Increasing evidence emphasizes the importance of chemokines and chemokine receptors as regulators of bone remodeling. The C–C chemokine receptor 3 (CCR3) is dramatically upregulated during osteoclastogenesis, but the role of CCR3 in osteoclast formation and bone remodeling in adult mice is unknown. Herein, we used bone marrow macrophages derived from adult male CCR3-proficient and CCR3-deficient mice to study the role of CCR3 in osteoclast formation and activity. CCR3 deficiency was associated with formation of giant hypernucleated osteoclasts, enhanced bone resorption when cultured on bone slices, and altered mRNA expression of related chemokine receptors and ligands. In addition, primary mouse calvarial osteoblasts isolated from CCR3-deficient mice showed increased mRNA expression of the osteoclast activator–related gene, receptor activator of nuclear factor kappa-B ligand, and osteoblast differentiation–associated genes. Microcomputed tomography analyses of femurs from CCR3-deficient mice revealed a bone phenotype that entailed less cortical thickness and volume. Consistent with our in vitro studies, the total number of osteoclasts did not differ between the genotypes in vivo. Moreover, an increased endocortical osteoid mineralization rate and higher trabecular and cortical bone formation rate was displayed in CCR3-deficient mice. Collectively, our data show that CCR3 deficiency influences osteoblast and osteoclast differentiation and that it is associated with thinner cortical bone in adult male mice.

During physiological bone remodeling, the actions of bone-forming osteoblasts and bone-resorbing osteoclasts cooperate in order to successively rebuild the skeleton, a highly balanced process that is crucial to preserve normal skeletal functions and to maintain bone mass. Osteoblasts and osteocytes express receptor activator of nuclear factor kappa-B ligand (RANKL), the key protein that promotes differentiation of cells of the macrophage/monocyte lineage into mature osteoclasts with bone resorptive capacity. In addition to hormones and mechanical loading, molecules that are primarily associated with the immune system, e.g., cytokines and chemokines, regulate the level of available RANKL and thus also control the bone remodeling process (1). Inflammation may disturb the prevailing equilibrium between bone formation and bone resorption, which most often manifest as a net bone loss because of excessive formation and activity of osteoclasts. Pathological bone loss is a hallmark of chronic inflammatory conditions, such as rheumatoid arthritis (RA), periodontitis, and loosened joint prosthesis and tooth implants (1).

Chemokines are key regulators of the innate and adaptive immune system. They are released by a wide variety of cell types in response to infection or tissue damage and form chemotactic gradients that attract leukocytes to sites of inflammation, which further facilitate the immune response by mediating angiogenesis (2). Besides the involvement of chemokines during pathological conditions, chemokines are also involved in homing of immune cells to lymph nodes during homeostasis. The mode of action relies on interaction between chemokines and their cognate receptors, a system that is highly complex as there are approximately 50 chemokine ligands and 20 chemokine receptors known to date (3). Hence, multiple ligands share the same receptor. Although the crossreactivity is comprehensive, most chemokines have a preferred high-affinity receptor (4).

Increasing evidence shows that chemokine signaling promotes crosstalk between the cells of the immune system and bone cells and thus plays a crucial role in the bone remodeling process during physiological and pathological conditions (5). We have previously shown that individuals with periodontitis, a condition that entails jawbone loss triggered by chronic inflammation in the gum, present with systemically and locally higher levels of the C–C motif chemokine ligand 11 (CCL11) (6). Further experimental studies revealed that CCL11 is highly expressed by osteoblasts during inflammation-induced bone resorption (7). In line with our findings, others have shown high CCL11 levels in conditions that are characterized by pathological bone loss, e.g., RA (8), osteoarthritis (9), and osteoporosis (10), strongly suggesting that CCL11 plays a decisive role in inflammation-induced bone resorption. CCL11 binds to the chemokine receptors CCR2, CCR3, and CCR5, with highest affinity to CCR3 (11, 12).

The importance of CCR2 and CCR5 signaling in the continuous balancing of bone mass and during osteoclastogenesis has
been demonstrated in chemokine and chemokine receptor deficiency in vivo models. Mice lacking either the receptor CCR2 or its primary ligand CCL2 display a reduction in osteoclast numbers and size, resulting in decreased bone resorption and severe osteopetrosis (13, 14). Mice lacking CCR5 are resistant to RANKL-induced bone loss, and osteoclasts from CCR5-deficient mice show defect adhesion, reduced locomotion, and resorptive capacity (15). Together, these results indicate essential roles for CCR2, CCR5, and their associated ligands in bone-destructive conditions through the influence on osteoclast function.

Despite the fact that CCR3 expression is highly upregulated during osteoclastogenesis, the importance of CCR3 signaling in normal bone remodeling and pathological conditions affecting the bone remains largely unknown. CCR3 can bind several ligands, of which CCL7 stimulates osteoclast differentiation in vitro (16) and CCL5 (also known as regulated on activation, normal T cell expressed, and secreted, RANTES) is chemotactic for osteoblasts and has an inhibitory effect on osteoclasts (17, 18). Interestingly, its most high-affinity ligand CCL11 is reported to increase preosteoclast migration and osteoclastic bone resorption (7, 17, 18).

Herein, we study the effect of CCR3 deficiency on osteoclast differentiation, bone resorption, and osteoblast gene expression in vitro. Furthermore, we analyze the bone phenotype of CCR3-deficient adult male mice.

Results

CCR3 deficiency results in increased osteoclast size and number of nuclei per osteoclast

To study if CCR3 deficiency affects osteoclastogenesis and osteoclast morphology, we cultured primary bone marrow macrophages (BMMs) from Ccr3<sup>−/−</sup> and Ccr3<sup>+/+</sup> mice in the presence of macrophage colony-stimulating factor (M-CSF) and RANKL. The osteoclasts formed after 48 h tended to be larger in size in the Ccr3<sup>−/−</sup> than in the Ccr3<sup>+/+</sup> cultures. This

Figure 1. Increased osteoclast (OC) size and number of nuclei in Ccr3<sup>−/−</sup> bone marrow macrophage (BMM) cultures. Mouse BMMs derived from C-C chemokine receptor 3 (CCR3)-deficient (Ccr3<sup>−/−</sup>) and wildtype (Ccr3<sup>+/+</sup>) mice were cultured on plastic, supplemented with macrophage colony-stimulating factor and receptor activator of nuclear factor kappa-B ligand and stained for tartrate-resistant acid phosphatase. A, Ccr3<sup>−/−</sup> BMMs developed into significantly larger OCs compared with Ccr3<sup>+/+</sup> BMMs. Representative images are shown. B, the number and size of Ccr3<sup>−/−</sup> and Ccr3<sup>+/+</sup> OCs was measured after 72 h incubation. There was no significant difference in total OC numbers. However, when the cells >10,000 μm² were stratified in three different size groups, the number of Ccr3<sup>−/−</sup> OCs was significantly higher in all groups compared with Ccr3<sup>+/+</sup> OCs, n = 8 wells per genotype. C, quantification of number of nuclei per OC in Ccr3<sup>−/−</sup> and Ccr3<sup>+/+</sup> cultures shows significantly greater number of nuclei in Ccr3<sup>−/−</sup> OCs, n = 3 wells per genotype. Data represent mean values ± SD. *p < 0.05; **p < 0.01; and ***p < 0.001.
size difference was even more pronounced after 60 and 66 h of culture. After 72 h, Ccr3−/− osteoclasts were highly oversized (Fig. 1A), which also could be verified quantitatively as the number of large osteoclasts (>10,000 μm²) was significantly higher in the Ccr3−/− osteoclast cultures (1.4-fold to 9-fold; Fig. 1B). Importantly, the Ccr3+/− osteoclasts did never reach the same giant size as Ccr3−/− osteoclasts, even though the culture time was extended (96 h; Fig. S1). Moreover, the number of nuclei per osteoclast was higher in Ccr3−/− osteoclast cultures (Fig. 1C). However, the total number of osteoclasts did not differ between the genotypes after 48 h or after 72 h of culture (Fig. 1B shows number of osteoclasts at 72 h). To analyze if even heterozygous osteoclasts present with an increase in cell size, osteoclasts derived from BMMs were cultured from Ccr3+/+, Ccr3−/−, and Ccr3+/− mice. The results show that oversized osteoclasts could be seen also in the Ccr3+/− cultures, although not as marked as in cells from the homozygous phenotype (Fig. S1).

**CCR3-deficient osteoclasts cultured on bone slices show increased size and bone resorption**

To explore if CCR3 deficiency also affects osteoclast formation and morphology when cultured on a physiological substrate, and to investigate if the bone-resorbing capacity is affected, BMMs derived from Ccr3−/− and Ccr3+/+ mice were cultured on bone slices. Similar to the results from BMM cultures on plastic, the Ccr3−/− derived osteoclasts on bone slices were larger in size compared with the Ccr3+/− derived cells, and the size difference visually increased over time (Fig. 2A). The amount of tartrate-resistant acid phosphatase 5b (TRAP5b) secreted into cell culture supernatants between days 3 and 5 was significantly higher in cultures of Ccr3−/− osteoclasts compared with cultures of Ccr3+/− osteoclasts (Fig. 2B). The release of C-terminal type I collagen (CTX-I, a marker of bone resorption) was significantly higher in cultures of Ccr3−/− osteoclasts compared with cultures of Ccr3+/− osteoclasts between days 5 to 7 and days 7 to 9 (Fig. 2C). Thus, osteoclasts lacking CCR3 show increased size and number of nuclei per osteoclast, higher TRAP5b release, and, when cultured on bone, an enhanced bone resorption capacity.

**CCR3 deficiency does not change expression of osteoclast-associated genes but coincides with upregulation of Ccr2 and Ccr5 expression**

To investigate whether the different osteoclast morphology, seen in the absence of CCR3, is related to altered expression of established osteoclastogenic and osteoclastic genes, we performed quantitative PCR (qPCR) analyses in Ccr3−/− and Ccr3+/+ BMM cultures supplemented with M-CSF or M-CSF and RANKL. RANKL upregulated all analyzed osteoclastogenic and osteoclastic genes downstream RANK in both genotypes (data not shown). However, no significant differences in mRNA levels of the pivotal osteoclastogenic transcription factors (Finkel–Biskis–Jinkins osteosarcoma oncogene [c-Fos] and nuclear factor–activated T cells c1 [Nfatc1]), the osteoclastogenic genes (receptor activator of nuclear factor–κB [Rank], Fc receptor γ subunit [Fcer1g], osteoclast-associated receptor [Oscar], alpha v beta 3 integrin [Itgav], DNAx adaptor protein 12 [DAP12]), and the osteoclastic genes (Trp/Acp5, cathepsin K [Ctsk], matrix metalloproteinase 9 [Mmp9], chloride channel 7...
[Clcn7], calcitonin receptor [Calcr], dentrocyte expressed seven transmembrane protein [Dcstamp] or ATPase, H+ transporting, lysosomal V0 subunit D2 (ATP6v0d2) were detected between the Ccr3−/− and Ccr3+/+ cells (Table S2).

Because the CCR3-related receptors CCR2 and CCR5 are both known to be important for osteoclast fusion and function, we also analyzed the mRNA expression levels of Ccr2 and Ccr5 and their high-affinity ligands Ccl2 and Ccl5. In the BMM cultures supplemented with M-CSF, we observed significantly higher levels of Ccr2, Ccl2, and Ccr5 mRNAs in Ccr3−/− than in Ccr3+/+ cells, and this difference was apparent up to 48 h (Ccr2 and Ccl2) and 72 h (Ccr5) after seeding (Fig. 3, A–C). Moreover, the Ccl5 expression levels were lower in the Ccr3−/− cells under these conditions (Fig. 3D). Although the addition of RANKL led to downregulation of the Ccr2, Ccl2, and Ccr5 mRNAs in both Ccr3+/+ and Ccr3−/− cells, the abundance of these mRNAs remained higher in the Ccr3−/− cells (Fig. 3, A–C). RANKL caused upregulation of Ccl5 in both Ccr3+/+ and Ccr3−/− cells (Fig. 3D), although the expression levels were lower in the Ccr3−/− cells. As expected, the expression of Ccr3 was upregulated by the RANKL stimulation in the Ccr3+/+ cells (data not shown). Taken together, CCR3 deficiency does not change the expression of osteoclast-associated genes in response to RANKL but coincides with a higher expression of Ccr2, Ccr5, and Ccl2 and lower expression of Ccl5.

Addition of CCL5 inhibits the formation of giant hypernucleated osteoclasts

Because Ccr3−/− preosteoclasts and osteoclasts show lower Ccl5 and higher Ccr5 mRNA abundance than the corresponding
CCR3 deficiency causes increased osteoclast activity

Ccr3<sup>−/−</sup> cells, we investigated if addition of recombinant CCL5 could influence the osteoclast in vitro phenotype. BMMs derived from Ccr3<sup>−/−</sup> and Ccr3<sup>+/+</sup> mice were cultured for 72 h, supplemented with M-CSF and RANKL, in the presence and absence of CCL5. As in previous osteoclast cell culture experiments, Ccr3<sup>−/−</sup>-osteoclasts became very large in size (Fig. 4A). Addition of CCL5 clearly inhibited the formation of hypernucleated giant osteoclasts in Ccr3<sup>−/−</sup> cultures (Fig. 4B) and also reduced the osteoclast size in Ccr3<sup>+/+</sup> cultures (Fig. 4, C–D).

Figure 4. Reduced formation of giant and hypernucleated Ccr3<sup>−/−</sup> osteoclasts after addition of CCL5. Bone marrow macrophages (BMMs) derived from Ccr3<sup>−/−</sup> and Ccr3<sup>+/+</sup> mice were cultured on plastic, supplemented with macrophage colony-stimulating factor and receptor activator of nuclear factor kappa-B ligand, in the presence and absence of CCL5 (1 μg/ml). A, in the absence of CCL5, Ccr3<sup>−/−</sup> BMMs developed into large and hypernucleated osteoclasts after 72 h of culture, whereas B, Ccr3<sup>−/−</sup> BMMs cultured in presence of exogenous CCL5 showed reduced capacity to form oversized osteoclasts. However, the overall number of multinucleated osteoclasts formed was not reduced. Similarly, C, Ccr3<sup>−/−</sup> BMMs developed into multinucleated osteoclasts after 72 h of culture and D, became smaller in size when cultured in presence of exogenous CCL5. Representative images are shown from one of two comparable experiments, n = 4 wells. CCL5, C-C motif chemokine ligand 5.

CCR3 deficiency causes increased mineralization rate of endocortical osteoid and higher trabecular and cortical bone formation rate in mouse long bones

Dynamic histomorphometry was performed to evaluate the mineral apposition rate (MAR), mineralizing surface (MS), and bone formation rate (BFR) in nondecalcified bone sections from Ccr3<sup>−/−</sup> and Ccr3<sup>+/+</sup> femurs. The trabecular MAR was not significantly affected by the lack of CCR3 (Fig. 6A), whereas the cortical MAR was 35% higher in Ccr3<sup>−/−</sup> mice (Fig. 6B). The augmented MAR was particularly found in the endosteal compartment with an increase of 56% in Ccr3<sup>−/−</sup> mice compared with Ccr3<sup>+/+</sup> mice (Fig. 6C). The periosteal MAR did not differ between the genotypes (Fig. 6D). Images of fluorescently labeled Ccr3<sup>−/−</sup> and Ccr3<sup>+/+</sup> distal femur bone sections show the larger interlabel distance and thicker fluorescent bone labels on the endosteal side of the cortical bone in the Ccr3<sup>−/−</sup> section (Fig. 6E). No difference in trabecular or cortical MS could be seen between the genotypes, whereas the trabecular and cortical BFR was significantly increased by 43% and 32%, respectively, in Ccr3<sup>−/−</sup> mice (Fig. 7, A–D). To further elucidate if the cortical bone phenotype in Ccr3<sup>−/−</sup> mice is correlated to alterations in the number of osteoclasts or differences in osteoclast size in vivo, static histomorphometric analyses were performed on TRAP-stained bone sections from Ccr3<sup>−/−</sup> and Ccr3<sup>+/+</sup> mice. The number of osteoclasts in trabecular and cortical bones showed no significant difference between the genotypes (Fig. 8, A–B). In addition, no difference...
in osteoclast surface covering the underlying bone tissue could be seen (Fig. 8, C–D).

Bone resorption and bone formation markers in serum are not affected by CCR3 deficiency

Because of the skeletal changes seen in the μCT and histomorphometry analyses, we analyzed bone turnover markers in blood serum from Ccr3−/− and Ccr3+/+ mice. There were no significant differences, neither in the serum levels of TRAP5b, the bone resorption marker CTX-I, nor in the bone formation marker amino propeptide of type 1 collagen between genotypes (Fig. 9, A–C, respectively).

CCR3 deficiency increases osteoblastic Rankl expression and the expression of osteoblast anabolic genes

To investigate if osteoblasts contribute to the disturbed bone remodeling in Ccr3−/− mice, we isolated osteoblasts from Ccr3+/+ and Ccr3−/− mice calvaria and cultured these primary osteoblasts for 6 to 12 days and performed gene expression analyses. The analyses showed that the osteoclast activator–related gene, Rankl, is upregulated in Ccr3−/− osteoblasts (Fig. 10A). Although the expression of the early differentiation transcription factor runt-related transcription factor 2 (Runx2) was comparable in Ccr3+/+ and Ccr3+/− osteoblasts (Fig. 10B), the level of Sp7 mRNA, coding for the regulatory transcription factor osterix, was higher in the Ccr3−/− osteoblasts after 6, 9, and 12 days of culture (Fig. 10C). Similarly, the level of Spp1 mRNA, encoding the bone matrix protein osteopontin, was significantly higher in the Ccr3−/− osteoblasts at all three time points (Fig. 10D). In addition, higher levels of the Alp and Bglap mRNAs (encoding the enzyme for mineral deposition alkaline phosphatase and the bone matrix protein osteocalcin, respectively) were detected in Ccr3−/− osteoblasts after 6, 12, and 9 days of culture, respectively (Fig. 10, E–F).

Discussion

In this article, we show that the absence of CCR3 receptors cause thinner cortical bone and an elevated mineral formation and BFRs in adult male mice. In vitro analyses reveal that CCR3 deletion is coupled to higher mRNA expression of Ccl2, Ccr2, and Ccr5, together with lower Ccl5 mRNA expression, in preosteoclasts and osteoclasts. In addition, we show that the osteoclast size and activity are increased when CCR3 is missing. Moreover, primary mouse calvarial osteoblasts isolated from CCR3-deficient mice show increased mRNA
Figure 6. Increased mineralization of endocortical osteoid in Ccr3−/− long bones. Quantification of mineral apposition rate (MAR) in sagittal bone sections from calcein-injected Ccr3+/+ and Ccr3−/− mice showed (A–D) no differences in trabecular MAR (Tb. MAR, μm/day) between the genotypes, whereas the cortical MAR (Cort. MAR, μm/day) was increased in Ccr3−/− mice compared with Ccr3+/+ mice. The augmented cortical MAR was foremost detected in the endosteal compartment (Es. MAR, μm/day), although the periosteal MAR (Ps. MAR, μm/day) was not significantly changed. E, representative images of fluorescent-labeled Ccr3+/+ and Ccr3−/− bone sections, where an increased interlabel distance and thickness of the fluorescent bone labels could be seen on endosteal side of the cortical bone in the Ccr3−/− section. Data represent mean values ± SD, n = 7 to 9 per genotype. **p < 0.01. ns, not significant.
expression of the osteoclast activator–related gene Rankl, as well as of genes encoding proteins associated with anchorage of osteoclasts and osteoblast differentiation.

We and others have shown that the Ccr3 receptor mRNA expression is upregulated during RANKL-induced osteoclastogenesis (7, 19), but it is unknown what significance the receptor has for osteoclast formation. In this study, we used CCR3-deficient mice and show that lack of CCR3 receptors causes formation of giant hypernucleated osteoclasts that display enhanced bone resorption in vitro. In vivo, however, we could not detect any difference in size of osteoclasts. One reason behind these discrepancies could be that two different units of measurements were compared, i.e., an area in vitro with a length in vivo, which generally does not reflect the whole three-dimensional size of the cells. The size of osteoclasts in vitro is estimated as an area measured in several wells of cell cultures, whereas in vivo as a length (millimeter) of osteoclasts estimated on one single section per mouse. Unfortunately, it is with current method impossible to estimate the size of an osteoclast and the number of nuclei per osteoclast on bone sections. In vitro, CCR3-deficient

![Figure 7. Increased bone formation rate (BFR) in Ccr3<sup>−/−</sup> long bones.](image-url)

Quantification of (A–B) mineralizing surface (trabecular—Tb. MS; cortical—Cort. MS) and (C–D) BFR (trabecular—Tb. BFR; cortical—Cort. BFR) in sagittal bone sections from calcein-injected Ccr3<sup>+/+</sup> and Ccr3<sup>−/−</sup> mice showed significantly increased BFR in Ccr3<sup>−/−</sup> mice compared with Ccr3<sup>+/+</sup> mice. Data represent mean values ± SD, n = 7 per genotype. *p < 0.01. ns, not significant.
osteoclasts seeded on bone substrate show large osteoclasts with many nuclei that very efficiently resorb bone. This might be coupled to enhanced recruitment and fusion of pre-osteoclasts and/or increased osteoclast adherence, motility, and/or resorptive capacity. If this corresponds to the in vivo situation with osteoclasts on bone surfaces (BSs), the thinner cortical bone detected in the CCR3-deficient mice could be due to increased osteoclast activity when CCR3 is missing.

Chemokine signaling commonly stimulates osteoclastogenesis and osteoclast activity in an indirect manner through upregulation of RANK and subsequent downstream signaling proteins (13, 14, 20). Most surprisingly, none of the analyzed genes associated with osteoclast differentiation or fusion were expressed differently in CCR3-deficient osteoclast cultures. Our finding of higher Ccl2, Ccr2, and Ccr5 gene expression could indirectly contribute to the increased fusion of osteoclasts, creating giant hypernucleated osteoclasts, and the heightened bone resorptive activity seen in CCR3-deficient osteoclast cultures. CCR5 is known to stimulate cellular locomotion and bone resorption activity of osteoclasts, which is associated with the arrangement of podosomes and adhesion complex molecules including Pyk2 (15). Thus, the increased in vitro bone resorption in the CCR3-deficient osteoclast cultures on bone slices may be explained by increased osteoclast locomotion as the osteoclast function genes are not upregulated. Consistent with this notion, increased

Figure 8. No differences in osteoclast number and surface per millimeter of bone in Ccr3+/− long bones. Quantification of tartrate-resistant acid phosphatase (TRAP)+ (A–B) osteoclast number and (C–D) osteoclast surface per millimeter of bone (trabecular—Tb. Os.N; Tb. Os.S; cortical—Cort. Os.N; Cort. Os.S) in Ccr3+/+ and Ccr3−/− bone sections showed no significant difference between the two genotypes. Data represent mean values ± SD, n = 8 to 9 per genotype. ns, not significant.
CCR3 deficiency causes increased osteoclast activity

**Figure 9.** No alterations in serum levels of bone resorption/formation markers in Ccr3−/− mice. Serum levels of (A) TRAP5b, (B) CTX-I, and (C) P1NP measured by ELISA showed no difference between the genotypes. Data represent mean values ± SD, n = 10 to 14 per genotype. CTX-1, C-terminal type I collagen; ns, not significant; P1NP, amino propeptide of type I collagen; TRAP5b, tartrate-resistant acid phosphatase 5b.

**Figure 10.** Increased mRNA expression of Rankl and the osteoblast differentiation-associated genes Sp7, Spp1, Alp, and Bglap in Ccr3−/− primary osteoblast cell cultures. Primary osteoblasts isolated from Ccr3−/− and Ccr3+/+ mice calvaria were cultured on plastic for 6 to 12 days before gene expression analysis. A–F, the mRNA expression of Rankl, Sp7, Spp1, Alp, and Bglap was significantly increased in Ccr3−/− osteoblasts compared with Ccr3+/+ osteoblasts, whereas no difference could be seen in the mRNA expression of Runx2 between the two genotypes. Data represent mean values ± SD, n = 4 wells per genotype. **p < 0.01; ***p < 0.001.

Osteoclastogenesis is previously described in mice lacking CCL5 (18), indicating that CCL5 inhibits osteoclast formation. Besides higher Ccr5 mRNA expression in our CCR3-deficient BMM cultures, we also detected lower Ccl5 mRNA levels. Furthermore, addition of exogenous CCL5 to CCR3-deficient BMM cultures completely abolished the formation of giant...
hypernucleated osteoclasts, implying that the increased Ccr5 expression and decreased Ccl5 expression drive formation of giant osteoclasts when CCR3 is absent. As for CCR2 and CCL2, Khan et al. (21) could show that osteoclast formation was impaired in mice lacking a functional copy of either gene. For instance, a lower number of osteoclasts, accompanied by a reduction in cell size and a lower number of nuclei per cell, were evident in CCR2- and CCL2-deficient mice. Moreover, addition of exogenous CCL2 increased the number of osteoclasts and number of nuclei seen in CCL2-proficient cell cultures and restored the number and size of osteoclasts in CCL2-deficient cultures. Interestingly, the changed osteoclast morphology seen in CCR2-deficient mice was accompanied by higher levels of CCR3, suggesting that CCR3 contributes to a reduction of osteoclast size and nucleation (21). Therefore, our observation of increased Ccl2 and Ccr2 expression in CCR3-deficient BMM cultures may explain the formation of oversized osteoclasts. However, whether the pervading formation of giant osteoclasts and increased osteoclast activity seen in the absence of CCR3 occur indirectly because of upregulation of CCL2 and CCR2/CCR5 receptors, and downregulation of CCL5, needs to be further investigated.

The thin cortical femur bones found in the CCR3-deficient mice could be due to increased bone resorption, decreased bone formation, or a combination of both. Interestingly, our dynamic histomorphometrical analyses demonstrate an increased cortical mineralization rate of osteoid, mainly in the endosteal compartment, and that the BFR was increased in both cortical and trabecular bones in CCR3-deficient mice. This may be a compensatory mechanism to meet a high bone resorption activity or a sign of accelerated bone turnover, which requires increased osteoblast recruitment and activity. Yano et al. (17) have shown that osteoblasts express CCR3 receptors, but the role of CCR3 signaling in osteoblasts is hitherto unexplored. We isolated and cultured mouse calvarial osteoblast from CCR3-proficient and CCR3-deficient mice and found that, during the whole culture period, the deletion caused higher mRNA expression of Rankl, the gene encoding the RANKL protein with a central role in osteoclastogenesis and activation of osteoclasts (1). Osteoblastic RANKL expression in vivo could contribute to increased preosteoclast recruitment and/or increased osteoclast activity. In this context, it is interesting that we found an increased expression of Spp1 (osteopontin) mRNA in the CCR3-deficient osteoblasts. Osteopontin is important for, e.g., anchorage of osteoclasts (22) and could, if osteopontin is enriched in the CCR3-deficient mouse skeleton, promote bone resorption. Interestingly, Sp7 (osterix), a transcription factor crucial for driving osteoblast differentiation, was enhanced in CCR3-deficient osteoblast, along with Spp1 (osteopontin), Alp (alkaline phosphatase), and Bglap (osteocalcin). Because the last two genes encode proteins that are coupled to mineralization of bone (23, 24), the CCR3-deficient osteoblasts show an increased anabolic capacity, which could contribute to the increased mineralization seen in our dynamic histomorphometrical analyses of osteoid mineralization rate and BFR in CCR3-deficient mice. Clearly, the osteoblastic gene expression is changed when CCR3 is missing, not only in a way that could contribute to increased osteoclast recruitment, adherence, and activity but also in a bone anabolic direction.

In 2019, Mohan et al. (25) demonstrated that young female mice lacking CCR3 have increased trabecular bone mass because of a greater number and thickness of trabeculae compared with wildtype littermates. This is in contrast to our findings and could be explained by the differences in gender and age of the mice used in our respective studies. Moreover, our observation of an overall increased metaphyseal tissue and medullary volume indicates that the CCR3-deficient mice may have an altered femoral shape because of a changed postnatal skeletal development and growth. Thus, as our study is limited to one single model at one time point, further analyses are needed to investigate if CCR3 deficiency affects bone modeling in young male mice.

Although our skeletal analyses of the CCR3-deficient mice show an altered bone remodeling, our analyses of serum bone metabolic markers do not reveal any significant differences compared with the levels in CCR3-proficient mice. One explanation could be that the cortical bone, where we see the most distinct differences, in general is very slowly remodeled. This makes it difficult to track serum level differences when measuring at one single time point. Another explanation could be that the adult bone phenotype in CCR3-deficient mice is mainly mirroring early modeling effects. Notably though, the TRAP5b serum levels vary greatly in CCR3-deficient mice compared with CCR3-proficient mice. This may reflect the large variation in osteoclast size seen in vivo, corresponding to our in vitro observation with no increase in osteoclast numbers in CCR3-deficient cultures, but an increase in osteoclast size and TRAP5b levels compared with CCR3-proficient mice.

The fact that the CCR3 expression is upregulated in circulating monocytes of osteoporotic individuals with low bone mineral density (26) and in peripheral blood leukocytes in patients with RA (27) suggests that the receptor is involved in the disturbed bone remodeling process. Further studies are needed to rule out if the increased CCR3 expression is a defense mechanism prohibiting recruitment of osteoclast progenitor cells. We and others have shown that the CCR3 ligand CCL11, which is not synthesized by monocytes or osteoclasts, is associated with inflammatory bone loss conditions (6, 8) and that CCL11 stimulates preosteoclast migration and bone resorption in vitro. However, it is not clear by which receptor these effects are mediated. Studying chemokine receptor signaling using antibodies is difficult as the crossreactivity is high. To address the role of different chemokine ligands, and the potential functional interplay between chemokine receptors, such as CCR2, CCR3, and CCR5, conditionally genetic deficient cell and animal models are needed.

Taken together, the present study further emphasizes the relevance of studying the role of a hitherto virtually unrecognized player in bone research, CCR3, in bone cell differentiation and activity.
Committee at Umeå University.

Animal care and experiments were approved and conducted in (Special Diet Service #801730). Only male mice were used. Animal care and experiments were approved and conducted in accordance with regulations of the Local Animal Ethical Committee at Umeå University.

BMMs were seeded in droplets of 40

Olympus BX41 light microscope. For gene expression analyses, were considered osteoclasts. Images were acquired using an instruction. TRAP-positive cells with three or more nuclei

Aldrich, St Louis, MO, USA), following the manufacturer

–TRAP using the leukocyte acid phosphatase kit (Sigma

were

nostic systems [IDS], Copenhagen, Denmark). The cells were left to adhere for 10 min whereafter

5

μ

l( 5 × 10^3 cells) in 96-multiwell plates (Nunc, Roskilde, Denmark). The cells were left to adhere for 10 min whereafter 200 μl of complete α-MEM supplemented with M-CSF (30 ng/ml) and with or without RANKL (4 ng/ml) (R&D Systems/Biotechne, Abingdon, UK) in Corning nontreated culture dishes (Corning Inc, Corning, NY, USA) to which BMMs adhere but not stromal cells or lymphoid cells. After 3 days of incubation, nonadherent cells were discarded, and the adherent BMMs were harvested and either used for immediate RNA isolation and qPCR analyses or seeded for continued culturing as described later.

Osteoclast formation in cultures on plastic dishes

BMMs as prepared previously were seeded in droplets of 5 μl (5 × 10^3 cells) in 96-multiwell plates (Nunc, Roskilde, Denmark). The cells were left to adhere for 10 min whereafter 200 μl of complete α-MEM supplemented with M-CSF (30 ng/ml) and with or without RANKL (4 ng/ml) (R&D Systems/Biotechne) and CCL5 (1 μg/ml) (R&D Systems/Biotechne) was added. The cells were incubated at 37 °C in 5% CO₂ for various times (designated in Results section), whereafter the culture media were harvested and stored in aliquots at −20 °C until analysis of the active isoform 5b of TRAP5b (Immunodiagnostic systems [IDS], Copenhagen, Denmark). Adherent cells were fixed at the bottom of the 96-well plate and stained for TRAP using the leukocyte acid phosphatase kit (Sigma–Aldrich, St Louis, MO, USA), following the manufacturer’s instruction. TRAP-positive cells with three or more nuclei were considered osteoclasts. Images were acquired using an Olympus BX41 light microscope. For gene expression analyses, BMMs were seeded in droplets of 40 μl (4 × 10^4 cells) in 12-multiwell plates (Nunc) and cultured as mentioned previously.

Osteoclast formation in cultures on bone slices

BMMs were also seeded on bovine cortical bone slices (IDS) placed at the bottom of 96-well plates (Nunc) (5 × 10^3 cells/well) in 200 μl of complete α-MEM, supplemented with M-CSF (30 ng/ml) and with or without RANKL (4 ng/ml) and incubated at 37 °C in 5% CO₂ for 9 days. Culture media were changed at days 3, 5, and 7. The media from day 5, day 7, and at study discontinuation at day 9 were harvested and stored in aliquots at −20 °C until TRAP5b and CTX-I analysis (IDS).

Primary osteoblast cell culture

Preosteoblast cells were isolated through enzymatic digestion of five calvaria from each genotype, using Worthington’s type 2 collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA). Equivalent number of cells from both genotypes were seeded in separate 75 cm² cell culture flasks and cultured in complete α-MEM in 37 °C and 5% CO₂. Media were changed after 1 and 3 days of culture. After 4 days of culture, the cells were washed with EDTA and detached with 0.25% trypsin–EDTA. Following centrifugation and resuspension in complete α-MEM, the cell suspension was diluted to 40,000 cells/ml and seeded in 24-well plates at a density of 20,000 cells per well. The next day media were changed—day 0. Cells were cultured in 500 μl of complete α-MEM. Half of the media were changed twice a week, and all media were changed once a week to compensate for evaporation.

RNA isolation and first-strand complementary DNA synthesis

Total RNA was extracted from cells using the RNAqueous Micro Kit (Ambion, Austin, TX, USA) and subsequently treated with DNase. Single-stranded complementary DNA was synthesized from 0.2 μg of total RNA using the High-Capacity complementary DNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), as per the manufacturer’s instructions.

Real-time qPCR

Real-time qPCR analyses were performed in a QuantStudio 6 Flex real-time PCR system (Applied Biosystems by Life Technologies, Carlsbad, CA, USA) using TaqMan Universal Master Mix (Applied Biosystems) and predesigned TaqMan gene expression assays (Table S4).

Samples were run in duplicates, and the data were normalized to mRNA levels of the endogenous control gene, β-2-microglobulin (B2m). The 2^ΔΔCT formula was used to analyze the relative differences in gene expression (29).

Microcomputed tomography

High-resolution μCT analysis was performed on femurs of 14- to 16-week-old male mice using Skyscan 1275 μCT (Bruker, Kontich, Belgium). Femurs were imaged with an X-ray tube voltage of 40 kV, a current of 250 μA, and with a 1 mm aluminum filter. The voxel size of 8 μm was used. NRecon software (version 1.7.3.1) was employed for image reconstruction. Region/volume of interest selection, segmentation of binary image, and morphometric analysis were all performed using SkyScan CT-Analyser (CTAn) software. Volume of interest for trabecular bone was selected at the distal femur within a conforming volume commencing at the
growth plate and extending a further longitudinal distance of 0.48 mm (60 image slices) in the proximal direction and for cortical bone—4 mm (500 image slices). A total of 1.6 mm (200 image slices) and 1.2 mm (150 image slices) were analyzed for trabecular and cortical bone, respectively.

**Serum biomarkers**

Blood serum was collected from 16-week-old mice. As a marker of bone resorption, serum levels of CTX-I were assessed using an ELISA RatLaps kit (IDS). To validate osteoclast activity, a determination of TRAP5b activity was performed using mouse TRAP5b ELISA kit (IDS). Serum levels of amino propeptide of type I collagen were analyzed as a marker of bone formation (IDS).

**Histomorphometry**

About 18- to 19-week-old Ccr3+/+ and Ccr3−/− mice were injected with fluorescent compound calcein (30 mg/kg) (Sigma–Aldrich) 10 days and 3 days prior to tissue collection. Dissected femurs from both genotypes were fixed in 4% phosphate-buffered paraformaldehyde (Histolab Products AB, Askim/Gothenburg, Sweden) and embedded in methyl methacrylate (Pharmastest Services Ltd, Turku). Sagittal tissue sections (thickness of 4 and 8 μm) of distal femurs were used for quantification of dynamic bone histomorphometric parameters after fluorescence microscopy (Olympus BX41; Olympus CellSens Standard). The amount of trabecular and cortical MS (Tb. MS; Cort. MS, %) was estimated as the coverage of BS by fluorescent double-labeled surface and single-labeled surface according to the equation MS = (double-labeled surface + [single-labeled surface/2] × 100)/BS. Trabecular and cortical MAR (Tb. MAR; Cort. MAR, μm/day), including the periosteal and endosteal surfaces separately (Es. MAR; Ps. MAR, μm/day), was estimated by the interlabel distance of the two consecutive calcein labels divided by the time interval between the injections of labels. Corresponding BFR (μm3/μm2/day) was calculated as BFR = (MS/BS) × MAR. Adjacent sagittal sections were stained for mineralized tissue with von Kossa phosphate 5b.

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**Data availability**

All relevant data are included in the article and the supporting information files. Original data are available on reasonable request.

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**Abbreviations**—The abbreviations used are: μCT, microcomputed tomography; α-MEM, minimum essential medium Eagle, alpha modification; BFR, bone formation rate; BMM, bone marrow macrophage; BS, bone surface; CCL, C–C motif chemokine ligand; CCR3, C–C motif chemokine receptor 3; CTX-I, C-terminal type I collagen; M-CSF, macrophage colony-stimulating factor; MAR, mineral apposition rate; MS, mineralizing surface; qPCR, quantitative PCR; RA, rheumatoid arthritis; RANKL, receptor activator of nuclear factor kappa-B ligand; TRAP5b, tartrate-resistant acid phosphatase 5b.

**Statistical analyses**

The statistical analyses were performed using Student’s t test, and all experiments were performed at least twice with comparable results. In the gene expression analyses, the ΔCt values were used (determined as the Ct value for the gene of interest minus the Ct value for the reference gene). All data are presented as means ± SD. Significance levels were set to p < 0.05 (*), 0.01 (**), and 0.001 (***)
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