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Brief Report

HEMATOPOIESIS AND STEM CELLS

Smoothened signaling in the mouse osteoblastoid lineage is required for efficient B lymphopoiesis

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KEY POINTS

- Hh signaling has been selectively extinguished in the mouse osteoblastoid lineage.
- Removal of Smo from osteoblasts results in a profound B-lymphopoietic defect.

The stromal signals that promote B lymphopoiesis remain poorly understood. Hedgehog (Hh) signaling promotes B lymphopoiesis in a non–cell-autonomous fashion in vitro, and depletion of the Hh effector Smoothened (Smo) from stromal cells is associated with the loss of osteoblastoid markers. These observations suggested that Hh signaling in the osteoblastoid lineage promotes B lymphopoiesis in vivo. To test this, we employed a mouse model for conditional ablation of *Smo* in the osteoblastoid lineage. Depletion of Smo from osteoblastoid cells is associated with profound and selective reductions in the number and proportion of bone marrow B-lymphoid progenitors. Upon partial bone marrow ablation, mutant animals exhibit delayed repopulation of the B-lymphoid compartment after the early lymphoid progenitor stage. Primary osteoblasts from mutant mice are defective in supporting B lymphopoiesis in vitro, whereas hematopoietic progenitors from mutant mice exhibit normal differentiation. We conclude that efficient B lymphopoiesis in vivo is dependent on the maintenance of Hh signaling in the osteoblastoid lineage. (*Blood*. 2018;131(3):323-327)

Introduction

The morphogen Hedgehog (Hh) plays critical roles in development and stem cell maintenance.[1] Hh signaling proceeds through the essential activator Smoothened (Smo), which in the absence of ligand is inhibited by the receptor Patched (Ptch); the binding of ligand relieves inhibition of Smo, which in turn stimulates downstream transcription.[2] Hh signaling has been implicated in the expansion[3] and differentiation[4,5] of hematopoietic stem cells, but cell-autonomous Hh signaling is not required for B lymphopoiesis in the mouse.[6-8] We have shown that extinction of Hh signaling in stromal cells impairs their ability to support B lymphopoiesis from hematopoietic stem progenitor cells in vitro. Moreover, depletion of Smo from stromal cells is associated with downregulation of markers associated with osteoblastoid identity.[6] Taken together, these observations prompted the hypothesis that B lymphopoiesis in the bone marrow (BM) is promoted by Hh signaling in stromal osteoblasts (OBs). We have now tested this by employing a mouse model in which Smo is selectively removed from the osteoblastoid lineage.

Methods

Animals

The transgenic mouse strain *B6.Cg-Tg(Sp7-tTa, tetO-EGFP/cre) 1Amc/J*, hereafter termed *Osx1-GFP::Cre*, was obtained from The Jackson Laboratory (Bar Harbor, ME). *Osx1-GFP::Cre*$^{+/0}$, *Smo*$^{fl/fl}$ and *Osx1-GFP::Cre*$^{+/0}$, *Smo*$^{+/+}$ mice were obtained by interbreeding *Osx1-GFP::Cre*$^{+/0}$ and *Smo*$^{fl/+}$ mice (gift of N. Watkins, Garvan Institute). C57BL/6 mice bearing a GFP cassette within the *Rag1* locus were gifts of K. Medina (Mayo Medical School, Rochester, MN). All mouse lines were constructed on a C57BL/6 background. For partial BM ablation, mice were treated with 5-fluorouracil (5-FU; APP Pharmaceuticals) at 150 mg/kg by intraperitoneal injection. Mice were housed in accordance with policies of the Johns Hopkins Animal Care and Use Committee.

Flow cytometry

All antibodies were from BD Biosciences. Data were collected using an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo v10.1 software (Tree Star).

Hematopoietic stem progenitor cell differentiation

Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) hematopoietic progenitors were purified from BM of 6- to 10-week-old mice.[6] Assays for B-lymphoid differentiation on primary OBs were performed as described elsewhere.[6,9]

Isolation of primary OBs

Bone cells were isolated from 6- to 10-week-old mice as described previously,[10] except that the final incubation was in medium containing 20% fetal bovine serum, 50 μg/mL ascorbic acid, and 10 mM β-glycerophosphate. OB differentiation was induced as described elsewhere.[11] For immunofluorescence analysis, cells were fixed in methanol, incubated with 1.5% blocking serum in phosphate-buffered saline for 1 hour, and then with anti-mouse osteocalcin antibody (Takara) overnight at 4°C. Primary antibody was detected with a phycoerythrin-conjugated secondary antibody.

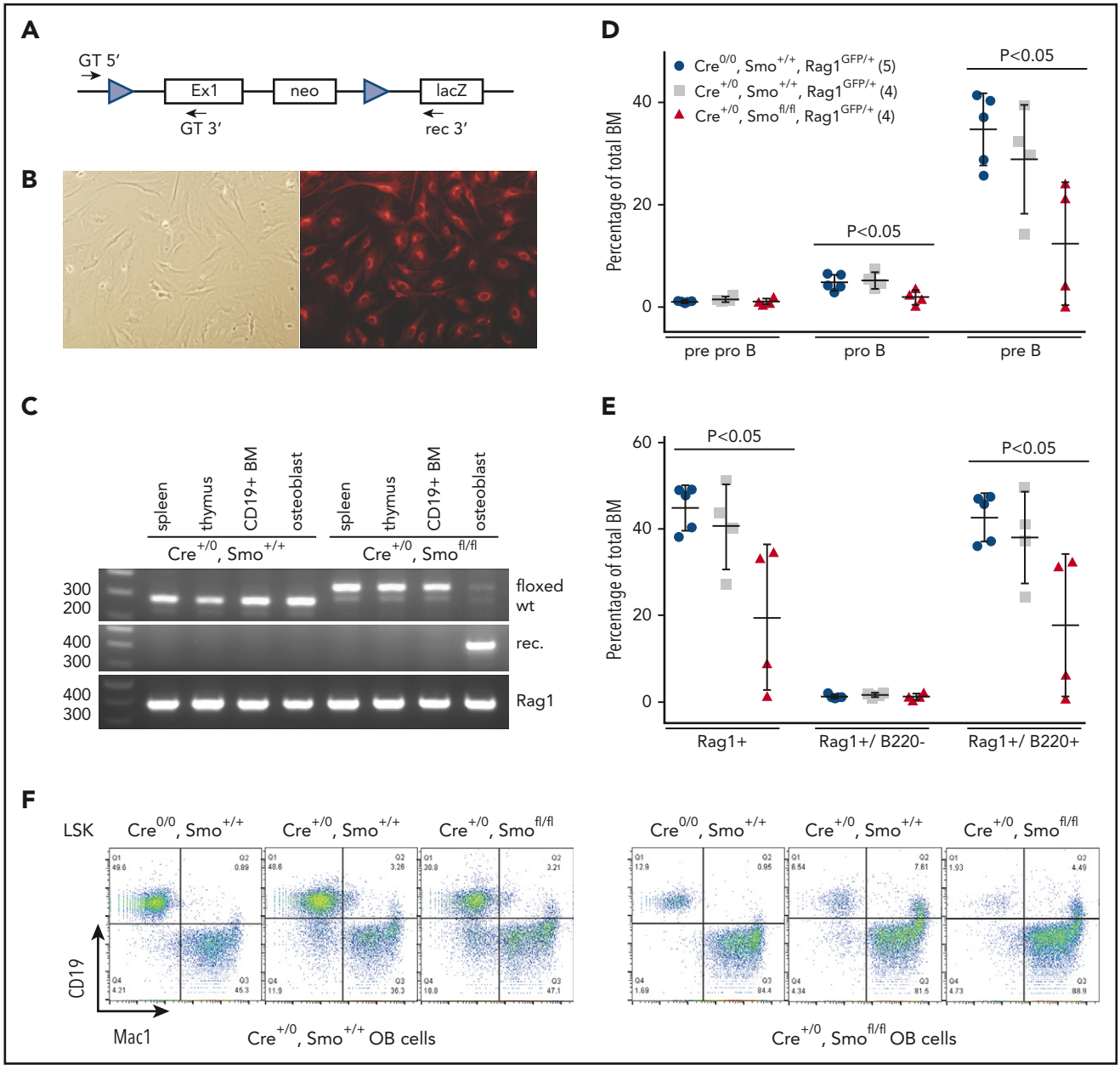

Figure 1. Osteoblastoid-specific ablation of Hh signaling impairs B-cell development. (A) The *Smo*^{fl} allele. Exon 1, neo cassette, and τ-lacZ are indicated. Blue arrowheads indicate *loxP* sites. GT5′ and GT3′, primers for amplification of wild-type and floxed alleles; rec3, reverse primer for detection of recombined allele. (B) Primary OBs examined under light-field (left) or immunostained with an osteocalcin-specific antibody (right). (C) Detection of *Smo* wild-type (wt), floxed, and recombined (rec.) alleles in spleen, thymus, CD19$^+$ BM cells, and OB cells from *Cre*$^{+/0}$, *Smo*$^{+/+}$ and *Cre*$^{+/0}$, *Smo*$^{fl/fl}$ mice. Amplification was performed with genomic DNA templates. The *Rag1* gene was amplified as a control. (D-E) Examination of B-lymphoid developmental subsets. BM was analyzed from 6- to 10-week-old mice of the indicated genotypes; the number of mice in each group is indicated in the inset (parentheses). Significant differences were determined by the Kruskal-Wallis test. (D) Percentages of pre-/pro-B (B220^{lo}CD19$^-$CD43$^+$), pro-B (B220^{lo}CD19$^+$CD43$^+$), and pre-B (B220^{lo}CD19$^+$CD43$^-$) subsets (mean and standard deviation [SD]). (E) Percentages of Rag1$^+$, Rag1$^+$ B220$^-$ (ELPs/CLPs), and Rag1$^+$ B220$^+$ subsets (mean and SD). (F) Impaired B lymphopoiesis from LSK progenitors maintained on Smo-depleted OB cells. LSK cells purified from mice of the genotypes indicated at top were maintained on *Cre*$^{+/0}$, *Smo*$^{+/+}$ or *Cre*$^{+/0}$, *Smo*$^{fl/fl}$ OB cells under conditions that promote B lymphopoiesis. Lymphoid and myeloid differentiation was assessed by flow cytometry.

Results and discussion

Disruption of Hh signaling in osteoblastoid cells specifically impairs B-cell development

To test the prediction that Hh signaling in OBs promotes B lymphopoiesis, we deleted *Smo* by Cre-mediated excision in the mouse osteoblastoid lineage (Figure 1A; supplemental Figure 1A-C, available on the *Blood* Web site). In these animals, Cre was expressed from the osteoblastoid-specific *Osx1* promoter.[12] OB cells prepared from *Osx1-Cre*, *Smo*$^{fl/fl}$ (*Smo*$^{Ob\text{-}ko}$) mice showed homogeneous staining for osteocalcin, a specific OB marker, and the identity of these cells was confirmed by flow cytometry (Figure 1B; supplemental Figure 2). Excision of floxed *Smo* alleles was observed in OB cells from *Smo*$^{Ob\text{-}ko}$ mice, but not in OB cells from *Osx1-Cre*, *Smo*$^{+/+}$ (*Cre* control) mice or in spleen, thymus, and BM B-lymphoid cells from either strain (Figure 1C). Primary OBs from Cre control mice expressed *Ptch1*, *Smo*, Hh ligands, and the Hh target *Gli1*; OBs from *Smo*$^{Ob\text{-}ko}$ mice exhibited a 10-fold

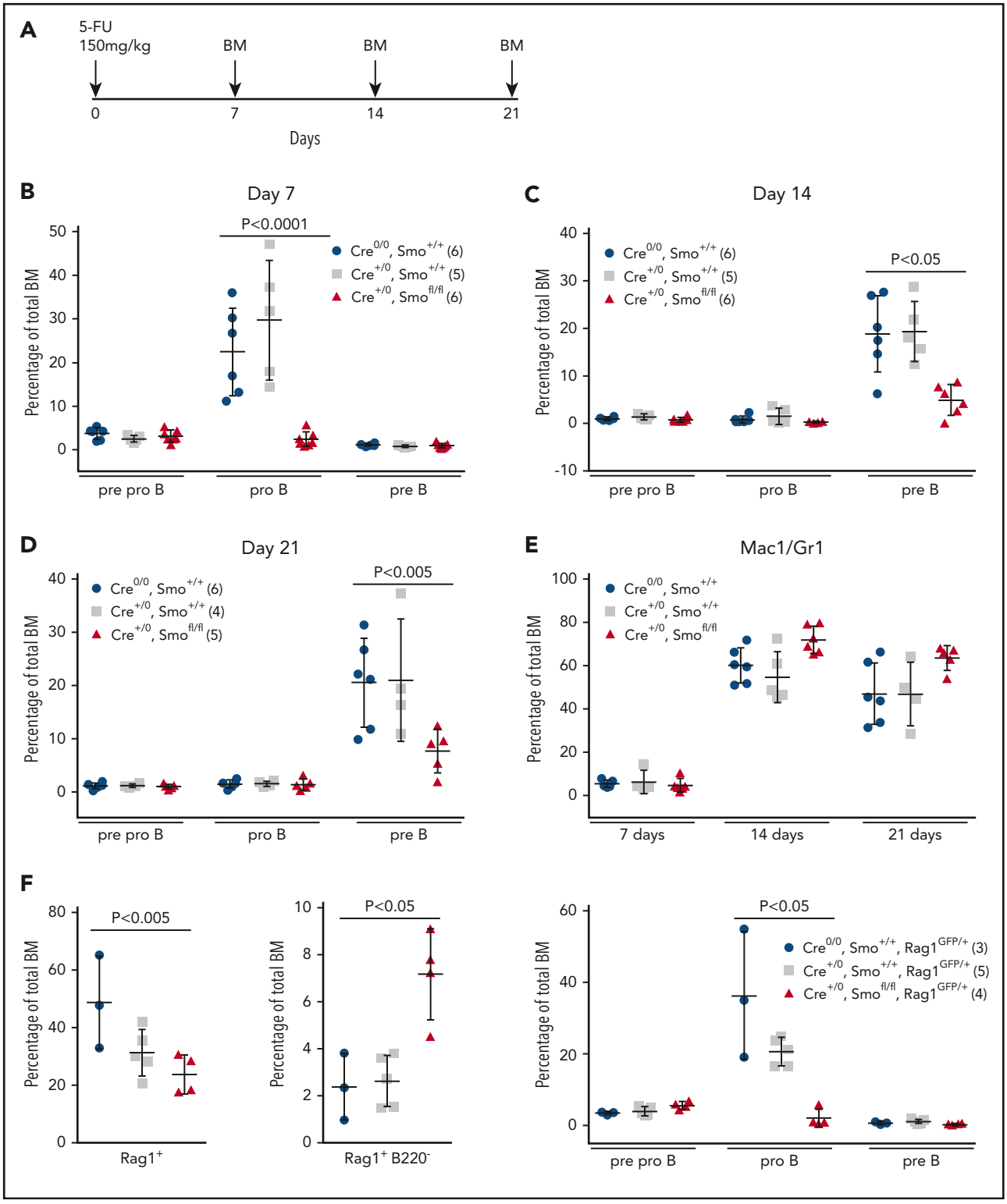

Figure 2. Osteoblastoid-specific depletion of Smo impairs reappearance of early B-lymphoid progenitors after BM ablation. (A) BM ablation protocol. Mice were injected intraperitoneally with 5-FU, and BM was collected as indicated. (B-D) Pre-/pro-B ($B220^{lo}CD19^{-}CD43^{+}$), pro-B ($B220^{lo}CD19^{+}CD43^{+}$), and pre-B ($B220^{lo}CD19^{+}CD43^{-}$) compartments in mice of the indicated genotypes were examined 7 days (B), 14 days (C) and 21 days (D) after 5-FU treatment. The number of mice in each group is indicated in the insets (parentheses). Significant differences were determined by the Kruskal-Wallis test. (E) BM myeloid cells ($Mac1^{+}Gr1^{+}$) were assessed 7, 14, and 28 days after 5-FU treatment in the same mice. The number of mice in each group is indicated in the insets of panels B-D (parentheses). Significant differences were determined as in B-D. (F) The percentages of $Rag1^{+}$ (left) and $Rag1^{+}B220^{-}$ (ELPs/CLPs) (middle) BM cells 7 days after 5-FU treatment were determined in mice of genotypes indicated at right. The number of mice in each group is indicated in the insets (parentheses). Pre-/pro-B ($B220^{lo}CD19^{-}CD43^{+}$), pro-B ($B220^{lo}CD19^{+}CD43^{+}$), and pre-B cell ($B220^{lo}CD19^{+}CD43^{-}$) compartments were also examined 7 days after ablation (right). Mean and SD are indicated. Significant differences were determined as in panels B-D.

reduction in *Smo* transcripts and a similar reduction in the expression of *Gli1* (supplemental Figure 3).

Smo$^{Ob\text{-}ko}$ mice exhibited reduced body weight (supplemental Figure 4A). Tooth elongation was impaired (supplemental Figure 4B) and femurs were osteopenic, with thin growth plates and decreased trabecular bone (supplemental Figure 4C). In *Smo*$^{Ob\text{-}ko}$ animals, splenic B-cell areas (the marginal zone and the periphery of the periarteriolar lymphoid sheath) were reduced, whereas the thymus showed a moderate decrease in cortical thymocytes (supplemental Figure 4D). Despite these abnormalities, the proportions of major lymphoid subsets in spleen and thymus of *Smo*$^{Ob\text{-}ko}$ mice were normal (supplemental Figure 5A-B); in blood, the proportions of B and T cells or CD4$^+$ and CD8$^+$ T-cell subsets were likewise unaffected (supplemental Figure 5C-D). In contrast, the percentage and number of steady-state B progenitors at pre-B and later developmental stages (supplemental Figure 1D)[13] were reduced in the BM of *Smo*$^{Ob\text{-}ko}$ mice (supplemental Figure 5E-F), whereas the proportion of myeloid progenitors was unaltered (supplemental Figure 5G).

To identify the developmental stage at which B lymphopoiesis is impaired in *Smo*$^{Ob\text{-}ko}$ animals, we examined B progenitors at steady state and during recovery from ablation. Early lymphoid progenitors (ELPs) and common lymphoid progenitors (CLPs) are marked by accumulation of *Rag-1* transcripts before stable acquisition of lineage identity.[14] We introduced a *Rag-1* allele bearing a GFP cassette (*Rag-1*^{GFP}) onto *Smo*$^{Ob\text{-}ko}$ and *Cre* control backgrounds (supplemental Figure 1C). In *Smo*$^{Ob\text{-}ko}$, *Rag-1*^{GFP} mice, BM pro-B and pre-B cells were significantly reduced (Figure 1D; supplemental Figure 6A), indicating an impairment of B lymphopoiesis at least as early as the pre-/pro-B to pro-B transition. The percentages and numbers of total GFP$^+$ (Rag-1$^+$) cells and GFP$^+$, B220$^+$ (Rag-1$^+$ B220$^+$) cells were also greatly diminished in *Smo*$^{Ob\text{-}ko}$, *Rag-1*^{GFP} mice, whereas under these steady-state conditions, GFP$^+$, B220$^-$ cells, which represent ELPs/CLPs, showed no obvious difference among genotypes (Figure 1E; supplemental Figures 6B and 7A).

Primary OB cells from *Cre* control and *Smo*$^{Ob\text{-}ko}$ mice were tested for their ability to support B-lymphoid differentiation from LSK progenitors in vitro. Control OB cells robustly supported B lymphopoiesis from LSK cells of wild-type, *Cre* control, or *Smo*$^{Ob\text{-}ko}$ origin (Figure 1F, left), whereas Smo-depleted OB cells were deficient in their support of B lymphopoiesis (Figure 1F, right). This indicated that Hh signaling in primary OB cells promotes B lymphopoiesis and suggested a causal relationship between the extinction of Hh signaling and the paucity of B-lymphoid progenitors in *Smo*$^{Ob\text{-}ko}$ mice.

B-lymphoid repopulation after BM ablation reveals an early developmental delay in *Smo*$^{Ob\text{-}ko}$ mice

We examined recovery of BM B progenitors after chemical ablation by 5-FU (Figure 2A). At 7 days, pro-B cells were 20- to 30-fold less abundant in *Smo*$^{Ob\text{-}ko}$ mice than in wild-type and *Cre* control animals (Figure 2B; supplemental Figure 6C). By day 14, pre-B cells had emerged but were markedly less numerous in *Smo*$^{Ob\text{-}ko}$ mice than in controls (Figure 2C; supplemental Figure 6D). The relative sizes of B progenitor subsets at day 21 (Figure 2D; supplemental Figure 6E) were similar to those observed at day 14, suggesting that repopulation had reached steady state. In contrast, myeloid repopulation was unimpaired in *Smo*$^{Ob\text{-}ko}$ mice (Figure 2E; supplemental Figure 6F).

At 7 days after BM ablation, the total GFP$^+$ population was reduced in *Smo*$^{Ob\text{-}ko}$, *Rag-1*^{GFP} mice relative to controls (Figure 2F, left; supplemental Figure 6G, left), consistent with a paucity of B progenitors (Figure 2B). Strikingly, ELPs/CLPs were overrepresented in *Smo*$^{Ob\text{-}ko}$ animals (Figure 2F, middle; supplemental Figure 6G, middle; supplemental Figure 7B). The distributions of B progenitors at 7 days after ablation were similar to those in mice lacking the *Rag-1*^{GFP} allele (Figure 2B,F; supplemental Figure 6C,G). These results confirm that extinction of Hh signaling in the osteoblastoid compartment impairs the emergence of pro-B cells. Moreover, the increase in accumulation of ELPs/CLPs in *Smo*$^{Ob\text{-}ko}$, *Rag-1*^{GFP} mice at 7 days after ablation suggests that Hh signaling in OB cells promotes the exit of B-lymphoid progenitors from the CLP compartment.

Taken together, our results indicate that Hh signaling in osteoblastoid cells promotes B lymphopoiesis; this requirement may be enforced as early as the transition from the CLP to the pre-/pro-B cell stage. Deletion of *Ptch1*, an inhibitor of Smo, either nonspecifically or in stratified epithelia has been associated with depletion of B- and T-cell progenitors.[15] Our study differs with respect to the lineage specificity of the deletion and the lymphopoietic defect, which was restricted to the B lineage. Although *Osx-1-Cre*–mediated deletion may occur in other BM cell types,[16] previous studies uncovered a critical role for OBs in B-cell development,[11,17] and we demonstrate that OBs from *Smo*$^{Ob\text{-}ko}$ mice are defective in Hh signaling and in promoting B lymphopoiesis. Our findings indicate that the ability of osteoblastoid cells to promote B lymphopoiesis is conferred, at least in part, by Smo, whose sustained activity is likely to support expression of factors essential for B-lineage development.

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Authorship

Contribution: W.L. designed and executed experiments, interpreted results, and wrote the manuscript; D.D. designed and executed experiments and interpreted results; D.H. performed histopathologic analysis; and S.D. designed experiments, interpreted the results, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

David Huso died on 27 January 2016.

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Footnotes

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