

## Distinctive Mesenchymal-Parenchymal Cell Pairings Govern B Cell Differentiation in the Bone Marrow

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### SUMMARY

Bone marrow niches for hematopoietic progenitor cells are not well defined despite their critical role in blood homeostasis. We previously found that cells expressing osteocalcin, a marker of mature osteolineage cells, regulate the production of thymic-seeding T lymphoid progenitors. Here, using a selective cell deletion strategy, we demonstrate that a subset of mesenchymal cells expressing osterix, a marker of bone precursors in the adult, serve to regulate the maturation of early B lymphoid precursors by promoting pro-B to pre-B cell transition through insulin-like growth factor 1 (IGF-1) production. Loss of *Osx*<sup>+</sup> cells or *Osx*-specific deletion of IGF-1 led to a failure of B cell maturation and the impaired adaptive immune response. These data highlight the notion that bone marrow is a composite of specialized niches formed by pairings of specific mesenchymal cells with parenchymal stem or lineage committed progenitor cells, thereby providing distinctive functional units to regulate hematopoiesis.

### INTRODUCTION

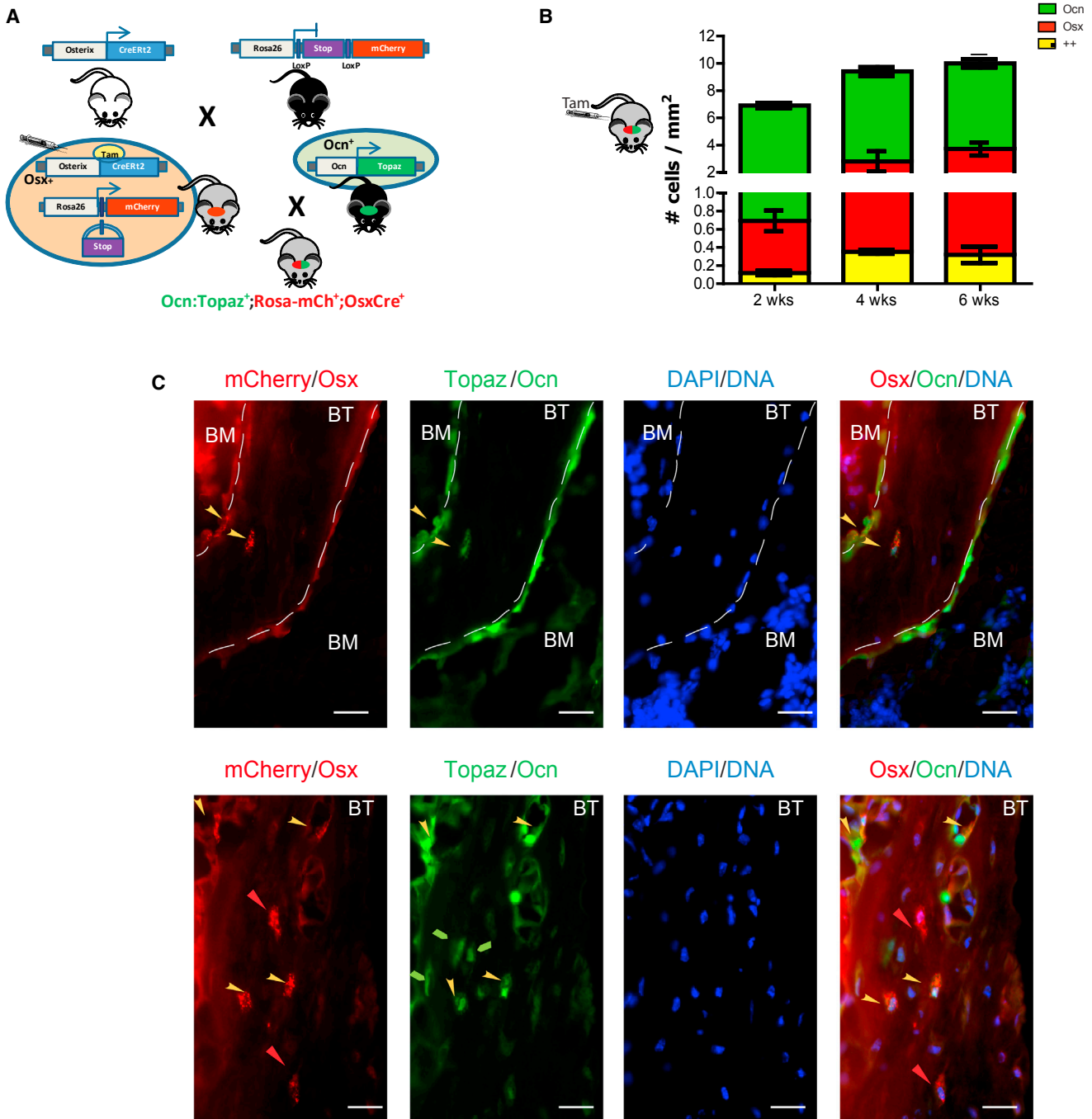
Characterization of the niche has provided insight into molecular pathways modulating stem cell function for tissue regeneration and cancer. Although the multitude of cells comprising bone marrow stroma remains incompletely defined (Yu and Scadden, 2016a), recent studies indicate that while primitive mesenchymal progenitors are more critical for hematopoietic stem cell (HSC) function, lineage-restricted mesenchymal cells control more committed hematopoietic progenitors (Mendez-Ferrer et al., 2010). Specifically, it was described that the deletion of a range of osteolineage cells or CXCL12 from these cells alters B cell progenitors (Greenbaum et al., 2013; Visnjic et al., 2004) or B and T cell counts (Ding and Morrison, 2013). Furthermore, deletion of osteocytes created systemic disruption of metabolism and affected thymic function (Sato et al., 2013).

These data suggest that niches are not limited to the governance of stem cells (Wu et al., 2009; Yu et al., 2015a), and the definition of “progenitor niches” may offer new ways to understand the regulation of tissue composition and function. We recently demonstrated that selective deletion of cells expressing the mature osteolineage cell marker *Osteocalcin* (*Ocn*) results in decreased T competent common lymphoid progenitors (CLPs) with a minimal effect on B cell-biased CLPs (Ly6D<sup>+</sup>) (Yu et al., 2015a) due to a defective generation of thymic-seeding progenitor cells. The mutant phenotype was recapitulated by se-

lective deletion of *DLL4* in *Ocn*<sup>+</sup> cells, or of its receptor and downstream signaling molecules in primitive hematopoietic cells; therefore *Ocn*<sup>+</sup> cells constitute a regulatory niche guiding the production and differentiation of hematopoietic cells contributing to T cell neogenesis.

Given that others using genetic constructs to disrupt a broad range of osteolineage cells showed a decrease in mature B cells (Ding and Morrison, 2013; Visnjic et al., 2004; Wu et al., 2008), that B cells are adjacent to CXCL12-abundant reticular (CAR) cells (Omatsu et al., 2010), and that continuous deletion of CXCL12 in development using *Osx*-Cre leads to reduced B cell production (Greenbaum et al., 2013), we hypothesized that *Osx*<sup>+</sup> cells may serve as a B cell differentiation modulator in adult animals. We sought to determine this using a cell-type-specific in vivo cell-ablation strategy.

The perceived hierarchy of bone cell differentiation begins from the multipotent mesenchymal stem cell, which matures to become the osteoprogenitor, the pre-osteoblast, the mature osteoblast and/or the lining cell, and eventually the terminally differentiated osteocyte. While less is known regarding the heterogeneity of the mesenchymal stem cell population (Yu and Scadden, 2016b) and multiple markers such as *Leptin* receptor (Zhou et al., 2014), *Nestin* (Ono et al., 2014), and *Gremlin1* (Worthley et al., 2015), and *Mx1* (Park et al., 2012) among others (Chan et al., 2015) had been recently proposed to label mesenchymal stem cell populations of various regenerative properties, markers for osteoprogenitor and mature osteoblast are



### Figure 1. A Triple Transgenic Mouse to Study the Dynamics of Osteolineage Subpopulations

(A) Osterix-Cre (*OsxCre*) mice were crossed with the *Rosa26-loxP-stop-loxP-mCherry* reporter mice (*Rosa-mCh*), which express mCherry fluorescent protein upon Cre-mediated excision of a stop sequence. *OsxCre<sup>+</sup>;Rosa-mCh<sup>+</sup>* mice were then crossed with the osteocalcin-Topaz (*Ocn:Topaz*) mice. Upon tamoxifen injection into the *OsxCre<sup>+</sup>;Rosa-mCh<sup>+</sup>;Ocn:Topaz<sup>+</sup>* mice, the *Osx<sup>+</sup>* cells and their progeny are labeled red, whereas the *Ocn<sup>+</sup>* cells are labeled green. Cells that express osteocalcin following tamoxifen injection and produce osteocalcin are yellow.

(B) *Osx<sup>+</sup>*, *Ocn<sup>+</sup>*, and ++ cells were enumerated in femur sections of *OsxCre<sup>+</sup>;Rosa-mCh<sup>+</sup>;Ocn:Topaz* mice at the indicated time points following tamoxifen injection.

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more clearly defined. *Osterix* (*Osx/Sp7*) is a zinc finger-containing transcription factor expressed in osteoprogenitors and serves as a key regulator of osteolineage differentiation (Mizoguchi et al., 2014; Nakashima et al., 2002). *Osteocalcin* (*Ocn*) is the most well-characterized extracellular matrix protein expressed by mature osteoblasts. However, these markers were mostly used in independent studies, rendering it difficult to study the dynamic relationship of osteolineage subsets in vivo. Here, we generated a triple transgenic mouse that differentially labels *Osx*<sup>+</sup> and *Ocn*<sup>+</sup> cells. We used this model to interrogate whether distinct osteolineage subsets serve to regulate different hematopoietic processes.

## RESULTS

We examined whether the *Osx*<sup>+</sup> cells could be distinguished from *Ocn*<sup>+</sup> cells in vivo for an interval sufficient to test their distinctive biological function. Mice carrying a fusion of Cre and modified estrogen receptor under the control of the *Osterix* promoter (*Osx*-CreERT2 [Maes et al., 2010]), hereafter called *Osx*Cre, were crossed with mice bearing a *Rosa26-loxP-stop-loxP-mCherry* (*Rosa*-mCh) transgene (*Osx*Cre;*Rosa*-mCh) (Strecker et al., 2013). Administration of 4-hydroxy-tamoxifen (4-OHT) to *Osx*Cre<sup>+</sup>;*Rosa*-mCh<sup>+</sup> mice resulted in Cre activation in *Osx*<sup>+</sup> cells followed by excision of the stop cassette and production of the mCherry fluorophore. Upon 4-OHT injection, the red fluorescence marks cells expressing OSX as well as their progeny. These mice were crossed with mice expressing the GFP, Topaz, driven by the *Osteocalcin* promoter (*Ocn*:Topaz) (Bilic-Curcic et al., 2005). In this triple transgenic model (*Osx*Cre<sup>+</sup>;*Rosa*-mCh<sup>+</sup>;*Ocn*:Topaz<sup>+</sup>), the OCN-expressing cells are green, the OSX-expressing cells (and their descendants) are red, and cells expressing both markers are yellow (Figure 1A). According to osteolineage ontology, we anticipated that the *Osx*<sup>+</sup> osteoprogenitors initially labeled red would become yellow as they express OCN. Six-week-old *Osx*Cre<sup>+</sup>;*Rosa*-mCh<sup>+</sup> mice were pulsed with an injection of tamoxifen (day 0) and fluorescent cells were quantified over time. In a 6-week chase, a modest number of dual-labeled (++) cells emerged (0.02% of total bone cells) (Figure 1B), but the majority of cells were either mCherry (*Osx*<sup>+</sup>) or Topaz (*Ocn*<sup>+</sup>) single positive (Figure 1C). Cells labeled as ++ were found at the metaphyseal region, located near the endosteal surface.

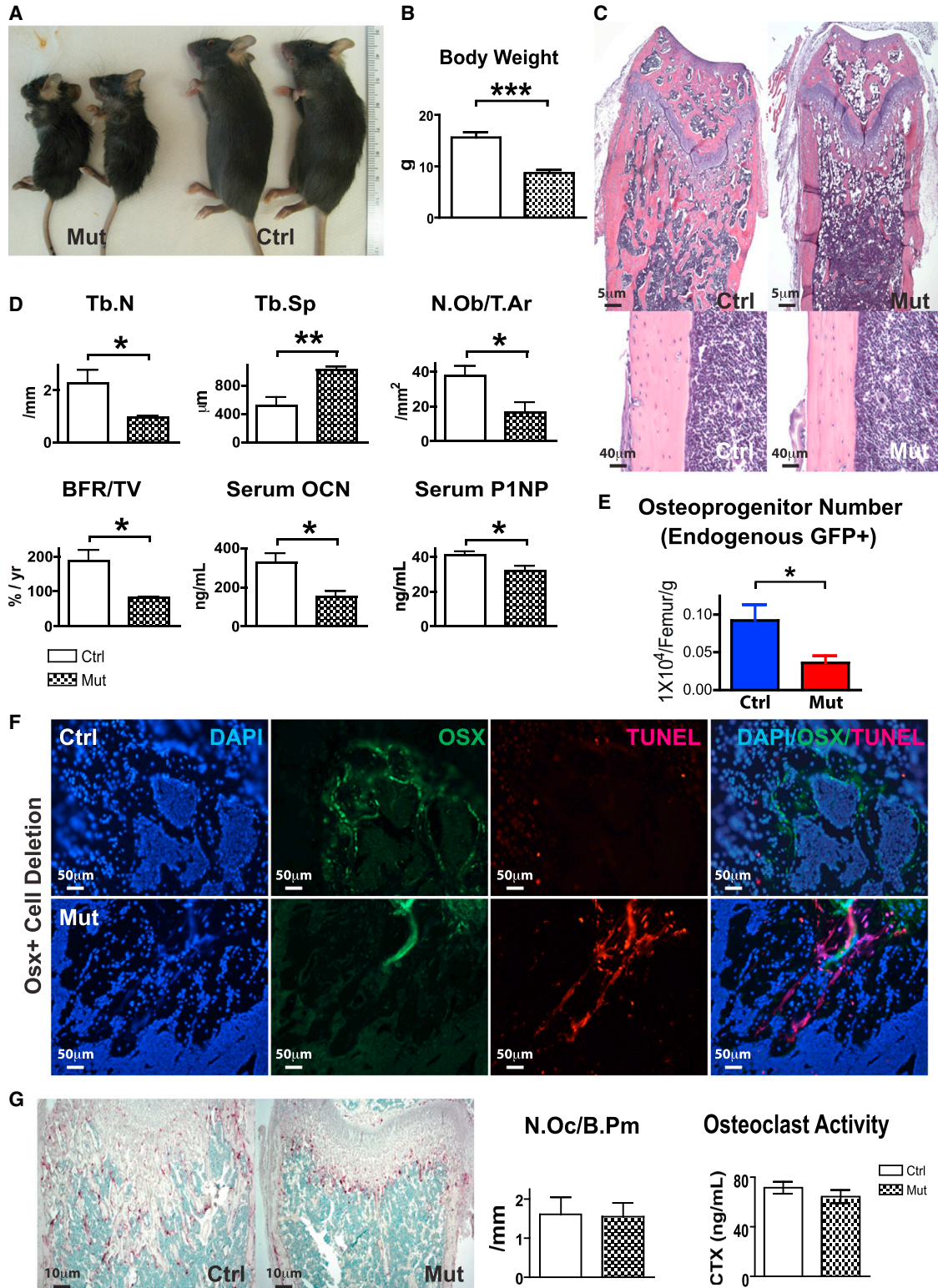
These data show that *Osx*<sup>+</sup> cells do not necessarily transition to *Ocn*<sup>+</sup> cells over a 6-week period, although a modest number of cells do. It may be that limited efficiencies of fluorophore expression underestimate the cells transitioning from *Osx* to *Ocn* expression. Also possible is that some OSX labeling occurs in cells that do not proceed to osteoblasts expressing OCN or that dually labeled cells are lost due to disadvantageous characteristics from dual fluorophore production. Nonetheless, this triple transgenic system enables us to isolate distinct subpopulations of the osteolineage within the same animal by flow cytometry and allows subsequent characterization of their molecular and functional profiles.

We then assessed the three labeled populations. At day 4 after 4-OHT treatment, *Osx*<sup>+</sup>, ++, and *Ocn*<sup>+</sup> cells were purified by fluorescence-activated cell sorting from bone cell suspensions isolated from the triple transgenic model, and subjected to gene-expression profiling by microarray. Principal component analysis of the 2,509 top genes clustered together *Osx*<sup>+</sup> and ++ cells as more similar compared with *Ocn*<sup>+</sup> cells (data not shown). Restrictive filtering revealed the top 25 differentially expressed genes among the three populations (Table S1). Gene ontology analysis revealed that genes upregulated in *Ocn*<sup>+</sup> cells were related to cell adhesion and cytokines, whereas *Osx*<sup>+</sup> cells highly transcribe genes involved in extracellular matrix interaction and Hedgehog-dependent pathways (Table S2). Interestingly, the ++ population represented a well-defined population with distinctive expression of pro-inflammatory cytokines and macrophage-related surface and secreted molecules. Microarray results were validated by RT-PCR on sorted *Osx*<sup>+</sup>, ++, and *Ocn*<sup>+</sup> cells (Figure S1A).

We investigated whether *Osx*<sup>+</sup>, *Ocn*<sup>+</sup>, or ++ cells have unique effects on hematopoietic stem and progenitor cell (HSPC) function ex vivo. Six days following 4-OHT injection into 4- to 6-week-old triple transgenic mice, FACS sorted *Osx*<sup>+</sup>, ++, or *Ocn*<sup>+</sup> cells were separately co-cultured for 5 days with purified HSPCs (LINEAGE<sup>lo</sup>, C-KIT<sup>+</sup>, SCA-1<sup>+</sup>, CD150<sup>+</sup>, CD48<sup>-</sup>) isolated from GFP<sup>+</sup> mice. Following co-culture, cells were transplanted into lethally irradiated recipients (Figure S1B). HSPCs co-cultured with each osteolineage subset maintained their long-term reconstitution and multilineage potential (Figure S1C). However, HSPCs co-cultured with *Osx*<sup>+</sup> cells displayed increased total chimerism compared with those co-cultured with *Ocn*<sup>+</sup> and ++ cells at 4 weeks, and improved chimerism compared with ++ cells at 16 weeks (Figure S1C).

(C) Representative sections of an *Osx*Cre<sup>+</sup>;*Rosa*-mCh<sup>+</sup>;*Ocn*:Topaz<sup>+</sup> mouse 4–6 weeks after tamoxifen injection showing the location of the different osteolineage subsets. Blue indicates DAPI, red mCherry (*Osx*<sup>+</sup> cells), and green Topaz (*Ocn*<sup>+</sup> cells). Yellow arrow indicates ++ cells and red arrow points to *Osx*<sup>+</sup> cells. BT, bone, trabecular; BM, bone marrow cavity. Note the punctated appearance of the mCherry fluorescence. Scale bar, 75 μm.

Experiment repeated once, n = 4/group/experiment (B and C).



**Figure 2. *Osx*<sup>+</sup> Cell-Specific Deletion In Vivo without Altering Osteoclastogenesis**

(A and B) Skeletal development in the *Osx*Cre;iDTR mutants compared with control littermates at 6 weeks of age. Three independent experiments; n = 9–15/group. \*\*\*p < 0.001.

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B lymphoid cells (B220<sup>+</sup> cells) were the major contributors to the increased engraftment from HSPCs co-cultured with *Osx*<sup>+</sup> cells (Figure S1D), while there was short-term enhanced reconstitution of T cells (CD4<sup>+</sup> and CD8<sup>+</sup> cells) at 8 weeks, and this effect disappeared at 12 and 16 weeks (Figure S1E). No difference was observed for the Mac/Gr1<sup>+</sup> subset (Figure S1F).

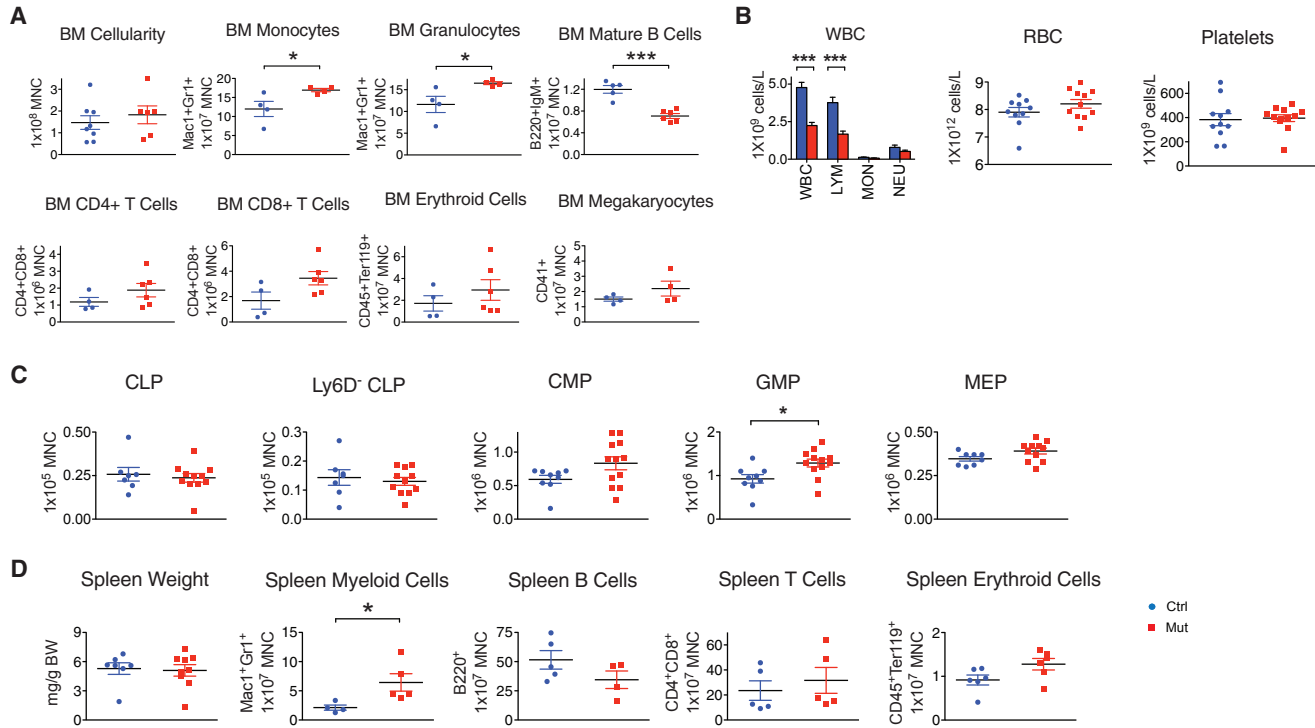
We next postulated whether *Osx*<sup>+</sup> cell regulates B cell populations in vivo using a selective cell-depletion model. We crossed the *Osx1-GFP::Cre* mice with the *iDTR* mice (hereafter *OsxCre;iDTR*), in which ubiquitous expression of the diphtheria toxin receptor (*iDTR*) is blocked by a *LoxP*-flanked STOP sequence. Cre-mediated excision of the STOP sequence allows expression of the *iDTR* in select cell populations that then become susceptible to killing upon peritoneal administration of diphtheria toxin (DT). We began daily DT injections into both control and mutant mice starting at 4 weeks of age. At 6 weeks, there was a striking difference of skeletal size when we compared the *OsxCre<sup>+</sup>;iDTR<sup>+/+</sup>* controls with the *OsxCre<sup>+</sup>;iDTR<sup>Fl/+</sup>* mutants (Figures 2A and 2B). *OsxCre<sup>+</sup>;iDTR<sup>Fl/+</sup>* mutants showed reduced bone mass (Figures 2C and 2D) and osteoblastic functions including reduced bone formation, serum levels of osteocalcin, and type I procollagen production (Figure 2D). Flow cytometry revealed a ~50% reduction of *Osx*<sup>+</sup> cells in mutants (Figure 2E). To assure targeted cell deletion, we examined DTR in mutant animals without toxin injection by immunohistochemistry. Expression of the DTR strongly overlapped with staining of OSX in the *OsxCre<sup>+</sup>;iDTR<sup>Fl/+</sup>* animals (Figures S2A–S2J). Of note, *OsxCre<sup>+</sup>;iDTR<sup>Fl/+</sup>* animals did not display extensive overlapping with OCN staining, confirming our observations from the lineage-tracing experiments. Staining of bone sections with OSX-specific antibodies in combination with TUNEL staining confirmed that the targeted osteolineage cells were correctly deleted (Figure 2F). Tartrate-resistant acid phosphatase (TRAP) staining and measurement of degraded collagen confirmed that osteoclastogenesis was not affected (Figure 2G). These data indicate that a distinct subset of osteolineage cells were depleted in the *OsxCre<sup>+</sup>;iDTR<sup>Fl/+</sup>* mouse and that we were able to selectively assess the impact of these *Osx*<sup>+</sup> cells for a time lapse following cell deletion. We asked whether selective dele-

tion of *Osx*<sup>+</sup> cells might lead to a compensatory expansion of more primitive bone progenitor cells, thereby confounding our interpretation. Colony-forming unit osteoblast (CFU-Ob) assays failed to detect any increase in primitive mesenchymal progenitors between controls and mutants (Figure S2K). However, other non-hematopoietic cell types might be affected by *Osx*<sup>+</sup> cell depletion and influence the hematopoietic phenotype observed.

Despite the dramatic changes in bone morphology in *OsxCre;iDTR* mutants, bone marrow cellularity was unaffected compared with their control littermates (Figure 3A). Red blood cell and platelet numbers were unchanged in the blood of the *OsxCre;iDTR* mutant, but there was a 50% decrease in the white blood cell count due to severe lymphopenia (Figure 3B). The mutant bone marrow had fewer mature B220<sup>+</sup>IgM<sup>+</sup> B cells, increased monocytes and granulocytes (Figure 3A), and a corresponding increase in granulocyte and macrophage progenitors (GMPs) (Figure 3C). Given the importance of the spleen for B lymphopoiesis and extramedullary hematopoiesis, we analyzed the spleens and noted no difference in weight, but similarly increased Mac1<sup>+</sup>Gr1<sup>+</sup> cells in the *OsxCre;iDTR* mutants (Figure 3D). In summary, *Osx*<sup>+</sup> cell deletion caused a loss of mature B cells and an increase in myeloid cells in both the bone marrow and spleen of mutant animals.

We assessed whether the GMP increase in the *OsxCre;iDTR* mutants was secondary to increased proliferation or decreased death of GMPs. There was change in neither cell-cycle status (Figure S3A) nor apoptosis (Figure S3B). The accumulation of GMP and Mac1<sup>+</sup>Gr1<sup>+</sup> cells could either be an osteoprogenitor-mediated hematopoietic effect or a consequence of inflammatory phagocytic-type immune response triggered by the death of osteolineage cells. To test this hypothesis, we co-injected indomethacin, a non-steroidal anti-inflammatory drug, together with DT in *OsxCre;iDTR* mutant mice for 1 week. Bone marrow analysis revealed normalization of the GMP, Mac1<sup>+</sup>, and Mac1<sup>+</sup>Gr1<sup>+</sup> cell populations in indomethacin-treated animals (Figure S3C). Therefore, the increase in Mac1<sup>+</sup>Gr1<sup>+</sup> myeloid cells could be due to inflammatory response to osteolineage cell death, although we cannot exclude an osteolineage cell-mediated myeloid effect.

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- (C) Histology of femurs from *OsxCre;iDTR* control and mutant mice. Three independent experiments; n = 9–15/group.
- (D and E) Bone histomorphometric measurement on trabecular number, trabecular separation, number of osteoblasts, bone formation rate, serum production of osteocalcin, and type I procollagen production. Three independent experiments; n = 9–15/group. \*p < 0.05, \*\*p < 0.01. (E) Flow cytometric quantification of *Osx*<sup>+</sup> cells in control and mutant groups. Three independent experiments; n = 9–15/group.
- (F) Bone sections were stained with osterix-specific antibody overlapped with apoptotic TUNEL staining to confirm targeted cell deletion. Three independent experiments; n = 9–15/group.
- (G) Measurement of osteoclast number as indicated by TRAP staining, and osteoclast activity, as indicated by the rate of collagen breakdown in sera. Two independent experiments; n = 22–24/group.
- Error bars represent ± SEM.



**Figure 3. *Osx*<sup>+</sup> Cell Ablation Causes B lymphopenia and an Increase in Monocytes and Macrophages**

(A) Flow cytometric measurement of bone marrow cellularity and multiple mature lineages in *OsxCre*;iDTR mutants compared with control littermates.

(B) White blood cell, red blood cell, and platelet counts in the peripheral blood of *OsxCre*;iDTR mutants versus controls.

(C) Enumeration of different progenitor subtypes in the bone marrow.

(D) Examination of the spleen as a major site for B lymphopoiesis and extramedullary hematopoiesis showed a similar increase in *Mac1*<sup>+</sup>*Gr1*<sup>+</sup> myeloid cells and attenuated mature B cell number.

Experiment repeated 2–3 times; n = 4–12/group. \*p < 0.05, \*\*\*p < 0.001. Error bars represent ± SEM.

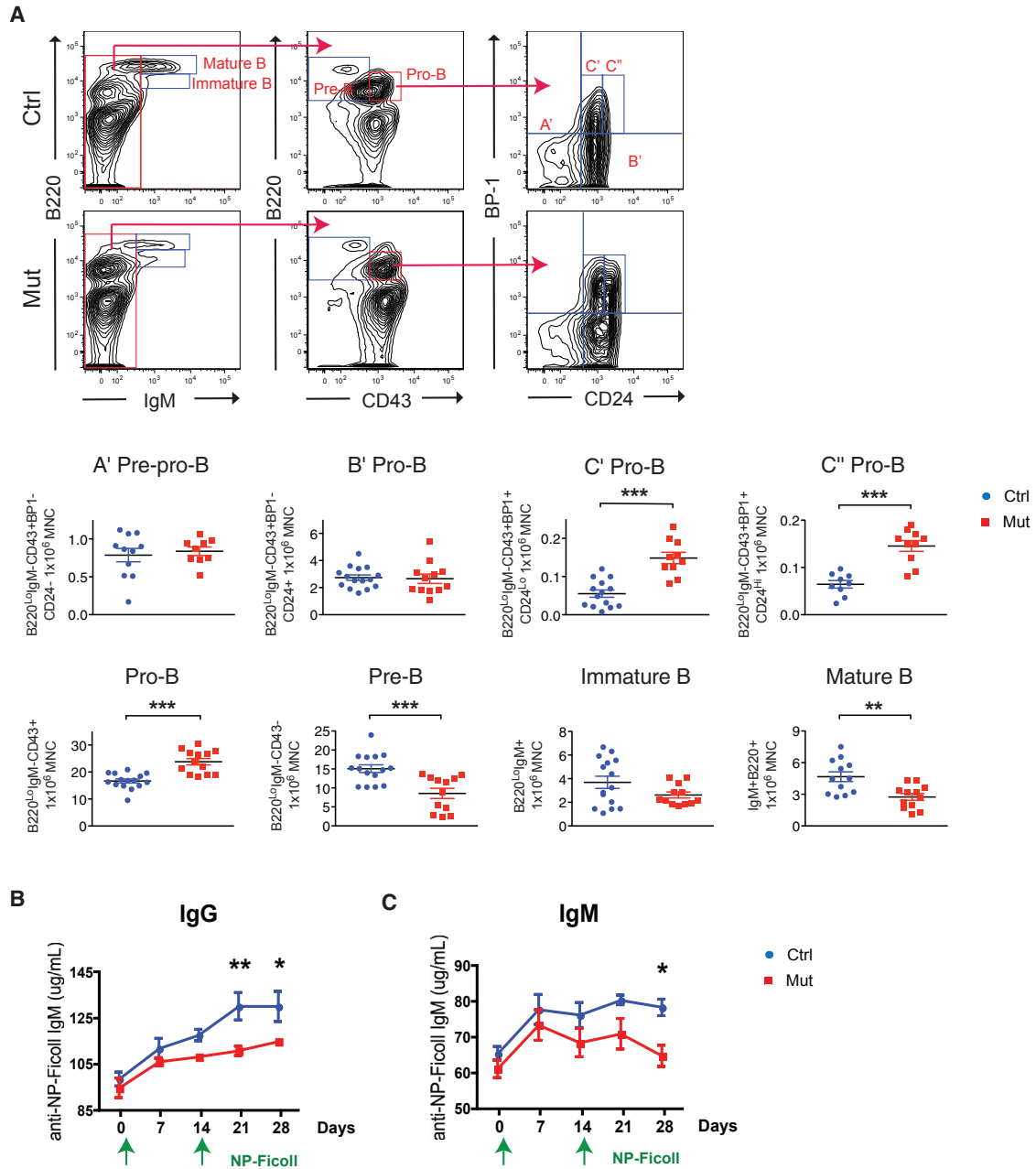
We asked whether HSC function was affected by the short-term deletion of *Osx*<sup>+</sup> cells. We observed no differences between the *OsxCre*;iDTR mutants and controls in long-term HSC (*LIN*<sup>lo</sup>*C-KIT*<sup>+</sup>*SCA-1*<sup>+</sup>*CD48*<sup>-</sup>*CD150*<sup>+</sup>) number (Figure S4A), proliferation (Figure S4B), or apoptosis (Figure S4C). There was an increase in *LIN*<sup>lo</sup>*C-KIT*<sup>+</sup>*SCA-1*<sup>-</sup> progenitor cell number and cell cycle (Figures S4A and S4B). However, no defect in HSC function was detected when bone marrow cells from were transplanted into primary and secondary recipients in a 1:1 ratio with congenic SJL competitor cells (Figure S4D). These data suggest that short-term deletion of *Osx*<sup>+</sup> cells did not affect HSC function.

Given the diminished production of mature B cells, we assessed CLPs and found no difference (Figure 3C). However, intermediate stages of B cell development were affected. Specifically, a decrease in both pre-B and mature B populations were noted while pro-B cells, especially during the later C' pro-B and C'' pro-B stages, were significantly increased (Figure 4A). These data strongly suggest that

B cell differentiation was impaired at the pro-B to pre-B juncture.

We next tested whether these changes affected the animal's immune response. Mice were challenged with NP-Ficoll to trigger a T cell-independent (Maizels et al., 1988) immune response and followed for 28 days. Data revealed that *OsxCre*;iDTR mutants could not sustain immunoglobulin G (IgG) and M (IgM) production over time compared with their control littermates (Figures 4B and 4C). These data indicate that the perturbation in B cell development was not simply immunophenotypic, but there was also a functional reduction in Ig production and immune response.

To determine whether the B cell differentiation defect was microenvironment-dependent, we transplanted 1 × 10<sup>6</sup> bone marrow cells from congenic SJL mice into lethally irradiated control or mutant *OsxCre*;iDTR recipients. A similar rate of reconstitution was seen in both control and mutants (Figure 5A). As wild-type SJL cells repopulated both control and mutant *OsxCre*;iDTR recipients over the



**Figure 4. *Osx*<sup>+</sup> Cell Deletion Leads to Blockade of pro-B to pre-B Transition and Impairs Adaptive Immunity**

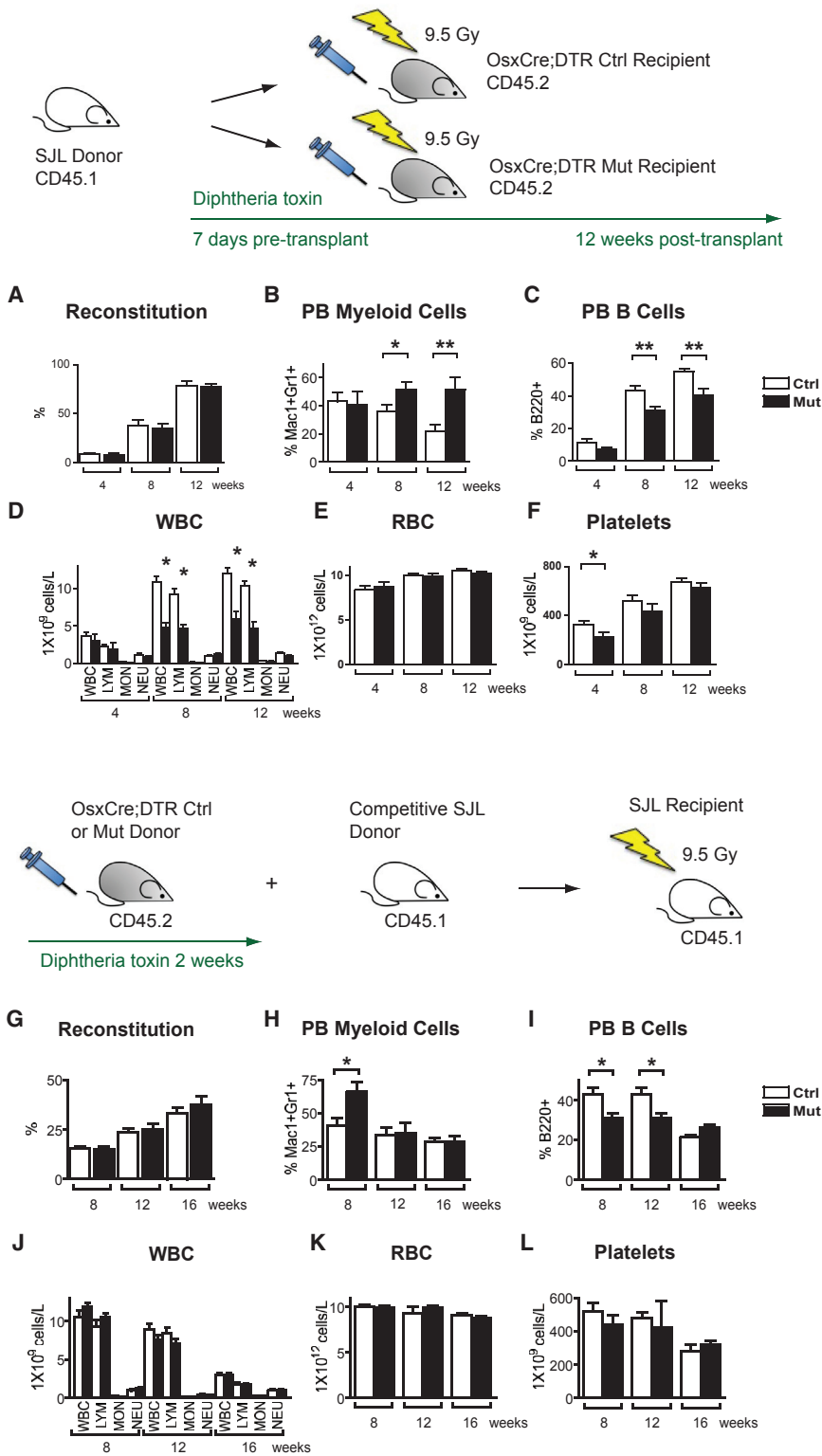
(A) Analysis of intermediate stages of B cell maturation in the *OsxCre*;iDTR mutants compared with control littermates.

(B and C) Animals were challenged with NP-Ficoll to produce immunoglobulins in a T cell-independent manner. Production of IgG (B) and IgM (C) were assessed over 28 days.

Three independent experiments; n = 12–16/group. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. Error bars represent ± SEM.

next 12 weeks, we observed a gradual recapitulation of the *OsxCre*<sup>+/-</sup>;DTR<sup>Fl/+</sup> hematopoietic phenotype in the mutant recipients but not in the controls (Figures 5B–5F). These data strongly suggest that the incomplete B cell differentiation observed in *OsxCre*;iDTR was caused by an altered bone microenvironment.

To confirm this, we transplanted mutant cells into wild-type hosts in a reverse transplant setting. Either 5 × 10<sup>5</sup> *OsxCre*;iDTR mutant or control bone marrow cells, competed with 5 × 10<sup>5</sup> SJL bone marrow cells in a 1:1 ratio, were transplanted into lethally irradiated SJL hosts. When cells from *OsxCre*;iDTR mutant mice were competed with



**Figure 5. *Osx*<sup>+</sup> Cell Controls B Lymphopoiesis through a Microenvironment-Dependent Effect**

(A–F) Transplantation of wild-type cells into mutant hosts.  $1 \times 10^6$  total bone marrow cells from wild-type congenic SJL mice (CD45.1) were transplanted into lethally irradiated control or mutant *OsxCre*;iDTR recipients (CD45.2). (A) A similar rate of reconstitution was seen in both control and mutant groups. (B–F) As wild-type hematopoietic cells repopulated the new hosts over the next 12 weeks, we observed a gradual re-creation of the *OsxCre*;iDTR mutant hematopoietic phenotype in the mutant recipients but not in the control group.

(G–L) Transplantation of mutant cells into wild-type hosts. Bone marrow cells ( $5 \times 10^5$ ) from either control or mutant *OsxCre*;iDTR mice were competed with wild-type  $5 \times 10^5$  SJL bone marrow competitors in a 1:1 ratio, and transplanted into lethally irradiated SJL hosts. (G) No functional disadvantage was seen in reconstitution when *OsxCre*;iDTR mutant cells competed with wild-type cells. (H–L) When hematopoietic cells from *OsxCre*;iDTR mutant donors were placed into a wild-type microenvironment, the hematopoietic defect was normalized over time. Two independent experiments;  $n = 10/\text{group}/\text{experiment}$ . \* $p < 0.05$ , \*\* $p < 0.01$ . Error bars represent  $\pm$  SEM.

cells from wild-type mice, no functional disadvantage was seen during reconstitution (Figure 5G). In addition, when hematopoietic cells from *OsxCre*;iDTR mutant donors

were transplanted into a wild-type host, the hematopoietic defect was normalized over time (Figures 5H–5L). Thus the hematopoietic defect observed in the *OsxCre*;iDTR





mutants was not cell autonomous, but caused by alteration in the microenvironment.

To assess which secreted factors released by osteoprogenitors mediate B cell differentiation, we compared local cytokines within the bone marrow of *OsxCre;iDTR* mutant and control mice by cytokine array. Since we observed a pro-B to pre-B differentiation blockade in the *OsxCre;iDTR* mutants, and interleukin-7 (IL-7) is critical for commitment to B cell fate (Nagasawa, 2006), we initially focused on changes in IL-7 production. Decreased *Il7* transcription was noted in *OsxCre;iDTR* mutant bones by qPCR (Figure S5A); however, IL-7 protein in the bone marrow sera of mutants was not statistically decreased (Figure S5B). This suggests that although *Osx*<sup>+</sup> cell expresses IL-7, other stromal cell types within the bone marrow likely produce IL-7 when *Osx*<sup>+</sup> cells are absent.

Among the measured cytokines, insulin-like growth factor 1 (IGF-1) was notably decreased in our screen (Figure S5B). We confirmed that *OsxCre;iDTR* mutant bones had decreased *Igf1* transcripts compared with controls (Figure S5A). To determine what kind of hematopoietic cells were the targets of the IGF-1 released by *Osx*<sup>+</sup> cells, we assessed flow sorted hematopoietic populations including LIN<sup>lo</sup>SCA-1<sup>+</sup>C-KIT<sup>+</sup> (LKS), CLP, common myeloid progenitor (CMP), GMP, megakaryocyte-erythroid progenitor (MEP), pro-B, pre-B, mature B, and LIN<sup>+</sup> mature cells by qPCR for IGF-1 receptor (IGF-1R) expression. IGF-1R showed higher expression in LKS, CLP, pro-B, pre-B, mature B, and LIN<sup>+</sup> cells but not in CMP, GMP, and MEP (Figure S5C), although the difference was not statistically significant.

To further assess whether IGF-1 promotes B cell differentiation, we evaluated hematopoietic cells from femurs of the *OsxCre;iDTR* mutant and control mice by methylcellulose assays. Cells were grown with (1) no supplement, (2) IL-7, (3) IGF-1, or (4) IL-7 + IGF-1 for a period of 14 days and scored for CFU-G, CFU-M, CFU-GM, CFU-GEMM, burst-forming unit (BFU)-E, and CFU-Pre-B (Figures S5D–S5I). CFU-Pre-B assays showed that IL-7 promoted pre-B colony formation, and the effect was further enhanced when IGF-1 was added (Figure S5I). No positive effect on the differentiation of other hematopoietic progenitors was observed even when the combination of IL-7 and IGF-1 was added (Figures S5D and S5F–S5H). However, the addition of IL-7 and IGF-1 had a negative effect on CFU-M (Figure S5E).

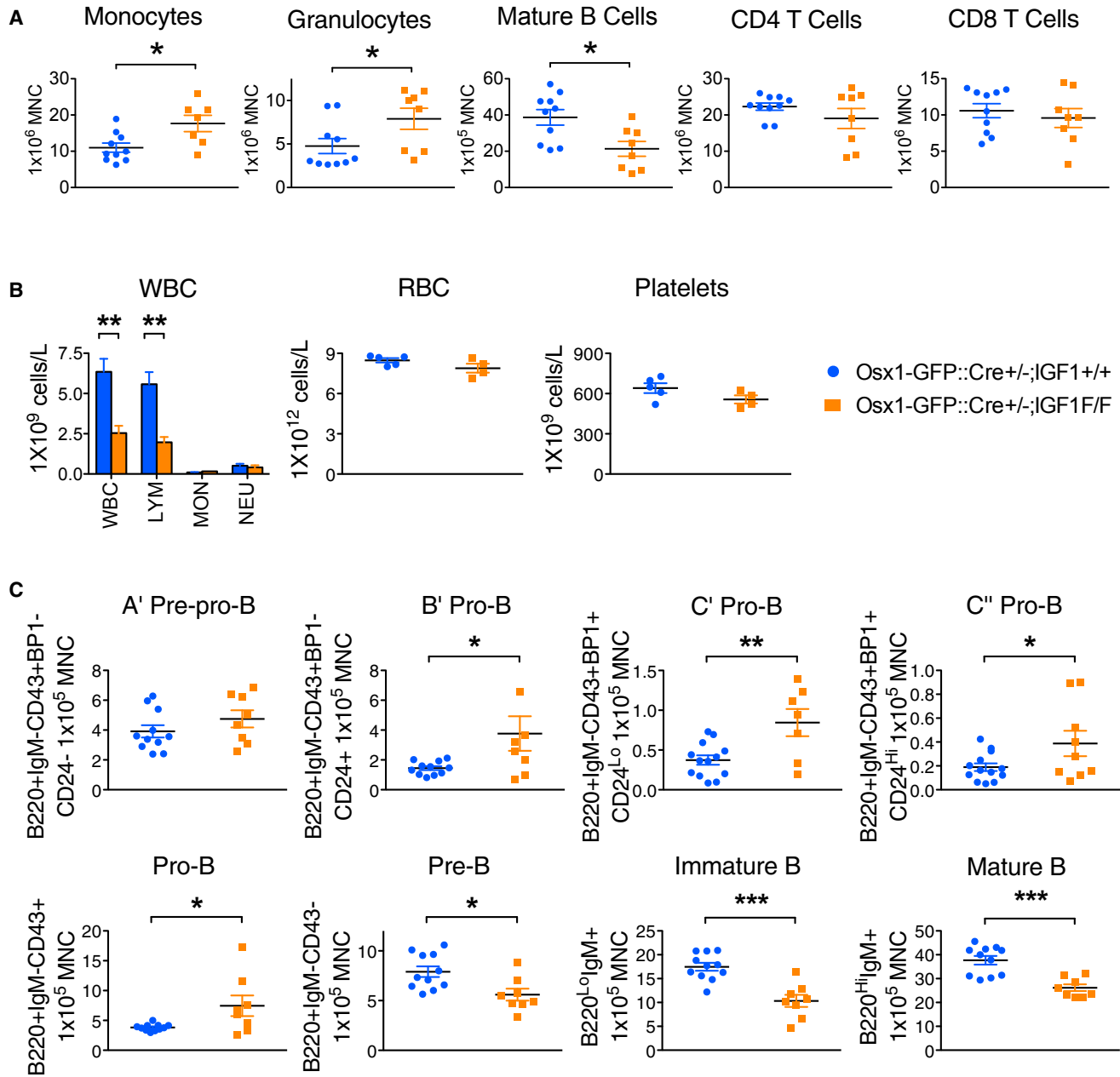
Our data suggest that *Osx*<sup>+</sup> cells express IGF-1 to mediate B cell differentiation. To validate this hypothesis in vivo, we crossed the *Osx1-GFP::Cre* mouse with the B6.129(FVB)-*Igf1*<sup>tm1Dlr/J</sup> (IGF-1<sup>F/F</sup>) strain, which has *LoxP* sites flanking exon 4 of the *Igf1* gene. Expression of Cre under the *Osx* promoter in *Osx1-GFP::Cre*<sup>+</sup>;IGF-1<sup>F/F</sup> mice specifically abolishes IGF-1 in *Osx*<sup>+</sup> cells (Figure S6). Interest-

ingly, *Osx1-GFP::Cre*<sup>+</sup>;IGF-1<sup>F/F</sup> mutants showed increased myeloid and decreased B lymphoid cells in the bone marrow (Figure 6A), and decreased white blood cell counts in the blood (Figure 6B), a phenotype reminiscent of the *Osx*<sup>+</sup> cell deletion mouse model. Strikingly, examination of B cell development showed that cell maturation was arrested at the pro-B to pre-B transition, a phenocopy of the *OsxCre;iDTR* mutants, and the effect was even more pronounced (Figure 6C). These data confirm that *Osx*<sup>+</sup> cell regulates B cell development by providing IGF-1 to enable B cell maturation.

Finally, we tested whether the B cell differentiation defect in the *OsxCre;iDTR* mutants could be rescued in vivo by intravenous administration of recombinant IL-7 and IGF-1. Both controls and mutants were subjected to daily injections of DT, DT + IL-7, or DT + IGF-1 for 12 days. At the end of the rescue regimen, bone marrow cells were evaluated by flow cytometry (Figures 7A–7H). IL-7 administration increased the C' and C'' pro-B populations (Figures 7C and 7D) but did not rescue later B stages (Figures 7F–7H). In contrast, IGF-1 had no effect at the early pro-B stages (Figures 7A–7E) but rescued the pre-B and mature B levels (Figures 7F–7H). These results suggest that while IL-7 supports the differentiation of early B precursors, IGF-1 is required for downstream B cell maturation and that *Osx*<sup>+</sup> cells regulate this process via production of IGF-1.

## DISCUSSION

These and our recent publication (Yu et al., 2015a) demonstrate that mesenchymal cell populations in the niche have stage-specific functional interactions with the hematopoietic system. Specifically, while primitive mesenchymal cells regulate HSC, we have recently shown that *Ocn*<sup>+</sup> cells modulate production of T cells through the regulation of thymus-seeding progenitors. Those data indicate that T lymphoid progenitor specification and production were diminished with *Ocn*<sup>+</sup> deletion; DLL4 was the identified *Ocn*<sup>+</sup> cell product responsible for the production of these T cell precursors. Here we show that specific steps of B cell differentiation are affected by the partial loss of *Osx*<sup>+</sup> cells. The importance of osteolineage cells in regulating B cells within the bone marrow has been known from reports documenting the roles of IL-7 and CXCL12, among other factors (Ding and Morrison, 2013; Greenbaum et al., 2013; Visnjic et al., 2004; Wu et al., 2009; Zhu et al., 2007). Previous studies reported that IGF-1 promotes B cell expansion in vitro (Taguchi et al., 2006) and that activation of the IGF-1 receptor is needed for immunoglobulin production (Baudler et al., 2005). Local IGF-1 production may be most important for marrow B lymphopoiesis, as prior studies of either liver or pituitary-specific IGF-1 deficiency only observed altered



**Figure 6. Deletion of IGF-1 in *Osx*<sup>+</sup> Cells Phenocopied *OsxCre*; *iDTR* Lymphoid Defects**

To delete IGF-1 in *Osx*<sup>+</sup> cells, we crossed the *Osx1-GFP::Cre* mouse with the *IGF-1<sup>F/F</sup>* strain.

(A) *Osx1-GFP::Cre<sup>+</sup>; IGF-1<sup>F/F</sup>* mutants showed increased Mac1<sup>+</sup> and Gr1<sup>+</sup> myeloid cells, and decreased B lymphoid cells in the bone marrow. Three independent experiments; n = 8–14/group.

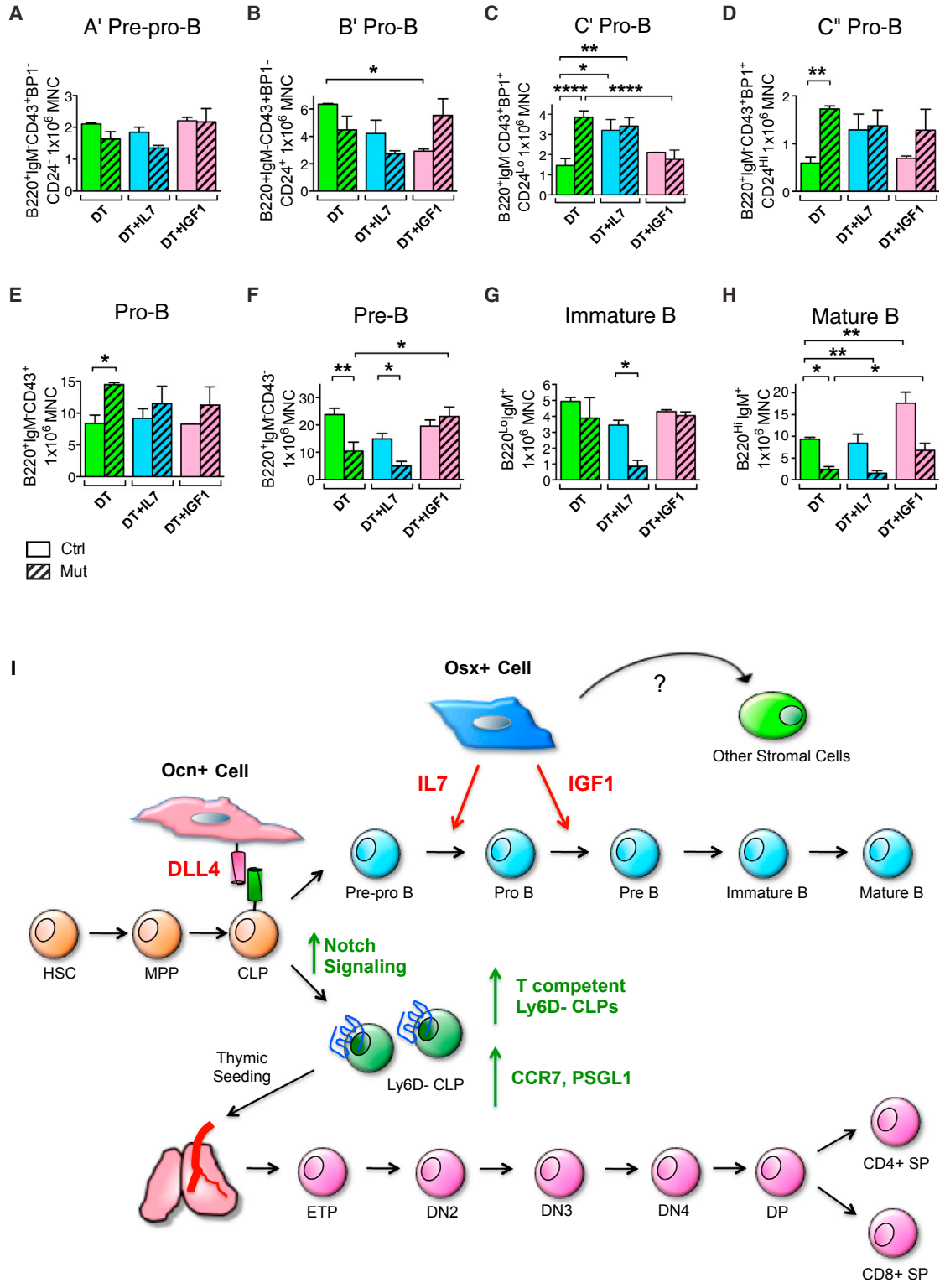
(B) Lymphopenia was also reflected in the peripheral blood. Experiment repeated once; n = 4–6/group.

(C) *Osx1-GFP::Cre<sup>+</sup>; IGF-1<sup>F/F</sup>* mutants showed a loss of mature B cells due to differentiation arrest at the pro-B to pre-B transition. Analysis of B cell differentiation showed accumulation of cells at the B', C', and C'' pro-B cell stages, but decrease in number at the pre-B, immature B, and mature B stages. Three independent experiments; n = 8–14/group.

\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. Error bars represent ± SEM.

B cell numbers in the spleen and not within the bone marrow (Montecino-Rodriguez et al., 1997; Welniak et al., 2004). Our data indicate that osteoprogenitors secrete IL-7

and IGF-1; both are necessary to support full B lineage differentiation in the bone marrow (Figure 7I). Deletion of *Osx*<sup>+</sup> cells lead to minimal changes in IL-7 serum levels



**Figure 7. *Osx*<sup>+</sup> Cell Produces IL-7 and IGF-1 to Regulate B Cell Differentiation**  
 (A–H) In vivo rescue experiment of *OsxCre*; *iDTR* lymphoid phenotype. *OsxCre*; *iDTR* control and mutant animals were subjected to either: DT, DT + IL-7, or DT + IGF-1 daily injections for 12 days. Bone marrow cells were harvested the next day following the last injection for flow cytometric analysis of B cell differentiation. Injection of IL-7 augmented the base level of C' and C'' pro-B (C and D), but failed to rescue (legend continued on next page)



despite significant knockdown of *Il7* transcripts. It is likely that other cell types compensate for the production of IL-7 in the niche. Rescue experiments showed that IGF-1 is required together with IL-7 to restore the full differentiation program of B cells. These data suggest that IL-7 controls early stages of B cell differentiation, in agreement with previous data (Tokoyoda et al., 2004), while IGF-1 is necessary downstream of IL-7 for the differentiation into pro-B and mature B cells. Taken together, our data suggest that marrow B lymphopoiesis requires localized IGF-1 production while extramedullary maturation is more affected by systemic IGF-1.

Having defined through the triple transgenic model that *Osx*<sup>+</sup> cells are distinctive molecularly from *Ocn*<sup>+</sup> cells, we tested their selective function. The model we used was intentionally of short duration to avoid the progression of *Osx*<sup>+</sup> cells to *Ocn*-expressing, ++ cells. In vivo, these cells are dynamic and are lost after ~90 days (Park et al., 2012). Our data demonstrate that the *Osx*<sup>+</sup> cells have no effect on CLP or the T competent progenitors, Ly6D<sup>-</sup>CLP (Figure 4C) in contrast to the *Ocn*<sup>+</sup> cells. Nor do they produce DLL4 at levels comparable with those in *Ocn*<sup>+</sup> cells. However, they do express abundant IL-7 and IGF-1 and through these molecules regulate the maturation of B cell through the pro-B cell stage. This maturation step is critical, as blunting B cell progression by deleting *Osx*<sup>+</sup> support cells resulted in B lymphopenia and functional compromise of immunoglobulin production.

Others have reported both B and T cell effects after osteolineage cell deletion (Ding and Morrison, 2013) but the promoter used in some of those studies, *Col1(2.3)*, extends through many cell stages of osteolineage progression. Here we define that these osteolineage cell states provide distinctive functions in support of hematopoiesis through secretion of specific regulatory molecules. Some of these effects are in specific lineage production as noted in *Ocn*<sup>+</sup> cell deletion (Yu et al., 2015a) and some are in lineage differentiation as in the *Osx*<sup>+</sup> deletion model.

It was notable that hematopoietic cell abnormalities in deletion of either of these osteolineage subsets appeared to be predominantly in the lymphoid compartment with

little or no perturbation of HSC function. Nevertheless, we encourage caution in interpreting the HSC data. Prior reports by us (Calvi et al., 2003; Ferraro et al., 2011; Fleming et al., 2008; Raaijmakers et al., 2010) and others (Visnjic et al., 2004; Zhang et al., 2003) indicate a functional role for osteolineage cells in HSPC regulation. A possible explanation for the observed differences is that our cell depletion was not 100% efficient. It is also possible that the lack of functional defects in HSC, as demonstrated by the serial transplantation experiment, could depend upon the limited time donor HSCs were exposed to the mutated microenvironment. Finally, we cannot rule out the possibility that other cell types might have compensated for the osteolineage cell loss, thus mitigating any HSC phenotype. In summary, whether HSC depend on osteolineage cells deserves further clarification.

Several markers, including LEPTIN receptor (LEPR), NESTIN, PDGFR $\beta$ , CXCL12, PRX-1, CD105, and OSX, have been described to label overlapping mesenchymal stem cell populations with broad differentiation potential in vivo (Yu and Scadden, 2016a, 2016b). In published studies using *OsxCreERT2*, *Osx*<sup>+</sup> cells did not overlap with *Nestin*<sup>+</sup> or *LepR*<sup>+</sup> cells after short induction, but overlapped highly after several weeks (Liu et al., 2013; Mizoguchi et al., 2014; Ono et al., 2014). In addition, *Osx*<sup>+</sup> cells have been shown to contribute to osteogenic, adipogenic, and chondrogenic lineages upon injury in vivo and in the neonate, but are restricted to the osteolineage in the adult (Mizoguchi et al., 2014). These inconsistent reports suggest that these markers, rather than being cell specific, might reflect functional states of mesenchymal cells that are dynamic during development and tissue regeneration. We examined the expression profile of these markers on *Osx*<sup>+</sup> cells in our model by microarray and flow cytometry. *Osx*<sup>+</sup> cells isolated from 6- to 8-week-old adults expressed low levels of LEPR, NESTIN, and CD105, and low to intermediate levels of PRX-1, CXCL12, and PDGFR $\beta$  (Figures S7A and S7B). We did not find extensive overlap in the *Osx*<sup>+</sup> cells with *LepR*<sup>+</sup> cells that might be considered CAR cells (Figures S7A and S7B), despite others (Greenbaum et al., 2013) reporting that some *Osx*<sup>+</sup> cells overlaps with CAR cells as defined by

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pre-B and downstream B cell maturation (F–H). In contrast, IGF-1 had no effect on earlier B cell differentiation (A–E), but rescued the pre-B and mature B cell loss in the mutant group (F and H). Three independent experiments; n = 6–9/group. \*p < 0.05, \*\*p < 0.01, \*\*\*\*p < 0.0001.

(I) Model of regulation of B and T lymphopoiesis by *Osx*<sup>+</sup> and *Ocn*<sup>+</sup> osteolineage cells. *Osx*<sup>+</sup> cell produces IL-7 and IGF-1, and both molecules are required to support full B lineage differentiation. While IL-7, also likely produced by other stromal cell types, mediates hematopoietic differentiation from early B cell precursors to pre-B cells, *Osx*<sup>+</sup> cell secretion of IGF-1 is necessary for further B cell maturation from pre-B to mature B. In comparison, *Ocn*<sup>+</sup> cell expresses the Notch ligand DLL4, which binds to cell-surface Notch receptor on the T cell-competent Ly6D<sup>-</sup>CLP population (Yu et al., 2015a). This engagement ensures T cell progenitor production, and expression of the chemotactic molecules CCR7 and PSGL1 for subsequent thymic seeding. The precise balance of *Osx*<sup>+</sup> and *Ocn*<sup>+</sup> osteolineage cells within the bone marrow niche is critical for both B and T lymphopoiesis, as perturbation of this balance leads to specific loss of B or T immune cells. Error bars represent  $\pm$  SEM.



the expression of CXCL12 (the age of the mice was not reported). Nonetheless, our data cannot exclude the possibility of *Osx*<sup>+</sup> cells being adipogenic in models with a longer time chase, after injury or during perinatal life, although we observed no evidence of adipocyte generation under the conditions we studied.

There is a growing appreciation of a woven functional architecture to the bone marrow space with mesenchymal stem cells providing support for HSC (Mendez-Ferrer et al., 2010), mature osteoblasts affecting HSC mobilization by granulocyte colony-stimulating factor (Ferraro et al., 2011), osteoprogenitors requiring microRNA processing to maintain integrity of early and late hematopoiesis (Raaijmakers et al., 2010), and *Osx*<sup>+</sup> and *Ocn*<sup>+</sup> cells having key roles in B lymphoid differentiation and thymic homing progenitors (Yu et al., 2015a). The emerging model is one of a highly interrelated system with “intermediate” populations of both skeleton and blood having very specific interactions. Whether these interactions are perturbed is of particular relevance in settings where specific subsets of cells are deficient, such as in particular blood disorders, or in the adaptive immune deregulation seen after allogeneic bone marrow transplantation. Furthermore, these heterologous cell interactions can now be explored in malignant processes such as lymphoma, leukemia, and bone metastatic processes. If the complexity of the relationship between mesenchymal subsets of bone and parenchymal subsets of blood is true in other organ types, defining mesenchymal cells that comprise organ “stroma” may provide insight into parenchymal regulation and dysfunction.

## EXPERIMENTAL PROCEDURES

### Osteolineage Cell Deletion Mouse Models

The *Osx1-GFP::Cre* mouse strain (Rodda and McMahon, 2006) was crossed to the *iDTR* strain (Buch et al., 2005) to achieve *Osx* cell deletion. These primers were used for genotyping: *OsxCre*-CTCTTCATGAGGAGGACCCT, *OsxCreR*-CAGGCAGGTGCCTGGACAT, *oIMR8052-GCGAAGAGTTTGTCCCTCAACC*, *oIMR8545-AAAGTCGCTCTGAGTTGTTAT*, *oIMR8546-GGAGCGGGAGAAA TGGATATG*. *OsxCre*<sup>+</sup>;*iDTR*<sup>+/+</sup> injected with DT or *OsxCre*<sup>+</sup>;*iDTR*<sup>Fl/+</sup> injected with PBS were used as controls while *OsxCre*<sup>+</sup>;*iDTR*<sup>Fl/+</sup> injected with DT were mutants. For most experiments, 100 ng of DT/g body weight (BW) was injected daily into both controls and mutants from 4 to 6 weeks old to achieve an acute deletion of specific osteolineage subsets. Mice were harvested the next day after the last dose of DT injection. Injection regimen had been prolonged to 4 weeks for phenotype comparison. For all experiments, littermates were used as controls. C57BL/6J and B6.SJL-*Ptprc*<sup>a</sup>*Pepc*<sup>b</sup>/BoyJ (SJL) strains were obtained from Jackson Laboratories. All animal use and procedures performed were approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital.

### Bone Histomorphometry

Paraffin sections of long bones were stained with H&E, von Kossa, and Goldner trichrome, and analyzed using BioQuant software. Statistical analysis was performed using the non-parametric Mann-Whitney test. TRAP staining was used to reveal osteoclasts. Osteolineage activity was assessed by measuring serum levels of OCN and N-terminal propeptide of type I procollagen (P1NP) using RatLaps ELISA kits. Bone resorption was assessed by measuring the C-terminal telopeptides of collagen type I fragments in mouse serum using the RatLaps EIA kit.

### Immunohistochemistry

Expression of the DT receptor was detected by immunohistochemistry using an anti-human heparin-binding EGF-like growth factor (anti-hHB-EGF) antibody. In brief, paraffin sections were treated with 0.1% trypsin and 3% H<sub>2</sub>O<sub>2</sub>, and blocked with reagent containing 5% animal serum in PBS + Tween 20 for 1 hr. Anti-hHB-EGF antibody (R&D Systems MAB391, 1:25 dilution) was used as primary antibody coupled with biotinylated IgG secondary antibody (Vector Laboratories BA-9500, 1:400 dilution). After staining, sections were treated with a Vectastain ABC Kit for biotin-streptavidin signal amplification and visualized by a DAB Peroxidase Substrate Kit. *Osx*<sup>+</sup> cells were detected by staining sections with anti-SP7 antibody (Abcam Ab22552, 1:2,000 dilution) coupled with secondary antibody conjugated to Alexa Fluor 488 (Thermo Fisher Scientific A27034, 1:1,000 dilution). *Ocn*<sup>+</sup> cells were recognized by anti-OCN antibody (Santa Cruz Biotechnology SC18322, 1:50 dilution) followed by secondary antibody conjugated to Alexa Fluor 488 (Thermo Fisher A11078, 1:1,000 dilution). Green fluorescent OSX- or OCN-antibody staining was then individually superimposed with red fluorescent TUNEL staining (In Situ Cell Death Detection Kit TMR Red, Roche Applied Science).

### Microarray Data

Microarray analysis was performed as previously described (Yu et al., 2015b). The Accession number is GEO: GSE66042.

### CFU-Ob Assay

The CFU-Ob assay was performed as previously described (Yu et al., 2015a).

### Flow Cytometry

Peripheral blood from each mouse was subjected to complete blood count. Tibias, femurs, iliac crests, spines, ulnae, radii, and humeri were harvested for bone marrow cells. Spleen and thymus were collected for lymphocyte staining. Flow cytometry staining for all hematopoietic subpopulations including T cell developmental stages was performed as previously described (Yu et al., 2015a). For B cell development, the following scheme was used: B220-Pacific Blue (BD Biosciences 558108), IgM-PE-Cy5 (eBiosciences 15-5790-82), CD43-fluorescein isothiocyanate (FITC) (eBiosciences 11-0431-82), CD24-APC (Biolegend 101814), and BP1-PE (eBiosciences 12-5891-83). Definitions of stages of B cell maturation were as follows: A' pre-pro-B (IgM<sup>-</sup>B220<sup>+</sup>CD43<sup>+</sup>BP1<sup>-</sup>CD24<sup>-</sup>), B' pro-B (IgM<sup>-</sup>B220<sup>+</sup>CD43<sup>+</sup>BP1<sup>-</sup>CD24<sup>+</sup>), C' pro-B (IgM<sup>-</sup>B220<sup>+</sup>CD43<sup>+</sup>BP1<sup>+</sup>CD24<sup>lo</sup>), C'' pro-B (IgM<sup>-</sup>B220<sup>+</sup>CD43<sup>+</sup>BP1<sup>+</sup>CD24<sup>hi</sup>), pro-B (IgM<sup>-</sup>B220<sup>+</sup>CD43<sup>+</sup>), pre-B (IgM<sup>-</sup>B220<sup>+</sup>CD43<sup>-</sup>),



B progenitors (IgM<sup>-</sup>B220<sup>+</sup>), immature B (IgM<sup>+</sup>B220<sup>lo</sup>), and mature B (IgM<sup>+</sup>B220<sup>hi</sup>). For cell-cycle analysis, bromodeoxyuridine-FITC and 7AAD, or Ki67-FITC and DAPI staining were coupled with staining of specific populations to reveal their cell-cycle status.

### Anti-inflammatory Assay

Indomethacin was injected at 2.5 mg/kg BW daily along with DT throughout the deletion regimen. Mice were euthanized the next day following the last injection. Bone marrow was harvested from femurs, tibias, iliac crests, and spines. After lysis of red blood cells, bone marrow cells were stained with antibodies against monocytes and granulocytes (Mac1, Gr1), macrophages (Mac1), and granulocyte macrophage progenitors (CD127, LIN, SCA-1, C-KIT, CD34, CD16/32) for flow cytometric measurement as previously described (Yu et al., 2015a).

### Immune Response Assay

After 1 week of DT injection (day 0), mice were injected with 0.1 µg/µl NP28-AECM-Ficoll/PBS intraperitoneally to trigger T cell-independent Ig production. In this assay, DT injection continued throughout the next 28 days. Mice were re-immunized with a second boost of NP28-AECM-Ficoll at day 14. Mice were bled at days 0, 7, 14, 21, and 28. Sera collected from control and mutant animals were subjected to measurement of IgG and IgM levels by ELISA following standard protocol.

### Transplantation

For transplantation of wild-type cells into mutant environment,  $1 \times 10^6$  bone marrow cells from 6-week-old SJL donors were transplanted into each of ten age-matched 9.5-Gy lethally irradiated *OsxCre<sup>+</sup>;iDTR<sup>+/+</sup>* control or *OsxCre<sup>+</sup>;iDTR<sup>Fl/+</sup>* mutant recipients. DT injection into both control and mutant recipients began at 7 days before transplantation and were maintained every alternate day until 12 weeks post transplant. Recipients were bled at 4, 8, and 12 weeks post transplant. Peripheral blood was subjected to complete blood cell count and reconstitution analysis as previously published (Yu et al., 2015a). All recipients were euthanized at 12 weeks post transplant. Bone marrow cells were harvested from femurs, tibias, and iliac crests, and subjected to lineage reconstitution analysis as described previously (Yu et al., 2015a). For transplantation of mutant cells into wild-type recipients,  $5 \times 10^5$  bone marrow cells from 6-week-old mutant *OsxCre<sup>+</sup>;iDTR<sup>Fl/+</sup>* or control *OsxCre<sup>+</sup>;iDTR<sup>+/+</sup>* donors were competed with  $5 \times 10^5$  bone marrow cells from age-matched SJL mice. For both controls and mutants, a total of  $1 \times 10^6$  mixed donor cells were transplanted into each of ten 9.5-Gy irradiated SJL recipients. Recipients were bled at 4, 8, 12, and 16 weeks post transplant. Peripheral blood was subjected to complete blood cell count and reconstitution analysis by flow cytometry as reported by Yu et al. (2015a). At 16 weeks post transplant, all recipients were euthanized. Bone marrow cells were subjected to lineage reconstitution analysis as described by Yu et al. (2015a).

### Cytokine Array

Bones with both ends excised were centrifuged at 12,000 rpm for 1 min for bone marrow extraction. Each sample was further centrifuged at 6,000 rpm for 10 min for serum isolation, and stored at  $-20^{\circ}\text{C}$  until use. For measurement of cytokines in circula-

tion, 200 µl of peripheral blood was collected, centrifuged at 6,000 rpm for 10 min for serum isolation, and stored at  $-20^{\circ}\text{C}$  until use. A 1/5 dilution of each serum sample was subjected to RayBio Mouse Cytokine Antibody Array G Series 4 (RayBiotech, AAM-CYT-G4-4) (n = 24 per group).

### qPCR

Positive hits of cytokine array were validated by qPCR using the GAPDH gene for normalization. Various sources were used for RNA extraction: total bone marrow cells of mutant or control mice, flushed femoral bone of mutant or control mice, and flow sorted cells of stained hematopoietic populations including LKS, CLP, CMP, GMP, MEP, Pro-B, Pre-B, Mature B, and Lineage<sup>+</sup> cells.

### Rescue Experiments

For in vitro rescue experiments, bone marrow cells were harvested from femurs and tibias of *OsxCre<sup>+</sup>;iDTR* controls or mutants and plated at  $2 \times 10^4$  in each well of a six-well plate with methylcellulose-containing medium, supplemented with 100 ng/ml IL-7, 100 ng/ml IGF-1, or both IL-7 and IGF-1. Cells were incubated at 5% CO<sub>2</sub> at 37°C undisturbed and enumerated for progenitor colonies by day 8 or day 14. For in vivo rescue experiments, control and mutant *OsxCre<sup>+</sup>;iDTR* mice were intraperitoneally injected with (1) 100 ng/g BW DT alone, (2) DT and 250 ng/g BW recombinant murine IL-7, and (3) DT and 1 µg/g BW recombinant mouse IGF-1 daily for 12 days. IL-7 and IGF-1 were injected intravenously. Mice were euthanized for bone marrow cell harvest. Cells were stained for GMP- and B cell development-specific antibodies for flow cytometric analysis.

### Statistical Analysis

Statistical analysis of all paired experiments was analyzed by two-tailed Student's t test, unless otherwise stated. Data in all graphs represent the mean and SEM where \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001. Statistical comparison of multiple parameters were analyzed by one-way ANOVA followed by Bonferroni post test, where \*p < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2016.06.009>.

### AUTHOR CONTRIBUTIONS

V.W.C.Y. designed, executed, and interpreted data for the majority of experiments in this project and wrote the manuscript. S.L. designed, and together with F.F., A.J., P.S., and R.V. carried out and analyzed the experiments involving the triple fluorescent mouse model. D.T.S. supervised the whole project and was involved in experimental design, data interpretation and manuscript editing. V.W.C.Y., S.L., and F.F. provided editorial input for the manuscript.

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