increases the populations of both $B220^+$ $BP\text{-}1^+$ pre-B cells and $B220^+$ IgM^+ immature B cells as effectively as IL-7 (23). Overexpression of TSLP in transgenic mice also produces a polyclonal mixed cryoglobulinemia, indicating that the cytokine can promote immunoglobulin production in vivo (25). Notwithstanding these data, we were unable to demonstrate any significant effect on B-cell maturation or T-cell development due to the removal of the TSLP-R alone. A reduction in peripheral $B220^+$ cells was observed, but only against a background in which the IL-7 pathway was effectively removed by the loss of Jak3. No significant changes in the number of IgM^+ $B220^+$ cells or in humoral antibody production were observed. Our results suggest that, despite the ability of TSLP to support B-cell maturation in vitro, the cytokine plays only a minor supporting role in murine lymphoid development in vivo, especially compared to IL-7. We favor the notion that TSLP is one of many factors that cooperate to modulate the size of the B-cell compartment in vivo, such that in the absence of functional TSLP-R, a variety of homeostatic mechanisms could ensure normal levels of B-lymphocyte production.

Published reports (4, 10, 12) concerning the mTSLP-R indicate that it phosphorylates Stat5 without activating any of the Jak kinases, although the box 1 region can substitute for that of the EpoR in activating Jak2 (4). The available evidence also suggests that the murine receptor cannot function as a homodimer (4, 15). Consistent with these reports, our EpoR–mTSLP-R chimeric receptor failed to support proliferation in transfected cell lines. In contrast to the mouse chimeric receptor, the EpoR–human TSLP-R did support proliferation in response to Epo, indicating that the human cytoplasmic domain can work as a homodimer. In addition, the human chimeric receptor activates both Jak2 and Stat5, further distinguishing it from its murine counterpart.

Human TSLP is produced by epithelial cells and keratinocytes and is emerging as an important trigger of allergic T-cell responses (5, 24). The human TSLP-R is expressed together with the IL-7 receptor α chain primarily in myeloid cells; it activates $CD11c^+$ dendritic cells and monocytes, but not other lymphoid or myeloid target cells. In mice, however, dendritic cells are unresponsive to TSLP (21, 24), and there are no reports of responses in myeloid cells. Consistent with this, we detected no changes in dendritic or other myeloid cell populations in TSLP-$R^{-/-}$ mice. However, mice that express a TSLP transgene from the *c-Fes* promoter develop splenomegaly and lymphadenopathy, with increased cell population predominantly myeloid in nature (B. Zhou and S. F. Ziegler, unpublished data). In summary, although the evidence from initial studies suggested a role for TSLP in murine B lymphopoiesis, its in vivo function remains to be fully elucidated.

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