Spleen serves as a reservoir of osteoclast precursors through vitamin D-induced IL-34 expression in osteopetrotic op/op mice

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Osteoclasts are generated from monocyte/macrophage-lineage precursors in response to colony-stimulating factor 1 (CSF-1) and receptor activator of nuclear factor-κB ligand (RANKL). CSF-1–mutated CSF-1\(^{-/}\)op mouse as well as RANKL\(^{-/}\) mice exhibit osteopetrosis (OP) caused by osteoclast deficiency. We previously identified RANKL receptor (RANK)/CSF-1 receptor (CSF-1R) double-positive cells as osteoclast precursors (OCPs), which existed in bone in RANKL\(^{-/}\) mice. Here we show that OCPs do not exist in bone but in spleen in CSF-1\(^{-/}\)op mice, and spleen acts as their reservoir. IL-34, a newly discovered CSF-1R ligand, was highly expressed in vascular endothelial cells in spleen in CSF-1\(^{-/}\)op mice. Vascular endothelial cells also expressed IL-34, but its expression level was much lower than in spleen, suggesting a role of IL-34 in the splenic generation of OCPs. Splenectomy (SPX) blocked CSF-1 induced osteoclastogenesis in spleen. However, IL-34 was expressed under the control of the CSF-1 promoter in CSF-1\(^{-/}\)op mice, suggesting the existence of cell-cycle–arrested RANK/CSF-1R double-positive cells as the direct OCPs in spleen (18). When RANKL was administered to RANKL\(^{-/}\) mice and CSF-1 to CSF-1\(^{-/}\)op mice, OCPs similarly differentiated into osteoclasts in bone tissue without cell cycle progression. OCPs were detected in the vicinity of osteoclastic cells in RANKL\(^{-/}\) mice, suggesting the existence of OCPs in bone in WT mice. However, our preliminary experiments showed that OCPs were not present in bone in CSF-1\(^{-/}\)op mice.

The active form of vitamin D\(_2\) [1α,25(OH)\(_2\)D\(_2\)] regulates calcium homeostasis by acting on various types of cells such as intestinal endothelial cells, renal tubular cells, and osteoblastic cells (19). Shevde et al. (20) reported that 2-methylene-19-nor(20)-1α,25(OH)\(_2\)D\(_3\) (2MD), a highly potent analog of 1α,25(OH)\(_2\)D\(_3\), strongly enhanced osteoclastic cell-mediated osteoclast formation and also induced bone formation in vitro and in vivo. We have synthesized a derivative of 2MD at carbon 2 (2α-methyl-19-nor(20)-1α,2β,25(OH)\(_2\)D\(_3\), 2-methyl-2MD) and showed that osteoclastic bone resorption is indispensable for the hypercalcemic action of the 2MD analog in vivo (21). Recently, increasing evidence has been accumulating in showing that 1α,25(OH)\(_2\)D\(_3\) directly regulates activities of vascular endothelial cells

platelet endothelial cell adhesion molecule 1-positive cells | osteoblasts | blood stream

Osteoclasts are bone-resorbing cells generated from monocyte/macrophage-lineage precursors. The differentiation of osteoclast precursors (OCPs) into osteoclasts is regulated by bone-forming osteoblasts. Osteoblastic cells express two cytokines responsible for osteoclastogenesis: one is colony-stimulating factor 1 (CSF-1, also called macrophage colony-stimulating factor [M-CSF]) and the other is receptor activator of nuclear factor-κB ligand (RANKL). OCPs express CSF-1 receptor (CSF-1R, also called c-Fms) and RANK receptor (RANKL) and differentiate into osteoclasts in response to CSF-1 and RANKL. The expression of RANKL is up-regulated by osteoclast-inhibiting factors such as parathyroid hormone (PTH) and 1α,25-dihydroxyvitamin D\(_3\) [1α,25(OH)\(_2\)D\(_3\)] (1, 2).

CSF-1 is the most potent growth factor for monocytes/macrophages (3), but its synthesis by osteoblasts occurs independently of PTH and 1α,25(OH)\(_2\)D\(_3\) (4). CSF-1–mutated mice cannot produce a functionally active CSF-1 (4), and therefore, exhibit monocytopenia and osteopetrosis (OP) (5, 6). However, several curious phenomena have been observed in CSF-1\(^{-/}\)op mice. First, osteoclasts are totally absent in young CSF-1\(^{-/}\)op mice, but appear in aged CSF-1\(^{-/}\)op mice (7). Second, osteopetrotic characteristics of CSF-1R\(^{-/}\) mice are more severe than those of CSF-1\(^{-/}\)op mice (8). Third, F4/80\(^+\) [F4/80\(^+\) IL-34] macrophages exist in the spleen of CSF-1\(^{-/}\)op mice as well as in WT mice, and their number is regulated by a mechanism independently of CSF-1 (9, 10). Fourth, the administration of vascular endothelial growth factor (VEGF) rescues osteopetrosis in CSF-1\(^{-/}\)op mice (11, 12), but VEGF cannot substitute for CSF-1 to induce osteoclast formation in vitro (13).

Recently, Lin et al. (14) discovered IL-34, as a new ligand for CSF-1R. The amino acid sequence of IL-34 was quite different from that of CSF-1, but IL-34 promoted macrophage colony formation like CSF-1 did. IL-34 was specifically expressed in splenic tissues, predominantly in the red pulp region. When IL-34 was expressed under the control of the CSF-1 promoter in CSF-1\(^{-/}\)op mice, the osteopetrotic phenotype was rescued (15). IL-34 in combination with RANKL induced osteoclastic differentiation of progenitor cells in mouse (16, 17) and human (17) cell culture systems. However, it remains unclear why IL-34 cannot substitute for CSF-1 in CSF-1\(^{-/}\)op mice in vivo.

Using RANKL\(^{-/}\) mice and CSF-1\(^{-/}\)op mice, we identified cell-cycle–arrested RANK/CSF-1R double-positive [RANK(+)CSF-1R(−)] cells as the direct OCPs in vivo (18). When RANKL was administered to RANKL\(^{-/}\) mice and CSF-1 to CSF-1\(^{-/}\)op mice, OCPs similarly differentiated into osteoclasts in bone tissue without cell cycle progression. OCPs were detected in the vicinity of osteoclastic cells in RANKL\(^{-/}\) mice, suggesting the existence of OCPs in bone in WT mice. However, our preliminary experiments showed that OCPs were not present in bone in CSF-1\(^{-/}\)op mice.

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through vitamin D receptors (22–24). These results suggest that 1α25(OH)2D3 plays a unique role in the vascular system beyond the classical role in calcium homeostasis.

In the present study, we examined how osteoclasts were formed in CSF-1op/op mice in response to various stimuli. We found that OCPs existed in spleen but not in bone in CSF-1op/op mice. OCPs in CSF-1op/op mice were transferred from spleen to bone and differentiated into osteoclasts in response to CSF-1, VEGF, and 2MD administrations, and also to aging. IL-34 appeared to play a pivotal role in the generation and storage of OCPs in spleen and osteoclastogenesis in CSF-1op/op mice. In addition, we have shown that the IL-34 gene in the vascular endothelial cells is a unique target of vitamin D.

**Results**

Immunohistochemical analysis showed that RANK(+) cells and CSF-1R(+) cells were present in the proximal region of tibiae obtained from RANKL−/− mice as well as from WT mice (Fig. 1A). Most of the RANK(+) cells expressed CSF-1R in WT mice. Cells double positive for RANK and CSF-1R [RANK(+)/CSF-1R(+) cells] were also detected in RANKL−/− mice and identified as the direct OCPs in vivo (18). Conversely, neither RANK(+) cells nor CSF-1R(+) cells were detected in bone tissue in CSF-1op/op mice (Fig. 1A, Right). In contrast, RANK(+) cells, CSF-1R(+) cells, and RANK(+)CSF-1R(+) cells (arrows) were detected in spleen in CSF-1op/op mice as well as in WT mice and RANKL−/− mice (Fig. 1B, Right). RANK(+)CSF-1R(+) cells were mainly observed in the red pulp region and marginal zones. These results suggest that OCPs do not exist in bone but do exist in spleen in CSF-1op/op mice.

We then examined the tissue distribution of IL-34 mRNA (Fig. 2A). Consistent with the previous report (14), IL-34 was expressed predominantly in spleen but slightly in bone (Fig. 2A). The osteoblast level of IL-34 mRNA was much lower compared with that of CSF-1 mRNA (Fig. 2B). Little IL-34 mRNA or CSF-1 mRNA was expressed in bone marrow (BM) macrophages and osteoclastic cells (Fig. 2B). IL-34(+) cells were mainly distributed in the splenic red pulp region and marginal zones (Fig. 2C), where RANK(+)CSF-1R(+) cells were detected (Fig. 1B). IL-34(+) cells were similarly distributed in the spleen in WT mice (Fig. S1 A and B). Most IL-34(+) cells expressed platelet endothelial cell adhesion molecule 1 (PECAM-1), a marker of vascular endothelial cells (Fig. 2 C–E and Fig. S1 A and B). Cells double positive for IL-34 and PECAM-1 [IL-34(+)PECAM-1(+) cells] were detected as endothelial cells in blood vessels. Most of the endothelial cells found in white pulp also expressed IL-34 (Fig. 2 C–E). Notably, endothelial cells in central arterioles robustly expressed IL-34 (Fig. 2D). Next, we examined the distribution of IL-34 expression in bone. PECAM-1(+) cells expressed IL-34 but alkaline phosphatase (ALP)(+) osteoblastic cells did not (Fig. 2F and Fig. S1C). The endothelial cell

![Fig. 1. Distribution of OCPs in bone and spleen in WT, RANKL−/−, and CSF-1op/op mice. (A) Localization of RANK (red) and CSF-1R (green) in proximal tibiae in WT, RANKL−/−, and CSF-1op/op mice. Nuclei were stained with DAPI (blue). Lower panels show magnified views of the boxed areas in the Upper panels. Dashed lines represent bone surface. Arrows indicate RANK(+)CSF1-1R(+) cells. (Scale bar, 400 μm (Upper), 5 μm (Lower)). (B) Localization of RANK (red) and CSF-1R (green) in spleen in WT, RANKL−/−, and CSF-1op/op mice. Lower panels show magnified views of the boxed areas in the Upper panels. WP, white pulp; RP, red pulp. (Scale bar, 400 μm (Upper), 10 μm (Lower)).](Image 123x183)

![Fig. 2. Distribution of IL-34 expression in WT and CSF-1op/op mice. (A) Real-time RT-PCR measurements of IL-34 mRNA expression in bone, liver, and spleen in 3-wk-old WT and CSF-1op/op mice. Data obtained from triplicate PCRs using RNA from different mice are expressed as the mean ± SD (n = 3). (B) Real-time RT-PCR measurements of IL-34 and CSF-1 mRNA expression in osteoblastic cells, BM macrophages, and osteoclasts. Data obtained from triplicate PCRs are expressed as the mean ± SD *P < 0.01. (C) Localization of IL-34 (red) and PECAM-1 (green) in spleen in CSF-1op/op mice. Dashed circles represent the white pulp. The outlines of the circles show the marginal zones and red pulp. (Scale bar, 100 μm.) (D) Magnified views of the boxed areas [a] red pulp and [b] white pulp] in C. Nuclei were stained with DAPI (blue). Arrows indicate IL-34(+)PECAM-1(+) cells. (Scale bar, 20 μm.) (E) Number of IL-34(+) cells and PECAM-1(+) cells in the red and white pulp. Values in red bars represent percentages of IL-34(+) cells among PECAM-1(+) cells. Data are expressed as the mean ± SD for four optical fields. (F) Localization of IL-34 (red) and PECAM-1 (green) (Left) and that of IL-34 (red) and ALP activity (green) (Right) in the proximal tibiae in CSF-1op/op mice. Dashed lines represent bone surface. Arrows indicate IL-34(+)PECAM-1(+) cells. Arrowheads indicate ALP(+) osteoblastic cells. (Scale bar, 20 μm.)](Image 123x183)
population was much lower in bone than in spleen, consistent with the result of real-time RT-PCR (Fig. 2A). These results suggest that IL-34 is involved in the generation of OCPs in spleen in CSF-1op/op mice.

Then, we examined the biological activities of IL-34 in several assays and found them to be similar to those of CSF-1 (Fig. S2). Consistent with the previous reports (14–17), IL-34 promoted not only the proliferation of BM macrophages (Fig. S2A), but also the formation of osteoclasts (Fig. S2B), both of which were similarly inhibited by adding aCSF-1R Ab. IL-34 as well as CSF-1 supported the survival of osteoclasts, which was similarly inhibited by adding aCSF-1R Ab (Fig. S2C). Thus, IL-34 is concluded to stimulate osteoclastogenesis through CSF-1R. These results suggest that splenic OCPs are transferred from spleen to bone in response to CSF-1/IL-34 administration in CSF-1op/op mice.

We next examined the effect of splenectomy (SPX) on osteoclast formation in CSF-1op/op mice (Fig. 3A). Three-week-old CSF-1op/op mice were subjected to SPX or a sham operation (Sham) and injected with CSF-1 4 d after the surgery. The CSF-1 injection produced tartrate-resistant acid phosphatase (TRAP, an osteoclast marker) (+) osteoclasts in bone in Sham CSF-1op/op mice, but not in SPX CSF-1op/op mice. RANKL−/− mice were also subjected to SPX or Sham and examined for osteoclastogenesis in response to CSF-1/IL-34 administration in CSF-1op/op mice. RANKL appeared to induce osteoclasts to form from RANK (+)/CSF-1R (+) cells preexisting in bone in RANKL−/− mice.

The administration of VEGF improved the phenotype of osteopetrosis in CSF-1op/op mice (11, 12), and a deficiency of VEGFRI1 worsened it (12). VEGF may stimulate the growth and IL-34 synthesis of vascular endothelial cells. Consistent with the previous reports (11, 12), the injection of VEGF-A120 (VEGF120) into CSF-1op/op mice increased the appearance of TRAP(+) osteoclasts (Fig. S3A). SPX prevented VEGF-A120-induced osteoclastogenesis in CSF-1op/op mice. However, the VEGF120 injection did not increase the expression of IL-34 mRNA in bone, liver, or spleen in CSF-1op/op mice (Fig. S3B). These results suggest that VEGF induces loosening of the endothelial cell contacts (25), and the subsequent entry of OCPs into the blood stream.

We next examined the possibilities of whether the spleen acts as a reservoir of OCPs in aged CSF-1op/op mice and whether IL-34 is involved in this process as well (Fig. 4). TRAP(+) osteoclasts were the exclusive localization of OCPs in bone in CSF-1op/op mice (Fig. 4A). The expression of IL-34 mRNA in bone and spleen but not in the liver increased with aging in CSF-1op/op mice (Fig. 4B). WT mice also exhibited an age-associated increase in IL-34 expression in bone. CSF-1 mRNA expression showed no correlation with aging in WT mice (Fig. 4B). Then, 5-wk-old CSF-1op/op mice were subjected to SPX or Sham. Five weeks later, tibiae were recovered and examined for osteoclastogenesis (Fig. 4C). Histomorphometric analysis of tibiae showed that SPX suppressed the age-associated appearance of osteoclasts, erosion surface, bone erosion volume (ES/BS), and increased bone volume (BV/TV) in aged CSF-1op/op mice (Fig. 4C). These results suggest that spleen acts as a reservoir of OCPs in the age-associated appearance of osteoclasts in CSF-1op/op mice.

We previously reported that a large amount of a 2MD analog, 2-methyl-2MD, induced hypercalcemia in WT mice, but not in c-Fos−/− mice (21). The potency of 2-methyl-2MD as well as 2MD in inducing osteoclastogenesis was 100 times higher than that of 1,25(OH)2D3 (21). In the course of investigating further, we found that administration of 2-methyl-2MD to CSF-1op/op mice induced hypercalcemia (Fig. S4). We, then, examined whether administration of the original 2MD induces osteoclastogenesis in CSF-1op/op mice. CSF-1op/op mice subjected to SPX or Sham were injected with a large amount of 2MD (Fig. 5A). The 2MD injection increased the appearance of osteoclasts in bone in Sham but not in SPX CSF-1op/op mice. 2MD increased erosion surface in parallel with the increase of osteoclast number in Sham but not in SPX CSF-1op/op mice (Fig. 5A). 2MD is known to induce RANKL expression in osteoclasts (20, 21). Then, RANKL was injected into CSF-1op/op mice, but neither the osteoclast formation nor the increase in erosion surface (ES/BS) was observed (Fig. 5A). The administration of 2MD stimulated the expression of IL-34 mRNA in spleen and bone in CSF-1op/op mice (Fig. 5B). The number of IL-34+ PECAM-1 (+) cells and that of RANKL(+)/CSF-1R (+) cells were also increased in response to 2MD administration (Fig. 5 C and D). We then performed siRNA-mediated knockdown of IL-34. Using fluorescence-labeled control siRNA, we confirmed that the siRNA was successfully delivered to spleen and bone (Fig. S5A). Then IL-34 siRNA or control siRNA was injected into CSF-1op/op mice 24 h before the administration of 2MD. The expression of IL-34 mRNA in spleen was reduced by up to 80% by adding IL-34 siRNA (Fig. S5B). IL-34 siRNA but not control siRNA suppressed the 2MD-induced osteoclastogenesis in CSF-1op/op mice (Fig. 5E). These results indicate that IL-34 is involved in the 2MD-induced mobilization of OCPs from spleen to bone in CSF-1op/op mice. We finally examined whether 2MD-induced up-regulation of IL-34 expression is mediated by the vitamin D receptor (VDR) using VDR−/− mice. Although comparable levels of IL-34 mRNA expression were detected in bone and spleen in VDR−/− mice, the IL-34 expression was not enhanced by 2MD administration (Fig. S5F).

**Discussion**

We have shown that spleen in CSF-1op/op mice acts as a reservoir of OCPs, which are transferred to bone and differentiate into osteoclasts in response to diverse stimuli (Fig. 6). The existence of OCPs in spleen seems to be supported by IL-34 expressed in vascular endothelial cells. The mysterious phenomena observed in CSF-1op/op mice (7–12) may be explained by the transfer of OCPs from spleen to bone.

The exclusive localization of OCPs in spleen was observed in CSF-1op/op mice but not in RANKL−/− mice. SPX prevented osteoclastogenesis in CSF-1op/op and RANKL−/− mice in CSF-1op/op mice. However, 2MD-induced osteoclastogenesis in normal mice was not impaired by SPX (Fig. S6). Therefore, it is unlikely that spleen acts as a reservoir of OCPs in osteoclastogenesis under the physiological condition in normal animals. However, 2MD administration increased the number of OCPs as
well as the expression of IL-34 in spleen in CSF-1\(^{op/op}\) mice. SPX suppressed the age-associated appearance of osteoclasts in CSF-1\(^{op/op}\) mice. These results suggest that OCPs are generated and maintained in spleen through IL-34 during the lifetime of CSF-1\(^{op/op}\) mice.

OCPs were released from spleen into the blood stream in CSF-1\(^{op/op}\) mice. Using an in vivo system of bone morphogenetic protein 2 (BMP-2)–induced ectopic bone formation in RANKL\textsuperscript{−/−} mice, we demonstrated that OCPs existed in the peripheral blood as well as in bone marrow (26). Circulating OCPs were cell-cycle–arrested cells committed to the osteoclast lineage. When CSF-1 was injected into CSF-1\(^{op/op}\) mice, osteoclasts detected in bone were generated from cell-cycle–arrested OCPs (18). Our findings also support the notion that the lineage-committed OCPs circulate in the blood stream and fix to the correct site for osteoclastogenesis. Ishii et al. (27) reported that an agonist of sphingosine 1 phosphate (S1P) increased the migration of OCPs between blood and bone. CSF-1 administration increased the mobilization of OCPs from spleen to bone, suggesting that CSF-1 as well as IL-34 plays important roles not only in the osteoclastic differentiation of OCPs in bone but also in the mobilization of OCPs from spleen to bone in CSF-1\(^{op/op}\) mice. Future studies will further clarify the mechanism by which OCPs are transferred from spleen into blood and home to bone.

The expression level of IL-34 was much lower in bone than in spleen. This may explain why OCPs and osteoclasts are absent in bone in young CSF-1\(^{op/op}\) mice. Osteoclasts appeared in aged CSF-1\(^{op/op}\) mice with concomitant up-regulation of IL-34 expression in bone. 2MD administration also enhanced IL-34 expression in bone. These results suggest that IL-34 generated by vascular endothelial cells contributes to osteoclastogenesis induced by aging and 2MD administration in CSF-1\(^{op/op}\) mice. VEGF also induced osteoclastogenesis in CSF-1\(^{op/op}\) mice. However, the ability of VEGF to induce osteoclastogenesis was much weaker than that of CSF-1 and 2MD, and VEGF failed to up-regulate IL-34 expression. These results suggest that IL-34 expressed in vascular endothelial cells in bone is essentially involved in osteoclastogenesis in CSF-1\(^{op/op}\) mice.

Administration of 2MD induced osteoclastogenesis with up-regulation of IL-34 expression in CSF-1\(^{op/op}\) mice. The RNA interference experiment further supported the notion that IL-34 is involved in the 2MD-induced osteoclastogenesis in CSF-1\(^{op/op}\) mice. When 2MD was administered to WT mice, the expression of IL-34 mRNA was significantly increased in WT mice (Fig. S7). The stimulatory effect of 2MD on IL-34 expression was not observed in VDR\textsuperscript{−/−} mice. Administration of a large amount of 1α,25(OH)\(_2\)D\(_3\) into CSF-1\(^{op/op}\) mice also induced osteoclastogenesis (Fig. S8). These findings suggest that the IL-34 gene is a unique target of 1α,25(OH)\(_2\)D\(_3\). Using the program Pattern Search for Transcription Factor Binding Sites (PATCH 1.0), we found five putative binding sites of VDR within the 2 kb upstream of the transcription start site of the mouse IL-34 gene. The expression level of IL-34 mRNA in bone and spleen in VDR\textsuperscript{−/−} mice was comparable to that in wild-type mice. These results suggest that VDR-mediated signals are not essential for IL-34 expression, but are involved in the up-regulation of IL-34 expression in endothelial cells.
We have not succeeded in showing the stimulation of IL-34 expression by 2MD in mouse splenic endothelial cell cultures. Vascular microenvironment or blood vascular networks may be required for 2MD-induced up-regulation of IL-34 expression. Vascular endothelial cells are shown to regulate the recruitment of monocytes/macrophage lineage cells through IL-34 expression (28-31). At the amino acid sequence level, IL-34 gene is more conserved than the CSF-1 gene during evolution (32). At the amino acid sequence level, IL-34 gene is more conserved than the CSF-1 gene during evolution (32). These results suggest that the vitamin D system and IL-34 may fundamentally work in normal angiogenesis.

Recently, it was reported that IL-34 was expressed in synovial tissues obtained from rheumatoid arthritis patients, and tumor necrosis factor α (TNFα) stimulated IL-34 expression in those synovial cell cultures (33, 34). Giant cell tumors of bone have been shown to express IL-34 (17). Therefore, we examined the effects of osteotropic factors such as 1α,25(OH)2D3, 2MD, TNFα, interleukin 1β (IL-1β) and prostaglandin E2 (PGE2) on IL-34 expression in mouse osteoblastic cells in culture (Fig. S9). Real-time RT-PCR analysis showed that TNFα, IL-1β, and PGE2, respectively, failed to increase IL-34 mRNA expression in osteoblastic cell lines that were used in this study (Fig. S9). Real-time RT-PCR analysis showed that TNFα, IL-1β, and PGE2, respectively, failed to increase IL-34 mRNA expression in osteoblastic cells in our cell culture conditions. 2MD and 1α,25(OH)2D3 significantly increased IL-34 mRNA expression in osteoblastic cells. However, it is unlikely that IL-34 expressed by osteoblastic cells in response to 1α,25(OH)2D3 can substitute for CSF-1 for osteoclastogenesis, because 1α,25(OH)2D3 failed to support osteoclast formation in cocultures of hematopoietic osteoclast precursors and CSF-1op/op mouse-derived osteoclastic cells (35). At present, the cause of the difference between our result and the results reported previously is not known. Further studies will elucidate the regulatory mechanism of IL-34 expression in fibroblastic cells as well as endothelial cells.

Both CSF-1op/op and RANKLop/op osteopetrotic mice develop splenic extramedullary hematopoiesis due to the impaired bone marrow microenvironment. This suggests that spleen acts as the reservoir of hematopoietic precursors under pathological conditions such as osteopetrosis. Recently, Miyamoto et al. (36) reported that the mobilization of hematopoietic stem and progenitor cells (HSPCs) occurring after granulocyte colony-stimulating factor (G-CSF) injection was comparable or even increased in osteopetrotic CSF-1op/op, RANKLop/op and c-Fosop/op mice, compared with that in WT mice. Contrary to OCPs, the mobilization of HSPCs was not suppressed by SPX in CSF-1op/op mice in the G-CSF treatment (36). These results suggest that spleen cannot act as the reservoir of HSPCs in CSF-1op/op mice, although HSPCs exist in the enlarged spleen. Swirski et al. (37) identified unique monocytes in spleen, which exited the spleen en masse, accumulated in the injured tissue, and participated in wound healing, in response to ischemic myocardial injury. CSF-1 has been shown to be involved in the coordinated dynamics of the tissue distribution of macrophages and dendritic cells (38). Thus, spleen plays important roles in the maintenance and tissue distribution of monocyte–macrophage lineage cells through IL-34 expression.

In conclusion, IL-34 plays pivotal roles in the maintenance and mobilization of splenic OCPs in CSF-1op/op mice. IL-34 and CSF-1 play dominant roles in determining the distribution of OCPs. The IL-34 gene in vascular endothelial cells is a unique target of macrophage lineage cells through IL-34 expression.
vitamin D. Clarifying how splenic OCPs enter the blood stream and reach bone may provide a unique strategy to control bone resorption.


Materials and Methods

Detailed protocols are given in SI Materials and Methods.

Animals. Breeding pairs of CSF-1<sup>−/−</sup> mice (B6C3Fe genetic background) were purchased from The Jackson Laboratory and F<sub>2</sub> mice were raised in our laboratory. Homozygous CSF-1<sup>−/−</sup> mice, identified by a lack of incisors at postnatal day 10, and RANKL<sup>−/−</sup> mice (C57BL/6 background) were fed a softened rodent Chow (Oriental Yeast) with water after weaning. VDR<sup>−/−</sup> mice (C57Bl/6 background) were generated by cross-breeding of VDR-floxed mice with CMV-Cre mice (40). VDR<sup>−/−</sup> mice were fed a high calcium diet (CE-2 supplemented with 2% (w/vt) calcium, 1.25% (w/vt) phosphorus, and 20% (w/vt) lactose; CLEA Japan) to normalize serum calcium level. Eight-week-old male df<sup>−/−</sup> mice and newborn df<sup>−/−</sup> mice (Japan SLG) were used as WT mice for determination of BM cells and osteoclastic cells, respectively. All experiments were conducted in accordance with the guidelines for studies with laboratory animals of the Matsumoto Dental University Experimental Animal Committee.

Splenectomy. Mice were anesthetized with isoflurane (Isofu; Dainippon Sumitomo Pharma) using a vaporizer (DS Pharma Biomedical). The spleen was identified after a transverse laparotomy incision just to the left of the spinal cord and removed after appropriate blood vessel ligation. Sham-operated animals underwent the laparotomy without a splenectomy.

Statistics. Statistical analyses were performed using the one-tailed Student t test and Fisher’s exact probability test, as appropriate. P < 0.05 was considered statistically significant.

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SI Materials and Methods

Administration of Drugs. Human colony-stimulating factor 1 (CSF-1) (Leukoprol; Kyowa Hakko) (1 μg/litter) was administered i.p. daily into 3-week-old CSF-1−/− mice for 4 d. Mice were killed 24 h after the last injection. GST-sRANKL was dissolved in water and PBS, respectively. Two-methylene-19-nor(20S)-1α,25(OH)₂D₃ (2MD; Sigma) was kindly provided by Masato Shimizu (Institute of Biomaterial and Bioengineering, Tokyo Medical and Dental University, Tokyo). A stock solution of 2MD or 1α,25(OH)₂D₃ (Sigma) dissolved in ethanol was diluted in propylene glycol at 1:9. 2MD (2 nmol/kg body weight, 40 μL per dose) or 1α,25(OH)₂D₃ (10 nmol/kg body weight, 40 μL per dose) was administered i.p. daily into 3-week-old CSF-1−/− mice twice 2 d apart. Mice were killed 48 h after the second injection. Under light ether anesthesia, mice were perfused with 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) through the left ventricle. Tibiae and spleens were recovered and immersed in the same fixative for 20 h at 4 °C.

Cell Cultures. Bone marrow (BM) macrophages, osteoclasts, and osteoblastic cells were prepared using ddY mice as described previously (1). To prepare BM macrophages, nonadherent BM osteoblastic cells were prepared using ddY mice twice 2 d apart. BM macrophages, osteoclasts, and BM macrophages were further cultured for 3 d in αMEM (Sigma) supplemented with 10% (vol/vol) FBS and human CSF-1 (Leukoprol) (10 μg/ml) in 48-well plates. To obtain osteoclasts, BM macrophages were further cultured for 3 d in the presence of human CSF-1 (10 ng/ml) and human RANKL (100 ng/ml; Peprotech). Osteoblastic cells prepared from newborn mouse calvariae (1 × 10⁵ cells per well) were cultured for 3 d in αMEM (Sigma) supplemented with 10% (vol/vol) FBS and human CSF-1 (Leukoprol) (10 ng/ml) and human RANKL (100 ng/ml; Peprotech). Osteoblastic cells prepared from newborn mouse calvariae (1 × 10⁵ cells per well) were cultured for 24 h in αMEM with 10% (vol/vol) FBS in 12-well plates. Then, cells were treated for 24 h with 2MD (10⁻⁴ M), 1α,25(OH)₂D₃ (10⁻⁸ M), mouse TNFα (25 ng/mL; R&D Systems), mouse IL-1β (25 ng/mL; R&D Systems), or PGE₂ (10⁻⁶ M; Sigma).

Real-Time RT-PCR. For preparing total RNA, cultured cells were lysed directly in TRIzol (Invitrogen). Tibiae were used for bone samples. Tissue samples were homogenized in TRIzol using TissueLyser II (Qiagen). RNA extraction was performed according to the manufacturer’s directions (Invitrogen). First-strand cDNA was synthesized from total RNA with an oligo (dT)₁₂–₁₈ primer (Invitrogen) and ReverTra Ace reverse transcriptase (ToYoBo). Real-time RT-PCR for the quantification of cDNA was performed using the Fast SYBR Green (Applied Biosystems) and StepOnePlus system (Applied Biosystems). The following temperature profile was used: 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. The Takara Bio probes in this study were for GAPDH (TaKaRa; MA050371), CSF-1 (MA066411), and IL-34 (MA097860) of mice. GAPDH was used as an internal control for normalization. Each expression level was calculated using a relative standard curve.

Histological Examination. For TRAP staining, tibiae fixed with 4% (wt/vol) paraformaldehyde were decalcified in 10% (wt/vol) EDTA (pH 7.5) for 3 wk at 4 °C. Then, the specimens were dehydrated in a graded series of ethanol solutions, embedded in paraffin, and cut into 4-μm-thick sections. Double staining for methyl green and TRAP was performed. TRAP(+) osteoclasts were detected as described previously (1). The number of osteoclasts per millimeter of trabecular bone surface was measured using Image J software (National Institutes of Health). For immunofluorescent staining, tibiae were frozen in −80 °C hexane using a cooling apparatus (PSL-1800; Tokyo Rakikakai) and embedded in a cryoembedding medium (Finetec). Five-micrometer-thick sections of tibiae and spleens were prepared using Kawamoto’s film method (cryofilm; Finetec) and fixed in ice-cold 5% (vol/vol) acetic acid in ethanol (2). Fluorescence-based staining for alkaline phosphatase (ALP) activity was performed using an ELF-97 phosphate substrate (Invitrogen). The sections were subjected to staining for RANK (biontinylated anti-RANK antibody [rRANK Ab; R&D Systems]), CSF-1R (rCSF-1R Ab; R&D Systems), PECAM-1 (BD Pharmingen), and IL-34 (R&D Systems) using specific antibodies. rCSF-1R Ab was labeled with FITC and dilAb-34 Ab with biotin using commercial kits (Dojin). The sections were subjected to a reaction with streptavidin–horseradish peroxidase (HRP), anti–FITC-HRP, or the corresponding dilgG Ab–HRP. The HRP-conjugated molecules were visualized with a tyramide signal amplification (TSA) kit (PerkinElmer). For the simultaneous identification of two different targets, HRP was inactivated using peroxidase blocking reagent (Dako) between each TSA reaction. Images were obtained using Plan-Neofluor 5×/0.15 and Plan-Neofluor 40×/0.75 objectives (Carl Zeiss) on a microscope (Axioplan 2 imaging; Carl Zeiss) with a digital camera (AxioCam HRc). Images were captured with AxioVision software. Figures were constructed using Photoshop (Adobe).

In Vivo Knockdown of IL-34 Expression Using Invivofectamine. First, 50 μL of 20 μg/μL IL-34 siRNA (Stealth siRNA; Invitrogen; MSS236361) duplex or Stealth siRNA negative control (Invitrogen) was incubated with 1 mL of Invivofectamine (Invitrogen) for 30 min at room temperature in an orbital shaker. To evaluate the delivery of siRNA molecules to target organs, 200 μL of 20 μM Block-iT Alexa Fluor control siRNA (Invitrogen) was also incubated with 1 mL of Invivofectamine in the same way. The Invivofectamine–siRNA mixture was diluted with 15 volumes (15 mL) of RNase-free 5% (wt/vol) glucose and concentrated using an Amicon Ultra-15 centrifugal device until the volume was reduced to ~0.5 mL. Then, the siRNA complex was recovered and adjusted to 1 mL with RNase-free 5% (wt/vol) glucose to prepare a 1 μg/mL siRNA solution. The siRNA solution (100 μL) was administered to 3-week-old CSF-1−/− mice through a retroorbital venous sinus injection, corresponding to 10 mg/kg body weight, under isoflurane anesthesia. For the evaluation of siRNA delivery, 200 μL of Block-iT Alexa Fluor 555 control siRNA solution was administered in the same way, and 2 h later, spleens and tibiae were recovered for histological examination. At 24 h post-siRNA treatment, 2MD (2 mmol/kg body weight, 40 μL per dose) was administered i.p. into the CSF-1−/− mice twice 2 d apart. For the evaluation of knockdown efficiency, spleens were harvested 24 h after a single 2MD injection, corresponding to 48 h post-siRNA treatment, and subjected to real-time RT-PCR analysis. For histological examination, tibiae were recovered 48 h after the second 2MD injection.


**Fig. S1.** Localization of IL-34(+) cells in WT mice. (A) Localization of IL-34 (red) and PECAM-1 (green) in spleen in WT mice. Dashed lines represent the edge of the white pulp (wp). (Scale bar, 100 μm.) (B) Number of IL-34(+) cells and PECAM-1(+) cells in the red and white pulp. Values in red bars represent percentages of IL-34(+) cells among PECAM-1(+) cells. Data are expressed as the mean ± SD for four optical fields. (C) Localization of IL-34 (red) and PECAM-1 (green) (Left) and that of IL-34 (red) and alkaline phosphatase (ALP) activity (green) (Right) in the proximal tibiae in WT mice. Dashed lines represent bone surface. Arrows indicate IL-34(+)/PECAM-1(+) cells. Arrowheads indicate ALP(+) osteoblastic cells. (Scale bar, 20 μm.)
**Fig. S2.** Evaluation of biological activities of IL-34 in vitro. (A) Effect of IL-34 on the growth of bone marrow cells. Nonadherent bone marrow cells (1 × 10^5 cells per well, 96-well plate) obtained from WT mice were cultured with CSF-1, IL-34, or GM-CSF in the presence or absence of a neutralizing αCSF-1R Ab (AFS98) or control IgG (1 μg/mL). Cell viability was measured by Alamar blue assay. Data are expressed as the mean ± SD (n = 8). (B) Effect of IL-34 on osteoclast formation in mouse BM cell cultures. Nonadherent bone marrow cells (1 × 10^5 cells per well, 96-well plate) were cultured with RANKL (100 ng/mL) in the presence or absence of IL-34 and CSF-1. Cultures were also treated with or without αCSF-1R Ab and control IgG (1 μg/mL). After culture for 6 d, cells were fixed and stained for TRAP (Right). TRAP(+) multinucleated cells containing more than three nuclei were counted as osteoclasts. Data are expressed as the mean ± SD (n = 8) (Left). (Scale bar, 100 μm.) (C) Effect of IL-34 on osteoclast survival. Osteoclasts isolated from cocultures of osteoblastic cells and bone marrow cells (375 ± 57 cells per well, 24-well plate) were cultured in the presence or absence of CSF-1 and IL-34. Cultures were also treated with or without αCSF-1R Ab and control IgG (1 μg/mL). After culture for 24 h, cells were fixed and stained for TRAP (Right). TRAP(+) multinucleated cells were counted as osteoclasts. Data are expressed as the mean ± SD (n = 8) (Left). (Scale bar, 100 μm.)
**Fig. S3.** VEGF-induced osteoclastogenesis in CSF-1op/op mice. (A) Effect of splenectomy (SPX) on VEGF-induced osteoclastogenesis in CSF-1op/op mice. Three-week-old CSF-1op/op mice were subjected to SPX or Sham. Four days later, mouse VEGF-A_120 (VEGF_120) (0.5 mg/kg) or vehicle was injected i.p. Mice were killed 4 d after the injection. Sections of tibiae were double stained for TRAP and methyl green (Left). Number of osteoclasts was counted (Right), Arrows indicate TRAP(+) osteoclasts. Data are expressed as the mean ± SD for four optical fields from four mice. *P < 0.01. (Scale bar, 50 μm.) (B) Effects of VEGF_120 administration on IL-34 mRNA expression. Three-week-old CSF-1op/op mice were injected with VEGF_120 (0.5 mg/kg) or vehicle, and killed 24 h later. The expression of IL-34 mRNA in bone, liver, and spleen was determined by the real-time RT-PCR. Data are expressed as the mean ± SD for triplicate PCRs using RNA from two mice.

**Fig. S4.** Effects of a 2MD analog (20-epi-1a) injection on serum calcium levels in WT mice, c-Fos−/− mice, and CSF-1op/op mice. WT mice, c-Fos−/− mice, and CSF-1op/op mice were injected daily with 2-methyl-2MD (2 nmol/kg) for 2 d. Blood was collected from the tail (day 0) and by heart puncture (day 2) under anesthesia. Serum concentrations of calcium were determined. Results for WT mice and c-Fos−/− mice have been reported previously (1).

Fig. S5. Delivery of siRNA molecules and efficiency of gene knockdown. (A) Delivery of siRNA molecules to spleen and bone. Alexa Fluor 555-labeled control siRNA together with Invivofectamin was administered i.v. into 3-wk-old CSF-1<sup>op/op</sup> mice. Mice were killed 2 h after administration and subjected to histological examinations. An intact CSF-1<sup>op/op</sup> mouse was used for the negative control. wp, white pulp; hz, hypertrophic zone of the growth plate. (Scale bar, 50 μm.) (B) Efficiency of the siRNA-mediated knockdown of the IL-34 gene. IL-34 siRNA (10 mg/kg) or control siRNA together with Invivofectamin was administered i.v. into 3-wk-old CSF-1<sup>op/op</sup> mice. 2MD (2 nmol/kg) or vehicle was injected i.p. 24 h after siRNA administration. Spleens were harvested 24 h after the 2MD injection and subjected to real-time RT-PCR analysis. Data are expressed as the mean ± SD for triplicate PCRs using RNA from two mice. *P < 0.01.

Fig. S6. Effect of splenectomy (SPX) on 2MD-induced osteoclastogenesis in WT mice. Three-week-old WT mice were subjected to SPX or Sham. Four days later, mice were injected with 2MD (2 nmol/kg) twice 2 d apart and killed 48 h after the second injection. Sections of tibiae were double stained for TRAP and methyl green. Number of osteoclasts was counted (Right). Data are expressed as the mean ± SD for four optical fields from four mice. *P < 0.01. (Bar, 50 μm.)
Fig. S7. Effect of 2MD on IL-34 mRNA expression in WT mice. Effects of the administration of 2MD on IL-34 mRNA expression in vivo. Three-week-old WT mice were injected with vehicle or 2MD (2 nmol/kg), and killed 24 h after the injection. Expression of IL-34 mRNA in bone, kidney, liver, and spleen was examined by real-time RT-PCR. Data are expressed for triplicate PCRs using RNA from two mice. *P < 0.01.

Fig. S8. Effect of 1α,25(OH)₂D₃ on osteoclastogenesis in CSF-1°°/°° mice. Effects of 1α,25(OH)₂D₃ on osteoclastogenesis. Three-week-old CSF-1°°/°° mice were injected with 1α,25(OH)₂D₃ (10 nmol/kg) twice 2 d apart and killed 48 h after the second injection. Sections of tibiae were double stained for TRAP and methyl green (Left), and number of osteoclasts (Center) and ES/BS (Right) was measured. Arrows indicate TRAP(+) osteoclasts. Data are expressed as the mean ± SD for four optical fields from four mice. (Scale bar, 50 μm.)

Fig. S9. Effect of 2MD, 1α,25(OH)₂D₃, TNFα, IL-1β, and PGE₂ on IL-34 mRNA expression in osteoblastic cells. Osteoblastic cells prepared from mouse calvariae were treated for 24 h with or without 2MD (10⁻⁹ M), 1α,25(OH)₂D₃ (10⁻⁸ M), TNFα (25 ng/mL), IL-1β (25 ng/mL), or PGE₂ (10⁻⁶ M). Cells were then processed for real-time RT-PCR analysis. Data are expressed as the mean ± SD for triplicate PCRs.