

# Impaired Bone Marrow Microenvironment and Immune Function in T Cell Protein Tyrosine Phosphatase-deficient Mice

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## Summary

The T cell protein tyrosine phosphatase (TC-PTP) is one of the most abundant mammalian tyrosine phosphatases in hematopoietic cells; however, its role in hematopoietic cell function remains unknown. In this report, we investigated the physiological function(s) of TC-PTP by generating TC-PTP-deficient mutant mice. The three genotypes (+/+, +/-, -/-) showed mendelian segregation at birth (1:2:1) demonstrating that the absence of TC-PTP was not lethal in utero, but all homozygous mutant mice died by 3–5 wk of age, displaying runting, splenomegaly, and lymphadenopathy. Homozygous mice exhibited specific defects in bone marrow (BM), B cell lymphopoiesis, and erythropoiesis, as well as impaired T and B cell functions. However, myeloid and macrophage development in the BM and T cell development in the thymus were not significantly affected. BM transplantation experiments showed that hematopoietic failure in TC-PTP -/- animals was not due to a stem cell defect, but rather to a stromal cell deficiency. This study demonstrates that TC-PTP plays a significant role in both hematopoiesis and immune function.

Protein tyrosine phosphorylation is regulated by the two antithetic gene families, protein tyrosine kinases (PTKs)<sup>1</sup> and protein tyrosine phosphatases (PTPs). These have been found in most signaling pathways and, in particular, in those occurring in immature as well as completely differentiated lymphoid cells involved in processes such as T cell receptor activation and secretion of cytokines by the stromal cells of the bone marrow (BM; reference 1). Protein tyrosine phosphorylation acts as a delicate switch modulating protein-protein interactions that are crucial for the immune system function. Several members of these two gene families have been directly implicated in lymphoid cell signaling. For example, the tyrosine kinases Lck, Fyn, Csk, and c-abl are essential for normal T and B cell activation and maturation (2–6). Conversely, the expression of several PTP members has been noted in lymphoid cells. Thus far, two PTPs have been directly associated with immune system functions: CD45, a receptor phosphatase, and the SH2 containing PTP, SHP-1 (7, 8). Gene targeting of the murine CD45 gene demonstrated a positive role for CD45 in

thymocytes and B cell development, in part through its action on the Lck kinase (9, 10). Furthermore, mutations in the SHP-1 PTPase cause severe autoimmunity and widespread hematopoietic failure in the motheaten (me) mouse mutant, demonstrating the importance of tyrosine dephosphorylation in the proper development and maintenance of a functional immune system (11, 12).

The mammalian T cell PTP (TC-PTP) was one of the first members of the PTP gene family identified. Although originally cloned from a T cell cDNA library, it is ubiquitously expressed at all stages of mammalian development and in most embryonic and adult tissues (13, 14). Yet, TC-PTP is found in higher amounts in lymphoid cell lineages suggesting that in addition to CD45 and SHP-1, TC-PTP could play an important role in immunity. Two major forms of human TC-PTP, designated TC-PTPa and TC-PTPb, are generated by alternative splicing at the 3' end of the gene (14, 15). These two forms, also referred to as PTP-S2 and PTP-S4 (16), differ in their COOH termini with TC-PTPa containing 382 amino acids (aa) that includes a unique 6 aa COOH tail end, PRLTDT, and TC-PTPb possessing a 34 aa hydrophobic tail. Immunofluorescence studies localize the TC-PTPa form to the nucleus and the TC-PTPb form to the endoplasmic reticulum (16–19). Importantly, the TC-PTPb levels vary among cell types and

<sup>1</sup>Abbreviations used in this paper: aa, amino acid; BM, bone marrow; ES, embryonic stem; nc, nucleotide; neo, neomycin; PFC, plaque-forming cell; PTP, protein tyrosine phosphatase; PTK, protein tyrosine kinase; SRBC, sheep RBC; TC-PTP, T cell PTP.

mammalian species, and it is generally the least abundant of the two forms where it represents a fraction of TC-PTP message in mice (14) and in rats and humans (16).

Expression of TC-PTPb in baby hamster kidney cells demonstrated that it is associated with the particulate cellular fraction, and is inactive in an in vitro assay unless it is first subjected to limited trypsinization (20). These results were further substantiated by the work of Zander et al. (21) who reported on the differential enzymatic activities and substrate specificities of the full-length and truncated forms of the TC-PTPb enzyme in which the COOH-terminal hydrophobic region is deleted. A recent report by Kamatkar et al. (16) also supported these studies and, in addition, showed that only the TC-PTPa form can bind nonspecifically to DNA in vitro. Thus, the hydrophobic COOH-terminal portion of human TC-PTP appears to regulate substrate specificity, enzymatic activity, and subcellular localization. Tillman et al. showed that the steady state levels of murine TC-PTPa messenger RNA fluctuate in a cell cycle-specific manner, with levels remaining low in all stages of the cell cycle except in late G1 where expression increases (18). In support of a function for TC-PTP in the cell cycle, Gould et al. demonstrated that human TC-PTPb can complement, at the nonpermissive temperature, a thermosensitive mutant of the dual-specific PTP p80cdc25 in *Schizosaccharomyces pombe* (22).

Since the physiological substrate(s) of either form of TC-PTP remains unknown and given the potential artefactual phenotypes caused by dephosphorylation of nonspecific substrates in transfected cells, we generated null mutant mice for TC-PTP using gene targeting technology in an attempt to further characterize its function in vivo. All homozygous null TC-PTP mutant animals appeared normal for the first 2 wk, exhibited severe weight loss after those 2 wk, and died by 5 wk after birth. Examination of lymphoid-derived cells indicated that TC-PTP-deficient mice displayed deficient T and B cell proliferative responses and a defect in B cell ontogeny and erythroid development in the BM. In addition, BM transfer studies demonstrated that the failure of hematopoiesis was not due to stem cell dysfunction, but rather to an inability of stromal cells to support normal hematopoiesis.

## Materials and Methods

**Genomic Library Screening and DNA Probe Preparation.** A  $\lambda$  DASH II<sup>TM</sup> (Stratagene Corp., La Jolla, CA) phage genomic library derived from 129/sv mouse female kidney DNA (a gift of Dr. Janet Rossant, Mt. Sinai Hospital, Toronto, Canada) was screened using plaque hybridization techniques as previously described (23) and a <sup>32</sup>P-labeled TC-PTP cDNA as probe. Plasmid DNA was prepared by alkali lysis and polyethylene glycol precipitation as previously described (24). DNA probes used in library screening, phage DNA mapping, and Southern and Northern hybridization analyses were obtained by restriction enzyme digestion of plasmid DNA followed by agarose gel electrophoresis and GeneClean<sup>TM</sup> (BIO/101, Vista, CA) purification of the excised DNA fragments. The DNA fragments were labeled with  $\alpha$ -[<sup>32</sup>P]dCTP (New England Nuclear, Boston, MA) using random primers and T7 DNA polymerase (T7 Quick-Prime<sup>TM</sup>; Pharmacia, Piscataway,

NJ). Removal of unincorporated nucleotides was achieved by Sephadex<sup>TM</sup> G-50 (Pharmacia) spin chromatography.

**Phage Mapping and Targeting Vector Construction.** The intron/exon boundaries of genomic clones were determined by Southern mapping and DNA sequencing. Dideoxy sequencing reactions were performed using  $\alpha$ -[<sup>35</sup>S]dATP (New England Nuclear) and Sequenase<sup>TM</sup> version 2.0 (Amersham Corp., Arlington Heights, IL) as stated in the manufacturer's instructions. The TC-PTP targeting construct as shown in Fig. 1 A was generated by subcloning, into pBluescript<sup>TM</sup> II KS(+) (Stratagene Corp.), the neomycin (neo) resistance gene cassette flanked by TC-PTP genomic sequences. pMC1neoPolyA (Stratagene Corp.) contains a 1-kb cassette with the neomycin resistance gene under the control of the Herpes simplex thymidine kinase gene promoter. The plasmid was digested with XhoI, the overhangs were blunt ended with deoxyribonucleotides (Pharmacia) and the Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA), and then digested with HindIII. The 1.1-kb fragment was gel purified and ligated using T4 DNA ligase (New England Biolabs) to pBluescript<sup>TM</sup> II KS(+) that had been digested with PstI, rendered blunt ended as described above, and digested with HindIII, generating the plasmid BSNEO.

A 3.1-kb genomic (NotI/ScaI) fragment, corresponding to the 3' end of an intron and the first 29 bp of the exon corresponding to nucleotide (nc) 226–328 and aa 54–63 in TC-PTP cDNA, was gel purified and ligated to NotI/SmaI-digested BSNEO as described above, generating the plasmid BSNEOA. The NotI site is from the polylinker sequence of the plasmid which contained subcloned genomic sequences. A 2.5-kb genomic HindIII fragment that included two exons (nc 429–771, aa 121–235 including the IHCSAGI domain in TC-PTP cDNA) was gel purified, rendered blunt ended, and ligated to XhoI/blunt ended BSNEOA, generating the final TC-PTP-targeting construct. The construct DNA was prepared by alkali lysis and polyethylene glycol precipitation as previously described (24) and linearized with NotI before electroporation.

**Embryonic Stem Cells, Electroporation, and Selection.** Techniques used were essentially as described elsewhere (25, 26). In brief, the 129/sv embryonic stem (ES) cell line J1 (27) was expanded on mitomycin C (10  $\mu$ g/ml)-treated mouse embryonic (BALB/c) fibroblasts (28). DMEM (28) was supplemented with leukemia inhibitory factor at 1,000 U/ml (ESGRO; GIBCO BRL, Gaithersburg, MD). Trypsinized cells ( $1\text{--}2 \times 10^7$ ) were washed and resuspended in 1 ml of  $1 \times$  PBS, mixed with 50  $\mu$ g of linearized plasmid DNA, and electroporated using a gene pulser (240 V, 500  $\mu$ F; Bio Rad Labs., Hercules, CA). After electroporation, ES cells were plated at a density of  $1.5\text{--}3 \times 10^5$  cells/60-mm plate on BALB/c-NEO<sup>r</sup> feeders, and 24 h later, selection was applied with DMEM containing G418 (400  $\mu$ g/ml powder; GIBCO BRL). 10–12 d after transfection, individual colonies were transferred to 96-well plates, expanded for freezing, and DNA extraction as described elsewhere (28).

**Generation of Chimeric and Heterozygous Mice.** BALB/c blastocysts were injected with 10–15 heterozygously targeted ES cells and transferred into appropriate foster mothers. Chimeric males were mated with normal BALB/c females, and genotyping of heterozygous animals was performed by Southern blot analysis.

**Southern Blotting Analysis of ES Cell and Mouse Genomic DNA.** DNA from individual ES cell colonies grown in 96-well plates and genomic DNA (10–50  $\mu$ g) isolated from mouse tails or from cells of the LN, thymus, or spleen were digested to completion with 20 U of NdeI and analyzed by Southern blotting as previously described (24). Following UV cross-linking, the membrane

was prehybridized and hybridized in hybridization solution (0.5 M  $\text{NaH}_2\text{PO}_4$ , pH 7.0, 1 mM EDTA, 7% SDS, 0.5% BSA; reference 29) in the presence of a  $^{32}\text{P}$ -labeled probe lying outside of the targeting vector but present in a novel restriction fragment length polymorphism (probe A, Fig. 1 A). Membranes were washed at  $65^\circ\text{C}$  in  $2\times$  SSC/0.1%SDS for 30 min (twice) and in  $0.2\times$  SSC/0.1% SDS for 30 min (twice), and were exposed to x-ray film (XAR-5; Kodak, Rochester, NY) with intensifying screens at  $-80^\circ\text{C}$  for several days.

**Northern Blotting Analysis.** RNA for northern analysis was extracted from wild-type (wt) and TC-PTP  $-/-$  spleen using the TRIzol<sup>TM</sup> reagent (GIBCO BRL) as previously described (30). 30  $\mu\text{g}$  of total RNA was electrophoresed on a 1% agarose gel containing formaldehyde (24). The gel was capillary transferred onto nylon membrane (Hybond-N+<sup>TM</sup>, Amersham Corp.). A  $^{32}\text{P}$ -labeled DNA fragment corresponding to the entire cDNA of TC-PTP was used as a probe. Hybridization and washing conditions were performed as described for Southern analysis. After exposure, the membrane was stripped (0.5% SDS at  $100^\circ\text{C}$  for 20 min, twice) and reprobed with a [ $^{32}\text{P}$ ] cDNA fragment derived from rat glyceraldehyde 3-phosphate dehydrogenase gene which served as a loading control (31).

**Western Blotting Analysis.** Spleen samples from wt or TC-PTP  $-/-$  mice were prepared in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.25% sodium deoxycholate, 1% NP-40, and 1 mM EGTA, and supplemented with a protease inhibitor mixture, Complete<sup>TM</sup>, Boehringer Mannheim, Indianapolis, IN). Equivalent protein amounts dissolved in SDS-PAGE sample buffer were subjected to SDS-PAGE on a 10% polyacrylamide gel. Gel was transferred onto polyvinylidene difluoride membranes by electrotransfer, and blots were processed for chemiluminescence detection as per manufacturer's recommendations (New England Nuclear, Boston, MA). Anti-TC-PTP monoclonal Ab 3E2 was used as the primary antibody (1:50 dilution of conditioned hybridoma culture media) followed by an anti-mouse IgG antibody conjugated to horseradish peroxidase (Amersham Corp.).

**Flow Cytometry.** On different days after birth, mice were killed and spleen, LN, and thymus were removed for preparation of cell suspensions in HBSS (GIBCO BRL) as previously described (32). BM cell suspension was prepared by aspirating the marrow of the tibia and femur. One million cells were labeled with the appropriate reagents and fixed in 1 ml of 1% paraformaldehyde as previously described (32). Fixed samples were analyzed by a FACScan<sup>®</sup> (Becton Dickinson, San Jose, CA), and 5,000 flow cytometry events were counted per sample. A selective gate, excluding cell debris, was set by using forward light scatter.

**Reagents for Flow Cytometry.** FITC-conjugated anti-mouse CD8 (clone YTS 169.4), PE-conjugated anti-mouse CD4 (L3/T4 clone YTS191.1), FITC-conjugated anti-sIgM, PE-conjugated anti-mouse B220 (CD45R, Ly5, clone RA3-6B2), PE-conjugated anti-mouse Gr1 (clone RB6-8C5, rat Ig<sub>2b</sub> isotype; Pharmingen, San Diego, CA), anti-F4/80 (gift from Dr. M.G. Baines, McGill University, Montreal, Canada), and the corresponding secondary antibodies were used for double immunofluorescence. All monoclonal antibodies were purchased from Cedarlane Labs. Ltd. (Hornby, Canada) unless otherwise stated. All reagents were diluted and used as recommended.

**Mitogen Assays.** Splenic T cell proliferative responses to Con A (Pharmacia) and B cell responses to LPS (Sigma Chemical Co., St. Louis, MO) were evaluated by [ $^3\text{H}$ ]thymidine incorporation, and quantitated on a beta counter (LKB Instrs., Uppsala Sweden) as previously described (32).

**Histological Analysis.** The spleen and axillary and brachial LNs

were carefully removed from each mouse and fixed in 4% formalin. The fixed tissues were embedded in paraffin, sectioned, stained with hematoxylin-eosin, and examined by light microscopy. The femurs from each mouse were decalcified before embedding and sectioning.

**Direct Plaque-forming Cell Assay.** Mice were immunized with a single intravenous injection of  $5 \times 10^8$  sheep red blood cells (SRBCs) in a volume of 0.3 ml. 4 d later, the direct splenic PFC response to SRBCs was assayed by the method of Cunningham and Szenberg (33) as modified previously (34).

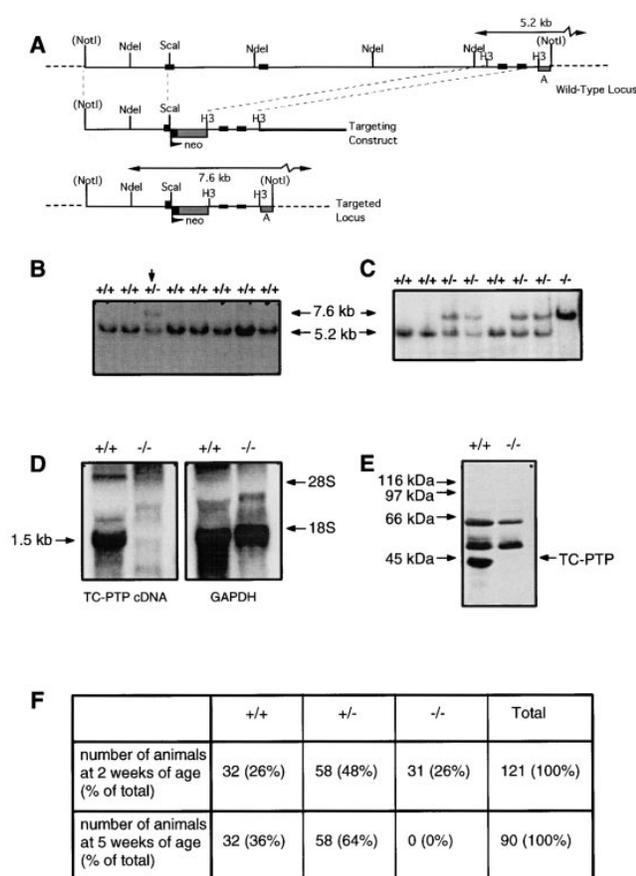
**Hematocrit Level.** Blood was collected from the retroorbital sinus of mice into two heparinized capillary tubes. The capillary tubes were centrifuged to determine the hematocrit.

**BM Reconstitution Experiments.** BM transplantation were performed in 129/sv inbred mice to avoid the complications of allograft rejection and graft-versus-host disease (32, 34). Inbred mice were generated by mating 129/sv female mice with chimeric male mice which gave rise to germ line offsprings. Since the ES cells used were also 129/sv, all the resulting offsprings would therefore be of 129/sv background. 3-wk-old wt and 2-wk-old TC-PTP  $-/-$  recipients (129/sv strain) were exposed to 900 rads of  $\gamma$  irradiation 18 h before a single intravenous injection of  $1.5 \times 10^7$  wt (3-wk-old) or TC-PTP  $-/-$  (10–12 d old) BM cells. Irradiation controls received HBSS without BM cells.

## Results

**Targeted Disruption of TC-PTP.** To isolate genomic sequences of the TC-PTP locus, a lambda phage mouse genomic DNA library was screened using TC-PTP cDNA as probe (see Materials and Methods). One phage clone with an  $\sim 17$ -kb insert was isolated with four exons mapped as shown in Fig. 1 A. The four exons correspond to nc and aa numbers, respectively, of TC-PTP cDNA (14) as follows: nc 227–328/aa 54–88, nc 329–428/aa 88–121, nc 429–561/aa 121–165, and nc 562–771/aa 166–235. The IHC-SAGI domain is within the fourth exon of this 17-kb fragment. The relative intron positions within the PTPase domains of TC-PTP and other transmembrane protein tyrosine phosphatases were published by Charest et al. (23).

To inactivate the TC-PTP gene, a targeting vector was constructed that contained 5.5 kb of TC-PTP genomic sequences flanking the neo resistance gene, as depicted in Fig. 1 A. After proper homologous recombination events,  $\sim 9$  kb of genomic sequence, including 1.5 exons encoding aa 64–121 inclusively, were eliminated from the TC-PTP locus, in effect rendering the gene product enzymatically nonfunctional. Standard ES cell techniques using the J1 parental ES cell line, were used (see Materials and Methods) to generate ES cell lines harboring a heterozygously inactivated TC-PTP gene locus. Approximately 1,200 NEO<sup>r</sup> ES cell clones were tested by Southern analysis for proper recombination events. Seven clones were positive for the desired recombination event, as shown in Fig. 1 B by Southern analysis using a probe lying outside the targeting vector (probe A; Fig. 1 A) that hybridizes to a 5.2-kb NdeI fragment representing the wt locus and a 7.6-kb NdeI fragment representing the targeted locus. ES derived from two independent clones were used for blastocyst injections.

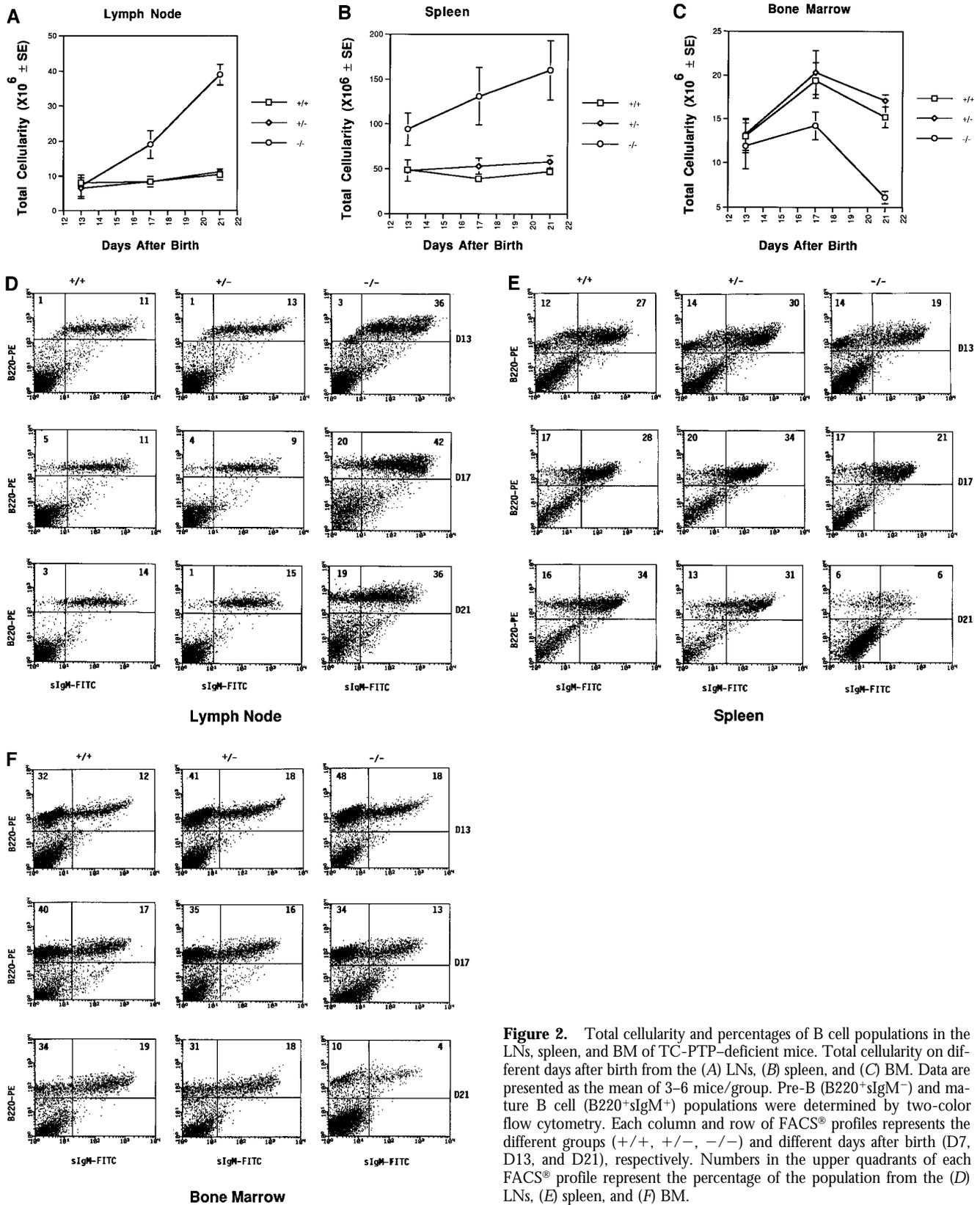


**Figure 1.** Generation of TC-PTP-deficient mutant mice by gene targeting. (A) Targeting construct of TC-PTP. Diagram of wt TC-PTP genomic locus encompassing four exons (nc 227–771, aa 54–235, in TC-PTP cDNA; reference 14). Probe A hybridizes to a 5.2-kb fragment generated by NdeI digestion. The targeting construct using a 5.5-kb genomic sequence with the neo resistance gene eliminates ~9 kb of genomic sequence including 1.5 exons. The resulting targeted locus after a correct homologous recombination event is shown. In the targeted allele, probe A hybridizes to a 7.6-kb fragment generated by NdeI digestion. *H3*, Hind-III. The thick line in the targeting construct diagram represents pBlue-script™ IIKS(+) backbone sequences. (B) Southern blot analysis of representative ES cell clones after electroporation. For each clone, DNA was extracted from one well of a 96-well plate and digested NdeI (+/+; wt; +/-, heterozygously targeted clone [arrow]). Hybridization was with probe A. Targeted allele and normal allele correspond to the 7.6-kb and 5.2-kb fragments, respectively. (C) Southern blot analysis of mouse tail DNA from progeny of heterozygous mouse matings. After DNA extraction, 50  $\mu$ g of tail DNA were digested with NdeI (+/+; wt; +/-, heterozygotes; -/-, homozygotes). Hybridization was with probe A. Targeted allele and normal allele correspond to the 7.6-kb and 5.2-kb fragments, respectively. (D) Northern blot analysis of total RNA extracted from spleen of wt (+/+) and homozygous (-/-) mice. Wt TC-PTP message was detected as a 1.5-kb transcript using TC-PTP cDNA as a probe. The same blot was stripped and reprobed with a glyceraldehyde 3-phosphate dehydrogenase probe to assess the level of loading. (E) Western blot analysis of total cellular protein extracted from spleen of wt (+/+) and homozygous (-/-) mice using the anti TC-PTP monoclonal antibody 3E2. TC-PTP migrates at 45 kDa. Other bands represent immunoglobulin chain subunits that are recognized by the secondary goat anti-mouse horseradish peroxidase conjugated antibody. (F) Table of progeny survival in littermates from the crossing of heterozygous mice for the TC-PTP disruption.

**Lethality of TC-PTP Disrupted Gene.** Mating of TC-PTP +/- mice gave rise to offsprings that followed Mendelian frequencies suggesting that the TC-PTP +/- genotype was not lethal in utero (Fig. 1, C and F). Both ES targeted cell lines gave rise to identical phenotypes. Northern blot analysis of total RNA showed that TC-PTP message was present in +/+, but was completely absent in -/- mice (Fig. 1 D). Western blot analysis using the anti TC-PTP monoclonal antibody 3E2 demonstrated that in the homozygous animals, no full-length TC-PTP protein was detectable (Fig. 1 E). These results confirmed the proper targeting and generation of a null mutant for TC-PTP. All newborn mice appeared healthy and displayed no physical abnormalities. TC-PTP +/+ and +/- littermates remained healthy throughout the duration of the experiments (i.e., 9 mo). Thus, there was no apparent dose-dependent effect caused by inactivation of a single TC-PTP allele. By 2 wk of age, TC-PTP -/- animals showed a slight growth retardation, but were otherwise healthy. However, between 3–5 wk of age, all TC-PTP -/- animals developed hunched posture, progressive closure of the eyelids, decreased mobility, and diarrhea, and no TC-PTP -/- mice survived beyond 5 wk of age (Fig. 1 F).

**Splenomegaly and Severe Anemia in TC-PTP -/- Mice.** To further investigate the phenotype of TC-PTP -/- mice, animals were killed at different days after birth. Gross anatomical observations indicated a striking splenomegaly and lymphadenopathy (data not shown) in mutant mice in the third week of life. The LNs of these mice showed loss of follicular demarcation due to hyperplasia of the follicles as a result of increased number of immunoblasts (data not shown). Hematocrit level of mutant mice decreased with increasing age, leading to severe anemia by day 21. Since the percentage of mature B cells was markedly increased in the LNs (Fig. 2 D), we tested the possibility that autoantibodies against erythrocytes were being produced and could therefore cause severe anemia. Using both flow cytometry and the Coomb's test (35), we failed to detect IgM autoantibodies in the circulation (data not shown). Histological analysis showed that, in the spleen of some TC-PTP -/- mice, the red pulp area was expanded (data not shown), consistent with increased sequestration of RBCs. These results suggest that the spleen of TC-PTP -/- mice could not compensate for the defective BM erythropoiesis leading to severe anemia that could contribute to the morbidity and mortality.

**B and T Cell Development and Function in TC-PTP -/- Mice.** The enlargement of the LNs and spleen in mutant mice reflected the marked increase in total cellularity that peaked on days 17–21 in these organs (Fig. 2, A and B). Flow cytometry demonstrated that the percentage of pre-B (B220<sup>+</sup>sIgM<sup>-</sup>) and mature B (B220<sup>+</sup>sIgM<sup>+</sup>) cell populations in the LNs of TC-PTP -/- mice were increased severalfold on days 13, 17, and 21 (Fig. 2 D). As a consequence of elevated cellularity (Fig. 2 A), the absolute number of pre-B and mature B cells in the LNs of mutant mice was markedly increased (3–10-fold) on all days studied,



**Figure 2.** Total cellularity and percentages of B cell populations in the LNs, spleen, and BM of TC-PTP-deficient mice. Total cellularity on different days after birth from the (A) LNs, (B) spleen, and (C) BM. Data are presented as the mean of 3–6 mice/group. Pre-B (B220<sup>+</sup>sIgM<sup>-</sup>) and mature B cell (B220<sup>+</sup>sIgM<sup>+</sup>) populations were determined by two-color flow cytometry. Each column and row of FACS<sup>®</sup> profiles represents the different groups (+/+, +/-, -/-) and different days after birth (D7, D13, and D21), respectively. Numbers in the upper quadrants of each FACS<sup>®</sup> profile represent the percentage of the population from the (D) LNs, (E) spleen, and (F) BM.

**Table 1.** Absolute Number of T Cell Subsets in the Different Lymphoid Organs of TC-PTP  $-/-$  Mice

Groups	Lymphoid organ	Absolute No. of T cell populations ( $\times 10^6 \pm SE$ )			
		CD4 <sup>+</sup> 8 <sup>+</sup>	CD4 <sup>+</sup>	CD8 <sup>+</sup>	CD4 <sup>+</sup> /CD8 <sup>+</sup> ratio ( $\pm SE$ )
+/+	Thymus	102.7 $\pm$ 16.9	17.5 $\pm$ 4.0	6.1 $\pm$ 1.2	2.8 $\pm$ 0.2
+/-	Thymus	155.9 $\pm$ 16.5	22.7 $\pm$ 1.6	7.9 $\pm$ 0.5	2.9 $\pm$ 0.3
-/-	Thymus	30.2 $\pm$ 9.3	14.5 $\pm$ 1.6	4.4 $\pm$ 0.2	3.5 $\pm$ 0.2
+/+	Spleen	NA	4.8 $\pm$ 2.7	2.1 $\pm$ 1.0	2.0 $\pm$ 0.2
+/-	Spleen	NA	7.8 $\pm$ 1.5	3.4 $\pm$ 0.8	2.4 $\pm$ 0.1
-/-	Spleen	NA	6.6 $\pm$ 1.6	3.1 $\pm$ 1.1	2.6 $\pm$ 0.3
+/+	LN	NA	6.8 $\pm$ 1.6	2.4 $\pm$ 0.5	2.8 $\pm$ 0.1
+/-	LN	NA	9.6 $\pm$ 0.8	3.4 $\pm$ 0.4	2.9 $\pm$ 0.2
-/-	LN	NA	4.3 $\pm$ 1.0	1.3 $\pm$ 0.3	3.2 $\pm$ 0.3

Mice were killed 21 d after birth. Absolute number was calculated as total cellularity  $\times$  percent cell population. The percent cell population was determined by flow cytometry using antibodies against CD4 and CD8 molecules. Data are presented as the mean of 3–5 mice/group. NA, not available.

when compared to TC-PTP  $+/+$  and  $+/-$  littermates. The splenic B cell populations in  $-/-$  mice was slightly but consistently lower than  $+/+$  and  $+/-$  mice on days 13 and 17, but by day 21 the percentage of splenic B cells was dramatically reduced (Fig. 2 E). No significant differences in the absolute number of LNs and splenic B cell populations were observed between heterozygous and wt animals (Fig. 2, A, B, D, and E). In addition, the macrophage (F4/80<sup>+</sup>) population was increased several fold in the spleen, but was unchanged in the LNs (results not shown), whereas the granulocyte (i.e., Gr1<sup>+</sup>) population remained unaltered in both peripheral lymphoid organs (data not shown).

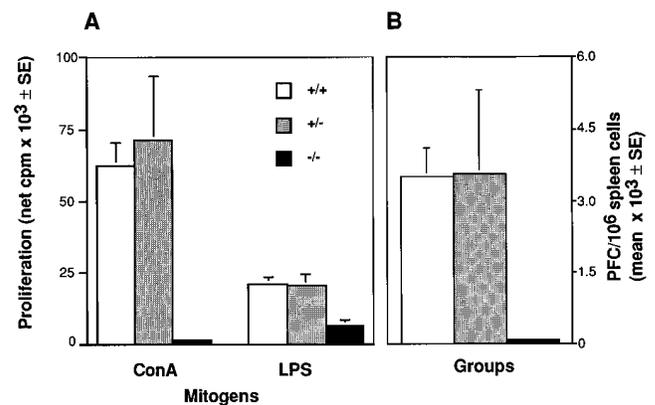
In contrast to the spleen and LNs, BM cellularity and the percentage of pre-B and mature B cell populations in the BM of mutant mice declined with age (Fig. 2, C and F). By day 21, there was at least a ninefold reduction in the absolute number of pre-B ( $0.4 \times 10^6$ ) and mature B ( $0.7 \times 10^6$ ) cells in the BM of TC-PTP  $-/-$  mice when compared to TC-PTP  $+/+$  and  $+/-$  littermates. No significant differences were observed in the absolute number of B cell populations in the BM of TC-PTP  $+/+$  and  $+/-$  littermates. Macrophage and granulocyte populations in the BM of TC-PTP  $-/-$  animals was not grossly altered (data not shown).

The thymus of TC-PTP  $-/-$  animals was involuted by day 21 owing to the marked decrease number of immature double positive (CD4<sup>+</sup>8<sup>+</sup>) thymocytes (Table 1). However, in both thymus and peripheral lymphoid organs, there was only a slight reduction of mature CD4<sup>+</sup> and CD8<sup>+</sup> thymocytes and the CD4<sup>+</sup>/CD8<sup>+</sup> T cell ratio of TC-PTP  $-/-$  animals was similar to TC-PTP  $+/+$  and  $+/-$  littermates (Table 1). These results suggest that lack of TC-PTP does not appear to affect the development of mature CD4<sup>+</sup> and CD8<sup>+</sup> T cells.

Since we observed thymic atrophy and detected a defec-

tive B cell population in the marrow, we investigated T and B cell function 21 d after birth. Splenic cells obtained from TC-PTP  $-/-$  mice responded poorly to both T and B cell mitogens, Con A, and LPS, respectively (Fig. 3 A). In addition, *in vivo* splenic antibody response to SRBCs was suppressed in the TC-PTP  $-/-$  animals (Fig. 3 B). Thus, the disruption of the TC-PTP gene led to impaired T and B cell functions, suggesting that TC-PTP is important for the development of immunocompetence of T and B lymphocytes.

*Defective Microenvironment of the BM of TC-PTP-deficient Mice.* Impairment of erythropoiesis and severe decline of pre-B and mature B cell populations in the BM of TC-PTP  $-/-$  mice implied that TC-PTP may play an important



**Figure 3.** Immune function of TC-PTP-deficient mice. (A) Proliferative responses of splenic cells to B- and T cell-specific mitogens, LPS, and Con A, respectively. Mice were killed 21 d after birth. Data are presented as the mean  $\pm$  SE of 3–6 mice/group. (B) Response of splenic PFCs against SRBCs. Mice of 21 d of age were injected intravenously with SRBCs and killed 4 d after immunization. Data are presented as the mean PFC  $\pm$  SE/ $10^6$  spleen cells.

**Table 2.** *Effect of Bone Marrow Reconstitution After Irradiation*

Recipients	No. of mice	Irradiation	Donor BM	No. of mice/ total mice	Survival
		<i>rads</i>			%
-/-	6	900	+/+	0/6	0
-/-	3	900	Media	0/3	0
-/-	7	None	+/+	0/7	0
-/-	2	None	Media	0/2	0
+/+	12	900	-/-	10/12	83.3
+/+	13	900	+/+	13/13	100
+/+	13	900	Media	0/13	0

Recipients were irradiated with 900 rads of  $\gamma$  irradiation 18 h before an intravenous injection of media or  $1.5 \times 10^6$  donor BM. Recipients that were not irradiated were injected intravenously with  $2.5 \times 10^7$  donor BM, as well as with an intraperitoneal injection of  $2.0 \times 10^7$  donor splenic cells.

role in hematopoiesis. To address this possibility, we attempted to restore normal hematopoiesis by transplanting +/+ BM into TC-PTP -/- recipients. To avoid graft rejection and graft-versus-host disease, 129/sv inbred TC-PTP +/+ and -/- mice were used (see Materials and Methods). Our data showed that BM from wt donors failed to rescue irradiated TC-PTP -/- recipients leading to 0% survival (0/6; Table 2). The death of the irradiated BM-transplanted TC-PTP -/- recipients could not be due to the viability of the wt BM graft since all irradiated wt recipients transplanted with wt BM survived (Table 2). The possibility that irradiation was involved in the death of the TC-PTP -/- recipients prompted us to reconstitute non-irradiated TC-PTP -/- recipients with wt BM. The transplantation of wt BM into nonirradiated TC-PTP -/-

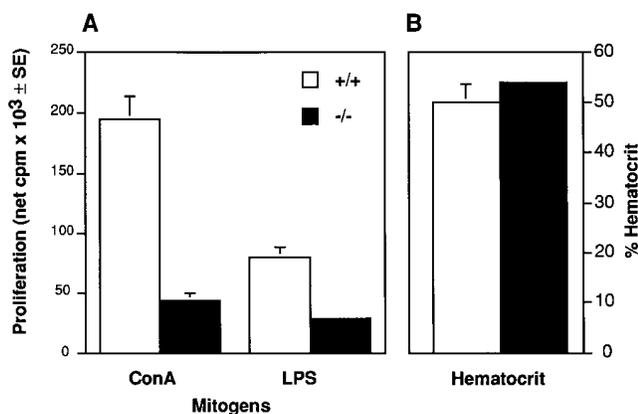
recipients also failed to rescue the recipients (Table 2). Taken together, the transplantation experiments suggested that the microenvironment of the BM of TC-PTP -/- mice was deficient and was unable to support hematopoiesis of the wt BM graft. To test this hypothesis, we transplanted BM from 10–12-d-old TC-PTP -/- donors into irradiated wt recipients. We reasoned that this experiment would test whether TC-PTP -/- mice produced stem cells capable of differentiating into the different hematopoietic lineages and hence would rescue irradiated wt recipients. Our results showed that TC-PTP -/- BM did indeed successfully rescue irradiated wt recipients (Table 2) implying that stem cell production and differentiation was normal.

To further examine the ability of TC-PTP -/- BM to reconstitute irradiated wt recipients, the BM transplanted mice were killed 2 mo after reconstitution. B and T cell populations expressed the neo gene, confirming that the lymphoid cells population were derived from the injected PTP -/- BM (data not shown). Gross observation revealed that the spleen and LNs of these recipients were not enlarged and were similar in size to the control group. Flow cytometry showed that, unlike the nonreconstituted, nonirradiated TC-PTP -/- mice (Table 1), the thymus of wt recipients reconstituted with TC-PTP -/- BM did not undergo atrophy (Table 3), and B cell production in the BM of the rescued recipients was similar to that of control irradiated wt recipients reconstituted with wt BM (Table 3). Moreover, the hematocrit and the number of pre-B and mature B cells in the spleen and LNs of the recipients reconstituted with TC-PTP -/- BM were also similar to the control groups (Fig. 4 B and Table 3). Although T and B cells developed normally in recipients rescued with TC-PTP -/- BM, T and B cell proliferative response to mitogens remained defective suggesting that TC-PTP is important for the development of functional competence of T and B cells (Fig. 4 A). Together, the above studies indicate

**Table 3.** *Lymphocyte Populations in Irradiated wt Mice Reconstituted with TC-PTP +/+ or -/- BM*

Donor BM	Organ	Cellularity ( $\times 10^6 \pm$ SE)	Absolute No. of different cell populations ( $\times 10^6 \pm$ SE)				
			CD4 <sup>+</sup> 8 <sup>+</sup>	CD4 <sup>+</sup>	CD8 <sup>+</sup>	B220 <sup>+</sup> sIgM <sup>-</sup>	B220 <sup>+</sup> sIgM <sup>+</sup>
+/+	Thymus	40.5 $\pm$ 11.3	29.6 $\pm$ 8.2	6.7 $\pm$ 2.2	1.8 $\pm$ 0.6	NA	NA
-/-	Thymus	60.3 $\pm$ 14.8	31.9 $\pm$ 4.2	11.7 $\pm$ 3.2	4.1 $\pm$ 1.1	NA	NA
+/+	BM	9.6 $\pm$ 1.6	NA	NA	NA	0.44 $\pm$ 0.07	0.27 $\pm$ 0.05
-/-	BM	5.7 $\pm$ 0.7	NA	NA	NA	0.37 $\pm$ 0.04	0.14 $\pm$ 0.02
+/+	Spleen	83.8 $\pm$ 9.9	NA	24.2 $\pm$ 4.3	6.0 $\pm$ 1.5	21.9 $\pm$ 2.4	13.4 $\pm$ 1.3
-/-	Spleen	83.3 $\pm$ 12.0	NA	14.7 $\pm$ 1.7	3.4 $\pm$ 0.9	27.4 $\pm$ 5.4	12.6 $\pm$ 2.4
+/+	LN	8.4 $\pm$ 1.8	NA	2.7 $\pm$ 0.6	0.59 $\pm$ 0.1	0.58 $\pm$ 0.18	0.39 $\pm$ 0.12
-/-	LN	11.9 $\pm$ 3.1	NA	2.3 $\pm$ 0.1	0.6 $\pm$ 0.1	1.5 $\pm$ 0.1	0.5 $\pm$ 0

All wt recipients were irradiated with 900 rads of  $\gamma$  irradiation 18 h before an intravenous injection of  $1.5 \times 10^6$  BM cells. Recipients were killed 2 mo after the BM transplantation. Data are presented as the mean of 3–4 mice/group. NA, not available.



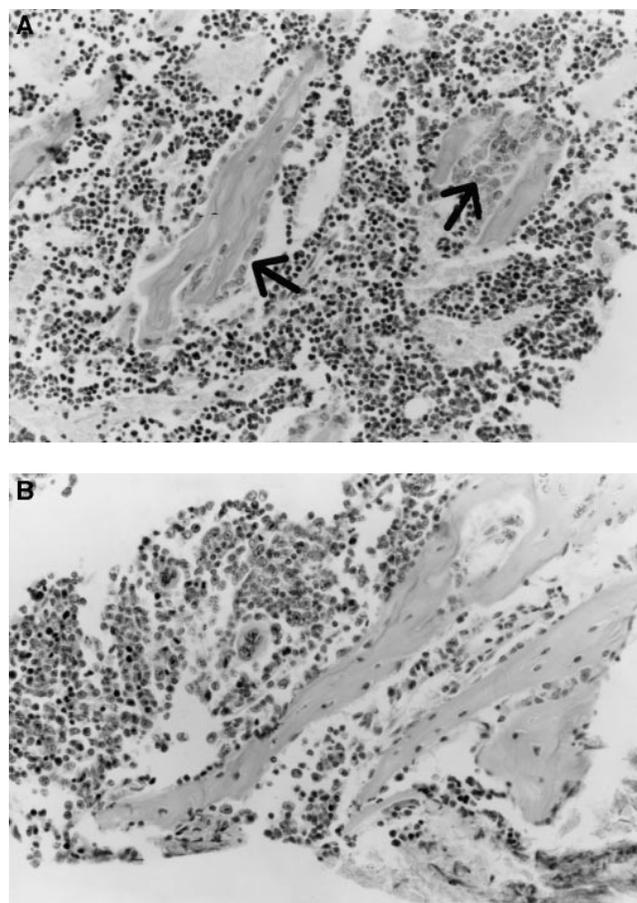
**Figure 4.** Immune function and hematocrit of irradiated wt recipients rescued with wt or TC-PTP-deficient BM. (A) Immune function of irradiated wt recipients reconstituted with TC-PTP wt or  $-/-$  BM. Immune function was assessed by the ability of splenic cells to proliferate in response to Con A and LPS. Data represents the mean  $\pm$  SE of 3–6 mice/group. (B) Hematocrit level of irradiated wt recipients rescued with TC-PTP  $-/-$  BM. Data are presented as the mean  $\pm$  SE of 3 mice/group.

that BM of TC-PTP  $-/-$  mice contains stem cells capable of differentiating and maturing in a normal BM microenvironment. In support of this hypothesis, histological analysis of the BM microenvironment demonstrated that the number of stromal cells in the BM of TC-PTP  $-/-$  mice was reduced when compared to wt and heterozygous animals (Fig. 5). Thus, the TC-PTP  $-/-$  mutation could lead to a quantitative and/or qualitative alteration in BM microenvironmental function in these mice.

## Discussion

In this report we demonstrated that TC-PTP exerts an important selective function on hematopoiesis. First, BM of TC-PTP  $-/-$  mice failed to support the development of B cells and erythrocytes; however, granulocyte and macrophage production was not affected. Second, even though T cells acquired a mature phenotype in both the thymus and the peripheral lymphoid organs, they remained functionally deficient. In the absence of B cell production in the BM, the spleen seemed to compensate, resulting in significant B cell production for about one week. However, as with T cells, they were nonfunctional. Stem cells from TC-PTP  $-/-$  animals were able to reconstitute erythropoiesis and B cell lymphopoiesis in a normal BM microenvironment, but T and B cell functions remained impaired in the reconstituted animals. It appears that TC-PTP is important for the functions of T and B cells and is critical for maintaining the integrity of the BM microenvironment that is crucial for hematopoiesis.

Although B cell development in the BM was deficient in TC-PTP  $-/-$  mice (Fig. 2 F), T cells appeared to develop nearly normally (Table 1). The thymus of mutant mice underwent thymic involution as a result of a selective marked decrease in the number of CD4<sup>+</sup>8<sup>+</sup> thymocytes; however,



**Figure 5.** Bone marrow histology. Histology of the BM microenvironment of nonirradiated TC-PTP-deficient mice. Analysis of the BM was performed on 21-d-old mice. (A) Stromal cells (arrows) are attached to, or clustered between bone trabeculae in  $+/-$  control mice. (B) By contrast, a marked paucity of stromal cells is observed in the BM of  $-/-$  mice in those areas normally rich in stromal cells. The hematopoietic cell population is comparable in both groups of mice. Periodic-acid Schiff stain; original magnification: 150.

single positive CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and CD4<sup>+</sup>/CD8<sup>+</sup> ratio in the thymus, spleen, and LNs were similar to  $+/+$  and  $+/-$  littermates (Table 1). These results suggest that phenotypic development of T cells is not markedly affected in the TC-PTP  $-/-$  mice. Despite a defect in B cell lymphopoiesis in the BM (Fig. 2, C and F), there was a marked increase in the number of pre-B and mature B cells in the LNs, and to a lesser degree in the spleen of TC-PTP  $-/-$  animals (Fig. 2, A, B, D, and E). The splenic changes are consistent with an expansion of the B cell compartment, as shown by the hyperplastic changes of the lymphoid follicles. The pattern of follicular expansion and the cytological features of the cells indicated that the proliferative changes represent lymphoid hyperplasia rather than lymphoma (data not shown). This implies that early B cell progenitors with limited renewal capacity migrate to the spleen, and in the absence of B cell production in the BM, these cells are able

to renew the B cell population for a limited period of time but eventually die off as their renewal capacity is exhausted.

The disruption of the TC-PTP gene led to severe compromised *in vitro* T and B cell mitogenic responses and antibody production against SRBCs (Fig. 3). We also demonstrated that although hematopoiesis was restored in irradiated wt recipients injected with TC-PTP  $-/-$  BM, T and B cell mitogenic responses remained suppressed (Fig. 4 A). These results provide the first evidence that TC-PTP is important for the development of functional competence of T and B cells. The effector functions of T and B lymphocytes are mediated by critical intracellular tyrosine phosphorylation and dephosphorylation events. Engagement of the T and B cell receptor rapidly activates PTKs of the src and non-src families (36–38). In addition, both the phosphorylation of PTKs and their targeted proteins are regulated by PTPs. In T and B cells, the dephosphorylation activity of CD45, a receptor-like transmembrane glycoprotein, is crucial for T and B cell activation (10, 39, 40). There is now increasing evidence implicating a critical role of nonreceptor-like cytoplasmic PTPs in the signaling pathway of lymphocytes after receptor engagement (41–43). Tailor et al. showed that SHP-2 participates actively in the activation of T cells (44), and Igarashi et al. demonstrated that a nonreceptor-like PTP is rapidly activated when B cell receptors are triggered (45). In light of these studies, it is possible that, since most TC-PTP is localized in the nucleus, it may provide further downstream modulation for lymphocyte activation. It is important to investigate the role of TC-PTP in lymphocyte activation and signaling, and these mice will provide us with the opportunity to carry out these studies.

The early normal erythropoiesis and lymphopoiesis followed by an almost total loss of these functions by 3–4 wk of age could be accounted for by the dramatic switch in hematopoiesis that occurs in different organs during mouse ontogeny. Primitive hematopoiesis occurs first in the yolk sac during embryonic development. By day 11 of fetal life, hematopoiesis is initiated in the fetal liver, presumably by the migration of hematopoietic stem cells from the yolk sac to the fetal liver which remains the main site of hematopoiesis during mid to late gestation. After day 15 of gestation, the fetal spleen also becomes an important hematopoietic organ and may remain functional for up to 2 wk of postnatal life. The BM initiates hematopoietic function during late fetal life and rapidly becomes the major site of hematopoiesis throughout life (46). It would appear that in the TC-PTP  $-/-$  animals, hematopoiesis may occur in a near normal fashion during fetal and early postnatal life (i.e., yolk sac, fetal liver, and spleen), but there is a failure in the BM to initiate hematopoietic function once it ceases in the fetal liver and the spleen. As a result there is an initial burst of hematopoiesis which is able to sustain the TC-PTP  $-/-$  animals for 2–3 wk. However, hematopoiesis declines in the fetal liver and spleen and it is not initiated in the BM, resulting in severe anemia and an arrest of B cell production in the marrow.

The precise reason for BM erythropoietic and B cell lymphopoietic failure is unknown. The inability of wt BM to rescue irradiated and nonirradiated TC-PTP  $-/-$  recipients strongly suggests that the BM microenvironment of the mutant mice is defective (Table 2). In support of this possibility, we demonstrated that the BM of TC-PTP  $-/-$  mice was able to rescue and restore hematopoiesis in irradiated wt recipients (Table 2). These results indicate that the mutant BM contains stem cells that can differentiate in a normal microenvironment. Although we have not yet identified the exact defect(s) in the BM microenvironment of TC-PTP  $-/-$  mice, several possibilities may exist. Histological analysis of the BM showed that the number of BM stromal cells of TC-PTP  $-/-$  animals were considerably reduced when compared to wt controls (Fig. 5). It has been well documented that the interaction of hematopoietic precursor cells with BM stromal cells is crucial for proper development and functions of the different hematopoietic cell lineages (47). Hence, the deficient number of BM stromal cells in mutant mice could likely affect the integrity of the BM microenvironment which consequently could lead to impaired B cell lymphopoiesis and erythropoiesis.

A second possibility is that the reduced number of remaining stromal cells fail to function properly. The adhesive interactions, via adhesion molecules, between progenitor cells and BM stromal cells and the extracellular matrix are also critical for hematopoiesis (47–50). Recently, a report has implicated the critical role of  $\alpha 4$  integrins in maintaining B cell lymphopoiesis in the BM since the disruption of the  $\alpha 4$  gene by homologous recombination resulted in severe impairment of B cell lymphopoiesis (51). However,  $\alpha 4$  integrins did not appear to be important during fetal and early postnatal hematopoiesis, but were critical for adult hematopoiesis, a phenomenon similar to the one that we observed in the TC-PTP  $-/-$  mice.

A third explanation for the defective hematopoiesis in TC-PTP  $-/-$  mice may be that cytokines produced by the BM microenvironment are inadequate. *In vivo* studies have shown that B cell lymphopoiesis markedly depends on the secretion of IL-7 by BM stromal cells (52). Similarly, IL-11 and -3, secreted by BM stromal cells, have been demonstrated to be potent stimulators of erythropoiesis (53). Thus, TC-PTP may be involved in the production of IL-3, -7, -11, and/or other hematopoietic cytokines. Alternatively, TC-PTP may be directly implicated in signaling pathways of the stromal cells. Jarvis and LeBien reported that direct contact of B cell precursors with BM stromal cells induced tyrosine phosphorylation of various proteins in the stromal cells (54). The induction of tyrosine kinases in the stromal cells may ultimately regulate expression of specific genes, such as cytokines and adhesion molecules, that are important for hematopoiesis. The exact defect(s) in BM microenvironment and lymphoid function in TC-PTP  $-/-$  mice has not been identified, however these animals provide us with an excellent model to answer these important questions.

We thank Ailsa Lee Loy and Rosmarie Siegrist-Johnstone for their excellent technical assistance. We also thank Dr. M.G. Baines for the generous gift of anti-F4/80 antibody, Anne Marcil and Dr. Dave Thomas for their help in the generation of the anti-TC-PTP 3E2 monoclonal antibody. We are thankful to Dr. Trevor Owens and Dr. Lee Wall for critical reading of the manuscript and for helpful suggestions.

E.S. Muise received studentships from the Canderel Foundation and the Fonds pour la formation de Chercheurs et l'Aide à la Recherche, and M.L. Tremblay is a Chercheur-Boursier from the Fonds de la Recherche en Santé du Québec. This work was supported by a Terry Fox research grant to M.L. Tremblay from the National Cancer Institute of Canada and by operating grants from the Cancer Research Society and from the Medical Research Council of Canada grant No. MT12466 to M.L. Tremblay, MT10315 to S. Jothy, and MT3526 to W.S. Lapp.

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Received for publication 17 April 1997 and in revised form 20 June 1997.

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