

Figure 3. Essential In Vivo Functional Roles of CAR Cells in HSCs

Bone marrow from wild-type and CXCL12-DTR-GFP mice was analyzed 2 days after DT administration.

- (A) Flow-cytometric analysis of the numbers of cells in the CD34⁺Lin⁻Sca-1⁺c-kit⁺CD150⁺CD48⁻ HSC population per two femurs and tibiae. n = 3; *p < 0.05.
- (B) The numbers of HSCs were estimated using repopulating units (RU), based on a competitive repopulation assay. n = 3; *p < 0.05.
- (C) Flow-cytometric analysis of the numbers of Ki67-positive cells in the HSC population per two femurs and tibiae. n = 3; *p < 0.05.
- (D) Relative mRNA expression levels of Evi-1, Pbx1, Cyclin D1, D2, A2, Cdc2a, Cdc6, and Mad1 in sorted cells in the HSC population by qRT-PCR analysis normalized to G3PDH levels. n = 4; *p < 0.05.
- (E) Flow-cytometric analysis of RNA content of cells in the HSC population by PY staining.
- (F) Flow-cytometric analysis of cell size of cells in the HSC population by FSC.
- (G and H) Sections of bone marrow from DT-treated CXCL12-DTR-GFP mice were stained with antibodies against CD150, CD48, and CD41. Cell size of CD150⁺CD48⁻CD41⁻ HSCs in contact with (G, left) or at a distance from remaining CAR cells (G, right) was determined using a confocal microscope and its software (H). n = 20; *p < 0.05.
- (I) Relative mRNA expression levels of PU.1 and M-CSFR in sorted cells in the HSC population by qRT-PCR analysis normalized to G3PDH levels. n = 4; *p < 0.05.
- (J) Generation of myeloid lineage cells from cells in the HSC population in culture at an early point of time. Sorted cells in the HSC population were cultured in the presence of SCF and CXCL12. Flow-cytometric analysis of the numbers of Gr-1^{hi}CD11b⁺ cells per input cell at day 7 of culture. n = 4; *p < 0.05. All error bars represent SD of the mean (A–D and H–J).

DT Does Not Act Directly on Hematopoietic Cells in CXCL12-DTR-GFP Mice

To rule out the possibility that DT acted directly on hematopoietic cells in DT-treated CXCL12-DTR-GFP mice, we infused bone

marrow cells from CXCL12-DTR-GFP or wild-type mice into lethally irradiated recipients in the absence of competitor cells. When bone marrow hematopoietic cells of chimeric mice were mainly derived from donor HSCs 12 weeks after transplantation,

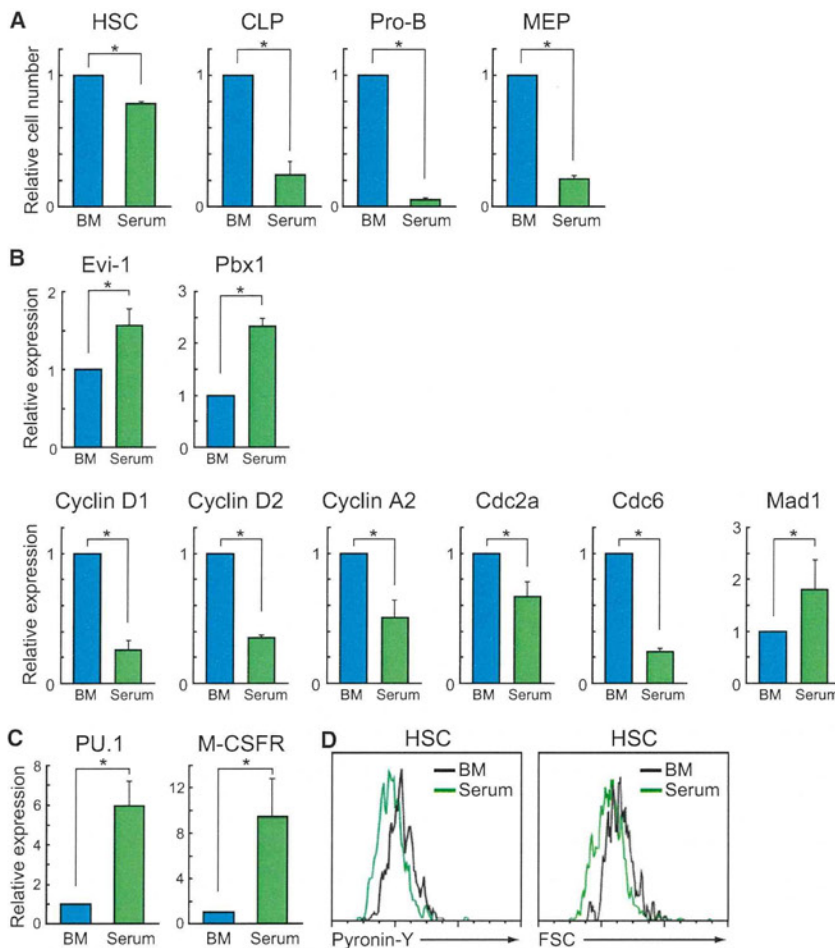


Figure 4. The Phenotypes of Wild-Type HSCs and Lympho-hematopoietic Progenitors Cultured in Serum Alone

(A) Single $CD34^{-}Lin^{-}Sca-1^{+}c-kit^{+}CD150^{+}CD48^{-}$ HSCs, CLPs, pro-B cells, and MEPs from wild-type mice were sorted and cultured in serum alone (1 cell/well). The numbers of living cells after 48 hr culture are shown (green bars). Control samples before culture have been normalized to 1. $n = 3$; $*p < 0.05$.

(B–D) $CD34^{-}Lin^{-}Sca-1^{+}c-kit^{+}CD150^{+}CD48^{-}$ HSCs from wild-type mice were sorted and cultured in serum alone. QRT-PCR analysis of mRNA expression of HSC-specific genes (B, upper) and genes involved in cell-cycle promotion or repression (B, lower) and early myeloid differentiation (C) in HSCs before and after 6 hr culture. Results are expressed as fold difference compared to the levels found in freshly isolated HSCs from wild-type mice (G3PDH normalization). $n = 3$; $*p < 0.05$.

(D) Left: flow-cytometric analysis of RNA content of HSCs before (black line) and after (green line) 6 hr culture by PY staining. Right: flow-cytometric analysis of cell size of HSCs before (black line) and after (green line) 12 hr culture by PY staining by FSC. All error bars represent SD of the mean (A–C).

we injected these chimeric mice with 100 ng/kg DT. Flow-cytometric analysis revealed that the numbers of donor-derived $CD34^{-}Lin^{-}Sca-1^{+}c-kit^{+}CD150^{+}CD48^{-}$ HSCs, CLPs, pro-B cells, MEPs, proerythroblasts, and GMPs, and the cell size of donor-derived HSCs in mutant chimeras, were comparable to those observed in control chimeras 2 days after DT treatment (Figures S2B and S3B). These results indicate that a 4-fold excess of DT does not act directly on CXCL12-DTR-GFP hematopoietic cells and that the failure of hematopoiesis in DT-treated CXCL12-DTR-GFP mice was because of environmental factors.

Phenotypes of HSCs Cultured in Serum Are Similar to Those from CAR Cell-Depleted Mice

To confirm further the role of bone marrow niches for HSCs and lymphoid and erythroid progenitors, we sorted $CD34^{-}Lin^{-}Sca-1^{+}c-kit^{+}CD150^{+}CD48^{-}$ HSCs, CLPs, pro-B cells, and MEPs from bone marrow of wild-type mice and cultured them in serum alone. After 48 hr of single-cell cultures, many HSCs remained viable, but the numbers of living CLPs, pro-B cells, and MEPs were progressively decreased over time (Figure 4A). QRT-PCR analysis revealed that the mRNA expression of Cyclin D1, D2, A2, Cdc2a, and Cdc6 was significantly reduced, although the mRNA expression of Evi-1, Pbx1, and Mad1 was elevated in the HSC population after 6 hr of culture (Figure 4B). In addition,

the mRNA expression of PU.1 and M-CSFR was markedly increased in the HSC population cultured in serum alone (Figure 4C). Flow-cytometric analysis of cultured HSCs revealed that PY fluorescence and the cell size was decreased compared with freshly isolated HSCs, although Annexin-V staining revealed no significant increase in apoptotic cells (Figure 4D and Figure S4). Together, these results demonstrate that phenotypes of wild-type HSCs and lymphoid and erythroid progenitors cultured in serum alone were similar to those from CAR cell-depleted bone marrow.

CAR Cells Are the Major Producer of SCF as Well as CXCL12 in Bone Marrow

To determine the mechanism by which CAR cells support HSCs and hematopoietic progenitors, we compared the mRNA expression of SCF and CXCL12, which are essential for the maintenance of HSCs, CLPs, and B cell and erythroid progenitors (Kohara et al., 2007; Miller et al., 1996; Sugiyama et al., 2006; Tokoyoda et al., 2004; Waskow et al., 2002), in CAR cells to other microenvironmental components of bone marrow. Flow-cytometric analysis of CXCL12-GFP mice has shown that CAR cells ($CXCL12-GFP^{hi}VCAM-1^{+}CD45^{-}Ter119^{-}$) constitute approximately 0.27% of nucleated cells in the bone marrow fraction. In the bone fraction, some $CD45^{-}Ter119^{-}$ nonhematopoietic cells have a much lower expression of CXCL12-GFP relative to CAR cells, and osteopontin (OPN)⁺ cells have been reported to include osteoblastic niche cells (Mayack and Wagers, 2008). QRT-PCR analysis of sorted CAR cells, $CD31^{+}Sca-1^{+}CD45^{-}Ter119^{-}$ endothelial cells, and

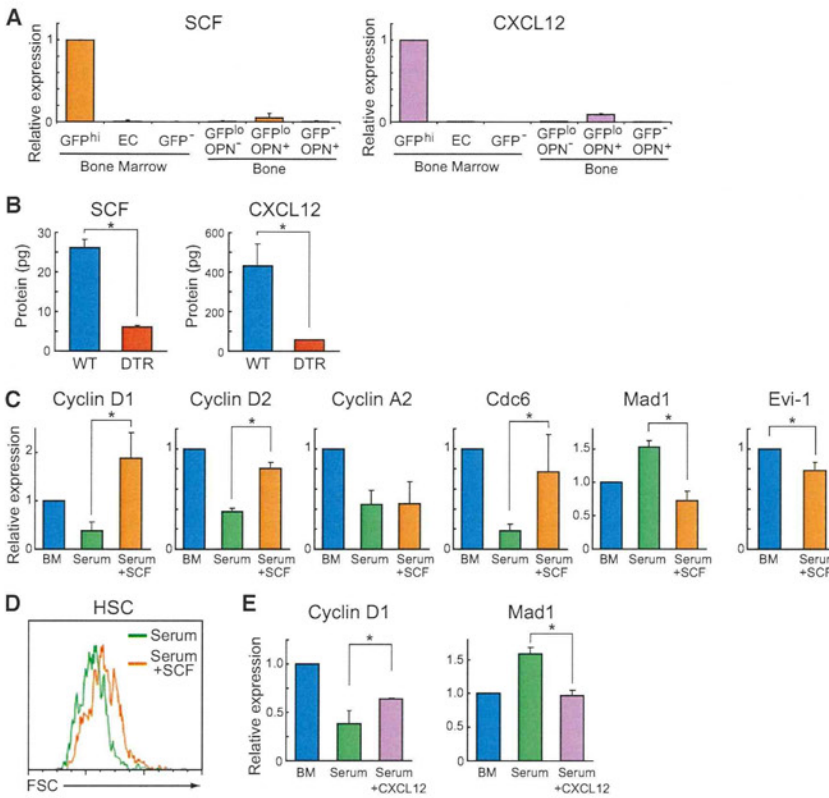


Figure 5. Preferential Expression of SCF and CXCL12 in CAR Cells

(A) QRT-PCR analysis of mRNA expression of SCF and CXCL12 in sorted CXCL12-GFP^{hi} CD45⁺Ter119⁻ CAR cells, CD31⁺Sca-1⁺CD45⁻Ter119⁻ endothelial cells (ECs), and CXCL12-GFP⁻CD31⁻Sca-1⁻CD45⁻Ter119⁻ cells from the bone marrow fraction and CXCL12-GFP^{lo}OPN⁻, CXCL12-GFP^{lo}OPN⁺, and CXCL12-GFP⁻OPN⁺ cells from the bone fraction of CXCL12-GFP mice. Results are expressed as fold difference compared to the levels found in CAR cell samples (G3PDH normalization). n = 4.

(B) The amount of SCF and CXCL12 protein per single femur from wild-type and CXCL12-DTR-GFP mice at 3 days after DT administration by enzyme-linked immunosorbent assay (ELISA). n = 3; *p < 0.05.

(C–E) CD34⁺Lin⁻Sca-1⁺c-kit⁺CD150⁺CD48⁻ HSCs from wild-type mice were sorted and cultured. (C) QRT-PCR analysis of mRNA expression of Cyclin D1, D2, A2, Cdc6, Mad1, and Evi-1 in HSCs before and after 6 hr culture in serum alone or with SCF. n = 3; *p < 0.05. (D) Flow-cytometric analysis of cell size of HSCs cultured in serum alone (green line) or with SCF (orange line) for 12 hr by FSC. (E) QRT-PCR analysis of mRNA expression of Cyclin D1 and Mad1 in HSCs before and after 6 hr culture in serum alone or with CXCL12. n = 3; *p < 0.05. Results are expressed as fold difference compared to the levels found in freshly isolated HSCs (G3PDH normalization) (C and E).

All error bars represent SD of the mean (A–C and E).

CXCL12-GFP⁻CD31⁻Sca-1⁻CD45⁻Ter119⁻ cells from the bone marrow fraction, and CXCL12-GFP^{lo}OPN⁻, CXCL12-GFP^{lo}OPN⁺, and CXCL12-GFP⁻OPN⁺ cells from the bone fraction of CXCL12-GFP mice revealed that the expression of SCF and CXCL12 was considerably higher in CAR cells than in other populations examined (Figure 5A). Consistent with this, the protein expression of SCF and CXCL12 was severely reduced in the bone marrow from CAR cell-depleted mice, supporting the idea that CAR cells are the major producer of SCF and CXCL12 in the marrow (Figure 5B). Single-cell RT-PCR analysis revealed that all individual cells (52 of 52; 100%) within the CAR cell population expressed SCF in the bone marrow from CXCL12-GFP mice, suggesting that individual CAR cells express high amounts of SCF as well as CXCL12. These results are in line with the facts that SCF augments the proliferation of B cell and erythroid progenitors in culture (Martin et al., 1990), that administration of a selective inhibitor (imatinib) of c-kit, c-abl, and platelet-derived growth factor receptor (PDGFR) tyrosine kinases led to a reduction in the numbers of CLPs, pro-B cells, MEPs, and proerythroblasts and to a modest reduction in the cell size of HSCs (Figure S5), and that the numbers of CLPs, pro-B cells, and proerythroblasts are severely reduced in mice lacking SCF receptor c-kit or CAR cells.

Like HSCs from CAR cell-depleted mice, wild-type HSCs cultured in serum alone revealed altered expression of genes involved in cell-cycle promotion, DNA replication, and early myeloid differentiation and had a decreased cell size, as

described above (Figures 4B–4D). SCF completely rescued the mRNA expression of Cyclin D1, D2, Cdc6, and Mad1 in HSCs (Figure 5C) and the cell size of HSCs (Figure 5D) in the culture for 6 hr. In contrast, SCF did not rescue the expression of Cyclin A2 and downregulated the expression of Evi-1 during SCF treatment (Figure 5C). In contrast, CXCL12 partially rescued the expression of Cyclin D1 and Mad1 (Figure 5E). These results suggest that SCF and CXCL12 partially contribute to the niche functions of CAR cells in HSC maintenance.

Individual CAR Cells Express both Adipogenic and Osteogenic Genes

We sought to identify the lineage nature of CAR cells. Flow-cytometric analysis of bone marrow from CXCL12-GFP mice revealed that CXCL12-GFP^{hi} CAR cells exhibited a largely homogeneous expression of cell-surface proteins, including VCAM-1, CD44, CD51 (αv integrin), PDGFR α and PDGFR β , suggesting that CAR cells consist of a relatively homogeneous population (Figure S6A). We analyzed mRNA expression of lineage-specific markers in sorted non-hematopoietic cell populations from the bone marrow and bone fractions of CXCL12-GFP mice. QRT-PCR analysis has shown that the expressions of C/EBP α and peroxisome proliferator-activated receptor γ (PPAR γ), which are essential for adipocyte differentiation (Barak et al., 1999), and Runx2 and Osterix, which are essential for osteogenic progenitors (Komori et al., 1997; Nakashima et al., 2002), were considerably higher in CAR cells than in other populations

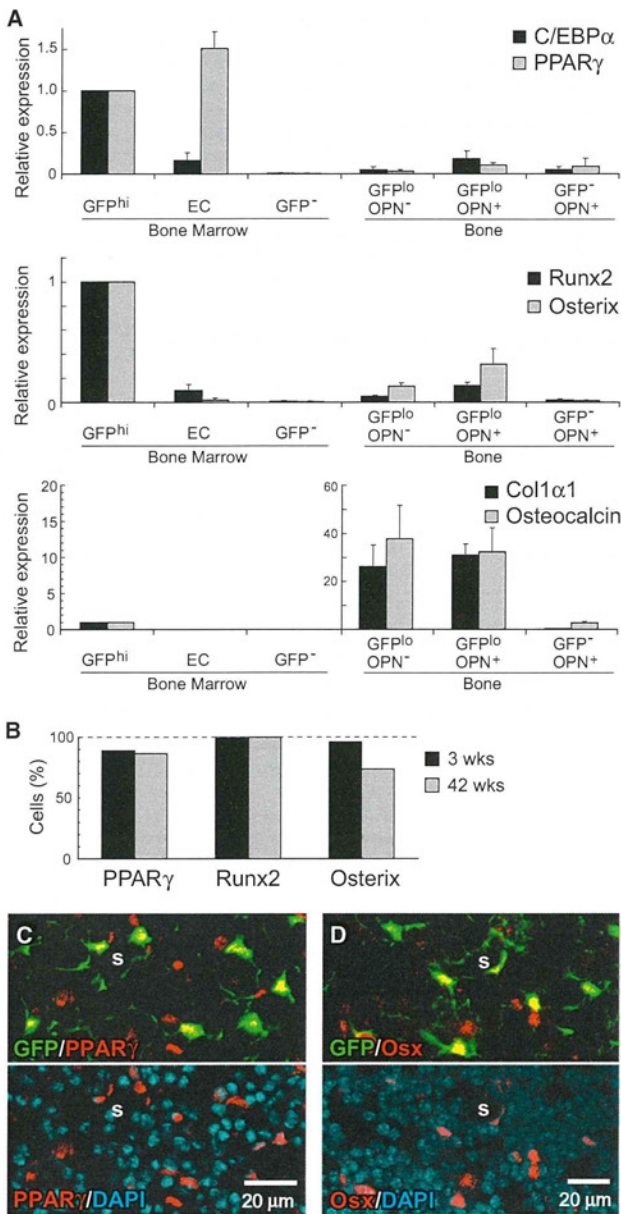


Figure 6. Expression of Requisite Factors for Adipogenesis and Osteogenesis in CAR Cells

(A) QRT-PCR analysis of sorted CXCL12-GFP⁺CD45⁻Ter119⁻ CAR cells, CD31⁺Sca-1⁻CD45⁻Ter119⁻ endothelial cells (ECs), and CXCL12-GFP⁻CD31⁻Sca-1⁻CD45⁻Ter119⁻ cells from the bone marrow fraction and CXCL12-GFP^{lo}OPN⁻, CXCL12-GFP^{lo}OPN⁺, and CXCL12-GFP⁻OPN⁺ cells from the bone fraction of CXCL12-GFP mice. mRNA levels of C/EBP α , PPAR γ , Runx2, Osterix, Col1 α 1, and Osteocalcin. CAR cell samples have been normalized to 1. Error bars represent SD of the mean; n = 4.

(B) Single-cell RT-PCR analysis of the frequency of cells expressing PPAR γ , Runx2, and Osterix in sorted CAR cells. n = 2.

(C) Bone marrow sections from 14-week-old CXCL12-GFP mice stained with antibodies against PPAR γ (red). Most CAR cells (green) express PPAR γ (yellow).

(D) Bone marrow sections from 3-week-old growing CXCL12-GFP mice stained with antibodies against Osterix (red). Most CAR cells (green) express

examined (Figure 6A). The expression of type I collagen and osteocalcin, which are abundant in osteoblasts, was much lower in CAR cells than in CXCL12-GFP^{lo}OPN⁺ or CXCL12-GFP^{lo}OPN⁻ cells from the bone fraction (Figure 6A). Furthermore, we observed a higher expression of BMPRIA and PPR, which regulate bone formation and HSC niche function (Calvi et al., 2003; Zhang et al., 2003), in CAR cells than in other cell populations (Figure S6B). To address the possibility that distinct adipogenic and osteogenic progenitors are included in the CAR cell population, we analyzed the expression of adipogenic and osteogenic genes in CAR cells at the single-cell level. Single-cell RT-PCR analysis revealed that most or all individual cells within the CAR cell population expressed PPAR γ , Runx2, and Osterix in bone marrow from CXCL12-GFP mice, suggesting that most CAR cells are adipo-osteogenic bipotential progenitors (Figure 6B). Consistent with this, immunohistochemical analysis of bone marrow revealed that most CAR cells expressed PPAR γ protein (Figure 6C). In addition, Osterix protein expression was observed in many CAR cells as well as bone-lining osteoblasts in 3-week-old growing CXCL12-GFP mice (Figure 6D and data not shown).

Individual CAR Cells Have Potential to Differentiate into Adipocytic and Osteoblastic Cells

We analyzed the ability of CAR cells to differentiate into adipocytic and osteoblastic cells in vitro. When CAR cells were sorted, most of these cells died immediately in culture. Thus, we cultured whole bone marrow cells from CXCL12-GFP mice in medium containing a PPAR γ activator, pioglitazone. In the cultures, most CXCL12-GFP⁺ CAR cells appeared to remain viable and many CAR cells (63%) differentiated into multivacuolar lipid-containing preadipocytes, as assessed by Nile Red staining (Figures 7A and 7B), suggesting that the majority of CAR cells have the potential to differentiate into adipocytic cells in vitro. Subsequently, we cultured whole bone marrow cells from CXCL12-GFP mice in the presence of BMP-2 and analyzed the expression of an osteoblast differentiation-related marker, ALP, in individual CAR cells. In the cultures, most CAR cells appeared to remain viable, and the majority of CAR cells (90%) exhibited high ALP activity (Figure 7C and Figure S7). Because histological analysis of bone marrow has shown that only bone-lining mature osteoblasts exhibited high ALP activity (Figure 1C), these results suggest that most CAR cells have potential to differentiate into mature osteoblasts.

Next, we analyzed the ability of CAR cells to differentiate into adipocytic cells in vivo. It has been shown previously that multivacuolar lipid-containing precursors of adipocytes appear in the bone marrow of humans and mice in the early aplastic phase following chemotherapy (Bianco et al., 1988). We injected CXCL12-GFP mice with 250 mg/kg of 5-fluorouracil (5-FU), which preferentially eliminates proliferating cells. Multivacuolar lipid-containing CAR cells, as assessed by Nile Red staining, were observed in the bone marrow 7 days after 5-FU injection (Figure 7D). Some of these multivacuolar lipid-containing CAR cells surrounded sinusoidal endothelial cells, and others did

Osterix (yellow). In (C) and (D), the nuclei of cells were labeled with DAPI dye (blue); S, vascular sinus.

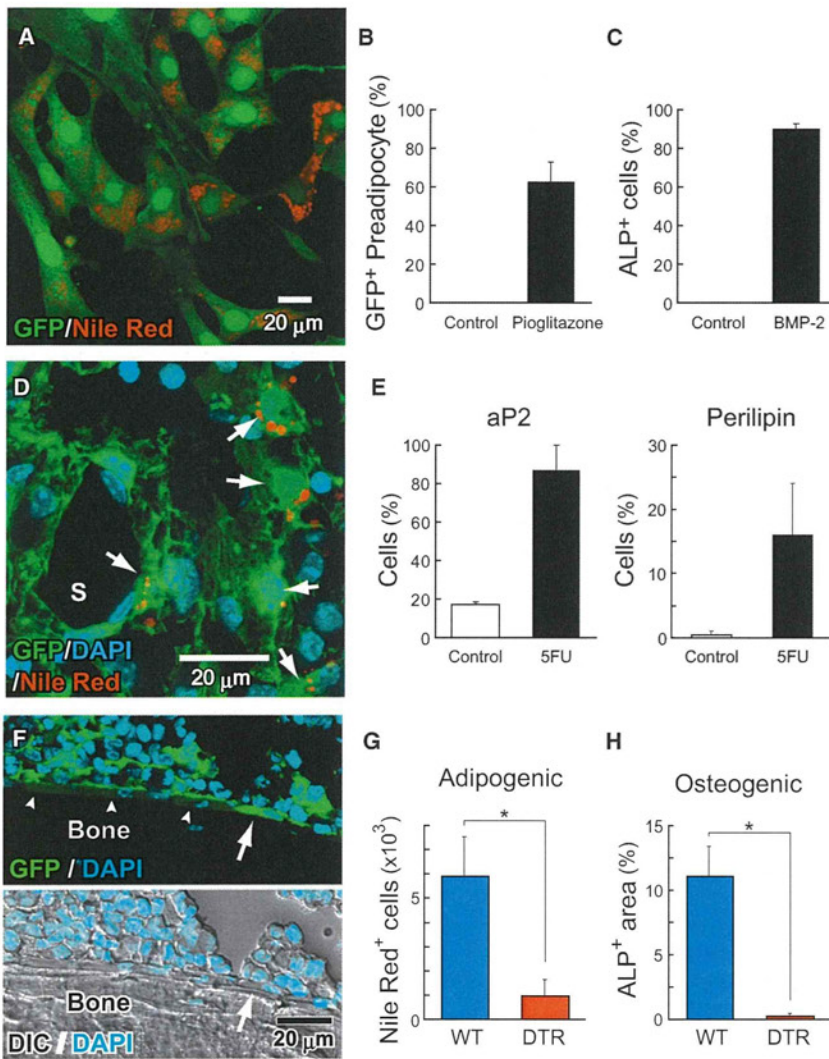


Figure 7. CAR Cells Have the Potential to Differentiate into Adipocytic and Osteoblastic Cells

(A-C) Bone marrow cells from CXCL12-GFP mice were cultured with pioglitazone (A and B) or BMP-2 (C). (A and B) After 7 days, cultures were analyzed by Nile Red staining. (A) Lipid inclusions (red) within the cytoplasm of CAR cells (green). (B) Frequency of lipid-containing cells in the whole CAR cells. (C) After 4 days, ALP activity was assayed by Fast Red staining. Frequency of ALP activity-containing cells in whole CAR cells. (D and E) CXCL12-GFP mice were analyzed 7 days after administration of 5-FU or control vehicle. (D) Bone marrow sections stained with Nile Red. Lipid inclusions (arrows; red) within the cytoplasm of some CAR cells (green). (E) Single-cell RT-PCR analysis of the frequency of cells expressing aP2 (left) and perilipin (right) in sorted CAR cells. (F) The bone marrow sections from 3-week-old growing CXCL12-GFP mice. Some CAR cells (green) are in contact with (arrowheads) or inserted into (arrow) the layer of mature osteoblasts lining the bone surface. (G and H) Whole bone marrow cells from wild-type and CXCL12-DTR-GFP mice at 2 days after DT administration were cultured with pioglitazone (G) or BMP-2 (H). (G) After 7 days, cultures were analyzed by Nile Red staining. Numbers of lipid-containing cells are shown. (H) After 4 days, ALP activity was assayed by Fast Red staining. The percentage of the ALP-positive area relative to the culture area is shown. All error bars represent SD of the mean; n = 4; *p < 0.05 (B, C, E, G and H). The nuclei of cells were labeled with DAPI dye (blue) (D and F).

not (Figure 7D). Additionally, single-cell RT-PCR analysis revealed that the frequencies of cells expressing aP2, an adipocyte-specific fatty acid binding protein, and perilipin, a protein coating lipid droplets of adipocytes, which is essential for adipogenesis (Martinez-Botas et al., 2000) within CAR cells, were elevated from 17% and 0.49% in untreated mice to 87% and 16% in mice 7 days after 5-FU injection, respectively (Figure 7E), suggesting that some CAR cells differentiated into adipocytes in vivo after 5-FU-induced bone marrow hypoplasia.

In addition, immunohistochemical analysis of 3-week-old growing CXCL12-GFP mice has shown that some CAR cells are in contact with or inserted into the layer of CXCL12-GFP⁻ mature osteoblasts lining the bone surface, raising the possibility that CAR cells near the bone surface differentiate into mature osteoblasts in vivo (Figure 7F). Together, these results suggest that the majority of CAR cells have the potential to differentiate into adipocytic and osteoblastic cells, and the differentiation of some CAR cells occurs in vivo.

Finally, we analyzed the ability of CAR cell-depleted bone marrow cells to differentiate into adipocytic and osteoblastic

cells. We cultured whole bone marrow cells from DT-treated CXCL12-DTR-GFP mice and control animals in medium containing pioglitazone or BMP-2. The numbers of lipid-containing adipocytic cells or marked ALP activity-containing osteoblastic cells were severely reduced in cultures from CAR cell-depleted bone marrow cells compared with cultures from control bone marrow cells, as assessed by Nile Red or Fast Red staining, respectively (Figures 7G and 7H and data not shown). These results support the idea that CAR cells are progenitors of adipocytes and osteoblasts within bone marrow.

DISCUSSION

In this study, we determined the in vivo essential role of CAR cells as the niche for HSCs and lymphoid and erythroid progenitors, using DT-treated CXCL12-DTR-GFP mice in which CAR cells were selectively lost but other candidate niches, including bone-lining osteoblasts, SNO cells, and endothelial cells, were still present. Together with the significant mRNA expression of Col1α1 in CAR cells, there is a possibility that CAR cells were decreased in the Col2.3ΔTK mice depleted of cells with the rat Col1α1 gene promoter driving thymidine kinase, in which B cells and erythrocytes were reduced (Visnjic et al., 2004). In addition,

because CAR cells exhibited high expression of BMPRIA and PPR, which have been reported to control HSC numbers, BMPRIA and/or PPR might regulate niche functions of CAR cells.

HSCs remained viable much longer than lymphoid and erythroid progenitors during cultures in serum alone and display similar phenotypes to HSCs from CAR cell-depleted mice. Considering that serum contains widely diffusible signals present in the circulation and marrow non-niche microenvironment, these results suggest that CAR cells are the major producer of specific niche signals for HSCs and hematopoietic progenitors in bone marrow. Because our results indicate that CAR cells are the major producer of SCF and CXCL12, which are essential for the maintenance of HSCs and lymphoid and erythroid progenitors, SCF and CXCL12 would contribute in concert with other factors to the niche functions of CAR cells.

Recent studies using label-retaining assays have identified a highly dormant small subpopulation (~30%) of HSCs, termed dormant HSCs (d-HSCs), dividing about every 145 days, and another rarely dividing activated HSC (a-HSCs) subpopulation (~70%) dividing around every 36 days, within the CD34⁺Lin⁻Sca-1⁺c-kit⁺CD150⁺CD48⁻ HSC population of bone marrow (Wilson et al., 2008). Considering that HSCs were reduced and more quiescent in the absence of CAR cells, this raises the possibility that a-HSCs were selectively lost or returned to a d-HSC status in CAR cell-depleted mice. Further studies will be needed to determine the role of CAR cells in the maintenance of d-HSCs and a-HSCs.

Our findings suggest that CAR cells maintain both HSCs and B cell and erythroid progenitors in a proliferative state; however, HSCs are cycling slowly, although B cell and erythroid progenitors are cycling much more rapidly. How can we explain the difference? Infrequent division of HSCs may be controlled by HSC-intrinsic mechanisms. An alternative explanation is that CAR cells or other microenvironmental components located near CAR cells, including endothelial cells, provide signals that slow the proliferation of HSCs, but not lymphoid or erythroid progenitors. Candidates include Angiopoietin-1, Wnts, and TGF- β s, which have been reported to slow the proliferation of HSCs (Arai et al., 2004; Fleming et al., 2008; Yamazaki et al., 2009). In contrast, critical lineage-specific soluble factors, including IL-7 and erythropoietin, augment the proliferation of lymphoid and erythroid progenitors, respectively, but not HSCs.

Although niche signals maintain stem cells in an undifferentiated state in *Drosophila* gonads (Xie and Spradling, 1998), it remains unclear whether vertebrate HSC niches employ an analogous mechanism. Our findings that HSCs from CAR cell-depleted mice have a markedly increased mRNA expression of PU.1 and generate substantial numbers of myeloid lineage cells in culture earlier than the wild-type suggest that CAR cells keep HSCs in an undifferentiated state. It may be that myeloid differentiation of HSCs is a default pathway in the absence of niche signals that maintain HSCs in a proliferative state. Alternatively, CAR cells may provide signals which inhibit the myeloid differentiation of HSCs controlled by non-CAR cell microenvironmental factors and/or HSC-intrinsic mechanisms.

At 2 days after the induction of CAR cell ablation, the numbers of HSCs were reduced about 50% in the marrow. Because it has been reported that only about 4% of HSCs are proliferating at

any given time (Kiel et al., 2005) and that HSCs divide every 36 or 145 days (Wilson et al., 2008), HSC reduction over 2 days in CAR cell-depleted mice cannot be explained simply by the results that CAR cells maintain HSCs in a proliferative state. It is likely that enhanced differentiation of some HSCs caused HSC reduction in CAR cell-depleted mice. In addition, although no increase in Annexin-V-positive apoptotic cells in the HSC fraction and the accumulation of HSCs in the circulation was observed in CAR cell-depleted mice, there is a possibility that CAR cells are also involved in supporting the survival and/or retention of HSCs within bone marrow.

CXCL12-DTR-GFP mice died probably from liver failure 5 days after DT treatment and it should also be noted that thrombopoietin (TPO), which plays a critical role in hematopoiesis is predominantly expressed in liver (Lok et al., 1994). However, at 2 days after DT treatment, the liver of CXCL12-DTR-GFP mice in which bone marrow hematopoiesis was affected appeared normal macroscopically and histologically, and had normal expression of TPO mRNA by qRT-PCR analysis (data not shown). In addition, there was no reduction of TPO protein in peripheral blood of the DT-treated CXCL12-DTR-GFP mice (data not shown). These results suggest that the liver of CXCL12-DTR-GFP mice did not cause the hematopoietic abnormalities 2 days after DT treatment. On the other hand, in the bone marrow of DT-treated CXCL12-DTR-GFP mice in which the numbers of CAR cells were reduced, the size of HSCs in contact with the remaining CAR cells was normal, although the size of HSCs which did not adjoin CAR cells was significantly reduced by histological analysis. Together, it is likely that phenotypes observed in the marrow of CXCL12-DTR-GFP mice were because of loss of CAR cells 2 days after DT treatment.

Our results do not rule out the possibility that other candidate niches, including SNO cells and endothelial cells, play an important role in hematopoiesis. The results that the numbers of MPPs, CCR9⁺MPPs, and CD122⁺NK1.1⁻ NK progenitors were normal, although CLPs and CCR9⁺CLPs (Zlotoff et al., 2010) were reduced in DT-treated CXCL12-DTR-GFP mice (Figure S2C), suggest that progenitors for distinct lymphoid lineages may inhabit different niches and have different requirements in bone marrow. Because many CAR cells are perivascular, interaction between CAR cells and endothelial cells may be important in the construction of HSC niches. Experiments using mice that allow the selective depletion of SNO cells or sinusoidal endothelial cells will be needed to determine the niche functions of these cells.

We have suggested that CAR cells are adipo-osteogenic progenitors, which produce large amounts of hematopoietic cytokines, CXCL12 and SCF, before they differentiate into mature cells producing large amounts of proteins for the storage of nutritional energy or bone formation. Previous studies have shown that adventitial reticular cells covering the endothelium of vascular sinuses give rise to adipocytes (Bianco et al., 1988) and that osteogenic progenitors are present in the intertrabecular space of bone marrow (Rouleau et al., 1990). Our results explain this discrepancy and suggest an essential *in vivo* role of adipo-osteogenic progenitors in bone marrow. In addition, our findings raise the possibility that differentiation of CAR cells into adipocytic and/or osteoblastic cells alters their niche functions. Together, our findings provide a basis for understanding

how microenvironmental niches regulate HSC maintenance and lympho-hematopoiesis within bone marrow.

EXPERIMENTAL PROCEDURES

Mice

CXCL12-GFP mice (Ara et al., 2003) and CXCL12-DTR-GFP mice were maintained on a C57BL/6 background. 5-FU (Kyowa Hakkō Kirin) was injected intraperitoneally into 12- to 15-week-old mice. Imatinib (Glivec; Novartis) was applied by oral gavage (twice a day, 200 mg/kg/day). All experiments were performed under guidelines of the animal ethics committee of Institute for Frontier Medical Sciences, Kyoto University.

Generation of CXCL12-DTR-GFP Mice

The targeting vector was generated as shown in Figure 1A. The neo cassette was removed from the recombinant allele by Cre-recombinase *in vivo*. CXCL12-DTR-GFP mice were backcrossed to C57BL/6 mice six times. DT (MBL) was injected intraperitoneally into 12- to 15-week-old mice.

Antibodies

The antibodies used were described in Supplemental Experimental Procedures.

Flow-Cytometric Analysis and Cell Sorting

Cell-cycle analysis was performed as described in our previous publication (Sugiyama et al., 2006). For the apoptosis assay, Annexin V-FITC Apoptosis Detection Kit I (BD Biosystems) was used according to the manufacturer's instruction. All flow-cytometric experiments and cell sorting were performed using a BD FACSAria (BD Biosystems). For sorting the nonhematopoietic cell populations, cells in bone marrow fraction were obtained from femurs and tibiae by flushing and collagenase (Sigma) digestion, and cells in bone fraction were obtained from marrow-depleted bones by mechanical disruption and collagenase digestion (Mayack and Wagers, 2008).

Competitive Repopulation Assay

Unfractionated 1/20 of bone marrow cells from two femurs and tibiae (C57BL/6-Ly5.2⁺ background) to be tested were mixed with 1×10^6 bone marrow cells of C57BL/6-Ly5.1⁺ mice as competitor cells and were transplanted into lethally irradiated (9 Gy) C57BL/6-Ly5.1⁺ mice. Peripheral blood cells of the recipient mice were taken 12 weeks after transplantation, and myeloid cells were analyzed by flow cytometer because their high turnover provides a good measure of HSC activity. Repopulating Units (RU) were calculated using Harrison's formula as described previously (Harrison et al., 1993).

Cell Culture and Analysis

For stromal cell-free culture, cells were sorted from wild-type mice and cultured in RPMI-1640 medium (Invitrogen) or S clone SF-3 medium (Sanko Junyaku) containing 10% fetal calf serum (FCS) with or without 20 ng/ml SCF (R&D Systems) or 1 μ g/ml CXCL12. Cultured PI⁻ viable cells were re-sorted and analyzed.

For adipogenic differentiation, bone marrow cell suspensions were cultured on a collagen-coated dish in DMEM supplemented with pioglitazone (Takeda Pharmaceutical) (10 μ M), 10% FCS, and 5×10^{-5} M 2- β -mercaptoethanol for 7 days. To visualize lipid droplets, PFA fixation and Nile Red staining were performed. For osteogenic differentiation, bone marrow cell suspensions were cultured in α -MEM supplemented with BMP-2 (R&D Systems) (300 ng/ml), 10% FCS, and 5×10^{-5} M 2- β -mercaptoethanol for 4 days. Osteoblastic phenotypes were evaluated by the activity of ALP using Fast Red staining.

Immunohistochemical Analysis

Bone marrow sections were analyzed by immunofluorescence as described previously (Sugiyama et al., 2006). To visualize lipid droplets, Nile Red staining was performed. Confocal microscopy was performed with a LSM 510 META (Carl Zeiss).

QRT-PCR Analysis

Total RNA was isolated from sorted cells using Isogen (Nippon Gene) and treated with DNase I (Invitrogen). cDNA was synthesized using SuperScript

VIL0 (Invitrogen) following the manufacturer's instructions. QRT-PCR analysis was performed with a Step One Plus (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems). Values for each gene were normalized to the relative quantity of *Gapdh* mRNA in each sample. The primers used for PCR are listed in Supplemental Experimental Procedures.

Single-Cell RT-PCR Analysis

Single cells were sorted into reverse transcription buffer containing 1 U/ μ l RNase Inhibitor (Toyobo) and 0.4% Nonidet P-40 (Nacalai Tesque) using flow cytometry. Cell lysates were reverse-transcribed using M-MLV reverse transcriptase (Toyobo) and gene-specific reverse primers. PCR was performed by the addition of premixed hot-start PCR enzymes and buffers (AmpliQ Gold; Applied Biosystems) containing the gene-specific forward and reverse primers designed to span introns to exclude genomic products. PCR was carried out in one round with 50 amplification cycles. The primers used for PCR are listed in Supplemental Experimental Procedures.

ELISA

Femurs were flushed with 0.3 ml of PBS containing a protease inhibitor cocktail (Sigma) and Nonidet P40 (1%). Cells were lysed by freeze and thaw, and the cell debris was removed by centrifugation and discarded. The concentrations of SCF and CXCL12 were measured using DuoSet ELISA Development kits (R&D Systems) according to the manufacturer's instructions.

Statistical Analysis

The significance of the difference between groups in the experiments was evaluated by analysis of variance followed by a two-tailed Student's *t* test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.immuni.2010.08.017.

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The Peptidomimetic CXCR4 Antagonist TC14012 Recruits β -Arrestin to CXCR7

ROLES OF RECEPTOR DOMAINS^{*§}

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CXCR7 is an atypical chemokine receptor that signals through β -arrestin in response to agonists without detectable activation of heterotrimeric G-proteins. Its cognate chemokine ligand CXCL12 also binds CXCR4, a chemokine receptor of considerable clinical interest. Here we report that TC14012, a peptidomimetic inverse agonist of CXCR4, is an agonist on CXCR7. The potency of β -arrestin recruitment to CXCR7 by TC14012 is much higher than that of the previously reported CXCR4 antagonist AMD3100 and differs only by one log from that of the natural ligand CXCL12 (EC₅₀ 350 nM for TC14012, as compared with 30 nM for CXCL12 and 140 μ M for AMD3100). Moreover, like CXCL12, TC14012 leads to Erk 1/2 activation in U373 glioma cells that express only CXCR7, but not CXCR4. Given that with TC14012 and AMD3100 two structurally unrelated CXCR4 antagonists turn out to be agonists on CXCR7, this likely reflects differences in the activation mechanism of the arrestin pathway by both receptors. To identify the receptor domain responsible for these opposed effects, we investigated CXCR4 and CXCR7 C terminus-swapping chimeras. Using quantitative bioluminescence resonance energy transfer, we find that the CXCR7 receptor core formed by the seven-transmembrane domains and the connecting loops determines the agonistic activity of both TC14012 and

AMD3100. Moreover, we find that the CXCR7 chimera bearing the CXCR4 C-terminal constitutively associates with arrestin in the absence of ligands. Our data suggest that the CXCR4 and CXCR7 cores share ligand-binding surfaces for the binding of the synthetic ligands, indicating that CXCR4 inhibitors should be tested also on CXCR7.

CXCR4 is a seven-transmembrane domain (7TMR)⁴ chemokine receptor of considerable clinical interest, involved in stem cell homing to bone marrow niches, cancer biology and metastasis, and HIV infection. Synthetic CXCR4 ligands are being developed, and the CXCR4 blocker AMD3100 has reached the clinic for the mobilization of hematopoietic stem cells from donor bone marrow. The natural CXCR4 ligand, the chemokine CXCL12 (also called SDF-1), has later been found to also bind the atypical chemokine receptor CXCR7, which in addition recognizes the chemokine CXCL11 (also called I-TAC) (1, 2). CXCR7 is atypical in that no classical heterotrimeric G-protein signaling pathways are observed and in that it does not induce chemotaxis of motile cells. However, CXCR7 sets off G-protein-independent signaling via the β -arrestin pathway, leading to activation of the Erk 1/2 kinases (3, 4). Functionally, CXCR7 has been implicated in cancer cell growth and transendothelial migration (2, 5, 6). Intriguingly, CXCR7 and CXCR4 can heteromerize, and this attenuates CXCR4 G-protein signaling (7, 8). Modulation of CXCR4 has been suggested as a major role of CXCR7 (9).

Given that CXCR4 and CXCR7 share a chemokine ligand, we have previously tested the effects of AMD3100 on CXCR7 and found that it induces arrestin recruitment at high doses on this receptor, contrary to its effect on CXCR4 (3). This finding prompted us to investigate another, structurally unrelated CXCR4 inhibitor. T140 is a horseshoe crab polyphemusin-derived peptidomimetic described as an inverse agonist of CXCR4 (10–12). Here we report that TC14012, a serum-stable derivative of T140 (13), recruits β -arrestin 2 to CXCR7. Using C terminus-swapping mutants of CXCR4 and CXCR7, we demonstrate that the CXCR7 core receptor, formed by the transmembrane helices and the connecting loops, but not the CXCR7 C terminus, is the determinant for the agonist activity of structurally unrelated synthetic CXCR4 blockers on the arrestin pathway mediated by CXCR7.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant CXCL12 was from PeproTech, AMD3100 was from Sigma, and [¹²⁵I]-CXCL12 was from PerkinElmer Life Sciences. TC14012 was synthesized as described (13).

Cell Culture and Transfections—Human embryonic kidney (HEK) 293E cells were cultured in Dulbecco's modified Eagle's

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⁴ The abbreviations used are: 7TMR, seven-transmembrane domain receptor; BRET, bioluminescence resonance energy transfer; Rluc, *renilla* luciferase.