The Hedgehog receptor Patched controls lymphoid lineage commitment


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A first step in hematopoiesis is the specification of the lymphoid and myeloid lineages from multipotent progenitor cells in the bone marrow. Using a conditional ablation strategy in adult mice, we show that this differentiation step requires Patched (Ptc), the cell surface-bound receptor for Hedgehog (Hh). In the absence of Ptc, the development of T- and B-lymphoid lineages is blocked at the level of the common lymphoid progenitor in the bone marrow. Consequently, the generation of peripheral T and B cells is abrogated. Cells of the myeloid lineage develop normally in Ptc mutant mice. Finally, adoptive transfer experiments identified the stromal cell compartment as a critical Ptc-dependent inducer of lymphoid versus myeloid lineage commitment. Our data show that Ptc acts as a master switch for proper diversification of hematopoietic stem cells in the adult organism. (Blood. 2007;110:1814-1823)

Introduction

Hematopoietic stem cells (HSCs) represent the common origin of all cell types within the immune system. According to the Akashi-Kondo-Weissman model, an early event of adult hematopoiesis is the differentiation of multipotent progenitors (MPPs) in the bone marrow (BM) into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs).1,3 CMPs further differentiate into megakaryocyte/erythocyte progenitors (MEPs), or into granulocyte/macrophage progenitors (GMPs), which give rise to megakaryocytes and erythrocytes, or to granulocytes and macrophages, respectively. CLPs develop into precursor B cells, early thymic T-cell progenitors (ETPs), and natural killer (NK) cells.

Discrete developmental stages of myelo- and lymphopoiesis have been defined based on the expression of stage-specific marker proteins (reviewed in Busslinger8). For example, HSCs normally express high levels of the surface receptors c-kit and Sca-1, which are both down-regulated as HSCs progress to CLPs. Formation of CLPs requires the transcription factors Spi-1/PU.1 and Ikaros together with the Fms-like receptor tyrosine kinase 3 (Flt3) and the α-chain of the IL-7 receptor (IL-7Rα). However, the details of early HSC differentiation are unclear, especially the specification of myeloid and lymphoid precursor cells. Recently, members of the Hedgehog (Hh) family of secreted signaling proteins have been shown to affect the proliferation of primitive hematopoietic cord blood cells5,6 and to play a role in proliferation, survival, and differentiation of early T cells and germline center B cells.5,7,11 How Hh affects T-cell development is unclear. The impact of this pathway on B-cell development and the specification of the myeloid lineage has not yet been investigated.

Three mammalian Hh proteins exist (Sonic, Indian, and Desert Hh), which are recognized and bound by the cell surface receptor Patched (Ptc). Binding of Hh to Ptc suspends the inhibition of its membrane-bound signaling partner Smoothened (Smo), which in turn initiates nuclear translocation and activation of the Gli family of transcription factors. This is followed by the expression of a plethora of downstream target genes including Bcl-2, cell-cycle regulators, and Ptc itself (reviewed in Ruiz i Altaba et al12). Hh/Ptc signaling is critical for cell-fate determination during embryogenesis and for cell growth and differentiation in the adult organism. An abnormal activation of the Hh/Ptc pathway results in developmental abnormalities and tumorigenesis.12,13 Because targeted disruption of either Hh or Ptc is embryonically lethal,14-16 analysis of Hh/Ptc signaling in adult tissues has been largely limited to in vitro analysis. By inducibly abrogating Ptc expression in the mouse, we show here that Hh/Ptc signaling in stromal cells is mandatory for the specification of both B- and T-cell lineages at the CLP progenitor stage, but not of the myeloid lineage in the BM.

Materials and methods

PtcΔlox/Δlox and PtcΔlox/ΔloxERT2/+/− mouse line generation

Genomic DNA clones were isolated as described in Hahn et al.16 To generate the PtcΔlox conditional mouse line, a targeting vector derived from pPNT4 described by Conrad et al17 was constructed, in which a loxp site was inserted into intron 7 of the Ptc gene. The second loxp site together with the neo-cassette of the pPNT4 vector was introduced into intron 9. The neomycin resistance cassette of pPNT4 is also flanked by frt sites to enable FLPe recombinase-mediated excision, if required.18 The linearized targeting vector was electroporated into R1 embryonic stem cells. Upon positive and negative selection of the transfectants,19 XhoI-digested genomic DNA was analyzed by Southern hybridization using a 5′ external probe to detect homologous recombination, which was confirmed by polymerase chain reaction (PCR) using the primers Neo-R/p910F, Neo-F/p1011R.2, or p910F.4/p1011R.2. Three positive clones were microinjected into blastocysts. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 USC section 1734.
C57BL/6 blastocysts, and obtained chimeric mice were mated to C57BL/6 females. The agouti offspring were tested by PCR and Southern blot analysis to confirm germ-line transmission of the Ptchflox conditional allele. Ptchfloxflox/+ siblings were intercrossed to generate Ptchfloxfloxoffspring. The Rosa26CreERT2 (ERT2) knock-in mouse strain (kindly provided by Dr Anton Berns, The Netherlands Cancer Institute, Amsterdam, The Netherlands) expresses a fusion gene encoding Cre recombinase and a modified ligand-binding domain for the estrogen receptor under control of the endogenous Rosa26 promoter (A. Berns, written personal communication, February 15, 2005). These mice were bred to Ptchfloxflox mice. The resulting Ptchfloxflox/ERT2+/- mice were crossed with Ptchfloxflox mice to obtain PtchfloxfloxERT2+/- mice. Eight-week-old PtchfloxfloxERT2+/- mice were injected intraperitoneally with 1 mg tamoxifen dissolved in a 1:10 ethanol-sunflower oil emulsion21 on 5 consecutive days to induce the Ptchdel mutation (named Ptch-/-) or with solvent alone. Mice with PtchfloxfloxERT2+/- genotypes were used to assess any unspecific effects of tamoxifen. To detect the Cre-mediated Ptchdel mutation, which removes the floxed exons 8 and 9, mice were genotyped by PCR using the primer combination Exon7-F/NeoR or Neo-F/p1011R.2. For location of primers, see Figure 1A. All experiments using animals were performed according to legal requirements.

Detection and quantification of CreERT2-mediated recombination at the Ptchflox locus

Two tamoxifen-treated PtchfloxfloxERT2+/- mice were killed at day 10 after the first injection and genomic DNA was isolated from several organs. PtchfloxfloxERT2+/- mice were used for generation of standard curves. Recombination efficiency was quantified on an ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA) by real-time PCR using forward primers hybridizing to intron 7 and intron 9, and a common reverse primer hybridizing immediately downstream of the second loxP site in intron 9. A FAM-labeled probe was used for detection of the deleted Ptchdel allele, whereas a Yakima Yellow–labeled probe detected the Ptchflox allele. A pelota gene-specific quantitative PCR assay was used for data normalization. Data were processed using the standard curve method for relative quantification. Separate standard curves were generated for each tissue. The deletion efficiency was calculated as the ratio of the values for the deleted allele to the total value from a Ptchflox mouse plus the deleted allele, and is expressed in percentage. All sequences of primers and probes together with detailed protocols are available upon request.

RNA isolation and reverse-transcription PCR

Total RNA was extracted from various tissues and embryos using TRIZOL reagent (Invitrogen, Carlsbad, CA). Reverse transcription was conducted with random hexamers and SuperScriptII reverse transcriptase (Invitrogen, Carlsbad, CA). Reverse transcription was conducted using forward primers hybridizing to intron 7 and intron 9, and a common reverse primer hybridizing immediately downstream of the second loxP site in intron 9. A FAM-labeled probe was used for detection of the deleted Ptchdel allele, whereas a Yakima Yellow–labeled probe detected the Ptchflox allele. A pelota gene-specific quantitative PCR assay was used for data normalization. Data were processed using the standard curve method for relative quantification. Separate standard curves were generated for each tissue. The deletion efficiency was calculated as the ratio of the values for the deleted allele to the total value from a Ptchflox mouse plus the deleted allele, and is expressed in percentage. All sequences of primers and probes together with detailed protocols are available upon request.

Blood and BM-cell analysis

Peripheral blood or single-cell suspensions of BM cells derived from femur and tibia of hind limbs as well as thymocytes and splenocytes were prepared and incubated for 15 minutes on ice with different combinations of the following antibodies: anti-CD3-FITC, anti-CD4-biotin, anti-CD8a-PE, anti-CD8a-PE-Cy7, anti-CD21-FITC, anti-CD24-PE, anti-CD25-FITC, anti-CD43-biotin, anti-CD44-PE-Cy5, anti-CD45R/B220-PE-Cy7, anti-Idg-FITC, anti-TcR-β-FITC (BD Biosciences Pharmingen, San Jose, CA), and anti-IgM-RPE (Southern Biotechnology Associates, Birmingham, AL). Binding of biotinylated antibodies was monitored by streptavidin-PE or streptavidin-PE-Cy5 (BD Biosciences Pharmingen) or by streptavidin-FITC (Southern Biotechnology Associates). Erythrocytes of peripheral blood samples were lysed with fluorescence-activated cell sorting (FACS) ysis solution (BD Biosciences Pharmingen). Annexin V-PE staining was carried out using an annexin V-PE apoptosis detection kit (BD Biosciences Pharmingen). Hematopoietic stem and progenitor cells from 107 BM cells were purified by depletion of lineage-positive (Lin+) cells using the EasySep mouse hematopoietic progenitor cell enrichment kit (StemCell Technologies, Vancouver, BC; depletion kit contains monoclonal antibodies toward murine CD5, B220, Mac-1, Gr-1, Ter119, and 7-4). Enriched lineage-negative (Lin-) BM cells were incubated for 15 minutes on ice with anti-CD117 (c-kit)-Biotin, anti-CD127 (IL-7Rα)-PE, and anti-Sca-1-FITC, and in a second staining step with streptavidin-PE-Cy7 or streptavidin-PE-Cy5 (all from BD Biosciences Pharmingen). For cell-cycle analysis, 10 μL of 10 mg/mL BrdU were injected intraperitoneally 3 hours before killing of the mice, and enriched Lin- BM cells were stained with anti-c-kit-Biotin (streptavidin-PE-Cy7), anti-Sca-1-biotin (streptavidin-PE), anti-BrdU-FITC, and 7-AAD (BD BrdU FITC Flow Kit; BD Biosciences Pharmingen). To avoid cross-reactions of unbound biotin binding sites in the biotin/streptavidin complexes, anti-c-kit-biotin and anti-Sca-1-biotin were mixed in separate reactions with the respective streptavidin conjugates that were used in excess. Probes were stained first for c-kit with anti-c-kit-biotin/ streptavidin-PE-Cy7 and after extensive washing with anti-sca-1-biotin/ streptavidin-PE. Cells were analyzed on an LSR II flow cytometer (BD Biosciences Pharmingen). Data acquisition and analysis were performed using software BD FacsDiva (BD Biosciences Pharmingen) and FlowJo (Treestar, Ashland, OR), respectively. EDTA blood samples were collected by heart puncture and analyzed on an Act 5 hematology analyzer (Beckman Coulter, Hialeah, FL) and by blood smear tests.

Clonogenic progenitor assay

Mouse clonogenic progenitor assays were performed by plating 2 × 103 Lin- BM cells into a methylcellulose medium (MethoCult 3434; StemCell Technologies) containing SCF, IL-3, IL-6, and Epo according to the manufacturer’s instructions. Differential colony counts were scored after 8 days by morphologic characteristics using an inverted microscope. ERT2-mediated Ptch inactivation in CFU colonies was analyzed by PCR as described in “Detection and quantification of CreERT2-mediated recombination at the Ptchflox locus.”

Adoptive transfer and repopulation assay

BM cells were isolated from control and Ptch-/- mice 19 days after tamoxifen injection and Lin- BM cells were purified as described above. Nine-week-old Rag-2-/- mice (Taconic, Germantown, NY) were irradiated with 7 Gy and engrafted with 400 000 Lin- BM cells from donor mice by tail vein injection.23 Seven and 25 weeks after injection of the cells, peripheral blood obtained from the retro-orbital sinus was analyzed by flow cytometry. Donor-derived T and B cells were detected using anti-CD4-biotin (streptavidin-FITC), anti-CD8a-PE-Cy7, and anti-B220-PE-Cy5 antibodies. T and B cells of Rag-2-/- mice reconstituted with control or Ptch mutant Lin- BM cells were isolated using magnetic-activated cell sorting (MACS) separation columns (Miltenyi Biotech, Auburn, CA), and ERT2-mediated recombination of Ptch alleles was analyzed as described.

Results

Postnatal inactivation of Ptch alleles

Proper genomic recombination of the Ptchflox targeting vector (pPtchflox) into the wt Ptch allele in R1 embryonic stem cells was analyzed by Southern blot hybridization and by PCR from genomic DNA (Figure 1B and 1C, respectively). Positive embryonic stem- cell clones were injected into blastocysts of C57BL/6 mice, and resulting chimeras were bred to wt C57BL/6 females. Heterozygous Ptchfloxflox offspring were intercrossed to obtain the homozygous Ptchflox mouse line. Ptchfloxflox mice were born at the expected Mendelian ratio. All heterozygous and homozygous mice remained healthy over an observation period.
mediated deletion of exons 8 and 9 of the recombinase activity. The recombination efficiency of Cre-mice (Figure 1D). Postnatal inactivation of expression of mutant confirmed by reverse-transcription (RT)–PCR and revealed spleen, thymus, and skin from Ptchflox/flox ERT2 (Figure 1D). Thus, tissue specificity and recombinase efficiency into Ptchdel compartments. More than 75% Ptchflox alleles (data not shown). These results were confirmed by reverse-transcription (RT)–PCR and revealed expression of mutant Ptchdel transcripts in the analyzed tissues (Figure 1D). Thus, tissue specificity and recombinase efficiency of Cre in ERT2 mice appear to be identical to those reported for Mx1Cre mice, which are frequently used for immunologic studies.

Thymic atrophy and defective T-cell development in tPtch mice

Fifteen days after the first tamoxifen injection, tPtch mice were strongly inactivated. Since both nonstromal and stromal cells contribute to whole BM, Ptch must have been deleted in both cell compartments. More than 75% Ptch alleles in liver, kidney, spleen, thymus, and skin from tPtch mice were converted into Ptchdel alleles. The recombination efficiency in lung, heart, and skeletal muscle was more than 50%, whereas in cerebellum and brain less than 25% of the Ptch alleles were converted into Ptchdel alleles (data not shown). These results were confirmed by reverse-transcription (RT)–PCR and revealed expression of mutant Ptchdel transcripts in the analyzed tissues (Figure 1D). Thus, tissue specificity and recombinase efficiency of Cre in ERT2 mice appear to be identical to those reported for Mx1Cre mice, which are frequently used for immunologic studies.

Thymic atrophy and defective T-cell development in tPtch mice

Fifteen days after the first tamoxifen injection, tPtch mice were marked reduced compared with control animals (Figure 2A,B). At day 19, the thymus of tPtch mice had lost its regular morphology and the cortex was no longer distinguishable from the medulla (Figure 2A and data not shown). Only a few thymocytes were left at this stage (Figure 2B,C left panel). Although a moderate reduction of thymocyte numbers was also observed in tamoxifen-treated Ptchdel/ERT2−/− control animals (Figure 2C left and middle panels), our data indicated a role of Ptch on thymocyte differentiation, which is consistent with previous reports. We thus monitored distinct T-cell populations by flow cytometry. Although the absolute numbers of all subpopulations were lowered in tPtch mice (data not shown), the relative numbers of immature double-negative (DN, CD4−CD8+) and mature single-positive (CD4+ or CD8+) T cells were strongly increased, whereas the double-positive (DP, CD4+CD8+) population was almost depleted in tPtch mice 19 days after tamoxifen injection (Figure 2C middle panel). This result...
suggested that DN T-cell precursors require thymic Ptch expression to progress to the DP stage.

Four early stages of DN-cell differentiation can be discriminated based on the expression of CD25 and CD44 surface markers. The DN1 population (CD25⁻/⁻CD44⁻/⁻) possesses multilineage potential, while DN2 (CD25⁻/⁻CD44⁻/⁻) and DN3 (CD25⁻/⁻CD44⁻/⁻) cells commit to the T-cell lineage before they further develop into DN4 cells (CD25⁻/⁻CD44⁻/⁻). In tPtch⁻/⁻ thymi, the DN1 population was considerably overrepresented, whereas DN2 and DN3 populations were diminished (Figure 2C right panel). The relative number of DN4 cells remained stable within the entire observation period. Collectively, our data show that upon Ptch ablation early thymocyte development is blocked at 2 stages (ie, at the transitions from DN1 to DN2 and DN4 to DP).

Ptch ablation compromises splenic B-cell development

We next assessed a possible role of Ptch ablation on peripheral B-cell subsets. Splenic B220-positive B lymphocytes can be distinguished based on their expression patterns of CD21, CD24, and the B-cell antigen receptor (BCR) of classes IgM and IgD.27 Transitional B cells of type 1 (T1, CD24⁺CD21⁺IgMhighIgDlow) are recent immigrants from the BM and develop into transitional type 2 B cells (T2, CD24⁺CD21⁺IgMhighIgDhigh). Mature B lymphocytes (CD24lowCD21lowIgMlowIgDlow) can be generated from both T1 and T2 B cells.27 In tPtch⁻/⁻ spleens, the absolute number of B220-positive cells was reduced compared with the respective controls (data not shown). This was due mainly to a reduction of the population of T1 B cells. This is also reflected by their relative numbers that are drastically diminished compared with vehicle- and tamoxifen-treated control animals (Figure 3). In contrast, the pool of the more mature T2 cells remained stable and mature B cells were overrepresented. This effect was detected already at day 15 after treatment onset (data not shown) and progressed until day 19. Thus, in the spleen of tPtch⁻/⁻ mice, the supply of immature B cells from the BM is severely impaired suggesting a role of Ptch on early B-cell differentiation.

Ptch is required for B-cell differentiation in the BM

B-cell lymphopoiesis in the BM can be monitored by cell-surface staining of B220, CD43, and IgM. B220 and CD43 are both expressed on early B-cell precursors prior to the pro-B-cell stage, which is defined as B220lowCD43⁻IgM⁻. Progression to the pre-B-cell stage is associated with loss of CD43 and
expression of the pre-BCR encompassing the μm heavy chain (B220\(^{\text{high}}\)CD43\(^{\text{low}}\)μm\(^{\text{low}}\)). B220 expression increases on immature B cells (B220\(^{\text{high}}\)CD43\(^{\text{low}}\)IgM\(^{\text{low}}\)IgD\(^{\text{low}}\)) and is maintained on immunocompetent recirculating B cells, which additionally express the IgD-BCR (B220\(^{\text{high}}\)CD43\(^{\text{low}}\)IgM\(^{\text{low}}\)IgD\(^{\text{high}}\)).28 The total lymphocyte fraction in the BM of tPtch\(^{-/-}\) mice was only slightly decreased compared with wt controls (Figure 4 left panel). Striking alterations were, however, observed for the individual BM B-cell subsets in tPtch\(^{-/-}\) mice. Cell-surface staining of B220 versus CD43 revealed an almost complete loss of CD43\(^{+}\) progenitor B cells (Figure 4 middle panel, fraction II), which in turn led to a dramatic loss of B220\(^{\text{low}}\) pre-B and immature B cells (right panel, fractions I and II). Conversely, the relative numbers of recirculating B lymphocytes, which are B220\(^{\text{high}}\) (Figure 4 middle and right panel, fraction III) and IgD positive (data not shown), were strongly increased. A similar distribution of BM B-cell subsets was observed when counting the absolute cell numbers (data not shown). A moderate effect of tamoxifen on the pro-, pre-, and immature B cells was observed at day 15 in all tamoxifen-treated control mice (data not shown); this effect was almost compensated at day 19 of treatment (Figures 3,4). Collectively, our data reveal a severe block of early B-cell development in tPtch\(^{-/-}\) mice prior to the pro-B-cell stage.

**Lineage specification of CLPs is defective in tPtch\(^{-/-}\) mice**

Since Ptch ablation affected both T- and B-cell lineages, we next analyzed their common precursors in our mutant mice. Within the pool of lineage-negative (Lin\(^{-}\)) cells in the BM, those with small and nongranule appearance harbor both HSCs and more committed descendant precursors.30 HSCs normally express high levels of c-kit and Sca-1, which are down-regulated in committed progenitors.30 As shown in Figure 5A (left panel), the HSC-containing Lin\(^{-}\)c-kit\(^{\text{high}}\)Sca-1\(^{\text{high}}\) cell population III was relatively increased in tPtch\(^{-/-}\) mice as was the fraction I of Lin\(^{-}\)c-kit\(^{\text{low}}\)Sca-1\(^{\text{low}}\) cells, which comprises most of the myeloid progenitors.28,31,32 In marked contrast, the CLP-containing fraction II (Lin\(^{-}\)c-kit\(^{\text{low}}\)Sca-1\(^{\text{low}}\)) was underrepresented and its relative cell number was reduced by half. Hence Ptch ablation appears to compromise CLPs. This notion was further confirmed by expression analysis of IL-7Rα chain (Figure 5A right panel), which is required for the generation of CLPs. In tPtch\(^{-/-}\) mice, the number of IL-7Rα-expressing cells within the CLP-containing fraction II (Lin\(^{-}\)c-kit\(^{\text{low}}\)Sca-1\(^{\text{low}}\)) was drastically reduced compared with controls. Moreover, BrdU/7-AAD stainings shown in Figure 5B revealed a markedly reduced

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**Figure 3. Splenic T1 B cells are lost in tPtch\(^{-/-}\) mice.** Splenocytes of vehicle-treated Ptch\(^{flox/flox}\)ERT2\(^{-/-}\) or tamoxifen-treated Ptch\(^{flox/flox}\)ERT2\(^{-/-}\) controls and of day-19 tPtch\(^{-/-}\) mice were analyzed by flow cytometry using antibodies against B220, CD21, CD24, IgM, and IgD to distinguish T1, T2, and mature (M) B cells. (Left panels) Within the B220-positive fraction, T1 cells are CD24\(^{\text{low}}\)CD21\(^{\text{low}}\), T2 cells are CD24\(^{\text{high}}\)CD21\(^{\text{high}}\), and mature B cells are CD24\(^{\text{high}}\)CD21\(^{\text{low}}\). (Right panels) Within the B220-positive fraction, T1 cells are IgM\(^{\text{low}}\)IgD\(^{\text{high}}\), T2 cells are IgM\(^{\text{high}}\)IgD\(^{\text{low}}\), and mature B cells are IgM\(^{\text{high}}\)IgD\(^{\text{high}}\)/B220\(^{\text{high}}\). The number of cells present in each fraction is presented as relative percentage of the total cell number plotted. Data are representative of 4 independent experiments (each experiment consisted of 1 vehicle-treated Ptch\(^{flox/flox}\)ERT2\(^{-/-}\), 1 tamoxifen-treated Ptch\(^{flox/flox}\)ERT2\(^{-/-}\), and 1 tPtch\(^{-/-}\) mouse).

**Figure 4. Early block of B-lineage commitment in the BM of tPtch\(^{-/-}\) mice.** BM cells from vehicle- and tamoxifen-treated control animals and of day-19 tPtch\(^{-/-}\) mice were analyzed by flow cytometry using antibodies against B220, CD43, and IgM (middle and right panels) to distinguish pro-B (fraction I), pre-B (fraction II), and the pool encompassing immature, mature, and recirculating B cells (fraction III) within the lymphocyte gate defined by the SSC/FSC plots (left panels). Data represent 4 independent experiments (each experiment consisted of 1 vehicle-treated Ptch\(^{flox/flox}\)ERT2\(^{-/-}\), 1 tamoxifen-treated Ptch\(^{flox/flox}\)ERT2\(^{-/-}\), and 1 tPtch\(^{-/-}\) mouse).
proliferative capacity of cells in fraction II from tPtch/H11002 mice (middle panel). This was also true for cells in fraction III (Lin−c-kit+Scakithigh), which contains HSCs and MPPs (right panel). Importantly, myeloid precursors of fraction I (Lin−c-kit−Scakitlow) proliferated normally (left panel).

Final evidence for a CLP deficiency in tPtch/H11002 mice was obtained by expression analysis of key transcription factors. The Ets-related transcription factor PU.1 is expressed in all Lin− cells and supports maintenance of HSCs.33 Transcription of PU.1 in Lin− cells of tPtch/H11002 mice was unaltered (Figure 5C), suggesting that the earliest stages of hematopoiesis do not require Ptc. In contrast, transcription of Ikaros, Flt3, and IL-7Rα, 3 marker genes known to be important for specification of CLP and also myeloid lineages,34 was hardly detectable, which is consistent with a loss of CLP upon Ptc ablation. The complete absence of transcripts for EBF and Rag-1/2 in Lin− BM cells of tPtch−/− mice demonstrated the inability of the progenitor cells to recombine antigen receptor genes and to initiate lymphocyte differentiation. In summary, our results reveal a Ptc mutation-induced specification defect of the lymphoid lineage at the level of CLPs in the BM. However, we found no induction of Gli transcription in Lin− BM cells of tPtch−/− mice (Figure 5C), which is usually associated with loss of Ptc-mediated Smo inhibition.12,35 It thus appears that upon tamoxifen treatment, Ptc-negative cells are rapidly lost from the BM.

Specification defect of the lymphoid lineage depends on Ptc function in the stromal cell compartment

So far our data established a mandatory role of Ptc for CLP formation. Targeted Ptc disruption in our mice is, however, not...
cell-type specific and hence we cannot discriminate between cell-autonomous and heterologous (ie, stromal cell–dependent) defects. To distinguish between these possibilities, Lin− BM cells were purified from 
tPtch−/− mice and adoptively transferred into lethally irradiated Rag-2−/− mice, which lack T and B cells and that express Ptc1 in the residual thymus as well as in BM stromal cells (data not shown). Lin− BM grafts from vehicle- or tamoxifen-treated mice served as controls. As shown in Figure 6A, repopulation of peripheral blood lymphocytes was achieved in all cases and with the same efficiency (left panel). The absence of inactivated Ptc1 alleles in T and B cells from reconstituted Rag-2−/− mice was detected by quantitative PCR (B). Data are representative of 3 independent experiments (each experiment consisted of the transfer of Lin− BM cells derived from 1 vehicle-treated Ptchflox/ERT2+/−, 1 tamoxifen-treated Ptchflox/ERT2+/−, and 1 Ptch−/− mouse).}

**Figure 6. Specification defect of the lymphoid lineage in tPtch−/− mice is dependent on stromal BM cells.** Lin− BM cells (400 000) from vehicle- and tamoxifen-treated control animals or day-19 tPtch−/− mice were injected intravenously into lethally irradiated Rag-2−/− mice. After 7 weeks, peripheral blood cells were analyzed by flow cytometry (A). Cells within the lymphocyte gate (left panels) were analyzed by staining with antibodies against either CD4 and CD8 (middle panel) or B220 to identify T and B cells, respectively. Nonreconstituted Rag-2−/− mice served as negative control (top row). The presence of inactivated Ptc1 alleles in T and B cells from reconstituted Rag-2−/− mice was detected by quantitative PCR (B). Data are representative of 3 independent experiments (each experiment consisted of the transfer of Lin− BM cells derived from 1 vehicle-treated Ptchflox/ERT2+/−, 1 tamoxifen-treated Ptchflox/ERT2+/−, and 1 Ptch−/− mouse).

### Normal specification of the granulocyte-macrophage lineage in tPtch−/− mice

So far we had no indication for myeloid deficiencies in tPtch−/− mice. To analyze this aspect in more detail, we investigated the myeloerythroid colony-forming activity within the Lin− fraction of the tPtch−/− BM 19 days after the first tamoxifen injection. We found no effect of Ptc1 disruption on the differentiation of the multipotent CFU-granulocyte/erythrocyte/megakaryocyte/macrophage (CFU-GEMM), bi- or unipotential CFU-granulocyte/macrophage (CFU-GM), CFU-granulocyte (CFU-G), CFU-macrophage (CFU-M) and burst-forming unit-erythroid (BFU-E) progenitors (Table 1). Efficient Ptc1 ablation in CFU-GEMM colonies derived from tPtch−/− mice was demonstrated by PCR (data not shown). We also investigated the morphology and distribution of all peripheral blood cells by automated blood cell distribution of all peripheral blood cells by automated blood cell counts, blood smear tests, and FACS analysis (Tables 2,3). Consistent with the specification defect of the lymphoid lineage, we found a reduction of peripheral lymphocytes in tPtch−/− mice compared with control animals. Except for an increase in the number of neutrophilic granulocytes, the number and morphology of all other blood cells remained normal. The high number of neutrophils in tPtch−/− mice is most likely a secondary effect of the Ptc1 ablation as this is associated with skin lesions and tumor formation (data not shown). However, these data show that Ptc1 is dispensable for development and specification of myeloid lineages.
Table 1. Cell counts of BM and of peripheral blood samples and clonogenic progenitor assay of Lin" BM cells from control and tPttch" mice

<table>
<thead>
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<th></th>
<th>Controls</th>
<th>tPttch&quot; (19 days)</th>
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<tr>
<td></td>
<td>Mean ± Deviation n</td>
<td>Mean ± Deviation n</td>
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<tr>
<td>CFU-GEMM</td>
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<td>CFU-GM</td>
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<tr>
<td>BFU-E</td>
<td>2.8 ± 1.8 5</td>
<td>2.3 ± 1.4 4</td>
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Progenitor activity of Lin BM cells from tPttch" mice and from respective controls was examined 19 days after the onset of tamoxifen treatment using the colony-forming assay. Lin" BM cells (2 x 10^6) were cultured in methylcellulose-containing medium on a 35-mm dish. Lin" BM cells of each animal were tested in triplicates. Numbers of colonies/dish were counted after 8 days. Data for controls were pooled from both vehicle- and tamoxifen-treated animals.

Discussion

With this paper, we have established a nonredundant inducer function of Pttch in the adult mouse severely compromises the CLP-containing organ. Second, the number of total progenitor activity of Lin" BM cells from tPttch" mice, the Lin" BM cells containing CLPs and CMPs, which are expressed on the cell-surface receptor Flt3, which are expressed on the cell-surface receptor Flt3, which are expressed on the cell-surface receptor Flt3, which are expressed on the cell-surface receptor Flt3, which are expressed on the cell-surface receptor Flt3, which are expressed on the cell-surface receptor Flt3, which are expressed on the cell-surface receptor Flt3, which are expressed on the cell-surface receptor Flt3, which are expressed on the cell-surface receptor Flt3, which are expressed on the cell-surface receptor Flt3, which are expressed on the cell-surface receptor Flt3, which are expressed on the cell-surface receptor Flt3, which are expressed on the cell-surface receptor Flt3, which are expressed on the cell-surface receptor Flt3, which are expressed on the cell-surface receptor Flt3, which are expressed on the cell-surface 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environment (ie, the stromal-cell compartment) that is necessary for lymphoid versus myeloid differentiation. One example of such a binary cell fate determinant on stromal cells is provided by the Jagged/Delta-like protein, which is the ligand for Notch expressed on thymocytes and directs T- versus B-cell development (for review see Germain9). However, we cannot formally exclude a cell-autonomous function of Ptc in developing T cells, which would be consistent with the reported expression of Ptc during all DN stages.7 The fate of Ptc-deficient BM cells, the Hh/Ptc effector mechanisms, and how exactly they regulate lymphoid lineage commitment remain to be elucidated in future studies.

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Authorship

Contribution: A.U. and K.D. designed and performed research, collected and analyzed data, and wrote the paper; F.N. designed and performed the research, and collected and analyzed data; R.D. designed and performed research; M.K. designed and performed the research, and collected and analyzed data; A.F. performed the research and collected data; A.Z. designed and performed research, and collected and analyzed data; I.A. designed and performed the research; M.N. designed and performed research; T.H. performed research and collected data; V.A. designed research; W.S.-S. designed research, contributed vital reagents, and collected and analyzed data; J.W. and H.H designed research, contributed vital reagents and analytical tools, and wrote the paper. A.U. and K.D. contributed equally to this work.

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References


The Hedgehog receptor Patched controls lymphoid lineage commitment