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Transcription Factor Zinc Finger and BTB Domain 1 Is Essential for Lymphocyte Development

Divya Punwani,*† Karen Simon,*† Youngnim Choi,‡ Amalia Dutra,‡ Diana Gonzalez-Espinoza,* Evgenia Pak,§ Martin Naradikian,*§ Chang-Hwa Song,*¶ Jenny Zhang,* David M. Bodine,† and Jennifer M. Puck*

Absent T lymphocytes were unexpectedly found in homozygotes of a transgenic mouse from an unrelated project. T cell development did not progress beyond double-negative stage 1 thymocytes, resulting in a hypocellular, vestigial thymus. B cells were present, but NK cell number and B cell isotype switching were reduced. Transplantation of wild-type hematopoietic cells corrected the defect, which was traced to a deletion involving five contiguous genes at the transgene insertion site on chromosome 12C3. Complementation using bacterial artificial chromosome transgenesis implicated zinc finger BTB-POZ domain protein 1 (Zbtb1) in the immunodeficiency, confirming its role in T cell development and suggesting involvement in B and NK cell differentiation. Targeted disruption of Zbtb1 recapitulated the T<sup>−</sup>B<sup>−</sup>NK<sup>−</sup> SCID phenotype of the original transgenic animal. Knockouts for Zbtb1 had expanded populations of bone marrow hematopoietic stem cells and also multipotent and early lymphoid lineages, suggesting a differentiation bottleneck for common lymphoid progenitors. Expression of mRNA encoding Zbtb1, a predicted transcription repressor, was greatest in hematopoietic stem cells, thymocytes, and pre-B cells, highlighting its essential role in lymphoid development. The Journal of Immunology, 2012, 189: 000–000.

Development of lymphocytes from hematopoietic stem cells (HSCs) is incompletely understood. Current models suggest that T, B, and NK cell precursors are predominantly derived from common lymphoid progenitors (CLPs) in bone marrow (BM) (1). CLPs can differentiate into all lymphoid progenitors (ALPs), which retain full lymphoid differentiation potential, forming both T cell progenitors that seed the thymus and B cell-biased lymphoid progenitors that become B cells (2). Notch-1 signals are required to redirect common lymphoid progenitors from the default B lineage to the T lineage (3, 4). After migration to the thymus, T lineage progenitors proceed through distinct CD4<sup>−</sup>CD8<sup>−</sup> (double-negative, or DN) thymic stages, DN1–DN4, after which they undergo TCR rearrangement and become CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) cells (5). DP cells undergo selection, with surviving self-tolerant CD8<sup>+</sup> or CD4<sup>+</sup> single-positive, naive T cells emerging from the thymus to populate peripheral lymphoid organs. The zinc finger BTB-POZ (Zbtb) transcription factor 7 (Zbtb7/Th-POK/cKrox) is crucial for switching a fraction of DP cells from the default CD8<sup>+</sup> pathway to form CD4<sup>+</sup> cells (6, 7). Identification of additional signaling factors at developmental branch points is required to understand lymphoid lineage commitment and differentiation in both mice and humans.

Human SCID syndromes, defined by profound defects in both cellular and humoral immunity, comprise experiments of nature that have revealed many nonredundant, critical functions in human lymphoid developmental pathways. Human SCID is caused by defects in a wide variety of genes encoding proteins necessary and specific for lymphocyte differentiation, proliferation, and activation (8–11). About two-thirds of SCID patients lack T cells but have B cells that fail to make specific Abs (T<sup>−</sup>B<sup>−</sup> phenotype); T<sup>−</sup>B<sup>+</sup> SCID can be caused by defects in the X-linked common γ-chain receptor for IL-2, -7, -9, -15, and -21; the IL-7 receptor α-chain; intracellular cytokine signal transducers JAK3 or STAT5; protein tyrosine phosphatase receptor CD45; or components of the CD3 receptor. Patients with these forms of SCID are in contrast to those with T<sup>−</sup>B<sup>−</sup>SCID, whose defects may lie in mediators of T and BCR gene rearrangement, including RAG1, RAG2, and Artemis, or in adenosine deaminase or purine nucleoside phosphorylase, which normally remove purine intermediates that are toxic to lymphocytes. Up to 10% of humans with SCID have other rare, or as yet unidentified, genetic defects.

In contrast to humans, the most studied mouse models for SCID have the T<sup>−</sup>B<sup>−</sup> phenotype, including the scid mouse deficient in the DNA-dependent kinase DNA-PK<sub>C</sub>, and Rag1/Rag2-deficient mice, all of which fail to complete V(D)J rearrangement of Ab and TCR genes during B and T cell development (12–17). These and the athymic nude mouse lacking transcription factor Foxn1...
Splenocytes were collected and cultured for 48 h as described (21). While the absence of peripheral T cells in our knockout mice confirms findings recently reported in the scanT mouse (22), our more detailed study of Zbtb1 and its role in the development of T, B, and NK cells.

Materials and Methods

Mice

Transgenic mouse line 26A with six tandem copies of mFas D231V has been described previously (21). The SCID phenotype in homozygotes was unexpected observed in homozygotes of one of five mouse lines transgenic for a Fas cDNA bearing a dominant death domain mutation (21). No off-spring of other transgenic founders lacked T cells, suggesting a recessive defect related to the insertion site rather than to the expression of mutant Fas. This mouse presented a unique opportunity to develop a phenotypic model similar to human T B NK SCID, to dissect requirements for lineage differentiation of lymphoid progenitors, and to identify a critical step in the decision of CLPs and ALPs to become B versus T versus NK cells. Moreover, the underlying defect turned out to be in Zbtb1, a transcription repressor of the zinc finger BTB domain family very recently recognized to be involved in lymphoid cell development (22).

While the absence of peripheral T cells in our Zbtb1 knockout mouse confirms findings recently reported in the scanT mouse with a missense variant of Zbtb1 (22), our more detailed study of hematopoietic stem cells, lymphoid progenitors, and lymphocyte subpopulations sheds light on Zbtb1 and its role in the development of T, B, and NK cells.

Lymphocyte subset enumeration

Cell suspensions were prepared from blood, thymus, spleen, and BM and stained according to standard protocols with mAbs (BD Biosciences, San Jose, CA; BioLegend/Bioscience, San Diego, CA). Flow cytometry was performed on an LSRII FACS machine (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR). Complete and differential white blood cells were analyzed with a BD Biosciences LSRII flow cytometer. Cytotoxicity was measured using the Promega CytoTox 96 kit (Promega, Madison, WI) following the manufacturer’s instructions.

BM transplantation

Donor BM cell suspensions were harvested by flushing femurs and tibia with PBS and straining. HSCs were enriched by depletion with biotinylated Abs (BD Biosciences) to lineage markers CD3, CD4, CD8, B220, Gr-1, and Ter-119, followed by addition of streptavidin microbeads and purification using a MACS column (Miltenyi Biotec, Auburn, CA). Two million cells were injected by tail vein into nine great irradiated recipients. Lineage engrafment was determined in blood and tissues after 4 mo. Experiments were performed in duplicate.

Bacterial artificial chromosome isolation, cytogenetics, and mapping

Metaphases from Con A-stimulated splenocytes from heterozygous transgenic (tg/+ ) animals were prepared as described (23). After fluorescence in situ hybridization (FISH) with the mFas plasmid labeled using cyochrome (yellow) showed a signal on chromosome 12C-D, PCR-amplified segments from genes in this region were used to screen murine RPCI-23 bacterial artificial chromosome (BAC) library filters (24). DNA from positive BAC clones containing Sos2 (RP23-353H11), Esz2 (RP23-351E10), Hspa2 (RP23-901C4), and Psen1 (RP23-291K4) was prepared and labeled by standard methods. Cytokine-labeled mFas DNA and BACs labeled with either rhodamine (red) or FITC (green) were used together as FISH probes.

At least 20 cells with each combination were scored.

Additional mouse BAC clones containing Hspa2 (RP23-191N23 and RP23-90K4) were obtained and aligned by PCR content in wild-type versus homozygous transgenic (tg/gt) mice. Primer pairs from nearby flanking genes and intragenic segments were used to walk from centromeric and telomeric undisturbed regions toward the insertion/deletion locus. PCR was performed using RTG PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ).

BAC transgenic mice for complementation

BAC DNA for transgenic injections was isolated by a NucleoBond BAC Maxi Kit (Clontech, Mountain View, CA) and verified by pulse field gel electrophoresis after Neo digestion. BAC clones containing 90K4 and 191N23 were linearized with BsiWI and Ascl, respectively. BAC DNA was injected into FVB/N fertilized eggs, and three resultant animals for each BAC with germline transmission were crossed to generate BAC-bearing tg/tg F2 mice for study. PCR with primers specific for genes located within the BACs confirmed BAC content. Animals were bred to determine fertility, immunophenotyping was carried out as above.

Retrovirus rescue of the SCID phenotype

A 2.3-kb EcoRI fragment containing Zbtb1 coding exon 2 was inserted into the 6.5-kb EcoRI-linearized murine stem cell virus (MSCV)-internal ribosome entry site (IRES)-GFP vector (provided by Art Nienhuis, St. Jude Children’s Research Hospital, Memphis, TN). Full sequencing assured forward orientation and integrity. MSCV-Zbtb1-IRES-GFP was transfected into AmphiPack 293 cells (Clontech) using a CellPhect kit (GE Healthcare/Amersham Biosciences, Piscataway, NJ), and supermamts were selected for in vitro transduction of G4P-E86 cells (25). GFP-positive clones expressing GFP were selected by FACS and expanded. Supernatants were titered on NIH 3T3 cells and analyzed by slot blot to determine the clone with the highest GFP expression. BM was harvested from t/m mice and their wild-type littermates 48 h after injection with 5-fluorouracil (Fluka/Sigma-Aldrich, St. Louis, MO) (26). BM cells were cultured and transduced with MSCV-Zbtb1-IRES-GFP as previously described (27). Tg/tg mice irradiated with 5 Gy received 2 x 10^5 cells in 200 μl by tail vein. Four months after BM transplantation (BMT), they were euthanized and immune phenotype was determined. DNA from Con A-stimulated T cells and peripheral blood was extracted using a Puregene kit (Qiagen, Valencia, CA). PCR was performed using vector primers MSCV-GFP (Clontech) cloned into pBluescript SK+ for assurance of G4P-E86 cells (25). Amplification conditions were 94°C for 2 min, followed by 94°C for 30 s, 65°C for 30 s, and 72°C for 45 s for 35 cycles, then 72°C for 5 min.

Production of Zbtb1 gene-targeted mice

Zbtb1 coding exon 2 was replaced by enhanced GFP (eGFP) using a pPNT-pgk-Neo vector. Homologous regions flanking Zbtb1 exon 2 were made from C57BL/6 genomic DNA by PCR using SuperMix High Fidelity (Invitrogen, Carlsbad, CA). The coding region of eGFP from pRRL2-GFP (Clontech) was cloned into pBluescript SK+ for assembly of G4P-E86 cells (25). For the 4.5-kb 5’ homologous segment located in the BAC with neo and BspHI digestion was ligated into the pBluescript-eGFP digested with Ncol and Xhol. The 2.4-kb 3’ homologous segment was ligated into pPNT using KpnI and EcoRI.
sites. Finally, the 5’ arm and eGFP fragment were released from pBlue-script with XhoI and SalI, gel purified, and ligated into the XhoI site of the pPNT construct, followed by electroporation into GeneHog competent cells (Invitrogen). Sequence-verified targeting construct DNA was linearized and injected at 1 µg/µl into C57BL/6 embryonic stem (ES) cells.

Injected ES clones under Neo selection were analyzed for homologous recombination by Southern blotting. Only one of three clones gave rise to germ line-transmitting chimeras following injection of ES cells into C57BL/6 blastocysts. Heterozygotes bred from this clone generated six wild-type, nine heterozygote, and eight homozygote offspring of both sexes, indicating that Zbtb1 is not necessary for embryonic development.

**Real-time PCR for Zbtb1 expression**

Expression levels of the two Zbtb1 splice variants were determined by quantitative real-time PCR. RNA was isolated from tissues and FACS-sorted subpopulations of BM, thymus, and spleen from 6- to 8-wk-old C57BL/6 mice. B cell subsets were sorted using markers described by Hardy and Hayakawa (28). RNA was prepared using the Qiagen RNeasy kit (Qiagen, Chatsworth, CA). All samples were treated with DNase I. RNA quality was assessed by A260/280 ratio (ND-100 spectrophotometer; NanoDrop Technologies, Wilmington, DE) and gel electrophoresis. A high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA) was used to prepare cDNA with 1 µg RNA from tissues or 200 ng from flow-sorted subpopulations. Absolute copy numbers of the two splice variant transcripts, Zbtb1T1 and Zbtb1T4, as well as those of housekeeping genes Pol2Ra and HPRT were assessed for normalization (primer sequences are available on request).

Amplified cDNA fragments were cloned into TOPO vector pCR2.1. Plasmids were prepared, sequence verified, and serially diluted to generate standards from 4 to 400 × 10^3 copies/well. Real-time PCR was performed in TaqMan Universal PCR Master Mix using 100 nM each primer and 200 nM FAM- and TAMRA-labeled oligonucleotide probe (primer and probe sequences are available on request) in an ABI Prism 7900HT system (Applied Biosystems). Cycle conditions were 10 min at 95˚C, followed by 95˚C for 30 s and 60˚C for 1 min for 40 cycles. All samples were analyzed in duplicate. Copy numbers were determined by SDS 2.1 detection software (Applied Biosystems), and data were normalized to obtain test gene copies per 1000 copies of HPRT and Pol2Ra.

**B cell proliferation assays**

Splenocytes were collected from Zbtb1 knockout, wild-type, and TCRα knockout mice, and T cells were depleted using the Pan T cell isolation kit (Miltenyi Biotec). B cells (1 × 10^5) were added to each well of a 96-well round-bottom plate and incubated with LPS (0.1 or 10 ng/µl), anti-IgM (0.1 or 10 ng/µl), or anti-CD40 (0.1 or 10 ng/µl) in RPMI 1640 with 10% FCS, penicillin and streptomycin, nonessential amino acids, and 5 µM BrdU (Invitrogen). BrdU incorporation after 48 h incubation was analyzed using a BD Biosciences LSRII flow cytometer.

**Serum Ig assays**

Serum collected in BD Microtainer separator tubes (Becton Dickenson, Franklin Lakes, NJ) had levels of IgM, IgG, and IgA assayed using an ELISA mouse Ig quantitation kit (Bethyl Laboratories, Montgomery, TX). Data were analyzed using an unpaired t test with a Welch correction (Prism 5; GraphPad Software, La Jolla, CA).

**Western blotting and immunoprecipitation**

Whole-cell, nuclear, and cytoplastic 293T cell extracts were prepared 24 and 48 h after transfection of cloned human or murine cDNA encoding Zbtb1T13 and Zbtb1664 isoforms with N- or C-terminal, FLAG, or myc epitope tags. Aliquots were subjected to electrophoresis, transfer, and immunoblotting using reagents and methods from eBioscience. Anti-FLAG (Sigma-Aldrich) or anti-myc (Santa Cruz Biotechnology, Santa Cruz, CA) mouse mAbs followed by HRP-anti-mouse IgG (Santa Cruz Biotechnology) were applied and detected by the SuperSignal substrate detection system (Pierce Protein Research Products/Invitrogen).

For immunoprecipitations, cell lysates from transfected 293T cells were mixed with protein A/G beads and rocked overnight with Sepharose beads conjugated to protein A. After washing the beads, protein was released by boiling in gel-loading buffer. Electrophoresis and detection were carried out as above.

**Results**

**SCID and infertility in a transgenic mouse**

Of five lines of FVB/N mice transgenic for mutant Fas D231V (21), one line, 26A, exhibited male infertility and profound immunodeficiency when the transgene was bred to homozygosity. Heterozygotes from all lines and homozygotes from lines other than 26A had mild hepatic infiltration with T cells, but did not have the phenotype of Fas-deficient MRL/pr mice or humans with autoimmune lymphoproliferative syndrome/autoimmunity, excess CD4+CD8− DN T cells, and defective apoptosis (29). Instead, the transgenic animals were healthy and indistinguishable from wild-type (+/+ ) mice. However, homozygotes of line 26A (tg/tg) had undetectable T and markedly reduced B and NK cells in peripheral blood, although B cells were present in spleens (Fig. 1A, 1B). Peripheral blood myeloid cells were slightly decreased in tg/tg mice whereas erythrocytes and platelets were normal (Supplemental Fig. 1A). Spleens in tg/tg mice were 50% of normal weight, with only 3% T cells but with an increased proportion of B cells to 60% (Fig. 1B) and normal absolute B cell numbers. Tg/tg mice showed poor proliferation to Con A and absent T cell cytotoxicity (Fig. 1C, 1D). They also had impaired isotype switching of the B cells, indicated by increased serum concentrations of IgM and decreased IgG and IgA (Fig. 1E). The phenotype was invariant with age up to 8 mo.

In contrast to their unaffected +/+ littermates, tg/tg mice had only a vestigial thymus (Fig. 1F). Typical of most human SCID cases, thymic epithelial and stromal cells were present, but cortical areas were undeveloped and lymphoid cells were rare to absent. Mesenteric lymph nodes in tg/tg animals, when located, were small; axillary and inguinal lymph nodes were not found. The infertility of male tg/tg mice was due to arrested spermatocyte development (Supplemental Fig. 1B).

**BMT to define the cellular lesion in tg/tg SCID mice**

To determine whether the SCID phenotype of tg/tg mice was HSC autonomous or attributable to a dysfunctional thymic environment, lineage-depleted BM from +/+ mice was injected in irradiated tg/tg mice as well as into irradiated control heterozygous littermates. After 4 mo, reconstitution in tg/tg mice was equivalent to that in controls (Supplemental Fig. 1D, Supplemental Table IA). Not only did CD3, CD4, and CD8 T cells appear in numbers equivalent to those of control mice, but also T cell proliferation to Con A and cytotoxicity against P815 target cells was normalized. Additionally, serum IgM, IgG, and IgA levels became normal, indicating recovery of isotype switching (data not shown).

When the reciprocal BMT was performed using lineage-depleted FVB/N tg/+ or tg/tg SCID mouse cells to reconstitute irradiated C57BL/6 × FVB/N F1 mice, the recipients developed fewer T and B cells than did recipients of tg/+ heterozygous BMT. Even though hemoglobin analysis indicated 100% donor engraftment, lineage chimerism using PCR with primers specific for C57BL/6 and FVB/N strains showed failure to engraft T and B cells (Supplemental Fig. 1E, Supplemental Table 1B). Proliferation and cytotoxic function remained poor, and IgM levels rose even through IgA and IgG levels were normal, with the latter attributed to radio-resistant recipient mouse plasma cells (data not shown). Thus, the SCID defect was intrinsic to HSCs, did not affect myeloid progenitors, and was limited to lymphoid lineages, mainly affecting the generation of T and NK cells.

**Mapping and identification of the genetic locus for immunodeficiency**

Labeled plasmid containing the 6-kb mFas transgene was used as a FISH probe to map the integration site on metaphase chromosome 3 spreads from heterozygous tg/+ animals; a single hybridization signal was found on mouse chromosome 12qC-D. BAC clones containing known mouse genes were used as probes in three-color hybridizations to narrow the region (Fig. 2A). The
transgene signal (yellow) was found between BACs containing Sos2 (green) and Psen1 (red) (Fig. 2Ai). Additional BACs within this interval were further used to refine the transgene location. A BAC containing Esr2 (red) was centromeric to the transgene, whereas one with Hspa2 (green) was replaced by the plasmid signal (yellow) on one copy of chromosome 12 (Fig. 2Aii), suggesting that partial or full deletion of Hspa2, perhaps with surrounding genes, had occurred when the transgene was integrated into mouse chromosome 12. We thus hypothesized that one or more genes disrupted by the transgene integration accounted for the SCID phenotype of tg/tg mice.

Hspa2, encoding a 70-kDa heat shock chaperone protein, had been knocked out in mice and found to be essential for male fertility (30, 31). However, a role for Hspa2 in the immune system had not been studied. Hspa2 knockouts, provided by Dr. Mitchell Eddy, had normal T and NK cells and normal Ab levels (data not shown), ruling out Hspa2 as a candidate gene for the tg/tg immunodeficiency. However, Hspa2 protein was absent in tg/tg mice as expected, shown by lack of staining with polyclonal anti-Hspa2 Ab, also provided by Dr. Mitchell Eddy (Supplemental Fig. 1C). Lack of Hspa2 thus explained the male infertility in tg/tg mice.

Boundaries of the tg insertion/deletion were established by PCR mapping with primer pairs selected from the mouse genome sequence (Fig. 2B). Positive PCR amplification in +/+ and tg/+ mice with failure in tg/tg mice indicated deleted sequences (Fig. 2B, hatched area, top). The tg allele was deleted for hypothetical genes AK133227, AK136613, and a portion of Plekhg3 on the telomeric side of Hspa2 and two genes in the Zbtb family, Zbtb1 and Zbtb25, on the centromeric side.

Implication of Zbtb1 in immunodeficiency

With Hspa2 ruled out as the immunodeficiency gene, we used a complementation approach with transgenesis of BAC clones 191N23 and 90K4, extending to the telomeric and centromeric sides of Hspa2, respectively (Fig. 2B), to evaluate the ability of the candidate genes to rescue immunity and fertility. After injections into blastocysts from the original tg/tg mice, three lines of mice with each BAC were produced and crossed to mFas tg/+ mice to generate tg/tg BAC-bearing animals. Tg/tg mice carry-
ing BAC clone 191N23 were fertile, as expected with restoration of \(Hspa2\), but retained the immunodeficient phenotype with absent T cells and T cell function, elevated IgM, and low IgG and IgA (Fig. 2C, right column). Only mice with the full-length 90K4 BAC were fertile with intact immunity, as shown in Fig. 2C (90K4 complete). In one mouse line, spontaneous breakage of BAC 90K4 resulted in restoration of the coding portion of \(Zbtb25\) (Fig. 2C, 90K4 partial). Crosses of the BAC transgenics with the \(mFas\) \(tg/tg\) mice revealed that triple transgenics homozygous for the original \(tg/tg\) insertion/deletion, and carrying the 191N23 BAC (restoring \(Hspa2\), AK133227, AK136613, and Plekhg3) plus the partial 90K4 BAC (possibly restoring \(Zbtb25\), but not \(Zbtb1\)), were fertile, but were affected with SCID. These experiments suggested that loss of \(Zbtb1\), or of both \(Zbtb1\) and \(Zbtb25\), was necessary and sufficient to produce T\(^+\)B\(^-\)NK\(^+\)SCID in the mouse.

To determine whether absent \(Zbtb1\) alone was the cause of SCID, we used a retroviral vector containing the single large coding exon of \(Zbtb1\) to transduce BM stem cells from \(tg/tg\) or \(+/+\) control donor mice and injected these transduced cells to rescue lethally irradiated \(tg/tg\) mice. At 4 mo after BMT, T cells were found in peripheral blood and spleen, although normal numbers were not completely restored (Supplemental Table IC). However, serum Ig levels were comparable to wild-type mice, and T cell proliferation and cytotoxicity were normal (Supplemental Fig. 1F), indicating that restoration of \(Zbtb1\) restored immunity, curing the SCID phenotype. **Targeted disruption of \(Zbtb1\)**

To prove that \(Zbtb1\) was necessary and sufficient to cause the observed immunodeficiency of \(tg/tg\) mice, we replaced exon 2 of \(Zbtb1\) with a GFP-pgk-Neo cassette to produce a gene-targeted mouse (Fig. 3A). The first exon of the \(Zbtb1\) gene is noncoding, with the ATG start of translation at nucleotide 19 in exon 2; therefore, disruption of exon 2 would obliterate \(Zbtb1\) gene expression. After transfection of C57BL6/J ES cells and injection into blastocysts, we obtained three founder mice, one of which passed the targeted disruption to offspring. \(Zbtb1\) knockout F\(^2\) mice were generated and were fertile (but unfortunately failed to express GFP). Whereas \(Zbtb1\) heterozygous (+/−) and wild type (+/+ ) littermates had normal T, NK, and B cell numbers in the periphery, \(Zbtb1\) knockout (−/−) mice showed nearly absent T cells, reduced NK cells, and normal B cells (Fig. 3B). Additionally, splenic T cells, which were very few in number, showed a complete lack of proliferative and cytotoxic function (Supplemental Fig. 2A). The thymus and lymph nodes were vestigial in \(Zbtb1\) knockout mice, and the spleen was half the weight of spleens of littermate controls (data not shown). Nonlymphoid cells in the peripheral blood were unaffected (Supplemental Fig. 2B). The phenotype of the \(Zbtb1\) knockout mice therefore replicated that of the original transgenic mice, as well as the recently reported \(scan\)\(^+\)mice with a missense mutation in \(Zbtb1\) (22). Detailed characterization of knockout mice enabled us to define the
phenotype resulting from complete absence of the Zbtb1 protein versus that resulting from published missense mutation C74R, therefore further delineating the role of Zbtb1 in hematopoietic stem cell differentiation and lymphoid development.

Investigation of embryonic thymi in Zbtb1-deficient mice

Because of the absence of mature T cells in the periphery and a hypocellular, vestigial thymus in adult knockout mice lacking Zbtb1, embryonic thymi were analyzed to determine at which stage T cell development was arrested. Thymocytes were identified according to their differentiation status, with the earliest arrivals from the BM being DN1 cells, CD4^+/CD8^- TCRβ^+ CD25^-CD44^-; successive maturation to DN2 CD25^+CD44^- and then DN3 CD25^+CD44^- cells was monitored by cell surface marker content (32), with progression beyond the DN3 stage indicated by rearrangement and expression of the TCRβ genes (33). When day 15.5 and day 17.5 mouse embryos were examined for thymocyte maturation,tg/tg mice had reduced but detectable DN1, but very few DN2 and DN3 cells (Fig. 4A) and essentially no DN4, DP, or single-positive T cells (data not shown). Further analysis of the DN1 population as described by Porritt et al. (34) (Fig. 4B, 4C) showed low, but detectable, numbers of DN1a (c-Kit/CD117^+, HA/CD24^-) and DN1b (c-Kit/CD117^+, HA/CD24^+) cells, which are thought to represent the major pathway for expansion and differentiation from BM progenitors to DN2 thymocytes. DN1c cells (c-Kit/CD117^+, HA/CD24^-), which are thought to retain B lineage developmental potential, were also detectable, but there were essentially no CD117^- DN1d or DN1e cells, which have poor proliferative potential and do not mature into T cells. This pattern indicates that T cell progenitors lacking Zbtb1, although present in the thymus in small numbers, were unable to progress through thymic differentiation.

Stem cell subpopulations in Zbtb1^-/- mice

To determine prethymic effects of knocking out Zbtb1, absolute numbers of cells comprising the different BM stem cell subsets, long-term HSCs (LSK, CD34^+,CD48^-), short-term HSCs (LSK, CD34^+,CD48^-), multipotent progenitors (MPPs) (LSK, CD34^+,CD48^-), and CLPs (LSK, CD127^+,CD115^+) were determined from the Zbtb1 knockout versus wild-type mice. Zbtb1 knockout mice had equivalent numbers of long-term HSCs but more cells of the other early subsets, short-term HSCs, MPPs, and CLPs (Fig. 4C). This could reflect feedback mechanisms in an attempt by the BM to make up for the reduced number of mature T cells in the periphery. Although the BM was able to produce lymphoid progenitors in the absence of Zbtb1, these progenitors did not appear in the thymus.

Our observations contrast with the report on scanT mice in which numbers and percentages of all hematopoietic subsets were equal to those of wild-type mice (22). This could be because the Zbtb1 protein, although mutated in the scanT mice, could still retain residual function and influence HSC numbers and differentiation, whereas in our knockout mice, the complete absence of Zbtb1 protein affects the maintenance of stem cell numbers.
BM and spleen B cell subsets in Zbtb1 knockout mice

Because the absence of Zbtb1 resulted in lack of T cells in the periphery and spleen, but did not prevent B cell development, B cell subsets (28) in both BM and spleen were analyzed to determine whether the absence of Zbtb1 affected their proportions. Wild-type and TCRα knockout mice were used as controls to evaluate the absence of T cell help as a contributor to any alterations in B cell subsets. No differences in pro-B cell frequencies in the BM were observed across the three mouse phenotypes. However, Zbtb1 knockout mice had fewer pre-B cells compared with TCRα knockout and wild-type mice (Fig. 5A, Supplemental Fig. 2Ci). When the B cell subsets in the spleens were analyzed, Zbtb1 knockout mice were found to exhibit higher proportions of marginal zone B cells and higher proportions of follicular B cells than did either wild-type or TCRα knockout mice (Fig. 5B, Supplemental Fig. 2Cii). These findings were consistent with the phenotype of the scantT mice (22). Thus, although B cell development appeared to be impaired at the pre-B cell stage in the absence of Zbtb1, suggesting that Zbtb1 is important during early B cell development, the number of mature B cells in the periphery was comparable to that in wild-type controls. This could be due to redundancy of the function of Zbtb1 in peripheral B cells and/or compensation for its functions by other members of the same protein family or through different signaling pathways.

It is difficult to distinguish a B cell-intrinsic defect from altered proportions of B cells at different stages of development. However, in vitro proliferation of B cells from Zbtb1 knockout mice, wild-type littermates, and TCRα knockout mice, in response to anti-IgM, LPS, and anti-CD40, showed that B cells from the Zbtb1 knockout mice proliferated at least as much or more than those from wild-type or TCRα knockout mice (Fig. 5C). The brisk proliferation of B cells from the Zbtb1 knockout mice could be accounted for by the increased proportion of marginal zone cells in their spleens (Fig. 5B), since marginal zone cells proliferate more than follicular B cells (37–39).

**FIGURE 5.** Analysis of B cells of knockout mice. (A) Relative proportions of BM B cell subsets (wild-type, Zbtb1−/−, and TCRα−/− mice) as defined by the Hardy and Hayakawa gating scheme: I, pro-B (B220+CD43+); II, pre-B (B220+CD43−IgD−IgM−); III, pre-B late (B220−CD43−IgD+IgM−); IV, immature B (B220−CD43−IgD+IgM−); V, mature recirculating B (B220+CD43−IgD+IgM−). (B) Relative proportions of splenic B cell subsets in wild-type, Zbtb1−/−, and TCRα−/− mice: I, immature B (B220−IgD+IgM+CD23−CD21/35−); II, marginal zone B (B220−IgD−IgM+CD23+CD21/35+); III, follicular B (B220−IgD−IgM+CD23−CD21/35−). Data represent means ± SD (four mice per genotype). *p < 0.1, **p < 0.05. (C) Wild-type, Zbtb1−/−, and TCRα−/− splenic B cell proliferation after stimulation with LPS (0.1 and 10 ng/μl), anti-IgM (0.1 and 10 ng/μl), or anti-CD40 (0.1 and 10 ng/μl), measured by BrdU incorporation at 48 h.
Tissue expression of Zbtb1

Copy number of Zbtb1 mRNA in lymphoid tissues of wild-type mice was determined by quantitative real-time PCR and normalized to the housekeeping gene HPRT (Fig. 6). Maximum tissue expression was found in thymus and spleen, followed by lymph nodes and PBMCs. Unfractionated and lineage-depleted BM had expression, but at lower levels (Fig. 6A).

Two isoforms were found, Zbtb1_713 and Zbtb1_644, that share an initial noncoding exon 1 and the 5’ end of exon 2. Zbtb1_713 is not further spliced and encodes a BTB/POZ domain followed by eight zinc finger domains in a single open reading frame (40). The shorter Zbtb1_644, formed by a splice from within exon 2 to an alternative exon 3, lacks the last three C-terminal zinc finger domains found in Zbtb1_713 and was less abundant in all cell types studied (Fig. 6B, 6C). Although DN1–DN4 thymocytes expressed both Zbtb1 isoforms, maximum expression was observed in the CD4⁺CD8⁺ DP population, in which Zbtb1_644 was 20% as abundant as the Zbtb1_713. CD4⁺ and CD8⁺ single-positive cells had lower expression of both isoforms (Fig. 6B).

Analysis of the expression of the two isoforms of Zbtb1 mRNA in BM revealed maximum expression in long-term HSCs (LSK, CD34⁺, CD48⁻), less in short-term HSCs (LSK, CD34⁺, CD48⁻), and still less in multipotent progenitors (LSK, CD34⁺, CD48⁻) (35, 36) (Fig. 6C).

B lymphoid subsets of the BM showed a striking increase in Zbtb1 expression from pro-B (B220⁺, CD43⁺) to pre-B (B220⁺, CD43⁻) cell stages, with levels then declining upon further differentiation into immature B cells (B220⁺, CD43⁻, IgD⁻, IgM⁺) and low levels in mature B cells (B220⁺, CD43⁻, IgD⁺, IgM⁺) (Fig. 6D). The increased expression of Zbtb1 mRNA in pre-B cells suggests that Zbtb1 plays an important role at this stage of B cell development and could possibly explain the above-described decreased number of pre-B cells observed in BM of Zbtb1-deficient compared with wild-type mice. In splenic B cells, overall Zbtb1 mRNA levels were 10-fold lower, with predominant expression in follicular (B220⁺, IgD⁺, IgM⁺, CD43⁻, CD5⁻, CD23⁻, CD21/35⁻) and marginal zone (B220⁺, IgD⁺, IgM⁺, CD23⁻, CD21/35⁻) fractions (Fig. 6E).

FIGURE 6. Zbtb1 mRNA expression measured by quantitative PCR in lymphoid populations in wild-type mice. (A) Spleen, PBMCs, thymus, lymph node, BM, and BM depleted of mature lineages (Lin⁻ve BM). (B) Thymocyte subsets, as described in Fig. 4A. (C) BM progenitor populations, as described in Fig. 4B. (D and E) BM and splenic B lineage subsets, as described in Fig. 5; y-axis shows copy numbers of Zbtb1 per 1000 copies of housekeeping gene HPRT. Data represent means ± SD (five mice per group).
Expression and nuclear localization of Zbtb1 protein

Expression vectors with human and mouse cDNAs encoding the 713- and 644-aa isoforms of Zbtb1 were constructed with epitope tags at either N- or C-terminal positions. Human Zbtb1 (713), with an N-terminal FLAG sequence following the initial methionine codon (N-FLAG-hZBTB1 (713)) was expressed in 293T cells. To determine the cellular localization of the protein, Western blotting was carried out with the nuclear and cytoplasmic protein fractions and blots were probed with anti-FLAG Ab to detect ZBTB1, as well as anti-histone deacetylase (HDAC1) and anti-α1a-tubulin Abs to document purity of nuclear and cytoplasmic extracts, respectively (Fig. 7A). The tagged protein was readily detectable in nuclear extracts (Fig. 7Ai). Consistent with its structural similarity to transcriptional repressors and its previously described nuclear location (41), we found the ZBTB1 signal with the anti-FLAG Ab confined to the nuclear fraction, and not in the cytoplasmic fraction (Fig. 7Aii).

Dimerization of Zbtb1 isoforms sharing identical BTB/POZ domains

We studied dimerization using Zbtb1 (713) tagged with FLAG and Zbtb1 (644) tagged with myc at either end to minimize the chance of steric hindrance (42). After transfection into 293T cells, N- and C-FLAG-Zbtb1 (713) were detected at 83 kDa using mouse anti-FLAG and HRP-anti-mouse IgG (Fig. 7B, upper panel); similarly, somewhat smaller N- and C-myc-Zbtb1 (644) were detected using mouse anti-myc (not shown). Upon reciprocal immunoprecipitation, the N- and C-FLAG-Zbtb1 (713) proteins were coprecipitated with either the N- or C-myc-Zbtb1 (644), and vice versa (Fig. 7B, middle and lower panels).

Thus, the epitope tags did not affect protein expression or interaction, and the short and long isoforms, with identical 5’ BTB dimerization domains, were shown to undergo dimerization. Thus, the short isoform, although lacking the three C-terminal zinc fingers and expressed at a considerably lower level than the predominant 713-aa isoform, may bind to the long form under physiologic conditions, potentially modifying the activity of Zbtb1 DNA-binding complexes.

ZBTB1 sequencing in human SCID patients

The phenotype of mouse Zbtb1 deficiency suggested that deleterious mutations in human ZBTB1 could cause T B+ immune deficiency in humans. We examined the sequence of ZBTB1 in 20 cases of human SCID with T B+ phenotype, but without demonstrable mutations in previously reported SCID disease genes. However, none was found to have variants in ZBTB1 that were predicted to be deleterious.

Discussion

Our results strengthen the evidence that Zbtb1 is essential for T cell development, as recently noted in scanT mice generated by chemical mutagenesis by Siggs et al. (22). The steps in T lineage differentiation from HSCs are not fully understood, and identification of the role of Zbtb1 in prethymic lymphoid pathways and migration to the thymus will further elucidate this process. Analysis of fetal thymi of our mice lacking Zbtb1 showed decreased numbers of the earliest DN1 thymocytes and failure to progress beyond the DN1 stage. A lymphocyte-autonomous defect rather than defective thymic microenvironment was demonstrated by reciprocal transplants showing that knockout thymus supported generation of wild-type BM into T cells, whereas the converse transplant of knockout BM into wild-type mice did not. Zbtb1 mRNA was expressed in wild-type thymocytes, increasing to a maximal level at the DP stage and then declining, and the total lack of maturing thymocytes in mice without Zbtb1 implies that the role of this factor in thymocyte differentiation is irreplaceable and cannot be compensated for by other members of the same protein family or by alternate signaling pathways. The much reduced NK cell number in all mice lacking Zbtb1 indicates that it is also important for NK cell development or expansion. The B cell phenotype of our knockout mice is similar to that of scanT mice (22); neither the scanT C74R missense mutation nor the complete absence of Zbtb1 in our transgenic mouse affected the number of B cells. Mature B cells were found in the periphery of Zbtb1-deficient animals. Their in vitro proliferation in response to...
to various stimuli was comparable to wild-type B cells, arguing against a B cell-intrinsic defect and suggesting that the observed inability to class switch could be due to absence of T cell help.

There are 49 known mammalian Zbtb gene family members (43). BTB/POZ domains at the N termini of these proteins mediate either homo- or heterodimerization with related proteins (44). All have multiple C2H2 zinc finger domains, which in canonical form have two Cys and two His residues in a C-2-C-12-H-3-H sequence. The 12 central residues, when stabilized by a Zn atom, form a projection that can interact with the major groove of a dsDNA helix, recognizing around five specific bp of a G-rich sequence (45, 46). Proteins belonging to the Zbtb family, including BCL6/ZNF51, Th-POK/ZFP67, PLZP, and PLZF/Zbtb16, to name a few, are transcription repressors implicated as switches at branch points between alternative developmental pathways. Their underexpression causes failure to branch out from a default pathway and alters homeostasis of hematopoietic cell compartments, whereas overexpression can also produce uncontrolled (often malignant) expansion of cells (6, 7, 44, 47, 48). Additionally, proteins with a similar structure have also been shown to mediate transcriptional repression and interact with components of histone deacetylase corepressor complexes (40, 48–51). The Zbtb25 gene located immediately adjacent to Zbtb1 is also implicated in T cell development and negatively regulates NF of activated T cells, NF-AT (52).

Zbtb1 is a small gene now known to encode a transcriptional repressor that may act through control of chromatin remodeling, which in turn leads to changes in gene expression (40). It is one of a few genes thought to be transcribed by spRNAP-IV, a single polypeptide nuclear polymerase expressed from an alternate transcript of the mitochondrial RNA polymerase gene POLRMT (53, 54). We have shown that both predicted isoforms of the protein are expressed, a 713-aa protein encoded by an unspliced open reading frame in exon 2 and an alternate 644-aa isoform produced by a splice from within the distal coding region of exon 2 to exon 3. Both isoforms share the N-terminal BTB/POZ dimerization domain and proximal five zinc finger domains (44), but the last three zinc finger domains are truncated from the alternately spliced isoform. The longer Zbtb1l133 is expressed at a higher level than Zbtb1l644 in all tissues investigated. The two isoforms not only form homodimers, as expected for members of the Zbtb family (44), but also can dimerize with each other. Although the physiologic targets of Zbtb1 transcription repression are unknown, the reduced complement of zinc finger domains in the shorter isoform might confer reduced repressive activity upon a heterodimeric Zbtb1l313/Zbtb1l644 complex, potentially modulating or fine-tuning its effect. Further functional studies with the two isoforms of the protein may also shed light on the regulation of repressive activity of other proteins with similar structures.

Detailed analysis of our Zbtb1 knockout mice has revealed a contrast between the scanT mice and the Zbtb1 knockout mice in the numbers of the BM progenitor subsets. Although the numbers and percentages of BM stem cell subsets in the scanT mice were reported to be comparable to wild-type mice (22), our Zbtb1 knockout mice had significantly increased numbers of short-term HSCs, MPPs, and CLPs. This difference could be due to residual function of the protein bearing a C74R mutation in the B1B domain of Zbtb1 in scanT mice. Complete absence of Zbtb1 may result in a loss of repression of branching into differentiation pathways, causing an increase in numbers of the stem cell subsets in default pathways in our knockout mice. Analysis of the transcriptional activity of truncated Zbtb1 proteins by Liu et al. (40), each containing one of the two major functional domains (BTB and ZNF), revealed that the ZNF domain is responsible for its nuclear localization and exhibits the strongest repressive activity. This suggests that some residual activity may therefore still be present in the scanT mice due to their unaffected ZNF domains.

An explanation for the increased number of HSCs in Zbtb1 knockouts could be that there is an attempt to compensate for increased apoptosis or otherwise decreased fitness that impairs lymphocyte development; our preliminary mixed chimera studies have suggested the latter, as did similar experiments with scanT mice (22). The increased number of HSCs and progenitors in the BM, but decreased thymocyte number in Zbtb1-deficient mice, could also reflect a defect in migration and homing to the thymus, a process facilitated by chemokine receptors CCR7 and CCR9 (55–57). Indeed, our preliminary experiments (not shown) suggest a decrease in expression of CCR9 mRNA in Zbtb1 knockout HSCs.

Activation of the cAMP signaling pathway has been shown to modulate T cell development, and stimulation of cAMP-dependent signaling in the thymus prevents differentiation of T cell precursors and results in increased apoptosis (58). It has been reported that one function of Zbtb1 is a dose-dependent repression of a cAMP response element, preventing it from binding to a CREB (40). Moreover, a human multiple malformation syndrome with hypoplasia of the thymus was recently associated with CREB1 mutation (59). Thus, the absence of Zbtb1-mediated repression of c-AMP signaling pathways could be one of the reasons why T cells are unable to develop in the thymus of both complete knockout and scanT mice.

The mechanism of action of Zbtb1 during early lymphoid determination could also be to repress the differentiation of B cell progenitors from the ALPs in BM (2), favoring the differentiation of ALPs into T cell precursors instead (Fig. 7C). Further experiments, such as with RNA expression arrays and chromatin immunoprecipitation, will elucidate the molecular targets of Zbtb1.

A precise understanding of the development of lymphocytes from HSCs is critical for treating humans with immunodeficiencies, blood diseases and cancers by transplantation, gene therapy, and discovery of new drugs. Zbtb1 is similar to transcription factors with proven roles in lymphocyte development and has now been shown repeatedly to be essential and specific for mouse thymocyte development (6, 7, 22). Our ability to elucidate and manipulate Zbtb1 functions in mice and ultimately in humans will facilitate understanding and treating human immune disorders. SCID and related primary immunodeficiencies in humans are rare, but life-threatening, and in many cases with genetic causes still unknown. Although to date we have not found mutations of human ZBTB1 in patients with typical T−B− SCID phenotypes, characteristics of humans with ZBTB1 defects may be distinct, and examining a wider range of human phenotypes, including those with multiple malformations associated with thymic hypoplasia, with deficient cortical T cells such as the CREB1 defect reported by Kitazawa et al. (59) may be fruitful. In any case, our new Zbtb1-deficient model will be useful to study the role of this transcription factor and its associations and targets in the development of T lymphocytes.

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