# INNATE IMMUNITY IN THE CONTEXT OF OSTEOIMMUNOLOGY,
## 2nd Edition

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# INNATE IMMUNITY IN THE CONTEXT OF OSTEOIMMUNOLOGY,
## 2nd Edition

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EDITORIAL
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# Editorial: Innate Immunity in the Context of Osteoimmunology

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**Keywords: osteoimmunology, innate immunity, fracture healing, bone regeneration, chemokines**

**Editorial on the Research Topic**

**Innate Immunity in the Context of Osteoimmunology**

The term "osteoimmunology" identifies the research field aimed at studying the crosstalk between cells of the skeletal and immune systems (1). The close relationship between these two systems is apparent based on the sharing of the same microenvironment (2), but it also extends beyond this through a network of signaling pathways and molecules acting in the pathophysiology of bone and immune cells (3). A large proportion of research in osteoimmunology has long focused on the effects elicited by adaptive immunity on bone, with rheumatoid arthritis as a prototypical disease condition (4). Only recently has the innate arm of the immune system received increasing attention in this framework (5). Indeed, osteonal macrophages, mast cells, and dendritic cells, in particular, have emerged as active players in skeletal remodeling and repair and in inflammation-induced bone loss (6–8). In parallel, novel concepts have been proposed regarding the capacity of bone cells to regulate immunity, suggesting, for example, the classification of osteoclasts as professional antigen-presenting cells and inflammatory osteoclasts as a different population compared to homeostatic osteoclasts (9, 10).

Hereby, the overall picture on the bone-immune system interplay gained additional complexity. This collection of articles reflects this topic and focusses on osteoimmunology with regard to innate immune cells/bone cells crosstalk.

## MACROPHAGES AND BONE FRACTURE HEALING

Bone healing is a prototype for a regenerative process; indeed, at least ideally, the injured tissue undergoes a complete *restitutio ad integrum* without scar formation through the contribution of different cell types; in particular, this context offers the stage to many osteoimmunological interactions. In this issue, Stefanowski et al. investigated early events of vascularization at sites of bone regeneration in a murine osteotomy model. The authors showed *in vitro* and *in vivo* that the newly generated vessels, expressing markers of type H endothelium, transiently accumulated far from the fracture site, close to osteoprogenitors and macrophages. In particular, $CX3CR1^{+}F4/80^{+}$ cells were the most abundant macrophage population, having progressively infiltrated the hematoma prior to functional vascularization and persisted until remodeling. Overall, this paper sheds initial light on the crosstalk between macrophages and endothelial cells.

In the same direction, Löffler et al. showed that disturbed bone regeneration in osteotomized aged rats was associated with impaired M2 macrophage function and consequent reduced revascularization of the bone callus. Accordingly, local infusion of $CD14^{+}$ macrophage precursors



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into the osteotomy gap of aged rats reduced fibrosis and improved vascularization and overall bone regeneration. This work paves the way to more extensive studies of macrophage dynamics in early phases of bone healing, in relation to outcome.

## MAST CELLS IN BONE HOMEOSTASIS

Mast cells (MCs), commonly referred to as tissue-resident immune cells promoting allergic reactions, have been demonstrated to also be involved in the pathophysiology of bone. Their effect is essentially elicited through the release of the content of secretory granules, that is, soluble mediators, including histamine, heparin, cytokines, growth factors and enzymes, which influence bone metabolism, potentiating either osteoclast or osteoblast activity. In this respect, Ragipoglu et al. provided a critical overview of the current knowledge of MC function in bone homeostasis and disease, specifically focusing on osteoporosis and bone regeneration. Owing to the lack of a relevant human condition lacking MCs, most of the literature has been derived from experimental murine models, and was sometimes contradictory. Despite the need for further investigation, the proposal to exploit MC-targeting drugs in the framework of bone diseases constitutes an attractive option.

## SOLUBLE FACTORS ON STAGE

In addition to the prototypical RANKL/RANK axis, other signaling pathways, like Wnt signaling, also have a clear osteoimmunological relevance. Goes et al. investigated this pathway in the context of periodontitis, an infectious disease of the alveolar bone and surrounding tissue in which an exacerbated inflammatory host response to an oral biofilm causes massive tissue destruction. In particular, the authors focused on the contribution of the osteocyte-derived Dkk1 molecule, a secreted inhibitor of the Wnt signaling induced by inflammatory mediators in the periodontal tissue, to disease progression. They found that in a model of experimental periodontitis, osteocyte-specific Dkk1 deletion dampened bone loss by acting both on osteoblast and on osteoclast parameters and limiting inflammatory infiltrates. This result underlined the role of the local milieu in determining periodontal bone regulation. Further investigation is required to clarify the immunomodulatory properties of Dkk1 and its possible role as a target in inflammatory bone loss conditions.

In the molecular crosstalk between bone and immune cells, an important role is played by chemokines, a large family of ligands (and corresponding receptors) commonly known to direct homing of immune cells, development, and inflammation. In addition to these functions, autocrine and paracrine chemokine signaling in the bone tissue regulate osteoblast and osteoclast functions in pathophysiological conditions. Brylka and Schinke reviewed the current knowledge on this topic, with major emphasis on the most established subsets of chemokines, for example, CCL2, CCL3, CCL20 and the CXCL12/CXCR4 axis. As envisaged by the authors, the scrutiny of ill-defined aspects of chemokine biology in the framework of bone metabolism can be clinically relevant, based on the pleiotropism of these molecules.

Similar considerations apply to the prototypical long pentraxin PTX3, mostly known for its role in innate immunity, inflammation and matrix remodeling, and recently emerging as an active player in bone pathophysiology. Parente et al. provided an overview of the novel *in vitro* and *in vivo* findings pointing to the role of PTX3 in stimulating osteogenic function. By contrast, evidence in humans and in experimental models suggests PTX3 may have pro-osteoclastogenic effects, particularly in inflammatory conditions and skeletal chronic diseases. The structural complexity of this molecule would indeed allow a wide range of (likely context-dependent) interactions, whose exploitation for specific therapeutic purposes could be of interest and foster research.

## GLUCOCORTICOIDS IN OSTEOIMMUNOLOGY

Endogenous glucocorticoids (GCs) represent a paramount stress response mechanism in the body. Based on their established immunomodulatory effect, GC are also abundantly exploited as drugs in different conditions. In addition, GC exert modulatory effects in diverse other contexts: Ahmad et al. reviewed direct and indirect effects of GC on bone and immune cells and on their crosstalk with each other and with vasculature and muscle. The authors paid specific attention to GC action in osteoporosis, inflammatory bone diseases and bone regeneration, and underlined the need for a more holistic approach in including all the players in the same picture.

## THE ENVIRONMENTAL CLUE

Bone tissue engineering translates the osteoimmunological principles into practice to face a growing medical need, particularly when considering the huge number of bone grafts implanted annually worldwide. Immunomodulatory properties are inherent to many components of endogenous extracellular matrices (ECM), including collagen fibers, hyaluronans, and heparin sulfate. García-García and Martin illustrated how material properties can be designed *ad hoc* for different purposes. In particular, they highlighted a new generation of biomaterials, that is, immunoinstructive ECM, able to direct the host immune cell behavior and to guide the spatiotemporal release of endogenous immunoregulators promoting efficient bone repair.

## CONCLUSION

At variance with the old-fashioned concept of bone as inert material with pure mechanical functions, the current view depicts the skeleton as a lively tissue actively interacting with all other tissues and organs in the body. In parallel, the innate immunity arm is now an established player with physiological relevance in bone homeostasis. A more thorough understanding of the interaction modes between these cell types and molecular cues might effectively impact on large population groups, thus warranting current and future efforts.

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REVIEW
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# Chemokines in Physiological and Pathological Bone Remodeling

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The bone matrix is constantly remodeled by bone-resorbing osteoclasts and bone-forming osteoblasts. These two cell types are fundamentally different in terms of progenitor cells, mode of action and regulation by specific molecules, acting either systemically or locally. Importantly, there is increasing evidence for an impact of cell types or molecules of the adaptive and innate immune system on bone remodeling. Understanding these influences is the major goal of a novel research area termed osteoimmunology, which is of key relevance in the context of inflammation-induced bone loss, skeletal metastases, and diseases of impaired bone remodeling, such as osteoporosis. This review article aims at summarizing the current knowledge on one particular aspect of osteoimmunology, namely the impact of chemokines on skeletal cells in order to regulate bone remodeling under physiological and pathological conditions. Chemokines have key roles in the adaptive immune system by controlling migration, localization, and function of immune cells during inflammation. The vast majority of chemokines are divided into two subgroups based on the pattern of cysteine residues. More specifically, there are 27 known C-C-chemokines, binding to 10 different C-C receptors, and 17 known C-X-C-chemokines binding to seven different C-X-C receptors. Three additional chemokines do not fall into this category, and only one of them, i.e., CX3CL1, has been shown to influence bone remodeling cell types. There is a large amount of published studies demonstrating specific effects of certain chemokines on differentiation and function of osteoclasts and/or osteoblasts. Chemokine signaling by skeletal cells or by other cells of the bone marrow niche regulates bone formation and resorption through autocrine and paracrine mechanisms. *In vivo* evidence from mouse deficiency models strongly supports the role of certain chemokine signaling pathways in bone remodeling. We will summarize these data in the present review with a special focus on the most established subsets of chemokines. In combination with the other review articles of this issue, the knowledge presented here confirms that there is a physiologically relevant crosstalk between the innate immune system and bone remodeling cell types, whose molecular understanding is of high clinical relevance.

**Keywords: bone remodeling, chemokines, osteoblasts, osteoclasts, osteoimmunology**

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# INTRODUCTION

## Skeletal Development and Remodeling

The skeleton consists of more than 200 differently shaped elements, which form by two distinct types of ossification. More specifically, whereas intramembranous ossification, involving direct differentiation of mesenchymal stromal cells into bone-forming osteoblasts, occurs primarily in the skull, most skeletal elements develop by endochondral ossification, where a cartilage intermediate is formed first (1). Here the mesenchymal cells condensate to form chondrocytes, which further differentiate into a hypertrophic state to produce a mineralized cartilage matrix. This initial step occurs in the center of the developing bones, and the subsequent replacement of cartilage by bone generates two zones, i.e., the growth plates, where chondrocytes continue to undergo a specific differentiation program from both sides toward the center (2). This program generates, similar to the initial step, hypertrophic chondrocytes producing mineralized cartilage, which is remodeled into bone by osteoclasts and osteoblasts. Importantly, this transition requires vascularization of these areas to allow invasion of the two cell types (3). Not only during skeletal development and growth, but also thereafter, there is a continuous remodeling of the bone matrix, which takes place throughout adult life (4). This steady renewal process, which is required to maintain skeletal integrity over decades, is mediated by two antagonistically acting cell types, i.e., osteoblasts and osteoclasts, which are fundamentally different in terms of progenitor cells, morphology, mode of action and regulatory molecules affecting their differentiation and function.

More specifically, osteoclasts represent a unique cell type with the ability to resorb mineralized matrix. Osteoclasts are generated by fusion of hematopoietic progenitors of the monocyte/macrophage lineage, which results in huge multinucleated cells and ensures, after attachment to mineralized bone, the formation of a large ruffled surface being required for proper resorption (5). The function of osteoclasts depends on two major mechanisms, i.e., extracellular acidification and secretion of matrix-degrading enzymes. Their dysfunction causes osteoclast-rich osteopetrosis, a severe disorder of early childhood, which requires immediate treatment (6). More specifically, the respective patients are strongly affected by impaired hematopoiesis and immunity, since their bone marrow is replaced by non-resorbed bone and marrow fibrosis. Importantly, if caused by an intrinsic osteoclast defect, which applies for the majority of cases, osteopetrosis is curable by hematopoietic stem cell (HSC) transfer. Besides osteoclast-rich osteopetrosis, there are additional patients, where osteoclasts are not generated. This specific disorder, i.e., osteoclast-poor osteopetrosis, can be caused by inactivation of genes encoding either the transmembrane protein receptor activator of nuclear factor κB (RANK) or RANK ligand (RANKL) (7). Confirmed by a huge number of *in vitro* and *in vivo* studies it is well-established that binding of RANKL, which is primarily expressed by osteoblast lineage cells, to RANK expressed by osteoclast progenitor cells is the most relevant trigger for osteoclast differentiation and bone resorption (8). Most importantly, *in vitro* formation of bone-resorbing osteoclasts does not occur in the absence of RANKL, and mice deficient for RANKL display severe osteopetrosis as they do not develop osteoclasts (9, 10). Moreover, the molecular interaction between RANK and RANKL can be physiologically counteracted by osteoprotegerin (OPG), a soluble protein acting as a decoy receptor of RANKL.

As stated above, osteoblast lineage cells are fundamentally different from osteoclasts and are physiologically regulated by other sets of molecules. Osteoblasts derive from mesenchymal progenitors residing in the bone marrow. They accumulate in larger groups of cells to simultaneously produce the extracellular matrix of bone, which is initially unmineralized. This matrix, termed osteoid, primarily consists of type I collagen, but also contains several additional proteins, such as serum-derived fetuin-A or locally produced matrix proteins, some of them selectively expressed by osteoblasts (11). During the process of matrix mineralization, which is still not fully understood at the molecular level, a subset of osteoblasts is embedded into the mineralized bone matrix to terminally differentiate into osteocytes (12). This third bone cell type is again unique in its morphology, since it forms long cytoplasmic extensions, which are connected to other osteocytes, but also to the bone surface. Osteocytes are known to regulate skeletal remodeling, for instance by producing sclerostin, a physiologically relevant inhibitor of osteoblast activity, whose mutational inactivation causes osteosclerosis, i.e., high bone mass due to excessive bone formation (13). The anti-osteoanabolic activity of sclerostin is molecularly explained by interaction with the transmembrane protein LRP5 (Low-density lipoprotein receptor-related protein 5), which physiologically promotes bone formation (14, 15). Although there are many other systemic or local regulators of bone formation known to date, it is evident that osteoclasts and osteoblasts have to be regarded separately when it comes to influences of specific molecules. Importantly however, there is hallmark evidence for a molecular communication between the two bone remodeling cell types, which is mediated by the RANKL/OPG system, but also by osteoanabolic molecules derived from osteoclasts (16).

The most prevalent bone remodeling disorder, i.e., osteoporosis, is characterized by systemic bone loss causing increased risk of skeletal fractures. Although there are various causes for osteoporosis in different patient groups, the disease is generally explained by a relative increase of bone resorption over bone formation. Given the differential regulation of osteoclasts and osteoblasts described above, there are two distinct options to treat osteoporosis, either inhibiting osteoclast differentiation and/or activity by anti-resorptives (RANKL neutralization or bisphosphonates) or stimulating osteoblast-mediated bone formation by osteoanabolic medication (teriparatide or sclerostin neutralization). With respect to osteoporosis management, it is also important to state that prolonged anti-resorptive treatment by interfering with physiological remodeling and renewal of the bone matrix may have adverse effects on skeletal integrity, i.e., increased fracture risk despite high bone mass. Therefore, osteoanabolic treatment options or their combination with anti-resorptives might be the preferable strategy for osteoporotic patients in the future (17). On the other hand, there are specific pathologies, where excessive osteoclastogenesis is the

primary clinical problem, which are most effectively treated by either bisphosphonates or antibody-mediated blockade of RANKL. These include multiple myeloma (MM), various skeletal metastases, but also different inflammatory disorders, as discussed below (18).

## Molecular Crosstalk Between Bone and the Immune Cells

An interaction between bone remodeling and the immune system is supported by several arguments. First, as discussed above, osteoclasts derive from hematopoietic progenitor cells and therefore represent a highly specialized immune cell. Second, the progenitors of both, osteoclasts and osteoblasts are located in the bone marrow, where they are in direct contact with progenitor or memory cells of the immune system. Third, the major pro-osteoclastogenic cytokine RANKL is not only expressed by osteoblast lineage cells, but also by activated T cells and B cells, and it not only promotes osteoclast differentiation, but also influences different immune cell types (19–21). Fourth, there are various reports showing that bone remodeling cell types affect immune cell differentiation, whereas many different cell populations of the innate and adaptive immune system were found to affect bone remodeling (22). Finally, there are several inflammatory disorders with a negative influence on bone mass, most of them associated with excessive bone resorption (23). Understanding the respective interactions at a molecular level is the focus of an emerging research area known as osteoimmunology, which has led to the discovery of specific cytokines with a remarkable influence of bone remodeling (24).

For example, there is hallmark evidence for a strong positive impact on osteoclastogenesis mediated by TNF-α, IL-1, IL-6, or IL-17. On the other hand, some cytokines were found to have an opposite effect, one of them IL-33, which inhibits osteoclast differentiation *in vitro* and *in vivo* (25, 26). It is important to state however, that there is a high complexity behind these influences, i.e., there are many conflicting results reported in the literature (22). Since this is potentially explained by different experimental settings and/or co-administration of other cytokines, these collective findings essentially suggest that the influence of inflammatory cytokines on bone remodeling cell types strongly depends on their maturation stage and the presence or absence of co-stimulatory signals. It is therefore even more important to refer to clinical data highlighting the specific role of certain cytokines in the context of osteoimmunology. For instance, the severe bone affection in patients with mutations of *IL1RN*, encoding an IL-1 receptor antagonist, essentially confirms the human relevance of IL-1 actions on skeletal cell types (27). Moreover, there is one particular cytokine, i.e., IL-17, where accumulating evidence over the last years strongly suggests a key role in the pathogenesis of bone loss in various inflammatory disorders. These include rheumatoid and psoriatic arthritis, periodontitis, inflammatory bowel disease and primary sclerosing cholangitis (28–32). At a molecular level, IL-17, primarily produced by Th17 cells, has been shown to promote osteoclastogenesis indirectly by inducing RANKL production in synovial fibroblasts or osteoblasts.

Since this cumulative knowledge has been summarized in various comprehensive review articles, the focus of the present article is solely related to another group of immune cells regulators, i.e., chemokines. More specifically, we will discuss the current knowledge regarding the impact of specific chemokines and their receptors on skeletal cell types. This includes direct or indirect influences on osteoclastogenesis and bone resorption, effects on osteoblast lineage cells and endochondral ossification. Moreover, since these interactions may be more relevant in the context of specific pathologies, we will further focus on the impact of chemokines on inflammatory bone loss, behavior of metastatic tumor cells and cancer-induced osteolytic lesions. In fact, certain cancers, such as breast, lung and prostate cancers, home predominantly to the bone marrow niche (33). Here the disseminated cancer cells can undergo dormancy and stay quiescent for up to several years until they start to proliferate again, colonize the bone marrow niche and form metastases (34). These bone metastases often cause osteolytic lesions by inducing osteoclasts to resorb bone. The underlying mechanisms of bone homing, dormancy and exit from dormancy, as well as osteolysis are not yet fully understood. There is however strong evidence showing that specific chemokines are involved in the homing of metastatic cancer cells to the bone marrow and also in osteolysis. Likewise, chemokines have also been shown to be involved in osteolytic bone destruction occurring in multiple myeloma, a type of cancer caused by uncontrolled proliferation of plasma cells in the bone marrow (35).

## Chemokines as Key Regulators of the Innate Immune System

Chemokines are homologous heparin-binding molecules with a molecular mass of 8–12 kDa, which are involved in many biological processes, including homing of immune cells, development, inflammation and angiogenesis (36–39). Almost 50 chemokine ligands are known, which are classified into four subfamilies according to their structure. The chemokine nomenclature refers to the first two highly conserved cysteine residues. The largest family is comprised by the C-C-chemokines in which the two cysteines are adjacent. The second largest group is represented by the C-X-C-chemokines, in which the cysteines are separated by one amino acid. CX3CL1/fractalkine, the only member of the C-X3-C family, contains three amino acids between the cysteines, whereas the two chemokines of the X-C family only have one cysteine. The nomenclature of the corresponding receptors is according to their chemokine ligands (however, note that CX3CL1 also binds CCL26). There are 19 classical chemokine receptors known, which are all G-protein-coupled receptors (GPRCs) containing a rhodopsin-like 7-transmembrane domain structure. The interactome between chemokines and their receptors is quite complex, due to receptor/ligand promiscuity and redundancy. Several different chemokines can bind to the same receptor, and some chemokines are able to bind to more than one receptor. Furthermore, chemokines can form homo- and heterodimers or oligomers, which can lead to different signaling responses compared to the monomer (36). Another level of complexity is added by

atypical chemokine receptors (ACKR), also known as chemokine decoy receptors. There are four atypical chemokine receptors (ACKR) known (ACKR1-ACKR4) (40–42). These receptors do not induce classical G-protein coupled signaling, but internalize the ligand and either induce ligand degradation, or transport the ligand to the other side of the cell. Similar to canonical chemokine receptors, ACKRs can dimerize and oligomerize with other chemokine receptors, and in this manner modulate chemokine signaling (42). Intriguingly, the central regulatory mechanism in osteoimmunology, i.e., RANKL/RANK signaling is also controlled by a decoy receptor, OPG.

Functionally, chemokines are known to form chemotactic gradients (with the exception of membrane-bound CX3CL1 and CXCL16) in order to guide cells toward the highest chemokine concentration (43). In this manner, they orchestrate cell migration in various biological processes. Chemokines can have major physiological functions, such as the well-known CXCL12/CXCR4 axis, which is crucial for homing of HSC in the bone marrow niche (44, 45). However, chemokines are mostly known for their regulatory functions of the immune system during inflammation, where they play important roles for the innate as well as the adaptive immune system (46, 47).

Importantly, the CXC-family of chemokines can be subdivided into two groups, depending on the presence of a specific motif which has functional implications. CXC-chemokines carrying a glutamate-arginine-leucine (ELR) motif near the N-terminus, are all agonists for the receptors CXCR1 and CXCR2, which can both be found on neutrophils (46, 48). Therefore, ELR-positive chemokines are crucial for neutrophil recruitment during wound repair or bacterial defense. Additionally, the presence of the ELR motif also determines their role in angiogenesis. Generally, chemokines containing the ELR motif are angiogenic, whereas ELR-negative chemokines are angiostatic, with the exception of CXCL12, which is an ELR-positive angiogenic chemokine (49). As most literature on chemokine function in angiogenesis focuses on the role of the CXC-chemokine family, CXC-chemokines are regarded as "the regulatory link between inflammation and angiogenesis" (50–53). However, CC-chemokines were shown to also regulate angiogenesis. For instance the pro-angiogenic chemokine CCL2 activates CCR2 on endothelial cells (54).

## INFLUENCE OF CC-CHEMOKINES ON BONE REMODELING IN HEALTH AND DISEASE

There are several chemokines of the CC-family, which were shown to influence skeletal remodeling in physiological and pathological conditions. The most established ones are CCL2, CCL3, and CCL20, which will be discussed separately below. Based on numerous publications from different investigators it is evident that these chemokines share the ability to promote osteoclastogenesis, which is supported by cell culture studies, analysis of mouse deficiency models and, to some extent, by patient analyses. On the other hand, our own

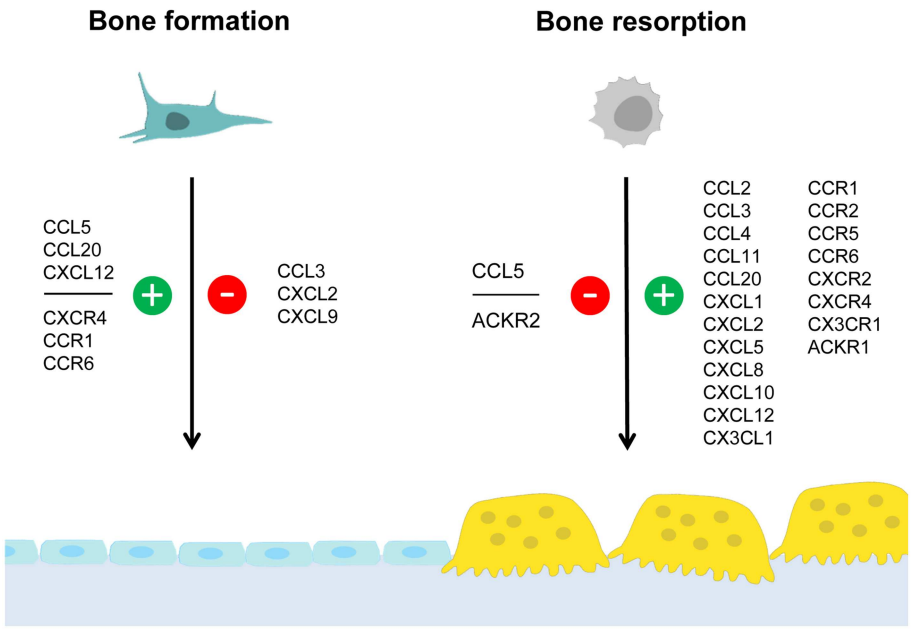


**FIGURE 1 |** Summary of chemokine influences on bone formation and/or resorption. Osteoblasts and osteoclasts are distinctive cell types required for bone formation and bone resorption, respectively. Whereas osteoblasts (left) derive from mesenchymal stem cells, osteoclasts (right) are generated by fusion of hematopoietic progenitor cells. This simplified schematic representation summarizes chemokines and chemokine receptors for which an influence on either bone formation and/or bone resorption was established. Positive influences are indicated in green (with the "+" symbol) whereas negative influences are indicated in red (with the "–" symbol). The data supporting these influences, the underlying mechanisms and the impact on pathological conditions are discussed in the text.

comparative analysis of *Ccl2*- and *Ccl5*-deficient mice revealed that these two chemokines have different influences on skeletal remodeling cell types. Since it was further remarkable that the osteoblast-related phenotype of *Ccl5*-deficient mice diminished with age, we will discuss these findings as an example of functional redundancy. Moreover, although the complexities of specific chemokine influences on either osteoclast or osteoblast differentiation are discussed in the following sections, we have summarized the current knowledge in a simplified schematic representation (**Figure 1**).

## CCL2

The pro-inflammatory chemokine CCL2 (also known as MCP-1), attracts dendritic cells, memory T cells and basophils via its receptor CCR2 (46). CCL2 plays a crucial role in bone remodeling, as demonstrated by studies involving mice deficient for *Ccl2* or *Ccr2* (55–57). Both mouse models show an increased bone mass due to decreased bone resorption, lower osteoclast numbers and a defect in osteoclast formation and function. *Ccl2*$^{-/-}$ mice have a milder phenotype compared to *Ccr2*$^{-/-}$ mice, which is probably due to the fact that CCR2 binds multiple ligands (55–57). The skeletal phenotype of mice deficient for *Ccr2* was shown to be solely caused by a decrease in bone resorption, as osteoblasts in these mice were not affected. The activation of CCR2 signaling in osteoclast progenitor cells was shown to stimulate NF-κB and ERK1/2 signaling, thereby increasing the expression of RANK and making the cells more susceptible to differentiate into mature osteoclasts (55). In line with this, it was shown that osteoclast progenitor cells from *Ccl2*-deficient mice exhibited a decreased expression of RANK and a decreased sensitivity toward stimulation with RANKL (56).

To further investigate the role of CCR2 signaling in bone, *Ccr2*$^{-/-}$ mice were subjected to ovariectomy (OVX). In wildtype mice, CCR2 expression was increased in osteoclast progenitor cells. Mice deficient for *Ccr2* were resistant to bone loss after OVX, suggesting a role for CCR2 signaling in estrogen-deficiency mediated osteoporosis. As both, *Ccr2*$^{-/-}$ and wildtype OVX mice, showed similar numbers of bone-marrow pre-osteoclasts, the recruitment of these cells was independent of CCR2. However, as *Ccr2*$^{-/-}$ OVX mice showed decreased bone marrow RANK expression compared to wildtype OVX mice, CCR2 plays a role in osteoclast formation in the bone marrow. Also, in *Ccr2*$^{-/-}$ OVX mice only CCL2 serum levels were elevated, but not those of other chemokines were altered. Thus, the reduction in bone resorption in *Ccr2*$^{-/-}$ OVX mice was mainly caused by a lack of CCL2/CCR2 signaling. Taken together, the enhanced differentiation of preosteoclasts to osteoclasts due to increased CCR2 expression and the hereby-resulting increased RANK expression induced systemic bone loss after ovariectomy. This finding might be clinically relevant, as *Ccl2* was shown to be among the most strongly induced genes in human osteoporotic bone (58). One way to treat osteoporosis is by injection of the bone anabolic peptide parathyroid hormone (PTH). PTH stimulates bone formation, but also induces bone resorption by osteoclasts through stimulation of M-CSF and RANKL expression. Interestingly, *Ccl2* was shown to be the most strongly induced gene in osteoblasts upon PTH treatment in rats (59). When *Ccl2*-deficient mice were treated with PTH, both the anabolic effect as well as the increase in osteoclast number were reduced, indicating that the anabolic effect depends on stimulation of osteoclast progenitor cells with both RANKL and CCL2 (59–61).

CCL2 was also shown to be involved in other pathological conditions. Osteoblastic CCL2 induced the migration of CCR2-expressing cancer cells and in this manner contributed to bone metastasis formation (62–64). Also cancer cells were reported to express CCL2, thereby increasing tumor growth and osteolysis (65, 66). Furthermore, CCL2 was shown, amongst other chemokines, to be a chemoattractant for MM cells and its expression levels in patients correlated with the occurrence of multiple bone lesions (67). Moreover, inflammatory mediators or bacteria were found to induce the expression of CCL2 by osteoblasts *in vitro* (68, 69) and *in vivo* (70, 71) and in this manner contribute to inflammatory bone loss.

A physiological role for CCL2 has also been suggested in the recruitment of osteoclast precursor cells during tooth eruption (72, 73). Moreover, the expression of CCL2 was shown to be induced in osteoblasts during bone repair in a rat model of ulnar stress fracture (74). In line with its role in osteoclast differentiation, fracture healing was delayed in *Ccr2*-deficient mice, as shown by decreased numbers of infiltrating macrophages at the fracture site combined with a defect in osteoclast function (75).

Taken together, these collective data strongly suggest that CCL2, at least in mice, is involved in promoting osteoclastogenesis and bone resorption by stimulating RANK expression in a CCR2-dependent manner. Although it is worthwhile to mention, that the high bone mass and decreased osteoclastogenesis phenotype of *Ccl2*-deficient mice has been reported in three independent studies (55–57), the impact of the CCL2/CCR2 axis for human bone remodeling, osteoporosis, cancer metastases, and/or osteolytic bone destruction remains to be established.

## CCL3

A role in bone resorption has also been suggested for CCL3 (also known as MIP-1α). CCL3 binds to the receptors CCR1 and CCR5 on lymphocytes, monocytes, macrophages, eosinophils, natural killer cells and dendritic cells and was originally isolated from macrophages, but is also expressed by active osteoblasts (76). Similar to CCL2, CCL3 induces osteoclast formation in a RANK/RANKL-dependent manner, as the injection of recombinant CCL3 increased osteoclast numbers in calvariae of wildtype, but not in *Tnfrsf11a*-deficient (RANK) mice (77). Furthermore, *in vitro* experiments showed that CCL3 stimulated osteoclastogenesis directly, and indirectly by inducing RANKL expression in stromal cells and osteoblasts (78–80). Moreover, CCR1, which binds CCL3 and several other chemokine ligands, was found to be induced by RANKL in bone marrow and in RAW264.7 cells during *in vitro* osteoclast differentiation (81, 82), while treatment with the CCR1-specific antagonist MLN3897 inhibited *in vitro* osteoclastogenesis (83). CCR1 and its alternative ligand CCL9 were further reported to be the major

chemokine receptor and ligand expressed in RANKL-stimulated mouse osteoclasts (84).

Similar to CCL2, CCL3 was shown to be involved in fracture healing (85). In a mouse model of femur fracture, *Ccl3* expression was increased at fracture sites, while neutralization of CCL3 delayed macrophage recruitment and fracture healing. There is also clinical evidence for a role of CCL3 in human bone remodeling. In line with its role in osteoclast differentiation, CCL3 expression in circulating monocytes correlated with low bone mineral density in patients (86). Furthermore, a cross-sectional study showed that postmenopausal osteoporotic women had elevated CCL3 serum levels compared to non-osteoporotic controls (87). CCL3 also plays a role in inflammatory bone loss, in particular in animal models of rheumatoid arthritis (RA). In an RA rat model, CCL3 expressed by macrophages recruited osteoclast progenitor cells to the distal tibia, leading to local bone destruction (88). In line with this, treatment with an anti-CCL3-antibody led to decreased disease severity in a mouse model of collagen-induced arthritis (89). Furthermore, one publication showed that B cell-derived CCL3 inhibits bone formation in RA (90). The authors demonstrated in two different RA mouse models, that B cells accumulated in subchondral bone and in the endosteal niche adjacent to osteoblasts and expressed CCL3 and other factors, which inhibited osteoblast function, while depletion of mature B cells attenuated bone loss in these mice. The authors confirmed the clinical significance of their finding by demonstrating that B cells from RA patients expressed increased levels of CCL3 and inhibited *in vitro* osteoblast differentiation.

Finally, CCL3 appears to play a major role in MM osteolysis. First of all, there is a direct causative link between MM and CCL3 expression. Malignant plasma cells overexpressing FGFR3 or with activating RAS mutation were shown to express increased levels of CCL3, as CCL3 is a downstream target of FGFR3 which signals through the RAS-MAPK pathway (91). Other studies identified CCL3 as an osteoclastogenic factor involved in the formation of osteolytic lesions in MM patients which directly affect migration and survival of MM cells (92, 93). In line with the role of the CCL3/CCR1 axis in osteoblastogenesis, CCL3 from MM cells was shown to inhibit osteoblast function, leading to uncoupling of bone formation and bone resorption (83, 94). Likewise, treatment of a humanized MM mouse model with the CCR1-specific inhibitor MLN3897, led to increased osteoblast function, decreased osteoclast formation, as well as reduced tumor burden (83). Similar studies of MM mouse models showed that the CCR1 antagonist CCX721 could decrease osteoclastic activity, osteolytic lesions and tumor formation (95). Moreover, administration of an anti-CCL3 antibody could reduce tumor growth and osteolysis (77).

In the context of the putative function of CCL3 in bone remodeling, it is further relevant to state that a remarkable bone remodeling phenotype was reported for *Ccr1*-deficient mice (96). In contrast to *Ccr2*$^{-/-}$ mice, which display increased bone mass due to impaired osteoclastogenesis, *Ccr1*$^{-/-}$ mice are characterized by low-turnover-osteopenia, i.e., decreased trabecular bone mass with low numbers of both, osteoclasts and osteoblasts. Furthermore, the *ex vivo* differentiation into the two cell types was impaired in *Ccr1*$^{-/-}$ cultured bone marrow cells, indicating that chemokine signaling through CCR1 affects both arms of bone remodeling. Importantly however, the authors provided additional evidence suggesting that CCL3, even though it is a major ligand of CCR1, was not involved in the development of this phenotype. More specifically, treatment of bone marrow cells with a neutralizing anti-CCL3 antibody did not affect osteogenic differentiation, in contrast to antibodies against other ligands, including CCL5 and CCL9. Therefore, although it remains to be established, which CCR1 ligands are involved in the bone-anabolic function of CCR1, it appears that CCL3 does not induce osteoblast differentiation, but rather inhibits it, as discussed above.

Collectively, there is strong evidence for a critical impact of the CCL3 on bone remodeling cell types. In contrast to CCL2, CCL3 does not only promote osteoclastogenesis, but also has a negative influence on bone formation by osteoblasts. Especially in the context of MM, where CCL3 expression might be of major clinical importance, studies in cultured cells and animal models have shown that CCL3 inhibits osteoblast function, and that this influence is mediated by CCR1. However, as *Ccr1*-deficient mice display a severe impairment in osteoblastogenesis, instead of increased bone formation, it still remains to established, if and how a CCL3/CCR1 interaction influences physiological bone remodeling. Regardless of these open questions, it is quite important that there is also clinical relevance for an impact of CCL3 in human bone pathologies.

## CCL5

CCL5 (also known as RANTES) can bind to different receptors (CCR1, CCR3-5). All of them were found expressed in primary osteoblasts, and it was demonstrated that CCL5 acts as a chemoattractant for osteoblasts *in vitro* (97). Based on an unbiased screening approach, where we identified CCL5 and CCL2 as transcriptionally regulated genes after short-term administration of Wnt5a (98), we analyzed the skeletal phenotype of both, *Ccl2*$^{-/-}$ and *Ccl5*$^{-/-}$ mice (57). Whereas *Ccl2*$^{-/-}$ mice, in line with previous findings by others (55, 56) displayed an increased trabecular bone mass with reduced numbers of osteoclasts, the bone remodeling phenotype of *Ccl5*$^{-/-}$ mice was remarkably different. More specifically, 6-month-old *Ccl5*$^{-/-}$ mice displayed osteopenia with increased osteoclast numbers, i.e., the opposite phenotype as observed in age-matched *Ccl2*$^{-/-}$ mice. Moreover, more than 80% of the endocortical bone surfaces in 6-month-old *Ccl5*$^{-/-}$ mice were not covered by either osteoblasts or bone-lining cells. Of note, this pathology was associated with an absence of F4/80$^{+}$ osteal macrophages, which were previously shown to promote osteoblast formation at endocortical bone surfaces (99). Although these data indicated that CCL5 plays a critical role in the recruitment of osteoblast progenitor cells, it is important to state that this phenotype was only transiently observed, as it diminished with age (**Figure 2**).

In our opinion, this comparative study is potentially relevant in several regards. First, it shows that the deficiency of individual chemokines can cause entirely different skeletal phenotypes, thereby demonstrating the specificity of chemokine functions. Second, it underscores the importance of analyzing different

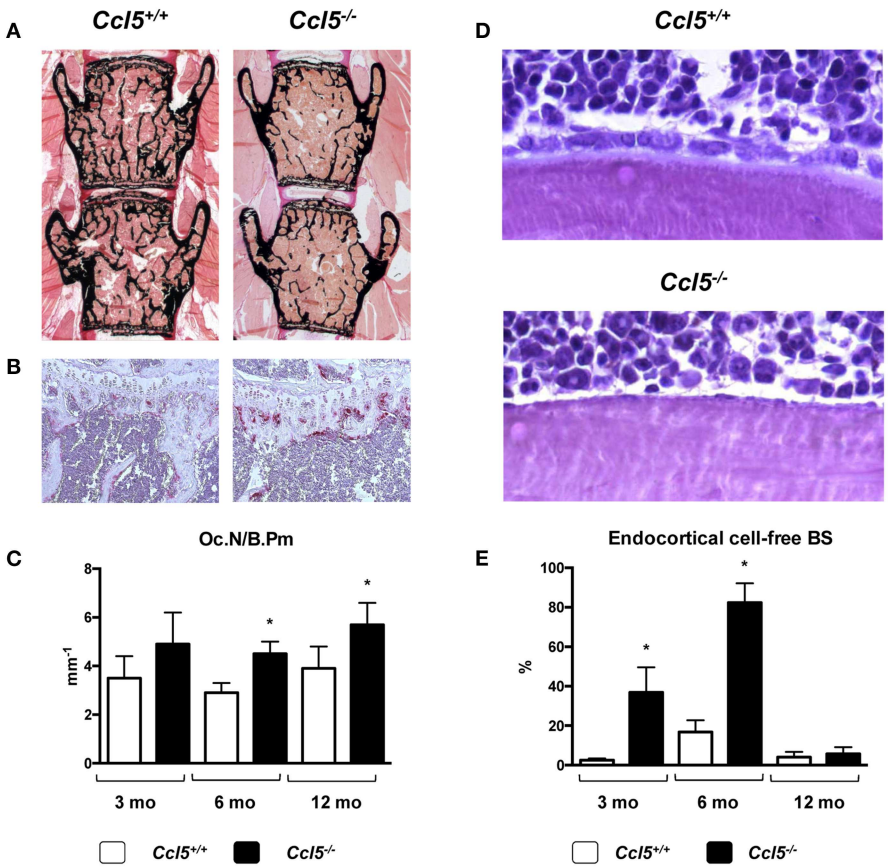


**FIGURE 2 |** Bone remodeling phenotype of *Ccl5*-deficient mice. **(A)** Representative images of undecalcified spine sections (von Kossa/van Gieson-staining, mineralized bone appears black) from 6-month-old littermate mice with the indicated genotypes showing reduced trabecular bone mass in *Ccl5*-deficient animals. **(B)** Representative images of tibia sections stained for activity of the osteoclast marker TRAP (tartrate-resistant acid phosphatase, red staining) from the same mice demonstrating increased osteoclastogenesis in *Ccl5*-deficient animals. **(C)** Histomorphometric quantification of the osteoclast number per bone perimeter (Oc.N/B.Pm) in wildtype and *Ccl5*-deficient littermate mice at the ages of 3, 6, and 12 months. Asterisks indicate significant differences (*$p < 0.05$). **(D)** Representative images of undecalcified tibia sections (toluidine blue staining) from 6-month-old littermate mice with the indicated genotypes show that the majority of endocortical bone surfaces in *Ccl5*-deficient animals are not covered by osteoblasts. **(E)** Quantification of the endocortical osteoblastic cell-free bone surface (BS) in wildtype and *Ccl5*-deficient littermate mice does not only demonstrate the severity of this phenotype at 3 and 6 months of age, but also that this pathology is normalized in 12-month-old animals. Asterisks indicate significant differences (*$p < 0.05$). These data are based on a published study (57).

skeletal elements and areas, since the phenotype of 6-month-old $Ccl5^{-/-}$ mice was much more pronounced in the cortical bone compartment of tibia sections than it was in the trabecular bone compartment of spine sections. Third, and most importantly, the transient nature of the $Ccl5^{-/-}$ phenotype, which is potentially explained by functional redundancy, raises the important question, if similar compensatory mechanisms exist in other mouse models and/or patients. If so, it might be required to study skeletal phenotypes of mouse models lacking specific chemokines or chemokine receptors at various ages and to identify, if possible, other chemokines with the ability to compensate a single gene deficiency. On the other hand, it is essentially not too surprising that inactivation of one specific chemokine does not translate into a severe and persistent bone pathology, which might also explain, why there is still no evidence for mutations in a chemokine-encoding gene as a cause of a monogenic skeletal disorder.

## CCL20

CCL20 (also known as MIP-3α), attracts T cells, B cells and dendritic cells via CCR6 and is important in the mucosal immune system. *In vitro* studies suggested a role for CCL20 in osteoclastogenesis. Here it was found that, upon stimulation with CCL20, primary human osteoblasts expressed elevated IL-6 levels (100). Likewise, treatment of human peripheral blood monocytes with conditioned medium from CCL20-treated osteoblasts induced osteoclast formation, which could be inhibited by neutralizing anti-IL-6 antibody. Thus, CCL20 indirectly affects osteoclastogenesis by inducing IL-6 expression *in vitro*. On the other hand, mice deficient for *Ccr6*, which encodes the sole receptor for CCL20, did not display a defect in osteoclast formation, indicating that this mechanism might not be relevant under physiological conditions (101). However, despite there was no phenotype related to osteoclastogenesis in either $Ccr6^{-/-}$ mice or $Ccl20^{-/-}$ mice, both models displayed

reduced trabecular bone mass (101). This was attributed to decreased bone formation, as these mice had reduced osteoblast numbers. Moreover, the authors found that the expression of *Ccr6* and *Ccl20* increased in the course of osteoblast differentiation, that osteoblast differentiation *in vitro* was delayed in cells from $Ccr6^{-/-}$ mice, and that CCL20 promoted the survival of wildtype osteoblasts. Thus, the CCL20/CCR6 axis seems to have a physiological role in the regulation of bone formation in mice, by regulating osteoblasts, but not osteoclasts.

On the other hand, studies of disease models and patients suggested, that CCL20 plays a role in pathological bone loss. For instance, breast cancer cells were shown to express CCL20 and this expression negatively correlated with survival in patients (102). In line with this, treatment of a breast cancer bone metastasis mouse model with a neutralizing anti-CCL20 antibody could inhibit metastasis and osteolysis (102). Furthermore, CCL20/CCR6 signaling was shown to play a role in MM. CCL20 expression in osteoblasts correlated with osteolytic lesions in MM patients, and MM cells were shown to induce osteoblastic CCL20 expression, leading to osteoclast recruitment (103). Besides in cancer, the CCL20/CCR20 axis was shown to be involved in inflammation-induced bone loss. Inflammatory mediators were shown to induce CCL20 expression in cultured osteoblasts and to stimulate the formation of pre-osteoclasts, while *in vivo* CCL20 was found to be induced in subchondral bone of RA patients (104).

While these data suggest a critical role of CCL20/CCR6 in pathological bone loss disorders, it is somehow surprising that the *Ccr6*-deficient mice only displayed reduced bone formation. Although this may raise critical questions about the suitability of mouse models for complex human pathologies, the comparative analysis of mice deficient in specific chemokines and their receptors is undoubtfully informative, especially since the discrepancy of the respective phenotypes clearly demonstrates that there is true specificity regarding chemokine influences on bone remodeling cell types.

## Additional CC-Chemokines With Putative Influence on Bone Remodeling

Besides the four CC-chemokines discussed above, there are additional studies providing evidence for other family members as regulators of bone remodeling cell types. Although their (patho)physiological impact needs to be further investigated, it is certainly relevant to refer to the respective studies in the present review article.

**CCL4** (also known as MIP1-β), which can bind to CCR1 and CCR5, was shown to be induced during osteoclast differentiation of RAW264.7 macrophages. Moreover, neutralization of CCL4 inhibited RANKL-induced osteoclast migration, but not their differentiation (105). In line with this observation, another study reported that treatment of mouse osteoclast progenitor cells with CCL4 did not influence RANKL-mediated osteoclastogenesis. However, the decrease in expression of its receptor CCR5 during osteoclast formation, was shown to be essential for osteoclastogenesis (106).

Finally, with respect to CC-chemokine receptors, there is evidence for a role **CCR3** in bone remodeling. CCR3, which binds several ligands, including CCL5 and CCL11, is highly expressed on eosinophils and basophils. Circulating human monocytes were also shown to express CCR3 and this expression was negatively correlated with bone mineral density (86). Therefore, the skeletal phenotype of mice deficient for *Ccr3* was evaluated (107). $Ccr3^{-/-}$ mice showed increased bone mineral density, and the authors hypothesized that this was due to effects on both, osteoclasts and osteoblasts. However, the study did not clarify the underlying cellular mechanisms. In another study it was found that the pro-inflammatory chemokine **CCL11** (also known as eotaxin), which predominantly binds to CCR3, is elevated in plasma of osteoarthritis patients (108). CCL11 was further identified to be the most significantly induced chemokine in the early phases of RA (109). In a bone inflammation mouse model, CCL11 was shown to be expressed by osteoblasts, concomitant with increased osteoclastogenesis and bone resorption, and that treatment of osteoclasts with CCL11 increased their resorptive activity on bone slices (110).

Taken together, there is huge complexity of the chemokine system, where certain receptors bind different ligands, and where deficiency of specific chemokines is potentially compensated by others. On the other hand, there are distinct bone phenotypes reported for various mouse models, where the lack of one chemokine or its receptor causes cell-specific impairments. Together with the data obtained in these models and/or patients with inflammatory bone loss or metastatic bone disease, the collective findings provide strong evidence that at least some CC-chemokines and their receptors are relevant in bone remodeling regulation. The same applies for CXC-chemokines, which will be discussed in the next section.

# INFLUENCE OF CXC-CHEMOKINES ON BONE REMODELING IN HEALTH AND DISEASE

Similar to the CC-chemokines there is also strong evidence for the impact of specific CXC-chemokines on skeletal cell types under physiological and pathological conditions. We will again focus on the most established and/or relevant ligands, i.e., CXCL2, CXCL9, and CXCL12 in the following paragraphs. Whereas, CXCL2/CXCR2 signaling has again been linked to osteoclastogenesis, CXCL9 may play a unique role in the coupling of angiogenesis and bone formation. Moreover, the probably best established chemokine receptor pair, CXCL12/CXCR4, plays a key role in recruiting specific cell types into the bone marrow microenvironment, which is particularly relevant in metastatic bone disease. Again, the impact of specific chemokine influences on either osteoclast or osteoblast differentiation are depicted in the simplified schematic representation (**Figure 1**).

## CXCL2

CXCL2 (also known as MIP2-α) recruits neutrophils during inflammation via its receptor CXCR2 and is mainly produced by monocytes and macrophages. CXCL2 was shown to stimulate

osteoclast formation *in vitro*, and the same was reported for an alternative CXCR2 ligand i.e., CXCL1 (111). Of note, this finding was made in the context of a study analyzing the role of CXCR2 signaling in marrow adipocyte-driven osteoclastogenesis (111). More specifically, adipose bone marrow, which commonly occurs in aging and obesity, was shown to induce osteoclast formation by expressing increased levels of CXCL1 and CXCL2, which in turn could be inhibited by antagonizing CXCR2. A different study reported that osteoclast precursor cells also expressed CXCL2 upon RANKL-stimulation and that osteoclast formation could be blocked by antagonizing CXCR2 (112). *In vivo* studies could confirm the pro-osteoclastogenic function of CXCL2. In mice, the injection of CXCL2 induced calvarial osteolysis (112), while osteolysis after LPS treatment was attributed to increased CXCL2 expression, since the LPS effect was be blocked with a neutralizing anti-CXCL2 antibody (113). The potential human relevance of CXCL2 is supported by two studies. In fact, CXCL2 was found to be induced in bone tissue surrounding bacterially infected implants (114), and patients with RA had elevated CXCL2 levels in their synovial fluids and sera (112).

A very recent publication demonstrated that CXCL2 might also inhibit osteoblast differentiation (115). In fact, osteoblasts in ovariectomized mice were shown to express increased levels of CXCL2 compared to sham operated controls, while injection of a neutralizing anti-CXCL2 antibody into the femoral cavity of these mice alleviated osteoporosis. Additionally, *in vitro* experiments showed that overexpression of CXCL2 in osteoblasts increased their proliferation at the expense of differentiation by inhibition of ERK1/2 signaling upstream of RUNX2, a transcription factor required for osteoblastogenesis. On the other hand, mice deficient for CXCR2 were smaller and lighter compared to wildtype littermates, had a lower trabecular bone volume with reduced cortical BMD and thickness, and their long bones had decreased mechanical properties (116). Also, the healing of calvarial defects in *Cxcr2*$^{-/-}$ mice was delayed. Surprisingly however, no differences in either number or activity of osteoblasts and osteoclasts were found in *Cxcr2*$^{-/-}$ mice. The authors argued that the role of CXCR2 in bone was rather related to its pro-angiogenic function and less to its effect on skeletal or immune cells. The fact that CXCR2 binds various chemokines with different functions (CXCL1-3, CXCL5-8), and is expressed by a variety of cells, might explain why the analysis of *Cxcr2*$^{-/-}$ mice provided contradicting results (43).

In conclusion, there is *in vitro* and *in vivo* evidence indicating that CXCL2 influences bone remodeling by promoting osteoclastogenesis and inhibiting osteoblast differentiation. Whether these effects are mainly mediated by CXCR2 remains to be established, and the same applies for the potential relevance of CXCL1/CXCR2 signaling for physiological and pathological bone remodeling in humans.

## CXCL9

CXCL9 (also known as MIG) is an ELR-negative, angiostatic chemokine which is strongly induced by interferon-ɣ (INFɣ). Similar to CXCL10 and CXCL11, CXCL9 exerts its immunological function through CXCR3, which is found on T cells and endothelial cells (117, 118). The main immunological role of CXCL9 is to attract CD4$^{+}$ Th1 cells and CD8$^{+}$ effector T cells to sites of inflammation. A recent publication by Huang et al. (119) has suggested an additional role for CXCL9 in the regulation of bone remodeling and vascularization. It was shown that osteoblasts constitutively express CXCL9 to regulate bone angiogenesis and osteogenesis. More specifically, in order to study the role of mammalian target of rapamycin complex 1 (mTORC1) signaling in bone remodeling, the authors generated mice with either constitutively activated or inactivated mTORC1 in mature osteocalcin-expressing osteoblasts. The major factor influencing osteogenesis and angiogenesis, which was positively regulated by mTORC1, was identified as CXCL9. It was further shown that CXCL9 inhibited angiogenesis by sequestering VEGF and preventing its binding to VEGFR. Moreover, CXCL9 was shown to inhibit osteoblast proliferation, differentiation and mineralization *in vitro* through a VEGF-dependent mechanism.

Of note, our own work related to the skeletal phenotype of mice deficient for fetuin-A (also known as α2-HS glycoprotein, encoded by the *Ahsg* gene), further suggested a critical role for CXCL9 during endochondral ossification (120). Fetuin-A is a hepatic plasma protein with high affinity to calcium phosphate, which explains its high abundance in the mineralized bone matrix (11, 121–123). Fetuin-A has been established as an important inhibitor of ectopic calcification (124), and shortened femoral bones in *Ahsg*$^{-/-}$ mice indicated a role for this protein in endochondral ossification (125, 126). We found that *Ahsg*$^{-/-}$ mice develop epiphysiolysis in their distal femora, which prompted us to perform a transcriptome analysis of the growth plates prior to growth plate slippage (120). The by far most strongly induced gene in *Ahsg*$^{-/-}$ growth plates was *Cxcl9* with an increase of >500-fold compared to wildtype littermates. In line with the findings by Huang et al. (119), we additionally identified a decreased number of capillary loops at the chondro-osseous junction in *Ahsg*$^{-/-}$ mice. These data suggest that excessive CXCL9 production in the growth plate of *Ahsg*$^{-/-}$ mice causes their epiphysiolysis phenotype, yet there are further experiments needed to demonstrate such causality.

In our opinion, the combined findings regarding CXCL9 expression in skeletal cell types, are potentially relevant, since recent studies have shown that vascularization not only serves the purpose of blood supply, but also fulfills very specific developmental and functional roles (127, 128). It was shown that a specific subset of bone sinusoidal endothelial cells, which are characterized by high expression of endomucin and CD31, actively promote osteogenesis and in this manner couple vascularization and bone formation (129, 130). As chemokines, in particular CXC-chemokines, regulate inflammation, bone remodeling as well as angiogenesis it would be highly interesting to study them in the context of endochondral ossification. In this regard, CXCL9 is a good candidate molecule, yet the skeletal phenotype of a corresponding mouse deficiency model has not been analyzed to date.

## CXCL12

CXCL12 (also known as SDF-1) and its receptor CXCR4 represent one of the best studied chemokine/receptor pairs in several regards. The CXCL12/CXCR4 axis is crucial during

development, as demonstrated by the fact that mice deficient for *Cxcl12* or *Cxcr4* die prenatally due to various defects in cardiac and brain development (131–133). Furthermore, CXCL12 is pro-angiogenic (despite being ELR-negative) and recruits CXCR4-expressing endothelial progenitors (134, 135). The CXCL12/CXCR4 axis is known as the most important pathway regulating the homing of HSC and developing innate immune cells into the bone marrow niche (136). In this manner, a pool of HSC is retained in the adult bone marrow niche, and adult mice with an induced deletion of *Cxcr4* have severely reduced numbers of bone marrow HSCs (136). Two back-to-back publications highlight the importance of osteoblasts and their progenitor cells in forming specific niches for HSC by specifically deleting *Cxcl12* in different cells of the bone marrow niche, including MSCs, osteoprogenitors or mature osteoblasts (44, 45). By expressing CXCL12, perivascular, endothelial and skeletal progenitor cells are crucial to maintain and support distinct subsets of hematopoietic progenitors in the bone marrow (137, 138). Bone marrow stromal cells, which can differentiate into osteoblasts, chondrocytes, adipocytes, and other different cell types, were shown to express CXCL12 and CXCR4, yet the expression of CXCL12 decreased with increased osteogenic differentiation (139). Of note, there is one cell type which expresses CXCL12 at very high levels, which is termed CXCL12-abundant reticular (CAR) cell. More specifically, CAR cells reside in the bone marrow niche surrounding sinusoidal endothelial cells, as well as in the endosteal niche. They are considered to be the major source of CXCL12 in the bone marrow (136). Furthermore, a specific subset of $CXCR4^{+}CD45^{-}$ pluripotent MSCs was identified in mouse bone marrow, which expresses high levels of CXCL12, but low levels of RANK and RANKL (140). The authors proposed that these cells represent a specific microenvironment, which supports osteoclastogenesis while not being directly involved in the RANKL signaling axis.

Apart from its roles in development, angiogenesis and stem cell homing, there is evidence from *in vitro* and *in vivo* studies that CXCL12 directly interacts with skeletal cells to regulate bone remodeling. RAW264.7 macrophages were shown to express CXCR4, and this expression decreased during RANKL-mediated osteoclastogenesis (141). Furthermore, CXCL12 acts as a chemoattractant for RAW264.7 cells, enhancing their migration through collagen, and increasing their MMP9 expression. An increased expression of MMP9 as well as an increased resorption of calcium phosphate chips was reported for human osteoclasts, which were differentiated in the presence of CXCL12 (142). CXCL12 was also shown to increase bone resorption in cultured human primary osteoclasts and induce resorption-related gene expression (*Ctsk*, *Mmp9*, and *Trap*), while this effect could be inhibited by the CXCR4-selective antagonist T140 (143).

The CXCL12/CXCR4 axis also plays important roles during bone loss induced by metastasis and MM. First of all, the CXCL12/CXCR4 interaction is critical for the recruitment of metastatic cancer cells into the bone marrow niche, since these cells, by expressing CXCR4, essentially hijack the homing mechanism for hematopoietic cells (144, 145). Furthermore, one study showed that MM patients had elevated plasma levels of CXCL12 which correlated with the occurrence of osteolytic bone lesions, and MM cells were shown to express significant amounts of CXCL12 (143). Interestingly, the CXCR4-specific inhibitor T140 reduced *in vitro* osteoclast formation which was stimulated by conditioned medium from an MM cell line, which contained high levels of CXCL12. Another study from the same group demonstrated a positive correlation between plasma levels of CXCL12 in MM patients and the bone resorption marker CrossLaps (146). It was further shown that intratibial injection of MM cell lines into mice induced focal osteolytic lesions proximal to the tumor, which could be reduced by T140, while osteolysis was increased when the tumor cells overexpressed CXCL12 (146). Taken together, by expressing CXCL12, MM cells recruit osteoclast precursors to the bone, thereby inducing osteolysis. Moreover, an involvement of CXCL12 in both RA and osteoarthritis has been demonstrated in numerous studies, where it affects synovial fibroblasts, immune cells and endothelial cells, and promotes the loss of bone and cartilage (147). The CXCL12/CXCR4 axis is therefore a promising drug target in RA, and treatment of mice with collagen-induced arthritis with the CXCR4-specific antagonist AMD3100 was shown to reduce disease severity (148).

Several studies demonstrated that the CXCL12/CXCR4 signaling pathway is not only involved in osteoclast formation, but also in osteoblast differentiation. It was shown that CXCR4 regulates osteoblast differentiation in cooperation with BMP signaling, and that mice with a conditional deletion of *Cxcr4* in osterix-expressing cells were osteopenic due to a defect in osteoblastogenesis (149). Moreover, primary osteoblasts from these mice were less responsive to treatment with BMP2 or BMP6, suggesting a coupling between BMP-signaling and the CXCL12/CXCR4 axis. In a subsequent study, it was shown that the expression of CXCR4 and CXCL12 in bone marrow-derived MSCs decreases with age, concomitant with decreased potential for *in vitro* osteogenic differentiation in response to BMP2 stimulation or osteogenic medium (150). Here the restoration of CXCR4 expression in bone marrow cells of old mice corrected their osteogenic differentiation defect. It was furthermore demonstrated that CXCL12 enhanced osteogenic differentiation of stromal cells which were transduced to express higher levels of CXCL12 (139). In line with this, mice with a deletion of CXCR4 in mature *Col1a1*-expressing osteoblasts were shown to have a decreased bone mass and decreased bone formation (151). Furthermore, a recent study showed that the deletion of *Cxcl12* in *Prx1*-expressing limb mesenchyme or osterix-expressing osteoblast progenitors, but not in mature osteoblasts, induced marrow adiposity and reduced trabecular bone volume (152). Thus, deletion of *Cxcl12* in osteoblast progenitor cells or early osteoblasts increased their adipogenic differentiation at the expense of osteogenic differentiation. Furthermore, expression of osteogenic markers, parameters of bone formation and osteoblast numbers were reduced in mice with a deletion of *Cxcl12* in *Prx1*-expressing cells, while osteoclast formation and activity were not affected. In contrast, deletion of *Cxcr4* in *Prx1*-expressing cells similarly led to a reduction in bone formation, but it did not increase marrow adiposity (152). Thus, limb mesenchymal cells regulate osteogenesis in a cell-autonomous manner through CXCL12,

while the modulation of adipocyte differentiation occurs through other mechanisms.

In line with these findings, CXCL12 has been shown to regulate fracture healing through BMP2 signaling (153). More specifically, BMP2 signaling controlled the spatial and temporal expression pattern of CXCL12 by $BMP2^+$ $CXCL12^+$ perivascular endosteal cells, which were recruited to the fracture site. Deficiency of *Bmp2* in mice led to an induction of *Cxcl12* expression, leading to a deranged angiogenic response during fracture healing, which could be corrected by treatment with AMD3100 (154). Furthermore, the role of CXCL12/CXCR4 signaling in bone healing was studied in a mouse femoral bone fracture model (155). Here, *Cxcl12* mRNA expression was shown to increase during fracture healing, especially in the periosteal region. Treatment with a CXCL12-neutralizing antibody or the antagonist TF14016, a more stable analog of T140, inhibited the formation of new bone (156). The study also showed that CXCL12 recruited MSCs for bone formation during fracture repair and was also important for vascularization during bone fracture healing. Another study showed that when *Cxcl12* was deleted in *Tie2*-expressing endothelial progenitor cells, the fracture callus was less vascularized and fracture healing was delayed (157).

Finally, the CXCL12/CXCR4 axis was shown to be involved in endochondral ossification. One study in E18.5 mice showed that CXCR4 was expressed by proliferative chondrocytes, while CXCL12 was expressed by prehypertrophic and hypertrophic chondrocytes in the growth plate (149). Conditional deletion of *Cxcr4* in osterix-expressing cells, which resulted in a 70% reduction in CXCR4-positive growth plate chondrocytes, led to a disorganization of the growth plate and a decrease in growth plate proliferation. Another publication showed that in newborn mice, CXCR4 was predominantly expressed by hypertrophic chondrocytes, while CXCL12 was expressed in the adjacent bone marrow (158). Here it was shown that CXCR4/CXCL12 signaling induced chondrocyte hypertrophy and that this was regulated in a positive feedback-loop, which was mediated by RUNX2.

Taken together, there is a huge amount of evidence, both *in vitro* and *in vivo*, showing that CXCL12 has remarkable influences in several aspects of skeletal biology (**Figure 3**). Through interaction with CXCR4 it promotes osteoclastogenesis, but it also induces osteogenic differentiation of mesenchymal stromal cells in cooperation with BMP signaling. The CXCL12/CXCR4 axis additionally regulates growth plate chondrocyte proliferation and hypertrophy during development, at least in mice. The most critical impact however is probably related to cancer metastases, since the respective tumor cells apparently hijack the CXCL12-mediated homing to the bone marrow by expressing CXCR4. In this regard, blockade of CXCR4 might be a valuable approach to prevent the detrimental interaction of cancer and bone remodeling cells and the development of osteolytic lesions. Currently, the most established CXCR4 antagonist is AMD3100 (Plerixafor) (154, 159–161). Originally developed as an antiviral agent against the replication of HIV, this drug is now widely used for the mobilization of HSC for autologous stem cell transplantation in lymphoma and MM patients. However, the low oral bioavailability of Plerixafor makes it less suitable for longer treatments. Therefore, the safety and efficacy of other CXCR4 antagonists is currently being evaluated in clinical trials (162, 163).

## Additional CXC-Chemokines With Putative Influence on Bone Remodeling

In addition to the three CXC-chemokines discussed above, it is again important to refer to studies on the putative impact of other CXC-chemokines as regulators of skeletal remodeling. In these cases the *in vivo* significance is less established so far, which however does not mean that the influences of the respective molecules on skeletal cell types are less relevant.

**CXCL8** (also known as IL-8) is a ligand for both, CXCR2 and CXCR1. Similar to CXCL2, it is secreted by macrophages and also by epithelial and endothelial cells. Its role in bone remodeling has mainly been studied *in vitro*. First, osteoblasts and osteoclasts were shown to express CXCL8 upon stimulation with inflammatory mediators (164, 165). Primary human osteoblasts stimulated with CXCL8 expressed elevated IL-6 levels and conditioned medium from these cells induced osteoclast formation in human peripheral blood monocytes, which could be inhibited by neutralizing anti-IL-6 antibody (100). Furthermore, treatment of human osteoclast precursor cells with CXCL8 in the presence of M-CSF was shown to induce the formation of $TRAP^+$ osteoclasts, and it was found that these cells were able to resorb bone in the absence of RANKL (166). Thus, CXCL8 stimulates bone resorption through direct and indirect mechanisms. A role for CXCL8 in bone metastatic disease was demonstrated in studies with breast cancer cells (166, 167). More specifically, the bone-tropic subclone MDA-MET derived from the human breast cancer cell-line MDA-MB-231 was found to secrete high levels of CXCL8. After tibial injection of MDA-MET, all recipient mice developed large osteolytic bone metastases, whereas treatment with a CXCL8-neutralizing monoclonal antibody prevented tumor formation in 85% of the mice (167). Finally, breast cancer patients with bone metastases were shown to have elevated CXCL8 plasma levels compared to patients without metastasis, and the CXCL8 plasma levels correlated with increased bone resorption (167). These data suggested that CXCL8 could be a promising drug target for breast cancer bone metastasis.

Like CXCL2 and CXCL8, **CXCL5** (also known as LIX) is a chemoattractant for neutrophils via the receptor CXCR2. *In vitro*, CXCL5 was found to be induced by IL-17 in osteoblasts (168). *In vivo*, increased CXCL5 expression was found in individuals with Paget's disease of bone (169), where a local dysregulation of bone remodeling causes high bone turnover (170). More specifically, these patients displayed a 180-fold higher expression of *CXCL5* in bone marrow cells, and a 5-fold increase of CXCL5 serum levels (169). By utilizing chromatin immunoprecipitation, the authors additionally found that CXCL5 increased RANKL expression in human bone marrow-derived stromal cells through the phosphorylation of CREB.

Finally, **CXCL10** (also known as IP-10), similar to CXCL9, also binds to CXCR3. A potential role for CXCL10 in bone remodeling

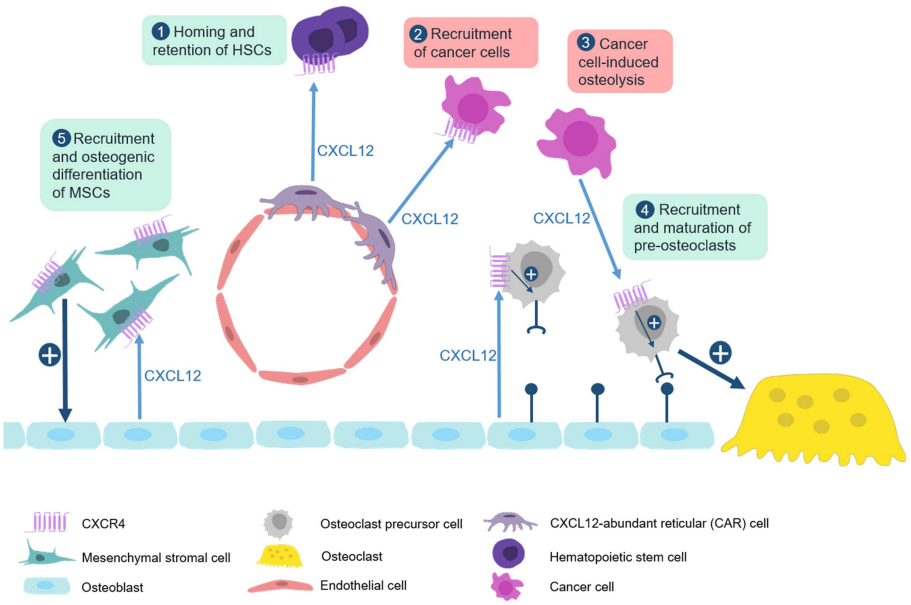


**FIGURE 3 |** The CXCL12/CXCR4 axis in physiological and pathological bone remodeling. Numerous studies have established that the CXCL12/CXCR4 axis is not only required for homing of hematopoietic stem cells, but also for the regulation of bone remodeling cell types in physiological and pathological conditions. (1) CXCL12, which is predominantly expressed by CXCL12-abundant reticular (CAR) cells, binds to CXCR4 on hematopoietic stem cells to recruit them to bone microenvironment. (2) This mechanism is also used by CXCR4-expressing metastatic cancer cells which explains their recruitment to the bone marrow niche. (3) CXCL12 expression by multiple myeloma cells enhances recruitment and maturation of pre-osteoclasts by inducing RANK expression. (4) Osteoblasts also express CXCL12 to physiologically regulate migration and maturation of osteoclast progenitor cells. (5) CXCL12 additionally cooperates with BMP signaling to promote osteogenic differentiation of mesenchymal stromal cells.

was identified in mice with an osteoblast-specific deletion of menin-1 (171), which develop an osteoporotic phenotype due to increased bone resorption. In an unbiased approach, it was shown that osteocytes from these mice express increased levels of CXCL10, and that treatment with anti-CXCL10 antibody could normalize osteoclast activity *in vivo*. In addition, it was reported that CXCL10 is involved in the recruitment of CXCR3-expressing cancer cells to the bone marrow leading to bone metastasis formation, induction of osteoclast differentiation and osteolysis, while treatment with anti-CXCL10 antibody decreased metastasis formation *in vivo* (172). Finally, CXCL10 has been shown to promote bone loss in a mouse model of collagen-induced arthritis (173).

Again, similar to the CC-chemokines, these latter examples illustrate that there are many different studies supporting a critical function of specific chemokines in physiological and pathological bone remodeling, most of them performed in cultured cells or in mouse deficiency models. The large amount of significant influences reported by many different investigators raises the critical question about the relative importance of the respective findings. Although it is evident that some ligand receptor pairs are better studied than others, it still remains to be established, which of these interactions are truly relevant for (patho)physiological skeletal remodeling regulation in humans. On the other hand, the same level of complexity applies for other key players in osteoimmunology, i.e., cytokines. In that case, it was indeed important that cumulative evidence was obtained in different groups of patients, thereby demonstrating, for instance, that IL17A does not only increase osteoclastogenesis in cell culture assays or mice, but also in specific patient groups (27–31). Based on these arguments, there is probably even more research necessary to clearly define chemokine receptor pairs, which could also serve as drug targets for patient treatment.

## CX3CL1

In addition to CC- and CXC-chemokines, there is one chemokine with pronounced influence on bone remodeling, i.e., CX3CL1, which does not fall into the two classical categories. Of note, CX3CL1 (also known as fractalkine) is a membrane-bound chemokine, which can be proteolytically processed to release a soluble domain that attracts cells expressing the receptor CX3CR1. Moreover, the uncleaved membrane protein can mediate a direct cell contact between *Cx3cl1*- and *Cx3cr1*-expressing cells. It was shown that CX3CL1 is expressed by osteoblasts, while its receptor CX3CR1 is present on osteoclast progenitors (174). Whereas, the soluble domain of CX3CL1 induces chemotaxis of osteoclast progenitors, the interaction of membrane-bound CX3CL1 expressed by osteoblasts with CX3CR1 on osteoclast progenitors was found to induce terminal differentiation of the latter. Moreover, administration of a CX3CR1-neutralizing antibody inhibited not only the osteoclastogenesis-promoting influence of co-cultured

osteoblasts, but also the number and activity of osteoclasts in wildtype mice (174).

The physiological relevance of these findings was supported by skeletal phenotyping of CX3CR1-deficient mice, which display moderately, yet significantly increased trabecular bone mass, mostly explained by reduced numbers of osteoclasts (175). *Ex vivo* experiments with primary CX3CR1-deficient osteoblasts and/or osteoclasts suggested that this phenotype can be explained by a dual mechanism, i.e. a reduced RANKL/OPG ratio produced by CX3CR1-deficient osteoblasts, and a cell-autonomous osteoclastogenesis defect of CX3CR1-deficient bone marrow cells. Another *in vivo* study of irradiation-induced osteoclastogenesis in mice showed, that circulating pre-osteoclasts, displaying high expression of CX3CR1, are attracted by vascular expression of CX3CL1 (176). More specifically, bone loss in these mice was less pronounced, when the transplanted bone marrow cells were derived from CX3CL1-deficient mice or when a CX3CR1-neutralizing antibody was injected. In line with these findings, the expression of CX3CL1 in synovial fibroblasts has further been linked to osteoclast-mediated bone destruction (177). Moreover, CX3CL1 expression in osteoblasts was found remarkably induced by inflammatory cytokines, and CX3CR1 was identified as a marker for inflammatory osteoclasts (178–180).

Overall, these data suggest that CX3CL1 promotes osteoclast-mediated bone loss. Importantly, a neutralizing antibody against CX3CL1 is already studied in clinical trials for the treatment of inflammatory disorders, including RA (177). So far it has been shown that this monoclonal antibody (E6011) is safe and well-tolerated in RA patients, yet its efficacy for reducing joint destruction remains to be studied in larger cohorts (181).

## ATYPICAL CHEMOKINE RECEPTORS

As stated in the introduction, the complexity of chemokine signaling is further enhanced by the existence of four atypical chemokine receptors (ACKR1-ACKR4), which do not induce classical G-protein coupled signaling (40–42). While ACKR1

**TABLE 1 |** Influences of the most established chemokines on physiological and pathological bone remodeling.

| Ligand | Receptor | Impact on physiological bone remodeling | Impact on pathological bone remodeling |
|---|---|---|---|
| CCL2/MCP-1 | CCR2 | • Stimulation of osteoclastogenesis (55–57) | • Fracture healing (74, 75)<br>• Osteoporosis (55, 58)<br>• PTH treatment (59–61)<br>• Bone metastasis (62–66)<br>• Multiple myeloma (67)<br>• Bacterial inflammation (69–71) |
| CCL3/MIP1-α | CCR1, CCR5 | • Stimulation of osteoclastogenesis (77–84) | • Fracture healing (85)<br>• Osteoporosis (87)<br>• Multiple myeloma (77, 83, 91–93)<br>• Rheumatoid arthritis (88, 89)<br>• Bacterial inflammation (114)<br>• Osteoarthritis (108) |
| CCL5/RANTES | CCR4, CCR5, CCR1 | • Osteoblast migration and bone formation (57, 97)<br>• Inhibition of osteoclastogenesis (57) | |
| CCL11/Eotaxin-1 | CCR3 | • Stimulation of osteoclastogenesis and bone formation (107) | • Rheumatoid arthritis (109, 110)<br>• Osteoarthritis (108) |
| CCL20/MIP3-α | CCR6 | • Stimulation of osteoclastogenesis (100, 101)<br>• Osteoblast differentiation (101) | • Bone metastasis (102)<br>• Multiple myeloma (103)<br>• Rheumatoid arthritis (104) |
| CXCL2/MIP2-α | CXCR2 | • Stimulation of osteoclastogenesis (111, 112, 116) | • Bacterial inflammation (113, 114)<br>• Rheumatoid arthritis (112) |
| CXCL5/LINX | CXCR2 | | • Paget's disease (169)<br>• Neutrophil recruitment (168) |
| CXCL8/IL-8 | CXCR1, CXCR2 | • Stimulation of osteoclastogenesis (100, 166) | • Bone metastasis (166, 167) |
| CXCL9/MIG | CXCR3 | • Inhibition of osteoblast differentiation (119)<br>• Inhibition of bone angiogenesis (119)<br>• Endochondral ossification (120) | |
| CXCL10/IP-10 | CXCR3 | | • Osteoporosis (144)<br>• Bone metastasis (145)<br>• Rheumatoid arthritis (173) |
| CXCL12/SDF-1 | CXCR4 | • Stimulation of osteoclastogenesis (141–143)<br>• Stimulation of osteoblastogenesis (139, 149, 150, 152)<br>• Endochondral ossification (149) | • Fracture healing (153, 155, 157)<br>• Bone metastasis (144, 145)<br>• Multiple myeloma (143, 146)<br>• Rheumatoid arthritis (142, 147, 148) |
| CX3CL1/fractalkine | CX3CR1 | • Stimulation of osteoclastogenesis (174–176) | • Rheumatoid arthritis (177–180) |

primarily acts by transporting the bound chemokine across the cell (182), ACKR2, ACKR3, and ACKR4 have been identified as scavenging receptors, which induce the degradation of the sequestered chemokine (42). Furthermore, ACKR2 and other scavenging ACKRs regulate the relocalization of β-arrestin from the cytoplasm to the cell surface (42), which in turn controls the activity and internalization of G-protein coupled receptors. Although there are only few studies so far, which evaluated the potential role of atypical chemokine receptors in bone remodeling, it is relevant to discuss these data, since ACKRs are now considered as key regulators of chemokine signaling.

As stated above, **ACKR1** (also known as the human blood group antigen Duffy antigen receptor for chemokines, DARC) does not induce ligand degradation, unlike ACKR2-4. Instead, after binding of the ligand, ACKR1 is internalized and transports the chemokine across the cell, a process known as transcytosis (182). This occurs for instance on endothelial cells, where ACKR1 transports chemokines across the endothelial cell barrier in order to regulate leukocyte transmigration (183). Since ACKR1 was identified as a quantitative trait locus for bone mineral density in mice, the skeletal phenotype of *Ackr1*-deficient mice was studied (184). These mice displayed a higher bone mineral density compared to wildtype controls possibly explained by reduced osteoclastogenesis. This conclusion was supported by the finding that an anti-ACKR1 antibody blocked the formation of osteoclasts *in vitro*. Moreover, when LPS was injected above the calvaria, *Ackr1*-deficient mice showed a decrease in monocyte recruitment and of TRAP-positive osteoclasts at the injection site compared to wildtype controls (185). Given the known biological function of ACKR1, this decoy receptor might be involved in the transcytosis of pro-inflammatory chemokines through the endothelial cell barrier and in this manner regulate osteoclast recruitment.

The scavenger receptor **ACKR2** (also known as D6), is internalized into the endosome and is transported back to the cell surface independent of ligand binding (186). When a chemokine is bound to ACKR2, it will detach inside of the endosome and is subjected to lysosomal degradation. As ACKR2 binds mostly pro-inflammatory chemokines, it functions to resolve chemokine-driven inflammation (187). One study investigated the role of ACKR2 during orthodontic tooth movement (OTM) (188). It was shown that ACKR2 was expressed during OTM in mature osteoclasts and early osteoblasts from wildtype mice. In *Ackr2*-deficient mice, osteoclast numbers, the expression of bone resorption markers and OTM were significantly increased. These findings are in principal agreement with the known biological function of ACKR2 as a scavenging receptor, and they suggest that therapeutic strategies increasing ACKR2 production might be useful to inhibit bone loss during inflammatory conditions.

**ACKR3** (also known as CXCR7) specifically binds CXCL12 and CXCL11 and can thus be regarded as a decoy receptor antagonizing the CXCR12/CXCR4 axis. As described above, mice deficient for *Cxcl12* or its receptor *Cxcr4* die prenatally due to various defects (131–133). Similarly, the majority of *Ackr3*-deficient mice died in the early postnatal phase due to cardiovascular defects, yet about 30% of these mice survived until adulthood (189). In reporter mice, ACKR3 was shown to be highly expressed in vascular endothelial cells, cardiomyocytes and also in osteocytes. Therefore, the skeletal phenotype was investigated at birth and at four weeks of age, however no differences between *Ackr3*-deficient mice and wildtype littermates were identified by μCT analysis. Moreover, no major differences were found after subjecting female mice to ovariectomy or male mice to orchidectomy. Thus, although ACKR3 was found highly expressed in osteocytes, it remains to be established, for instance by generating mice with cell-specific *Ackr3* deficiency, if this is linked to a functional role in bone remodeling.

Taken together, there is only a limited number of publications so far that addressed the influence of atypical chemokine receptors on physiological and pathological bone remodeling. Since ACKR2 mostly binds to proinflammatory chemokines, which were found to mediate a pro-osteoclastogenic influence, the respective findings can be regarded as the most promising ones. From a therapeutic perspective however, it would be advantageous to target a more specific interaction, as it is mediated by ACKR3.

## CONCLUDING REMARKS

As summarized in this review article, there is a huge amount of literature demonstrating that several chemokines and their respective receptors impact skeletal remodeling under physiological and pathological conditions. While the relevance of some influences needs to be supported by additional evidence, there are specific ligand-receptor pairs, which are truly established as regulators of bone remodeling cell types, based on the combined efforts by various investigators (**Table 1**). Despite the huge complexity of the chemokine system and probable functional redundancy, it is quite remarkable that many mouse models lacking specific ligands or receptors display a distinct impairment of their bone remodeling status. On the other hand, there is so far no evidence for mutations in specific genes encoding either chemokines or their receptors that would cause a monogenic skeletal remodeling disorder. Therefore, it is reasonable to speculate that chemokine signaling rather affects human bone remodeling in specific situations associated with either inflammation or the presence of tumor cells in the bone microenvironment. Since such diseases are highly prevalent, the accumulated knowledge summarized here could provide novel treatment options, by targeting chemokine signaling, for a large number of affected individuals. Based on these arguments it is still required to expand this research area in order to identify the most critical chemokine receptor pairs playing a role in human (patho)physiology.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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# Extracellular Matrices to Modulate the Innate Immune Response and Enhance Bone Healing

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Extracellular matrices (ECMs) have emerged as promising off-the-shelf products to induce bone regeneration, with the capacity not only to activate osteoprogenitors, but also to influence the immune response. ECMs generated starting from living cells such as mesenchymal stromal cells (MSCs) have the potential to combine advantages of native tissue-derived ECMs (e.g., physiological presentation of multiple regulatory factors) with those of synthetic ECMs (e.g., customization and reproducibility of composition). MSC-derived ECMs could be tailored by enrichment not only in osteogenic cytokines, but also in immunomodulatory factors, to skew the innate immune response toward regenerative processes. After reviewing the different immunoregulatory properties of ECM components, here we propose different approaches to engineer ECMs enriched in factors capable to regulate macrophage polarization, recruit host immune and mesenchymal cells, and stimulate the synthesis of other immunoinstructive cytokines. Finally, we offer a perspective on the possible evolution of the paradigm based on biological and chemico-physical design considerations, and the use of gene editing approaches.

**Keywords: tissue engineering, extracelullar matrix, immunomodulation, bone repair, innate immune system, mesenchymal stromal cell, regenerative medicine**

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## INTRODUCTION

Bone disorders have a worldwide prevalence since they can be derived from multiple causes, including orthopedic trauma, cancer or congenital diseases. Since it emerged in the early 90s, bone tissue engineering has aimed to develop innovative biological materials to improve bone repair and regeneration (1, 2). Among different biomaterials, extracellular matrices (ECMs) have been proposed as one of the best candidates to fabricate grafts for bone regeneration (3). Native tissue-derived ECMs represent a physiological solution providing not only structural support, but also multiple biomolecules capable to modulate the behavior of both resident and recruited cells in the context of bone healing (4–6). However, they exhibit limited reproducibility in their composition, can lead to pathogen transmission and lack the possibility of customization. Furthermore, native ECMs are rich in immunogenic molecules that can trigger an uncontrolled response and affect graft integration (7). Synthetic ECMs, typically in the form of hydrogels, have been developed as tunable alternatives, with promising results also in the context of bone repair (8). However, they still rely on the presentation of a limited set of signals, in ways which do not entirely recapitulate physiological processes. ECMs could be also generated from living cells, e.g., mesenchymal stromal cells (MSCs), using typical tissue engineering paradigms, and afterwards decellularized (9, 10). The resulting ECMs would in principle combine the advantages of a physiological system with the possibility

of standardization (e.g., through the use of immortalized cell lines) and tunability (e.g., by genetic modification of the cells used) (11). Decellularization of the MSC-generated ECMs can also be designed to improve the immunogenic properties of the resulting material (12, 13).

Despite many advances, the need for quality improvement of engineered ECM (either synthetic or MSC-generated) for bone healing is still quite large (3). Along this line, ECMs might be enriched in morphogens or angiogenic factors to enhance bone regeneration. Importantly, multiple evidences have revealed that a proportionated and coordinated immune system response is essential to critically promote bone healing. Indeed, many studies in the past years have revealed a broad crosstalk between the skeletal and immune systems through many shared cytokines, molecular pathways and transcription factors. All these findings have contributed to define the so-called osteoimmunology field, in which engineering ECMs to modulate immune signals has become one of the spearhead (14). In this context, current strategies do not aim to suppress the immune response, but rather engineer ECM-derived materials to present osteoimmunomodulatory factors and instruct the inescapable immune response in favor of bone regeneration (15).

In this review, we describe firstly key aspects of the interplay between innate immunity and bone healing. Then, we highlight how some ECM components are able to modulate the innate immune response. Finally, we summarize different strategies proposed for ECMs enriched with innate immunoinstructive factors to improve bone regeneration.

## INNATE IMMUNE SYSTEM IN BONE REPAIR

All bone substitute materials, as any other foreign structure, trigger a host immune reaction after implantation, which recapitulates the first steps of the classical immune response after bone injury (16, 17). In addition, implantation surgery is not more than a controlled injury. Therefore, understanding the immune cascade following bone injury is key to generate immunoinstructive scaffolds capable to enhance bone regeneration.

Immediately after any bone injury, vascular disruption generates a hematoma and triggers a quick and potent inflammatory reaction. Multiple blood and interstitial fluid proteins [e.g., Factor XII and tissue factor (TF)] adsorb the injury site and activate the blood coagulation cascade as well as the complement system (18). In this context, activated platelets play a critical role producing prothrombinases, which activate thrombin serin protease and allow the amplification of the coagulation process (19). All these proteins lead to a transient fibrin clot formation that constitutes the matrix for the recruitment of the first immune cells. In contrast with later stages, the onset of the acute inflammatory response is mostly governed by the innate immune system, whose main players are polymorphonuclear leukocytes (PMNs, neutrophils), monocytes and tissue-resident macrophages (20).

Circulating PMNs are quickly recruited by this chemoattractant protein matrix to the injury site. While they might contribute to fibrin clot formation (21), their main roles involve the release of proteolytic enzymes to promote tissue remodeling, and inflammatory cytokines (such as IL1β, TNFα, IL8, MCP1, or MIP1β) to recruit other myeloid cells and MSCs (15). Recruited monocytes release more cytokines and differentiate into macrophages. Both monocytes derived- and tissue resident macrophages have been revealed essential for successful bone formation (22). The relevance of this cell type resides in its capacity to exhibit different functional phenotypes in response to environmental cues (23). Initially, the inflammatory storm upon bone injury polarizes macrophages toward an activated M1 phenotype. M1 macrophages release more inflammatory cytokines to contribute to cell recruitment and dead cell clearing. At later stages, macrophages are alternatively polarized toward an anti-inflammatory M2 phenotype. These cells secrete tissue repair factors (IL10, IL1ra, TGFβ1, or VEGFα) to resolve the inflammation, recruit MSCs, promote angiogenesis and induce endochondral bone formation (24). Recruited MSCs undergo chondrogenic differentiation adjacent to the fracture site to form bone by endochondral ossification, while direct intramembranous ossification takes place under the periosteum (25). Interestingly, they also play a crucial paracrine role releasing immunosuppressive cytokines to resolve site inflammation. Human MSCs suppress innate immune cells migration, proliferation and differentiation through multiple pathways including Notch and PGE-2 signaling (26). Therefore, the coordinated crosstalk between MSCs/osteoprogenitor cells and macrophages is critically required for successful bone healing.

Following these principles, several studies have attempted to improve bone regeneration modulating either macrophage number or their polarization toward M1 or M2 phenotypes (27). On the one hand, it has been reported that the expression of some pro-inflammatory signals right after injury significantly improves bone healing. As examples, TNFα promotes postnatal intramembranous bone repair through the induction of osteoprogenitor cell recruitment or osteogenic cell activation (28), while IL1β administration could favor endochondral bone formation after injury (29, 30). Similarly, IL-6 family signaling was shown to stimulate bone formation during the inflammatory process (31). On the other hand, different studies have proposed that an anti-inflammatory M2 environment is more suitable for human MSC activity (32) and delivers osteoinductive signals (33). In this regard, IL4 administration could decrease bone degradation after joint replacement (34).

Accumulating evidences suggest that an appropriate transition from the inflammatory M1 to the anti-inflammatory M2 phenotype favors bone regeneration by endochondral ossification (24, 35). However, macrophage activation and polarization are very complex *in vivo*, since the exposition to multiple signaling leads to activation of macrophages with mixed functions. This is especially prominent in pathological conditions, where abnormal signaling might prime macrophages toward a profibrotic phenotype (36). Indeed, macrophage

activation nomenclature has been recently revised to unify criteria for the diverse experimental scenarios (37).

In the context of ECM engineering, some researchers have used myeloid cells to improve ECM-derived grafts integration after implantation and/or promote bone healing after trauma or bone degeneration. Although the supplementation of ECM-derived grafts with peripheral blood monocytes did not seem to increase bone regeneration by itself (38), peripheral blood-derived macrophages were reported to be essential in the degradation and remodeling of ECM-based materials (39). Other studies have developed strategies to generate immunoinstructive ECMs by modulating macrophage polarization during bone healing and promote bone formation (40). However, the success of these approaches is often subjected to several variables like patient health, trauma size or ECM composition.

## ECM COMPOSITION AND INNATE IMMUNITY

Many endogenous ECM components exhibit important immunomodulatory features that can decisively influence the innate immune response *in vivo* (41, 42). For example, the collagenous network is, together with the proteoglycans, the main component of bone tissue ECM that defines its mechano-physical features. However, collagen fibers exhibit motifs that can interact with some immune cell receptors. In particular, macrophages can specifically adhere to denatured forms of collagen type I fibers through their scavenger receptors (43). Furthermore, collagen fibers have been reported to affect metalloproteinase 9 (MMP9) secretion on the macrophage-like U937 cell line (44).

Hyaluronic acid is one of the most important glycosaminoglycan of native ECMs and it has been proposed to play a dual immunomodulatory role based on its molecular weight. Whereas, intact high molecular weight hyaluronic acid has a prominent anti-inflammatory effect inducing IL10 production by macrophages, damaged low molecular weight hyaluronic acid promotes a pro-inflammatory phenotype stimulating TNFα expression (45). Interestingly, this immune cells-hyaluronic acid crosstalk seems to be bidirectional, since monocyte activation can modulate its binding to hyaluronic acid too. More specifically, TNFα promotes monocytes-hyaluronic acid interactions through CD44 receptor, while IL4 administration is sufficient to abrogate this effect (46). Heparan sulfate, another important glycosaminoglycan that binds to ECM proteins to form proteoglycans, can also interact with the immune system to regulate cell adhesion, the availability of immune cytokines and leukocyte migration (47).

Importantly, not only components of native ECM have been reported to modulate the innate immunity. Fibrin is a molecule often used to build synthetic ECMs, which has been also shown to modulate macrophages behavior. This protein derives from fibrinogen after thrombin proteolytic activity and it is involved in the hemostatic clot formation after injury (48). Several studies have reported that fibrin could facilitate or block macrophages migration depending on its abundance in the matrix (49), and inhibit their pro-inflammatory properties (50). In contrast, fibrin degradation products induce leukocyte recruitment (51) and promote pro-inflammatory (IL1β, IL6) cytokines secretion by monocytes *in vitro* (52).

ECMs can also contain cryptic domains very similar to immune cytokines that are only exposed after proteolytic activity by metalloproteinases. In non-physiological conditions, the aberrant expression of these domains by exacerbated tissue remodeling can influence immune cell activation and survival (53, 54). Moreover, the decellularization step followed to generate non-immunogenic off-the-shelf grafts could also condition the immunomodulatory properties of ECM components. Pioneering work from Badylak using the bladder system showed that decellularized grafts preferentially induce an anti-inflammatory macrophage polarization, while cellular components trigger a pro-inflammatory polarization (55, 56).

Furthermore, different types of ECMs seem to induce a different innate immune response *in vivo*. For example, decellularized bone-derived ECM has a higher capacity to induce monocytes recruitment than cardiac ECMs, which might reflect the differential molecular composition of these matrices (57).

In summary, ECMs exhibit intrinsic immunomodulatory features which are mostly determined by their molecular composition. Therefore, a precise knowledge of the components of ECMs is essential to further develop their immunomodulatory properties with extrinsic factors.

## EXOGENOUS DELIVERY OF SPECIFIC IMMUNOREGULATORS IN ENGINEERED ECMs TO MODULATE THE INNATE IMMUNE RESPONSE

In order to modulate the innate immune response upon implantation, pro-inflammatory or anti-inflammatory cytokines can be directly delivered into the grafts. To antagonize the pro-inflammatory effect of IL1β, inhibitors of IL1R1/MyD88 signaling were covalently cross-linked into fibrin matrix to improve MSC-based bone regeneration in mice (58).

Immune cytokines could be also delivered sequentially in order to facilitate the transition between the inflammatory and anti-inflammatory phases during bone healing. For instance, Spiller et al. physically adsorbed IFNγ onto the scaffolds and attached IL4 using biotin-streptavidin binding to drive the sequential polarization of macrophages from M1 to M2 phenotype. These scaffolds also exhibited increased vascularization upon *in vivo* implantation, which proved their functionality (59). Along the same line, another study confirmed that IL4 released from a nanometer-thickness coating is critical promoting the M1-to-M2 transition during bone tissue repair and improving implant integration (60). Recently, Schlundt et al. further demonstrated the importance of M2 macrophages to induce endochondral ossification in the context of bone healing. Indeed, they added IL4 and IL13 to the collagen scaffolds prior to insertion in an osteotomy model. In this way, they

stimulated M2 macrophage polarization and improved bone regeneration (24).

In addition to interleukins, synthetic peptides represent an alternative way to modulate the immunomodulatory features of ECMs. The peptide Arg-Gly-Asp (RGD), contained in basement membranes components such as entactin or presented in photopolymerizable poly(ethylene glycol) (PEG)- based hydrogels, has been shown to enhance myeloid cells adhesion to the ECM (61, 62), while it induces macrophage polarization toward an anti-inflammatory profile via integrins interactions (63). As another example, a synthetic peptide binding to LAIR1, a receptor expressed in multiple immune cells, has been reported to reduce pro-inflammatory cytokines release by BM-derived macrophages. Interestingly, this effect was only observed when the peptide was linked to the scaffold surface (64). On the other side, TP508, a synthetic 23-aminoacid peptide representing a receptor-binding domain of human thrombin, promotes bone healing in a rat femoral fracture model by inducing inflammatory mediators release and angiogenesis (65). Adsorbed fibrinogen or scaffolds made of this material could also elicit a favorable immune response and improve the osteogenic capacity in a critical size bone defect in rats (66, 67). Among lipid compounds, specific prostaglandin agonists administration could enhance bone formation after injury avoiding systemic inflammation induction (68, 69). For example, prostaglandin E EP4 receptor agonist was shown to synergize with BMP2 and activate osteoprogenitor cells when delivered in a biodegradable copolymer composed by poly-D,L-lactic acid with random insertion of p-dioxanone and polyethylene glycol (70).

The anti-inflammatory properties of glucocorticoids are well-known. In particular, dexamethasone delivery in polydimethylsiloxane-based 3D scaffolds has been used to promote macrophage polarization toward an anti-inflammatory (M2) phenotype and suppress inflammatory pathways during the first week post-implantation (71). Dexamethasone delivery using poly (lactic-co-glycolic acid) microsphere/polyvinyl alcohol hydrogel composites has been shown to elicit an anti-angiogenic effect which could be overcame by co-administering VEGF (72).

Different approaches have been here discussed to deliver immunoregulatory factors into ECM in order to instruct the innate immune response *in vivo*. Nevertheless, the delivery of exogenous factors is subjected to several drawbacks including poor matrix penetration, diffusion, enzymatic degradation and thus uncontrolled doses. In addition, the delivery of few specific agents has been revealed inefficient in triggering a complete immune response *in vivo*. For this reason, different strategies have been developed to control the spatial and temporal delivery (73–75). Among them, 3D multilayer systems and intelligent hydrogels have been tested for the sequential release of several factors to ECM-based scaffolds (76, 77). Biomimetic biomaterials, like hydrogels, have been developed to achieve a molecular-level modulation. This includes strategies to immobilize incorporated factors by cross-linking and approaches based on protease-dependent degradation to release them (78). Other options to engineer immunoinstructive ECMs directly target MSCs or immune cells to modulate the natural production and release of immune factors by these cells.

## ECM-DRIVEN ENDOGENOUS SYNTHESIS OF IMMUNOREGULATORS BY HOST CELLS TO MODULATE THE INNATE IMMUNE RESPONSE

Aiming to generate ECM grafts instructed to trigger a more physiological immune response, many researchers have tried to use several biological agents to stimulate host MSCs and/or immune cells to deliver key immune cytokines and enhance bone formation. Macrophage recruitment is critical for dead tissue clearance and modulate the inflammatory cascade in bone healing. Kim et al. used a sphingosine-1 phosphate agonist in combination with platelet-rich plasma to sequentially induce pro-inflammatory (TNFα) and anti-inflammatory (OPG, IL10, and TGFβ1) signals in order to promote macrophages recruitment and enhance bone healing (79). In contrast, adding high sulfated hyaluronan to collagen I-enriched ECMs impairs the secretion of IL1β, IL8, IL12, and TNFα, while it enhances the production of IL10 and CD163 expression in macrophages (80).

Interestingly, inorganic compounds like magnesium-doped calcium phosphate cement are also able to elicit a favorable innate immune reaction modulating macrophage activity to improve osteogenesis and angiogenesis. This compound represses TNFα and IL6 expression while it upregulates TGFβ1 in macrophages (81). Beyond macrophage activation, immunoregulators have been also used to modulate MSC behavior. For example, the combination of RGD peptide and 3D hyaluronic acid hydrogels can influence MSC integrin expression (82).

To sum up, these studies attempt to improve bone regeneration by targeting endogenous MSC/immune cells to produce themselves the cues critical for an orchestrated repair upon bone injury (83).

## CONCLUSIONS AND PERSPECTIVES

In this work, we have reviewed some relevant aspects of the interplay between the innate immune system and osteogenesis in the context of bone healing. Then we have focused on the interactions between ECM components and innate immune cells to finally discuss some strategies followed to immune-instruct ECMs. However, many other critical aspects have not been discussed here.

As previously mentioned, the innate immunity plays an essential role during the initial phases after bone injury, promoting cell immunorecruitment and modulating the inflammatory environment (M1-to-M2 paradigm). Importantly, the adaptive immune response takes slowly part in this regulation to instruct the bone formation phase. Multiples studies have attempted to engineer ECM-based materials to modulate the adaptive immune response, specially targeting T cells (84). Indeed, many efforts are currently conducted to better

coordinate the activity of both branches of the immune response after engineered graft implantation.

We have discussed how different ECM components, even in the absence of immunomodulatory factors, could modulate the innate immune response. The works studying these interactions reveal that ECM composition is an important factor to consider prior to any further immunoregulatory engineering. However, beyond its chemical properties, ECM physical features can also decisively modulate the immune response *in vivo* (85). Therefore, strategies to enrich ECMs in immunoinstructive factors should be coupled with the engineering of endogenous physical and chemical properties of the ECM used (86).

Different approaches have been proposed to improve the spatiotemporal delivery of growth factors to engineer "smart" ECMs. However, in most cases they only focus on osteogenic and angiogenic factors. Immunomodulatory ECM-like microspheres have been recently used to improve IL4 delivery and accelerate bone regeneration modulating macrophage polarization (87). Future studies should aim to a coordinated delivery of osteogenic, angiogenic and immune factors according to the natural stages of bone healing.

Genetic manipulation of MSCs has also emerged as an alternative to better control the dose and temporal delivery of osteogenic and angiogenic factors into engineered ECMs to improve bone regeneration process (88). Genetically modified MSCs could contribute directly to bone formation promoting osteoprogenitor cells differentiation, but also indirectly enhancing host cells recruitment. The most followed approaches involve the expression of the osteoinductive bone morphogenetic protein (BMP) family factors to stimulate bone repair. In particular, BMP2-overexpressing cells have been successfully used to speed up the repair of critical-size bone defects in rodent models (89, 90). Other overexpressed factors like Osterix aimed to induce osteogenic differentiation (91, 92). As a master regulator of angiogenesis, VEGF has been overexpressed in different cell types to favor tissue vascularization (93). A VEGF-overexpressing MSC line gives rise to ECMs with high VEGF content and superior vasculature in an ectopic implantation model (11). In addition to its angiogenic properties, VEGF could also modulate the immune response (94). Similarly, sphingosine 1-phosphate has been reported to enhance vascularization and bone formation (95), but at the same time it also plays multiple roles in the innate immunity (96). These works represent examples of how MSC can be genetically engineered to generate ECMs enhancing osteogenesis and vasculogenesis. An analogous approach could be pursued to overexpress specific osteoimmunomodulatory factors and thus generate immunoinstructive ECMs (**Figure 1**). In this context, MSCs overexpressing IL4 and IL10 have been proposed as promising tools to mitigate chronic inflammation diseases (such as arthritis) and promote tissue regeneration (97, 98). However, their capacity to generate immunoinstructive ECMs have not been yet explored. Moreover, the development of inducible cell lines might represent an interesting refinement to control the temporal expression of these key genes (98). Delivering candidate genes efficiently into the cells without viral vectors (which may carry safety concerns) remains an open challenge (99).

In summary, important advances have been achieved in the last years to improve the quality of immunoinstructive ECM-derived grafts and their immunogenicity after implantation.

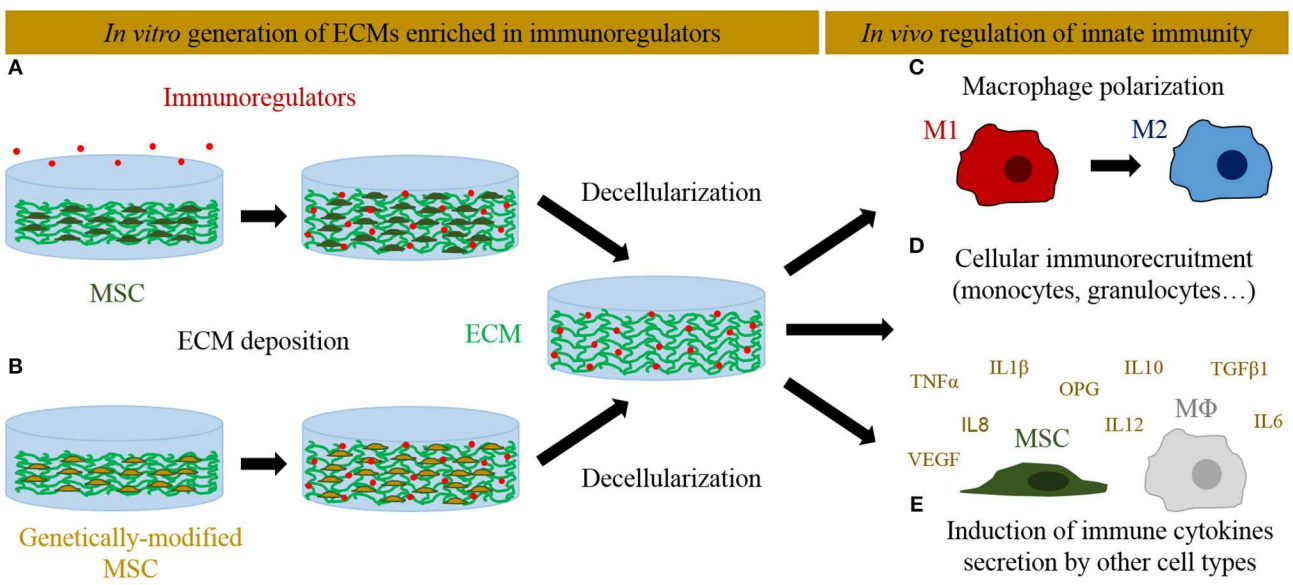


**FIGURE 1 |** Different *in vitro* approaches followed to deliver immunoregulators into ECM-derived scaffolds and their interactions with the innate immune system *in vivo*. **(A)** Immunoregulators can be directly supplemented and anchored into MSC-derived ECMs. **(B)** Alternatively, MSCs can be genetically edited to overexpress immunoregulators and seeded on scaffolds, where they will produce an ECM enriched in those factors. The tissues are later decellularized to generate cell-free ECMs. *In vivo*, these immunoinstructive ECMs can activate innate immunity at different levels: **(C)** induce macrophage polarization toward an anti-inflammatory M2 phenotype, **(D)** recruit immune cells, and **(E)** induce the secretion of immune cytokines by recruited mesenchymal stromal cells and macrophages (MΦ).

In the context of ECM engineering, immunoregulators can be exogenously delivered to enrich the biomaterial in specific cytokines and/or stimulate the endogenous synthesis of other factors by host cells. In this perspective, genetically modified MSCs represent a relevant alternative to control the spatiotemporal delivery of immunoregulators in order to engineer immunoinstructive ECMs promoting efficient bone repair.

## AUTHOR CONTRIBUTIONS

AG-G and IM prepared the figures and wrote the manuscript.

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REVIEW
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# A Jack of All Trades: Impact of Glucocorticoids on Cellular Cross-Talk in Osteoimmunology

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Glucocorticoids (GCs) are known to have a strong impact on the immune system, metabolism, and bone homeostasis. While these functions have been long investigated separately in immunology, metabolism, or bone biology, the understanding of how GCs regulate the cellular cross-talk between innate immune cells, mesenchymal cells, and other stromal cells has been garnering attention rather recently. Here we review the recent findings of GC action in osteoporosis, inflammatory bone diseases (rheumatoid and osteoarthritis), and bone regeneration during fracture healing. We focus on studies of pre-clinical animal models that enable dissecting the role of GC actions in innate immune cells, stromal cells, and bone cells using conditional and function-selective mutant mice of the GC receptor (GR), or mice with impaired GC signaling. Importantly, GCs do not only directly affect cellular functions, but also influence the cross-talk between mesenchymal and immune cells, contributing to both beneficial and adverse effects of GCs. Given the importance of endogenous GCs as stress hormones and the wide prescription of pharmaceutical GCs, an improved understanding of GC action is decisive for tackling inflammatory bone diseases, osteoporosis, and aging.

**Keywords: glucocorticoids, glucocorticoid receptor, osteoporosis, arthritis, inflammation, fracture healing, conditional knockout mice**

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## INTRODUCTION

Glucocorticoids (GCs) form one major axis of the stress response (1) and are used as immunosuppressive therapeutics in a variety of inflammatory bone diseases (2, 3). Strong impact on innate immune cells, namely macrophages, dendritic cells, and mast cells, contribute to the inhibition of inflammation. On the other hand, GCs are known to cause the most frequent secondary osteoporosis at conditions of high GC exposure. In this process myeloid cells, osteoclasts, and mesenchymal cells and their derivatives, chondrocytes, osteoblasts, and osteocytes are affected. Whereas, the cell-autonomous roles of GCs acting via the nuclear glucocorticoid receptor (GR) had been investigated intensively, the knowledge about the influence of GCs on cross-talk between innate immune cells, mesenchymal cells, and bone cells is scarce. How GCs act on cellular interactions in the osteo-immunological network is currently unraveled and is subject to this review.

## GLUCOCORTICOIDS (GCs), STRESS HORMONES AND ANTI-INFLAMMATORY AGENTS

Two different axes initiate the human physiological reaction to stress. While the activation of sympathetic-adrenal medulla (SAM)-axis starts a short-term stress reactions, long-term stress responses are mediated by the hypothalamus-pituitary-adrenal (HPA)-axis. Stress exposure results in the releases of corticotrophin-releasing hormone (CRH) from the hypothalamus, causing the synthesis of adrenocorticotropic hormone (ACTH) in the anterior pituitary gland, which activates the production of GCs in the adrenal cortex via induction of key enzymes of steroid synthesis (4).

Under long-term stress conditions GC release from the adrenal cortex also results in diverse physiological adaptations. Cortisol activates gluconeogenesis in the liver, decreases pancreatic insulin secretion, and promotes the release of glucagon. Furthermore, blood pressure elevates, the effect of catecholamines is potentiated, and a mild sodium/water-retention induced (5).

Since the first successful treatment of arthritis (6), GCs have been in frequent use and approximately 3% of the elderly population are being treated with GCs (7, 8), to reduce inflammatory symptoms in acute and chronic inflammatory diseases, including rheumatoid and osteoarthritis.

Adverse side effects of GCs on the human body have been observed upon extended treatment with daily prednisolone-doses of 7.5 mg and above. Besides the Cushingoid phenotype and osteoporosis, metabolic side effects as peripheral insulin resistance, type 2 diabetes and dyslipidemia are predominant (1). In addition, atrophy of skin and impact on the central nervous system can occur. To a similar extent, long-term GC treatment affects the cardiovascular system, resulting in hypertension, thrombotic stroke or myocardial infarction (9). These well-known side effects often preclude long-term treatment and cause occasional severe long lasting damage to the patient. Given the strong acute action of GCs to reduce inflammation, however, side effects are accepted to a certain extent in clinical praxis.

At the molecular level, intracellular GC-activity depends on the enzymes 11β-hydroxysteroid dehydrogenase type 1 and 2 (11β-HSD1 and 11β-HSD2). 11β-HSD1 catalyzes the conversion of cortisone into active cortisol, 11β-HSD2 mainly induces the reverse reaction by inactivating cortisol (10). A specific ratio of both isozymes is given in different tissue types, for example 11β-HSD1 being predominant in liver and adipose tissue (11). Molecular actions of GCs are initiated by binding to the mineralocorticoid receptor (MR) and the GC receptor (GR). Due to the wide expression of GR compared to MR and the inactivation of GCs by 11β-HSD2 in MR high expressing tissues, most of the GC effects are mediated by the GR as evident from knockout studies. However, the role of MR in inflammation is becoming more recognized and is reviewed elsewhere (12). The GR belongs to the nuclear receptor superfamily and acts as a ligand-induced transcription factor, resulting in transactivation or transrepression of genes (10). The GR structure is constituted by four domains: the transactivation domain AF1/2 (docking station for co-regulators and regulative enzymes), the DNA-binding-domain, the ligand-binding domain (binding locus for GCs) and the hinge-region (involved in translocation of GR) (10). When located in the cytoplasm GR, is in a state of high affinity to GCs and captured in a complex with immunophilins (FKBP51), heat-shock-proteins (Hsp90) and p23 (13). GC binding leads to an exchange of FKBP51 into FKBP52, resulting in translocation of the protein complex via interaction with the microtubules (10, 13). In case of nuclear transactivation, the GR tends to dimerize and bind to specific motives on target DNA, the GC response element (GRE). The ability of GCs to downregulate genes is mediated in part by GR-binding to negative GREs and consecutive recruitment of corepressors; all leading to deacetylation of histones and decrease of gene transcription [reviewed in (10, 14)]. A "tethering mode" whereby a GR-monomer interacts with DNA-bound inflammatory transcription factors (NF-κB, AP-1, STAT3, IRF3) instead of directly binding to DNA was observed for the repression of genes encoding pro-inflammatory mediators, such as cytokines and matrix metalloproteases (15). This way of cytokine-transrepression eventually leads to immunosuppression. Furthermore, crosstalk exists between DNA-bound GRs and NF-κB or AP-1 bound to transcription-factor binding-sites in the vicinity. However, both mechanisms—transactivation via dimerized GRs and transrepression via tethering of monomeric GR—are obligatory for complete anti-inflammatory GC actions (16). Non-genomic GR-effects can be observed under high-dose GC-application and modulated by GR-interaction with membranes or mitochondria (3).

Short term rise in physiological levels of GCs can stimulate the immune function, whereas immunosuppression resulting from chronic stress, favors infections or tumorigenesis (17). The immunomodulatory actions of GCs are amongst other functions achieved by priming of innate immunity. Under physiological stress conditions macrophage phagocytosis, natural killer-cell activity and cytokine production are increased (17). Furthermore, a wide range of stress-effects on leukocytes is observed: ranging from enhanced proliferation and distribution in the lymphatic system or better endothelial adhesion, to leukocyte margination and transmigration into the inflamed tissue (17). In contrast, chronically elevated GCs levels impair leukocyte proliferation and redistribution and cytokine and prostaglandin synthesis (17).

Accordingly Frank et al. (18) showed that GCs play an important role as an alarmin in neuroinflammatory priming. Stress induced high GC levels result in NLRP3 inflammasome priming, whereby the innate immune system (e.g., microglia) switches into activation mode (18). Frank et al. describe this paradox GC-induced neuroimmune activation under neuroinflammatory conditions to be an adaptive way of preparing against potential neuronal injuries or infections (18).

Thus, GCs via the GR suppress inflammatory reactions, but may also stimulate them, depending on pharmacological conditions. Whereas, for immune suppression several molecular mechanisms of the GR, transactivation of anti-inflammatory acting genes and repression of pro-inflammatory acting

genes is required, the mode of action for immune priming remains elusive.

How the different modes of action of the GR impact osteoimmunological cross-talk by influencing bone and immune cells is discussed in this review.

# GLUCOCORTICOID (GC) ACTION ON BONE: DIRECT EFFECTS AND THE MODULATION OF THE CROSSTALK OF BONE CELLS

## Cell Autonomous Effects of GCs on Bone Cells

Previous research focused on cell-autonomous effects of GC and GR action within bone cells toward bone homeostasis and insights were provided by the use of cell type specific mutant mouse strains compromising GC signaling.

Intriguingly, GCs at the physiological levels have anabolic effects on bone. They promote the formation of osteoblasts from mesenchymal progenitor cells and are essential for maintaining bone homeostasis (19). This is evident from patients (20), since fracture risk is increased during adrenal insufficiency (21) and was shown experimentally through the use of mice that have either impaired GC metabolism in the osteoblast lineage or a selective deletion of the GR. Overexpression of the GC inactivating enzyme 11β-HSD2 in mice in early differentiated osteoblasts (22–24), but not at late differentiation stages (25) led to a reduction of cortical and trabecular bone mass in adult mice. Furthermore, a defective mineralization in the calvaria was observed which was associated with diminished Wnt Signaling (26). A reduced trabecular bone mass was also seen in mice lacking the GR in the osteoblast lineages using the Runx2 as a driver for the cre expression in the cre-loxP system (27). Furthermore, GR deficient cells displayed strongly diminished differentiation potential *in vitro*. Since osteocytes are also mutant in $GR^{\text{Runx2Cre}}$ mice, currently it remains unclear how much the GR in osteocytes contributes to the bone mass at physiological conditions. Taken together, endogenous GC signaling via the GR promotes osteoblastogenesis. However, the GR is not essential for osteoblast generation. The embryonic lethal GR knockout mice (27) and mesenchymal specific GR knockout mice (28) displayed no absence of calcification in late stage embryos. Thus, GR is a positive modulator of osteoblastogenesis, but not a crucial factor. In contrast to the GR deletion in mesenchymal cells, deletion of GR in myeloid cells including macrophages, neutrophils, and osteoclasts, does not affect bone in adult mice in the absence of inflammation, indicating that osteo-immunological cross-talk in the absence of inflammation at physiological GC levels plays a minor role in controlling bone mass (27).

This becomes strikingly altered at conditions with high exposure of GCs as it occurs in steroid therapy. GC-induced osteoporosis is among the most common so-called secondary osteoporosis (29), when bone loss is induced as side effects by medication. Here exogenous GCs have contrasting effects to endogenous GCs on osteoblasts, which decreases their proliferation (30), differentiation, and induce apoptosis and modulate autophagy (25, 31–35). Whereas, induction of autophagy seems not to be decisive for inhibition of osteoblast and osteocyte function *in vivo* (36), an impaired differentiation and induction of apoptosis likely lead to decreased bone formation rate (27, 33). The molecular mechanisms of the pharmacological effects on osteoblast function are partially understood. The inhibition of proliferation and differentiation is supposed to be due to inhibition of growth factors (IGF-1, WNT proteins, BMPs), expression and inhibiting the activity of their downstream signaling pathways [reviewed in (10, 19)]. The molecular mechanisms of this inhibition involves in part the induction of inhibitory molecules such as DKK1, Sclerostin, secreted frizzled and WIF1, all antagonizing Wnt signaling (19, 37). Furthermore, negative interference of the activity of the transcription factors AP-1 and Notch had been proposed (27, 38). Recently, the involvement of miRNAs was suggested (39, 40). This was challenged by a study showing that the abrogation of dicer dependent processing of miRNAs did not inhibit decreased bone formation by GCs in osteoblast specific mutant Dicer mice (41). The induction of osteoblast and osteocyte apoptosis, another cellular phenotype associated with decreased bone formation was attributed to suppression of the pro-survival gene Bcl-XL and increase of pro-apoptotic genes BIM and BAK (42–44). Additionally the generation of reactive oxygen species by rapid activation of pro-active kinases Pyk2, and JNK were suggested (45) (**Figure 1**).

GCs also directly act on osteoclasts stimulating initial resorption after high GC exposure (46), which then declines with prolonged GC exposures. These effects are known to be mediated through the stimulatory actions of GCs on proliferation and differentiation of osteoclast precursors as well as by prolongation of their longevity (47–49). In contrast, early progenitors are attenuated by GCs (48, 49). This latter effect might explain the decline of resorption at very long GC treatments. Nonetheless, once the osteoclasts had been formed GCs lead to enhanced longevity (46, 50), apoptosis could be suppressed, and the effects of receptor activator of nuclear factor kappa-B ligand (RANKL) potentiated. Importantly, this was abrogated in osteoclasts from GRA485T ($GR^{\text{dim}}$) knock-in mice, with impaired GR dimerization (50, 51). This indicates that in contrast to GC-mediated suppression of bone formation for the increase of resorption, GR dimer dependent genetic programs are required.

## GCs Affecting Cellular Cross-Talk of Bone Cells

Since the observation that bone formation and bone resorption are functionally coupled at the bone remodeling unit (52), cross-talk of cells in bone was considered as a hall mark of bone metabolism. This observation was supported by the discovery that osteoblasts and osteocytes are regulating bone resorption by triggering osteoclastogenesis via the induction of the pro-osteoclastogenic factor RANKL (53, 54) following exposure to M-CSF. This occurs in response to systemic hormones, such as PTH. RANKL on the other hand is counteracted by OPG. GCs seem to affect this cross-talk in part as well,

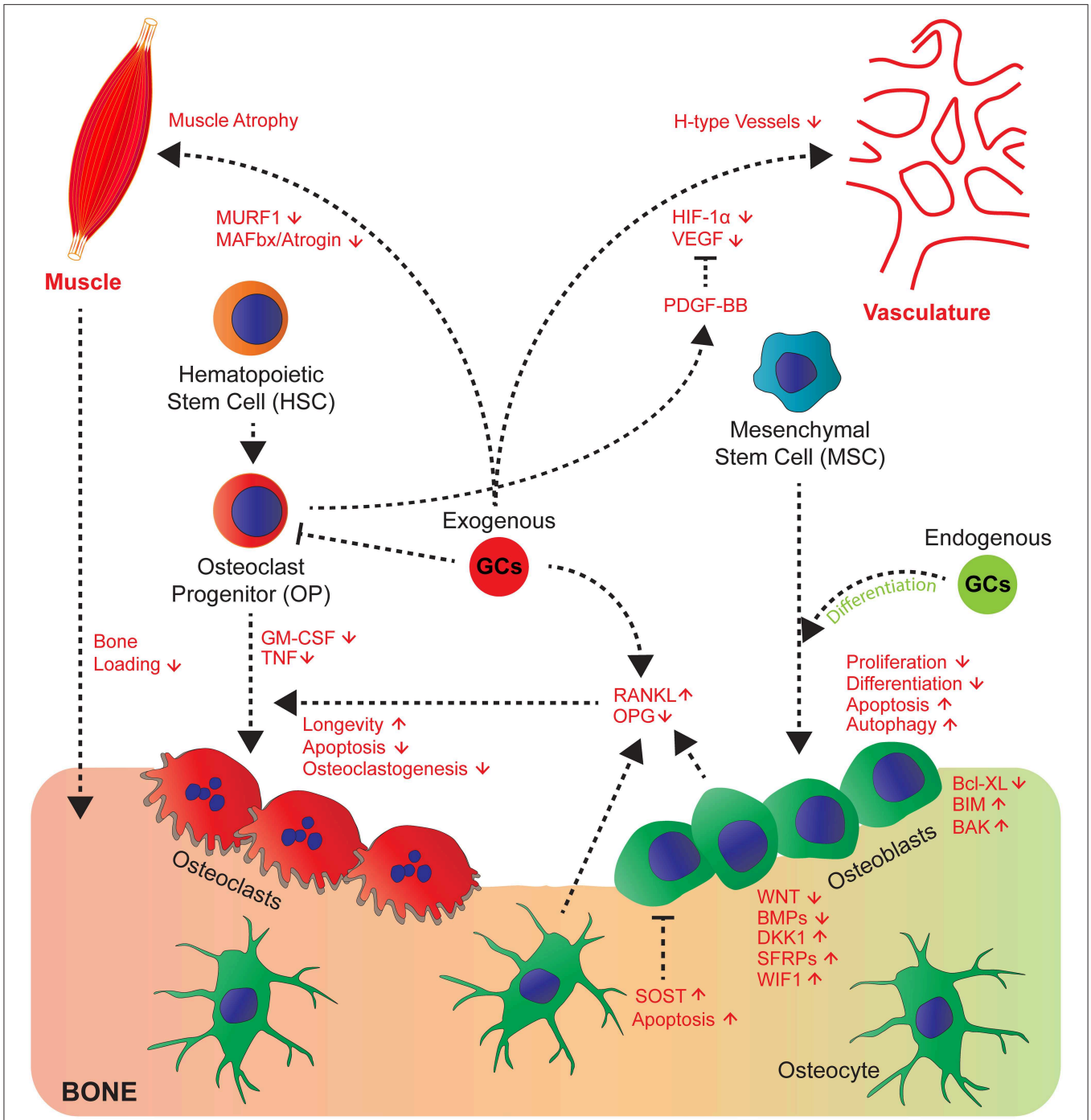


**FIGURE 1 |** GCs affect cross-talk of bone cells and their communication with muscle, vasculature and myeloid cell-derived osteoclasts. GCs act directly and indirectly on bone, hematopoietic and mesenchymal cells and tissues that affect bone integrity. Endogenous GCs (green) rather favour differentiation of osteoblasts, whereas exogenous (red) rather decrease proliferation, differentiation and enhance apoptosis and autophagy of osteoblasts and osteocytes by differential regulation of signalling molecules of the Wnt and BMP pathway and pro- and anti-apoptotic molecules. Direct effects on osteoclasts are differential concerning longevity, apoptosis, osteoclastogenesis (for details see text) and indirect by altering RANKL/OPG ratio. GCs regulate cross-talk of vasculature toward bone and muscle toward bone by exerting modulatory effects on both systems (muscle atrophy) and likely impairing H-type vessels, since respective signalling molecules (VEGF and PDGF-BB are regulated by GCs).

since GCs induce RANKL and suppress OPG in osteoblastic cells, affecting bone resorption (27, 55–57) (**Figure 1**). RANKL inhibition by Denosumab in humanized mice improved some, but not all parameters of bone loss to GC effects (57). For the increase of osteoclasts in cortical bone, RANKL expression in osteocytes is decisive as shown by Piemontese et al. using

mice with a conditional deletion of RANKL using *Rankl*$^{Dmp1Cre}$ mice (58).

Besides, the crucial soluble factors RANKL and M-CSF, TNF and TREM2 ligands play a decisive role in commitment, fusion and maturation of osteoclasts (59). Of these, TNF expression is strongly reduced by GCs at a transcriptional and post-transcriptional level. GM-CSF itself is reduced by the GR via interaction with NF-AT/AP-1 binding sites in the enhancer of the GM-CSF gene (60) (**Figure 1**). This is consistent with the observation that the onset of osteoclastogenesis is inhibited by GCs, which depends on cell autonomous effects (48, 49) and the down regulation of extracellular mediators. The latter was shown by coculture experiments where GCs strongly suppressed osteoclastogenesis dependent on the GR in osteoblasts despite the GR deficiency in osteoclast progenitors (27). This might play in particular a role during inflammation, where osteoclastogenesis and resorption is usually enhanced, and might be beneficially counteracted by GCs. Whether other osteoclast regulatory extracellular factors are under the control of GCs and whether this matters for osteoclastogenesis and activity is still unexplored.

Even less is understood, whether GCs affect osteoclast signals toward osteoblasts or osteocytes. This is still due to the paucity of knowledge of osteoclast-derived factors influencing osteoblasts and osteocytes. Among these identified are ephrinB2, the D2 isoforms of vacuolar (H+) ATPase (v-ATPase) V0 domain (Atp6v0d2), the complement component 3a, semaphorin 4D and microRNAs [reviewed in (61)]. It is not known whether any of these are regulated by GCs to our knowledge. Regulation of microRNAs had been shown for cell-autonomous effects in osteoblasts and osteoclasts, respectively, but whether the osteoclast-osteoblast communication or *vice versa* is affected is unknown. Thus, for this type of cross talk there is tremendous scope for research.

### GCs Influencing Cross-Talk of Vasculature and Bone Cells

Bone is highly vascularized and previous work demonstrated that vascularization and angiogenesis is coupled with bone growth and bone homeostasis (62–64). GCs have a profound inhibitory action on vasculogenesis in bone accompanied by inhibition of HIF-1α and its target gene vascular endothelial growth factor (VEGF) (65). This is accompanied by edema formation in the femoral head in mouse bone, an area with considerable amount of vessel remodeling. In OG2-11β-HSD2 transgenic mice, overexpressing the GC inactivating enzyme 11β-HSD2 in osteocalcin expressing cells, the decrease of vasculature volume was in part prevented (62, 65). Recent studies identified the presence of a subtype of vessels, so-called H-Type vessels, positive for CD31 and endocmucin being associated with bone formation (63). These H-Type vessels were found to be reduced by GC excess, a process that could be prevented by addition of platelet-derived growth factor-BB (PDGF-BB) (66). Since PDGF-BB is in part derived from osteoclast progenitors (67), PDGF-BB could be a factor targeted by GCs.

Taken together, the precise contribution of GC signaling in cells of the vasculature vs. osteoclasts, osteoblasts and osteocytes remain to be determined, which will be of importance to decipher the effects of GC excess on bone integrity.

### GCs Influencing Cross-Talk of Muscle and Bone

Since GC excess does not only influence bone strength, but also leads to muscle atrophy, this increases the risks of falls and reduces load on bone, thus accelerating bone loss and increasing fracture risk (68). GCs induce protein degradation in muscles associated with induced FoxO-dependent expression of E3 ubiquitin ligases atrophy F-Box [MAFbx/atrogin and muscle RING finger 1 (MURF1)], which is mediated in part through the GR in muscle (69–71). Surprisingly, some of these genes are also regulated in bone by excessive GC amounts (68), suggesting that some deleterious pathways might be shared between bone and muscle. The cross-talk between muscle and bone exist beyond the mechanical load. Kim et al., discovered that the muscle derived hormone Irisin binds to alphaV class of integrins in osteocytes and might stimulate resorption and increased sclerostin expression (72). Whether further soluble factors participate in this muscle bone cross-talk and whether they or Irisin signaling itself, are a target of GCs remains to be investigated. Nonetheless, both direct effects on muscle and on bone cells accelerate weakness of bone.

Interestingly, in the absence of inflammation, models of GC induced osteoporosis so far provide no clear evidence of regulation of the cross-talk between bone cells such as osteoblast/osteocytes with innate immune cells, except osteoclasts and their progenitors. This does not mean that GC mediated regulation of this cross-talk does not exist. However, this has not been addressed so far with appropriate cell conditional mouse models. This is completely different for conditions of inflammation in bone described below, where regulation of cross-talk emerges as a major theme for limiting inflammation at least in arthritis.

## GC EFFECTS ON INFLAMMATORY BONE DISEASES—DIRECT EFFECTS AND EFFECTS ON STROMAL-IMMUNE CELL CROSS-TALK

### Effects of GCs on Innate Immune Cells

Innate immune cells, in particular mast cells, tissue macrophages, neutrophils and other cell types secrete inflammatory mediators (cytokines and vasodilator agents) during chronic inflammation, as it occurs e.g., during tissue damage. GCs are known to suppress the production of inflammatory mediators partially by acting on Toll-like receptor (TLR) signaling (73, 74). They also act on macrophages to inhibit the production of eicosanoids, which are lipid mediators that promote vascular dilation and permeability (75, 76). GCs also reduce the blood flow to inflammatory sites by sensitizing endothelial cells to vasoconstrictors and by inhibiting the production of vasodilators (77). In addition, GCs attenuate leukocyte extravasation by inhibiting transcription of integrins and their ligands, intercellular adhesion molecule 1 (ICAM1) as an example (78, 79). Finally, GCs inhibit the expression of

many pro-inflammatory cytokines and chemokines. Mice with conditional GR ablation in macrophages or dendritic cells (DCs), produced higher levels of IL-1β, IL-6, TNF, and IL-12, and exhibited greater mortality during experimentally induced sepsis (80–82). Whereas, downregulation of chemokines, such as CC-chemokine ligand 2 (CCL2), CCL3, CCL5, restrains leukocyte migration, and deficiency of macrophage-recruiting molecule MCP-1 in mice (also known as C-C motif chemokine receptor 2 [CCR-2]), led to compromised fracture healing (83).

Interestingly, GCs reduce mast cell number, maturation and activation (84–87) and stabilize mast cells dose-dependently by inhibiting their exocytotic process. This effect is ascribed to the non-genomic actions of GCs, acting via the GR present in the plasma membrane of mast cells, and directly influencing the intracellular $Ca^{2+}$ signaling pathway (88). In a mouse model of 11β-HSD1 deficiency, reduced intracellular GC action in mast cells correlated with increased activation demonstrating a clear influence of 11β-HSD1 on mast cell degranulation (89).

Despite suppressing inflammatory activity of immune cells, the concept emerges that GCs terminate inflammation by polarizing cells toward an anti-inflammatory phenotype. This has been thoroughly investigated in macrophages. Several studies demonstrated that GCs induce specific differentiation of monocytes with an anti-inflammatory phenotype and promote their survival, contributing majorly to the resolution of inflammation (90–93).

The induction of anti-inflammatory acting immune cells is decisive for resolution of inflammation during fracture healing and arthritis and is subject to GC action.

# GLUCOCORTICOIDS (GCs) AND FRACTURE HEALING

## Cells Involved in Fracture Healing

The role of GCs during fracture healing, a process that requires multiple communication steps between different cell types, is not well-understood. Fracture healing involves close interaction between bone cells and immune cells. Bone injury causes the onset of inflammation. A fracture hematoma is formed containing DAMPs and PAMPs (danger/pathogen-associated molecular patterns), erythrocytes, inflammatory cytokines and cells of the innate immunity. The inflammatory phase is followed by the repair phase where a cartilaginous callus is formed and then remodeled by osteoblast and osteoclasts (94).

Several innate immune cells are present in the early fracture hematoma such as neutrophils, macrophages and mast cells (95–98). Activated mast cells release inflammatory mediators, including histamine, KC, IL-1β, TNF, and IL-6, as well as various chemokines attracting other immune cells (99, 100). Neutrophils and macrophages migrate to the injury site in response to inflammatory mediators to phagocytose debris and pathogens (96, 97, 101, 102) (**Figure 2**).

Depletion of neutrophils, leads to impairment of fracture healing in mice (95, 103), and a reduction of mesenchymal tissue repair in a rat model of growth plate injury (104). Macrophages persist during all phases of fracture repair (96, 97), where they are important for bone healing (105). In a mouse model of femoral fracture, Raggatt et al. showed that inflammatory macrophages were required for the initiation of the fracture repair, while both inflammatory and osteomacs, specialized resident bone macrophages, promoted anabolic processes during endochondral callus formation (106). Mast cell deficiency in mice, however, causes a reduction of the inflammatory response after fracture and disturbed callus remodeling. In the same study, *in vitro* investigation suggested histamine as a major mediator of mast cells action on osteoclastogenesis (98) (**Figure 2**).

During the repair phase, mesenchymal precursors, close to the site of the fracture, differentiate into chondrocytes and start the process of endochondral ossification. A cartilaginous soft callus is formed in order to stabilize the fracture (107). Under stable mechanical conditions the vascularization of the callus is initiated and subsequently followed by its mineralization and its conversion into bone (108). Finally, the callus is remodeled by osteoclasts and osteoblasts and the original bone architecture is restored (109).

Cells of the immune system influence the process of endochondral ossification. Tissue resident and infiltrating macrophages, in particular M2 macrophages enhance vascularization by secreting VEGF at the fracture site (97). They also release TGF-β that plays a pivotal role in chondrogenic differentiation of mesenchymal stem cells for callus formation (110). Monocytes, neutrophils, DC, and B and T lymphocytes produce RANKL and subsequently influence osteoclast and osteoblast activity (111, 112) (**Figure 2**).

## Effects of GCs on Fracture Healing

The injury represents a stress stimulus that triggers endogenous GC release to control the inflammation. We have previously shown, in a mouse model of fracture, that mice with an induced global deletion of the GR, including bone and immune cells, had an impaired fracture healing. The presence of the GR had a protective role in our model partially by shaping the inflammatory response (113).

Few studies investigated the effects of synthetic GCs on fracture healing. It was shown that short-term treatment with GCs had minor effects on bone repair (114) while long-term treatments significantly impaired the healing process (115, 116). In a medaka fish fracture model, although both chronic and acute GC treatment affected osteoclast recruitment and osteoblast accumulation, only chronic GC treatment significantly delayed the healing (117).

The role of GCs on endochondral ossification in fracture healing hasn't been widely investigated. In a model of glucocorticoid-induced osteoporosis, endochondral ossification was impaired after fracture as chondrocyte hypertrophy was delayed (118). In a tibial metaphyseal fracture model, GR deletion in chondrocytes attenuated endochondral bone healing by momentarily increasing the cartilage content of the callus, but didn't impact negatively on the healing outcome (119). In contrast, treatment with dexamethasone had an inhibitory effect on healing in the femur shaft fracture in comparison to metaphyseal fracture, suggesting a more important role of GCs in endochondral rather than intramembranous ossification (120).

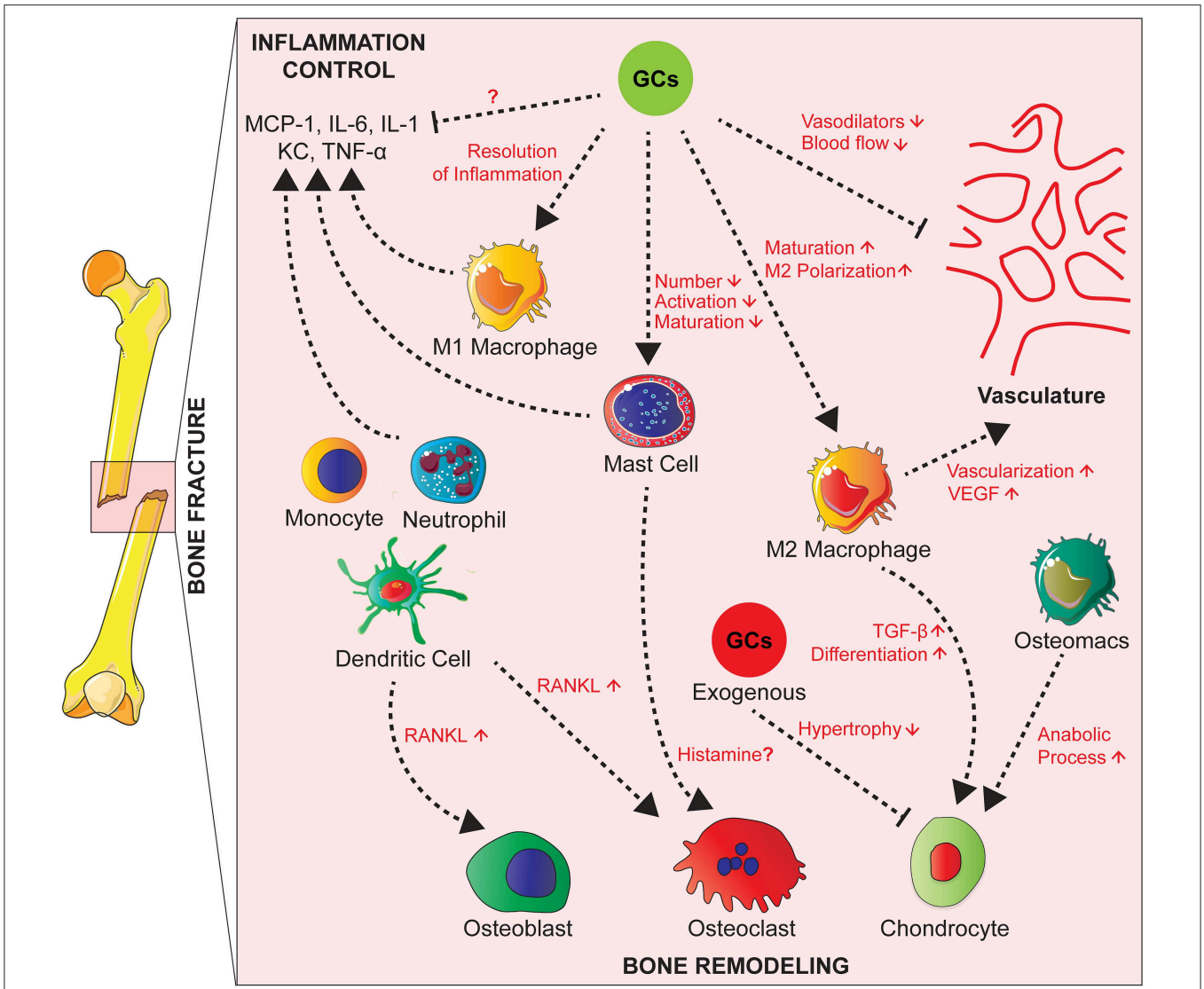


**FIGURE 2 |** Effects of GCs on the cross-talk between cells of the innate immunity, bone cells, and vascularization during fracture healing. During fracture healing, cells of the innate immunity such as neutrophils, macrophages and mast cells produce pro-inflammatory cytokines and attract other phagocytes to remove debris. GCs act on these cell types to control the inflammation and resolve it partially by polarizing macrophages toward an anti-inflammatory phenotype that will in turn promote tissue repair by increasing vascularization. Presumably GCs also inhibit M1 macrophages and cytokine expression, which is not proven yet. On the other hand, GCs have counterbalancing effects by inhibiting the production of vasodilators in order to control the inflammation. Innate immune cells act on bone cells by secreting factors such as RANKL affecting then osteoblast and osteoclast activity. Also, tissue resident and infiltrating macrophages play a role in endochondral ossification by promoting chondrocyte differentiation for callus formation. Chronic GCs treatment delays chondrocyte hypertrophy and attenuates endochondral bone healing.

Given the distinct roles of GCs on cross-talk of immune, bone and stromal cells, and on vasculature and muscle during osteoporosis and arthritis, it is very likely that GCs shape different aspects of fracture healing positively and negatively. The exact interplay requires intensive investigations.

## GLUCOCORTICOIDS (GCs) IN OSTEOARTHRITIS

### GC Effects on Osteoarthritis

Osteoarthritis (OA) is the most common form of arthritis and the leading cause of pain and disability in elder people (121). The clinical picture includes not only a process of "wear and tear" but also an unbalanced remodeling of the joint associated with inflammatory processes (122). Among the main risk factors for OA are obesity, gender and age (123).

Degeneration of joints occurs as damage in articular cartilage and subchondral bone, accompanied by ectopic bone formation, so-called osteophytes. The slow turnover of extracellular matrix is dramatically enhanced in OA due to secretion of degrading proteinases and consequent loss of proteoglycans and collagen (124). This process is likely triggered by a vicious cycle of cross-talk of inflammatory cells and stromal cells, such as chondrocytes and synovial cells.

The role of endogenous GCs in this process is obscure, a recent study of Tu et al., however, showed that overexpression of the GC inactivating enzyme 11β-HSD2 in osteoblasts in transgenic mice attenuates OA in a model of destabilization of the medial meniscus (DMM) in older mice (125). This indicates that in bone cells GCs might trigger the inflammatory and erosive process (**Figure 3**).

Administered GCs are accepted as short term, but not as long term agents for intra-articular injections of knee OA with few side effects [reviewed in (10)]. In the literature, the mechanisms are attributed to effects on stromal cells, by increasing the expression of ECM molecules and reduction of degradative proteases. This had been investigated in OA models, cartilage explant cultures and in cartilage cell lines [reviewed in (10, 126)] (**Figure 3**).

Macrophages are part of the inflammatory infiltrate in OA (127). Interestingly, a depletion of synovial macrophages led to the augmentation of OA in a model of destabilization of the medial meniscus (DMM) combined with high fat diet (128). The absence of macrophages caused intriguingly an increased numbers of T cells indicated a hyperinflammation. This indicates that anti-inflammatory polarized macrophages are essential to prevent aberrant progression of OA.

The precise contribution of GR in immune cells for GC effects on OA has not been addressed mechanistically so far. Furthermore, the suppression of VEGF by GCs (129), strongly suggests that effects on the vasculature, could be beneficial to facilitate repair processes during acute GC exposure. Long term effects on the vasculature could also be non-favorably and trigger further OA progression.

Overall, the GC action in OA is not completely understood and requires further elucidation given that GCs are frequently used for treatment, and that obesity, stress and age are known risk factors for the development of this pathology.

## GC Effects on Osteophytes in Arthritis

Beside the effects of GCs on joint erosion, not much is known about their protective effects against ectopically grown bone, so-called osteophytes. In both, inflammatory and osteoarthritis, osteophytes can be observed (130, 131) and result in pain and loss of function of joints (132). Osteophytes arise from periosteal mesenchymal stem cells (MSCs) that undergo chondrogenic differentiation, mature and produce a cartilaginous scaffold that is replaced by bone in the end-stage of osteophyte formation (133), a process closely related to endochondral ossification (130, 134–137). GCs are shown to suppress osteophyte formation (138–140), as well as endochondral ossification (141), however, it is uncertain whether the same mechanisms are involved. Interestingly, besides MSCs and chondrocytes, cross-talk with components of the innate immunity are shown to play an important role in the initiation of osteophytes (142–144). Osteophytes often develop in close proximity to synovial lining (144) and synovial inflammation is considered a key contributor to osteophyte formation (145). In this regard, it was shown that a single low-dose of avidin-conjugated dexamethasone (Dex) suppress synovial infiltration and osteophyte formation in post-traumatic OA (138). Especially, synovial macrophages, as part of the synovial infiltrate, are considered key players in osteophyte formation as their depletion significantly suppress osteophytes in two different mouse models of OA (142, 144) and GC-mediated inhibition of synovial macrophages might be beneficial to prevent osteophyte formation. Interestingly, inhibition of TNF does not result in the reduction of osteophytes in patients with psoriatic arthritis or mouse models of inflammatory arthritis (146, 147). Thus, GC-mediated suppression of pro-inflammatory cytokines alone might not be sufficient to suppress osteophyte formation. On the other hand, damage-associated molecules derived from degrading cartilage (142) can also activate synovial macrophages and depending on dosage and duration, GC treatment can protect against this cartilage degradation in OA (140, 148) and inflammatory arthritis (149). In this regard, experimental reduction of cartilage degradation reduces formation of osteophytes in mouse models of OA (150, 151).

The most prominent pathways involved in synovial macrophage activation and osteophyte growth are transforming growth factor β (TGFβ) and bone morphogenetic proteins (BMP-2/-4) (142, 144, 152, 153). TGFβ and BMPs initiate chondrogenic differentiation from periosteal MSCs and co-cultures of MSCs and macrophages enhanced spheroid formation after TGFβ treatment when compared to MSCs alone (144). Interestingly, macrophage-specific delivery of liposomal packed prednisolone results in down regulation of TGFβ in inflammatory arthritis (154) and Dex treatment was shown to suppress BMP-signaling and induce BMP-antagonists at least in osteoblast cell lines (155, 156). In addition, blockage of the hedgehog-signaling pathway also resulted in the suppression of TGFβ and BMPs and completely prevented osteophyte formation without affecting synovial inflammation (157). Thereby, GC-mediated control of TGFβ and BMPs might counteract osteophyte formation. Surprisingly, intra-articular injections of triamcinonone acetonide (TA, another GC) were associated with a higher macrophage activity, using folate-based radiotracers, but also resulted in a significant reduction of osteophytes (158). *In vitro* results of Siebelt et al. (158) suggested that the induction of CD163, folate receptor-β and interleukin-10 by TA might play a role in osteophyte suppression (158), however, this needs to be validated *in vivo*. Besides TGFβ and BMPs, dickkopf-1 (Dkk1), a master regulator of bone remodeling is strongly regulated by GCs (159) and is involved in osteophyte formation (160). Inhibition of Dkk1 results in osteophyte formation in an inflammatory mouse model that does not initially develop osteophytes (160). In addition, patients with spondylarthritis (SpA) arthritis that do develop osteophytes, show lower levels of Dkk1 (161), whereas rheumatoid arthritis (RA) patients that do not develop osteophytes have higher levels of Dkk1 (162). GCs, however, strongly induce Dkk1 expression and thereby inhibiting osteoblast differentiation and bone formation (163, 164), which might be beneficial to suppress osteophyte growth. Accordingly, overexpression of Dkk1 in the osteoblast-lineage significantly reduces osteophyte size in OA (165).

In contrast to exogenous GCs, disruption of endogenous GC signaling in the osteoblast-lineage reduces osteophyte formation in an age-related OA mouse model suggesting an osteophyte-promoting role of endogenous GC (125). Further experimental work is needed to discriminate the endogenous vs. the exogenous

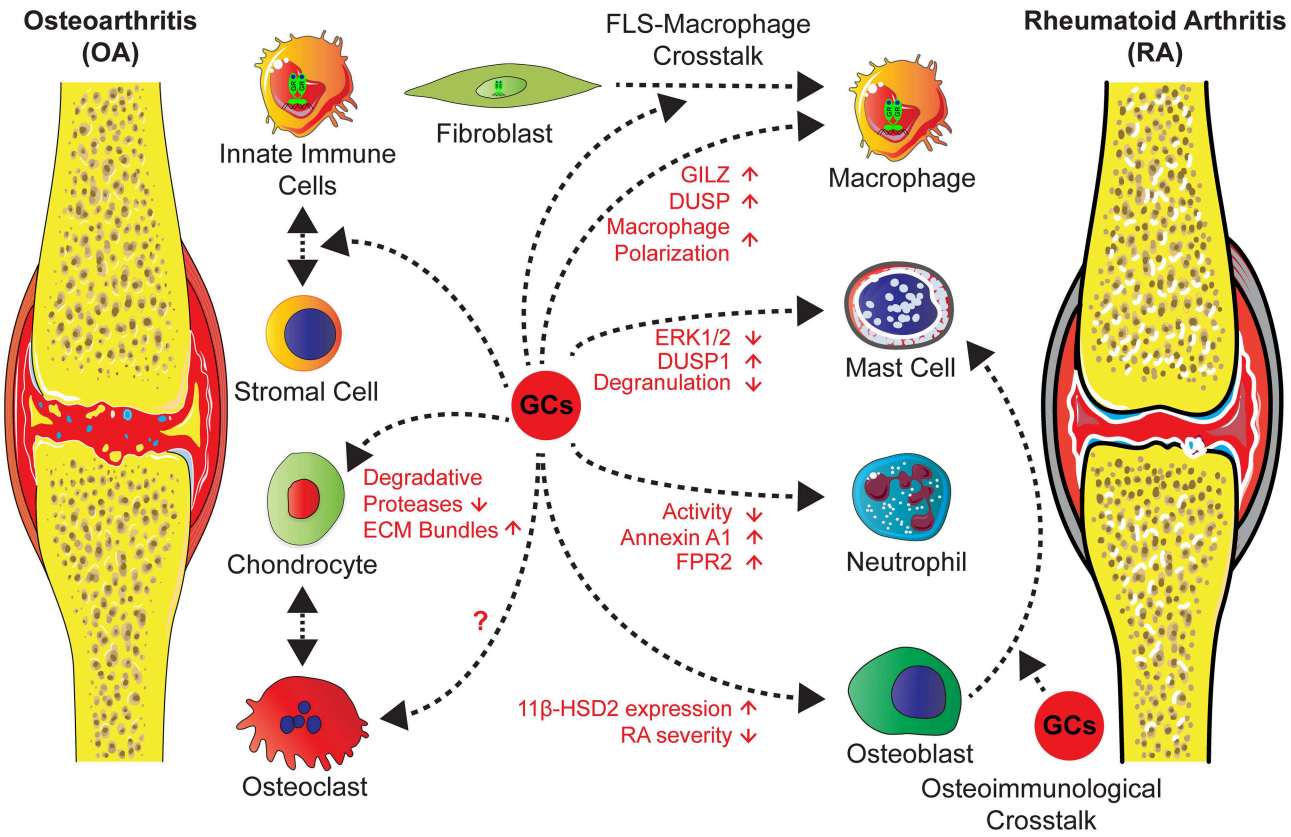


**FIGURE 3 |** GCs administration in the treatment of OA and RA exert direct effects on different cell types and influence stromal-immune cell crosstalk. Actions of GCs on neutrophils and mast cells lead to an attenuated inflammation and an induction of anti-inflammatory mediators. GCs operate on macrophages either directly, causing increased levels of GILZ and decreased inflammation, or indirectly via FLS through a cross-talk between both, leading to a shift of macrophage polarization toward an anti-inflammatory phenotype and an increased efferocytosis activity. Further effects on stromal cells, in particular chondrocytes are a reduction of degradative protease levels and an increase of ECM molecules. Concerning cross-talk between osteoclasts and chondrocytes in OA or in RA the influence of GCs are unknown. Strikingly, in RA osteoblasts GC effects might lead to inflammatory and erosive processes, since the overexpression of the GC inactivating enzyme 11β-HSD2 in osteoblasts, results in an attenuated disease severity by a non defined cross-talk.

effects of GCs on osteophyte formation and to validate potential pathways involved in GC-mediated suppression of osteophytes to better understand the crosstalk of bone and immune cells involved in this process.

## RHEUMATOID ARTHRITIS (RA)

Rheumatoid arthritis (RA) is a chronic, autoimmune disease with a worldwide prevalence of 0.5–1% (166). It affects all types of patients with the highest occurrence in elderly women and a female to male ratio of 3:1 (167). RA is associated with several contributing factors, such as genetics, smoking, obesity and the environment (168). A hallmark of RA is synovial inflammation and the destruction of cartilage and bone, which makes RA a bona-fide disease of osteo-immunological interactions. The etiology is still to a certain extend unclear, but involves rheumatoid factor and anti-citrullinated peptide antibodies (ACPAs), which are at least predictive for the development of RA. The expression of pro-inflammatory mediators, like TNF and IL-6 activating the innate immune system concomitant with aberrant T- and B-Cell regulation finally leads to the development of autoantibodies (169). In the joints, osteoclasts activated by citrullinated autoantibodies, lead to bone damage. This further results in cytokine release by local cells and activation of synovial fibroblasts and macrophages (170, 171), exaggerating the inflammatory and destructive response.

Since the discovery of their anti-inflammatory action 70 years ago, GCs are still one of the most frequently used medications to treat the acute inflammatory response in RA.

Our knowledge of the mechanisms of action of GCs rely on different animal models that comply with certain aspects of the inflammatory phase in arthritis, such as collagen-induced arthritis (involving aspects of T-cells, mast cells and macrophage functions), antigen-induced arthritis (strictly T cell dependent), serum transfer-induced arthritis (T-cell independent) and TNFα transgenic mice (involving multiple cell types).

In the serum transfer-induced arthritis (STIA) and TNF-transgenic model of arthritis, it could be shown that a deficiency of 11β-HSD1 leads to an increase of inflammation, suggesting attenuation of endogenous GC action (172, 173). However, in

another model of collagen-induced arthritis (CIA), 11β-HSD1 deletion caused an attenuation of inflammation indicating a pro-inflammatory role of GC activation in this model.

A clear anti-inflammatory role for the GR could be demonstrated in these models (**Figure 3**). For this, the capacity of the GR for dimerization seems to be required for suppression of inflammation in all arthritis models tested so far. GRA458T ($GR^{dim}$) knock-in mice with attenuated GR dimerization (51), but intact monomer activity, were found refractory in arthritis models of antigen-induced arthritis, glucose-6 phosphate isomerase (G6PI)-induced arthritis and STIA (174, 175). Thus, GR dimerization-induced gene regulation seems to be a general mechanism and is in accordance with animal models with disturbed GR dimer-dependent target genes of the GR such as mitogen-activated protein kinase phosphatase 1 (MKP1), Glucocorticoid-induced leucine zipper (GILZ), and Annexin A1 (**Figure 3**).

GILZ interacts with several crucial signaling pathways, such as NF-κB signaling and T-cell activation (176). GILZ is constantly produced in macrophages and is stimulated by GCs and IL-10, thereby mediating the deactivation of macrophages and thus a decrease of macrophage infiltration (177). This regulation affects the balance between intensified immune reactions and immune tolerance. In mice with CIA and in human patients with RA, it could be shown that GILZ was upregulated in the synovium after the administration of GCs. Furthermore, in cultured RA synovial fibroblasts, an overexpression of GILZ inhibited the release of IL-6 and IL-8 (178).

DUSP1/MKP1 is induced by the GR dimer (179), and an important mediator of anti-inflammatory actions of the GR (81, 180, 181). It inhibits MAP Kinase signaling and DUSP-1 knockout mice have an earlier onset and higher score in CIA (182).

Annexin A1 is associated with the adaptive and the innate immunity. The anti-inflammatory effects of GCs are partly regulated by the release of Annexin A1 and the activation of its receptor formyl peptide receptor 2 (FPR2, also known as ALXR) in neutrophils and macrophages (183). Annexin A1 deficient animals render resistant to GCs in STIA (184), indicating a pivotal role for inhibition of inflammation.

## Cell Type Specific GC Action and Crosstalk Between Immune— and Stromal Cells

Depending on the model used different cell type specific requirements for GC signaling and the GR were suggested to attenuate arthritis. For the T-cell dependent antigen-induced arthritis indeed the GR in T cells is absolutely essential for GC-mediated immune suppression in part by suppressing the generation of IL-17 producing T-cells (174). In contrast in the STIA model the deletion of GR in T-cells does not attenuate the response toward GCs (175). Strikingly, in both models GR deletion in macrophages in $GR^{LysMCre}$ mice hardly affected the efficiency of suppression of inflammation (174, 175). This is surprising, since there is multiple evidence for macrophages to respond to GCs during inflammation in general and the requirement of the GR in models of systemic inflammation, contact allergy and acute lung injury (80, 81, 185). In addition in STIA the presence of alternative activating macrophages is decisive for resolution of inflammation (186).

Despite other immune cells, such as type 2 innate lymphoid (ILC2) cells or others, have not been exploited yet for their functional relevance of anti-inflammatory efficacy, a new theme is emerging demonstrating the role of GR in non-immune cells.

Genetic inhibition of GC signaling in osteoblasts by overexpression of 11β-HSD2 lead surprisingly to an attenuated STIA (187). The mechanism is not clear yet, but maybe in accordance to the global 11β-HSD1 deletion in CIA.

In contrast, deletion of GR in chondrocytes in GR $Col2a1CreER^{T2}$ mice leads to an accelerated inflammation in both CIA and STIA model (188). This was accompanied by an increased CXCR2 expression in the joint suggesting that GR controls chondrocyte-immune cell cross-talk on the level of CXL2/5 CXCR2 chemokine axis involved in leukocyte recruitment.

A recent study showed that GC actions in stromal cells are decisive and GR expression in immune cells alone is not sufficient to suppress inflammation in STIA (175) (**Figure 3**). Experiments in bone marrow chimeric mice lacking the GR in the hematopoietic compartment showed no differences in the onset or progression of STIA, nor the responsiveness to GC treatment compared to chimeric mice with a functional GR in immune cells. Furthermore, a reverse approach with chimeric mice lacking the GR globally except for the hematopoietic system revealed that GR expression in stromal cells is essential for the anti-inflammatory actions of GCs. More precisely, the study showed that for these anti-inflammatory actions, the homodimer form of the GR in stromal cells is critical. Interestingly, deficiency of GR dimerization in these cells had no effect on the suppression of inflammatory cytokines upon GC treatment. This indicates that their decrease alone is not sufficient to suppress inflammation. Additionally, GR dimers in stromal cells induce non-classical, anti-inflammatory macrophages while the levels of classical macrophages are not altered. Several anti-inflammatory markers, associated with enhanced phagocytosis and efferocytosis activity, are increased only in wildtype (wt) but not in GR dimer deficient stromal cells. This suggests an insufficient clearance of apoptotic cells after GC treatment, which leads to a persisting inflammatory condition. Finally, the study suggests that the induction of anti-inflammatory macrophages may be indirectly guided by actions of stromal cells, in particular fibroblast-like synoviocytes (FLS), since cocultures of macrophages and FLS showed an elevated efferocytosis competence when compared to cocultures of macrophages and GR dimer-deficient FLS. In addition to that, the levels of macrophage associated chemokines macrophage inflammatory protein−1α and−1β (Mip-1α / Mip-1β) are decreased in wt but not in GR dimer-deficient FLS after GC treatment. Taken together, this indicates a GC-mediated, GR dimer-dependent cross-talk between FLS and macrophages that induces an increase in the anti-inflammatory macrophage population and thereby a suppression of inflammation and STIA itself (175) (**Figure 3**).

## OVERALL CONCLUSION/OUTLOOK

Overall GCs and the GR have complex actions in bone diseases. The power of conditional mouse genetics demonstrated that GC signaling and GR action in distinct cell types of the immune system, stromal cells and bone cells have different contributions to the overall effects of GCs. Moreover, going away from this simplistic approach of interpreting cell type specific—cell autonomous effects, the field is now moving toward understanding the impact of GCs on interactions of distinct cell types or even organs.

Other issues that remain unexplored are the interplay of GC triggered immune cells in the normal pathology of postmenopausal and age-related osteoporosis. This is striking since the immune cells from the bone marrow need the bone as a niche, therefore strong interactions of immune and bone cells occur as a normal physiological process.

Given that GCs are part of the neuroendocrine regulatory network that also control inflammation and healthy bone homeostasis, a more holistic view will be needed. With the technologies of high content analysis, single cell sequencing and systemic approaches in combination with organoid models and carefully interpreted animal models, our understanding will substantially increase about the influence of these versatile hormones on the immune-metabolic crosstalk.

## AUTHOR CONTRIBUTIONS

MA, YH, FM, KP, MK, and JT wrote individual sections of the article. MA, YH, and KP generated the figures.

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# Compromised Bone Healing in Aged Rats Is Associated With Impaired M2 Macrophage Function

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Fracture repair is initiated by a multitude of immune cells and induction of an inflammatory cascade. Alterations in the early healing response due to an aged adaptive immune system leads to impaired bone repair, delayed healing or even formation of non-union. However, immuno-senescence is not limited to the adaptive immunity, but is also described for macrophages, main effector cells from the innate immune system. Beside regulation of pro- and anti-inflammatory signaling, macrophages contribute to angiogenesis and granulation tissue maturation. Thus, it seems likely that an altered macrophage function due to aging may affect bone repair at various stages and contribute to age related deficiencies in bone regeneration. To prove this hypothesis, we analyzed the expression of macrophage markers and angiogenic factors in the early bone hematoma derived from young and aged osteotomized Spraque Dawley rats. We detected an overall reduced expression of the monocyte/pan-macrophage markers CD14 and CD68 in aged rats. Furthermore, the analysis revealed an impaired expression of anti-inflammatory M2 macrophage markers in hematoma from aged animals that was connected to a diminished revascularization of the bone callus. To verify that the age related disturbed bone regeneration was due to a compromised macrophage function, CD14+ macrophage precursors were transplanted locally into the osteotomy gap of aged rats. Transplantation rescued bone regeneration partially after 6 weeks, demonstrated by a significantly induced deposition of new bone tissue, reduced fibrosis and significantly improved callus vascularization.

**Keywords: bone regeneration, macrophage, monocyte, CD14+ cells, aging, angiogenesis, compromised healing**

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## HIGHLIGHTS

- Compromised bone regeneration in aged rats is connected to a reduced expression of the pan-macrophage markers CD14 and CD68.
- Anti-inflammatory M2 macrophage markers are decreased in the early callus from aged animals.
- Macrophage mediated angiogenesis is impaired in the early callus of aged animals.
- Transplantation of CD14 macrophage precursors rescues impaired bone regeneration in aged rats partially.

## INTRODUCTION

Fracture repair is a highly orchestrated process that involves a distinct pro- to anti-inflammatory signaling cascade in the hematoma, angiogenesis, coordinated extracellular matrix deposition and progression toward endochondral ossification (1). Comorbidities associated with an altered immune response, such as advanced age, diabetes, or rheumatoid arthritis, have been shown to reduce the initial biological potential of the fracture hematoma and may impair regeneration (2–4). In addition, disturbances in the revascularization or unbalanced expression of angiogenic growths factors can delay bone regeneration and eventually lead to the formation of atrophic pseudarthrosis (1, 5–8). Several studies report on the interconnection of immune cells, inflammation, and angiogenic processes. Especially monocytes and macrophages, cells from the innate immune system, are reported to regulate bone homeostasis and repair, as well as tissue vascularization (9, 10). In this context, optimal fracture repair is steered by a collaboration from infiltrating and bone-resident macrophages (11). In dependence of their surroundings, activated macrophages can adopt different functions that are characterized by a pro-inflammatory M1 (classically activated), and an anti-inflammatory M2 phenotype (alternatively activated). M1 macrophages produce large amounts of pro-inflammatory cytokines, as TNFa and IL-1b, and induce Th1 responses. In contrast, the so called M2 phenotype produces IL-10, IL-1 receptor type α, and TGF-β, induces Th2 immune responses, and contribute to angiogenesis, wound healing progression and granulation tissue maturation (12–16). Particularly M2 macrophages have shown to be highly diverse in their functionality and activation patterns. Over the last years, several M2 subsets have been identified (M2a, M2b, and M2c), even repolarization toward M1 phenotypes has been observed, highlighting the great plasticity of macrophages (17, 18).

However, detailed information on the diverse functions of even the more simplified concept of M1 and M2 macrophages during bone repair are rare. While Loi et al. demonstrated that a transition from M1 toward M2 phenotypes highly promotes bone formation *in vitro*, studies analyzing the impact of the different macrophage phenotypes in biologically compromised healing situations, as advanced age, are missing (19). We hypothesized that biologically impaired bone regeneration is connected to disturbances in macrophage functionality and alterations in the M1/M2 macrophage populations. To prove this hypothesis, we investigated the impact of M1/M2 macrophages on bone healing in aged rats more in detail.

## MATERIALS AND METHODS

### Animal Studies

For the *in vivo* animal studies 3 and 12 month old female ex-breeder Sprague Dawley rats from Charles River WIGA Deutschland GmbH were used. These aged rats, that had a minimum of 3 L served as models for biologically impaired fracture healing that develop a non-union, when no additional treatment is applied (4, 20–22). Animal experiments were conducted in compliance with the ARRIVE guidelines and according to the policies and principles of the Animal Welfare Act, the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the National Animal Welfare Guidelines. All animal experiments were approved by the local legal representative (Institutional Animal Care and Use Committees, LaGeSo, G0120/14, G0172/15). Animals were anesthetized with 0.3 mg/kg Medetomidin DomitorH and 60 mg/kg Ketamin by intraperitoneal injection prior to surgical procedure. Additionally, 20 mg/kg Tramadol was administered as analgesia. Forty-five milligram per kilogram Clindamycin was administered by subcutaneous injection and eyes were prevented from drying out by application of eye balm. A longitudinal skin incision was made over the left femur. The bone was exposed by blunt fascia dissections. An in-house developed unilateral external fixator was mounted to stabilize the bone, made of stainless steel and titanium as published previously (20, 23). For an exact placement of the four wire holes, a drilling template was used for every procedure. After incision of the titanium wires, the external fixator bar was placed on the wires and a standardized 2 mm gap was sawn by osteotomy into the femoral bone. To ensure reproducibility of the gap size a sawing template was used at all times. Muscle fascia and skin were closed using absorbable and non-absorbable sutures, respectively. Animals received an anesthetic antagonist and were placed under red light until awakening. Post-surgical analgesia was given by addition of Tramadol (25 ml/l) to the drinking water for 3 days. Fracture healing was assessed after 3 and 7 days, as well as after 6 weeks by euthanization and femur dissection. Animal IDs, weights and group sizes can be found in **Table 1**.

### Intraoperative Cell Transplantation

For the intraoperative cell transplantation of PBMCs or CD14+ cells into the osteotomy gap, 15 ml cardiac blood were drawn from 12-month-old donor rats. Subsequently, PBMCs were isolated by application of a density gradient using Histopaque-1083 (Sigma-Aldrich). The CD14+ subset was further extracted from the PBMC population using positive Magnetic Activated Cell Sorting by application of a murine CD14+ antibody (clone: biG, Abnova), combined with anti-mouse IgG microbeads from Miltenyi Biotec. Per blood clot $2 \times 10^5$ of either PBMCs (including CD14+ cells) or CD14+ cells were re-suspended in 200 µl autologous blood, that was drawn just prior to the surgical procedure (including 10 µl sodium citrate, to prevent clotting). The osteotomy procedure was performed as described above. For cell transplantation groups, blood clotting was induced right before the clot was placed into the osteotomy gap by adding 7 µl $CaCl_2$ 12%Thrombin (Baxter). The lid of a 1.5 ml Eppendorf tube served as a forming device for the artificial hematoma. The clot was designed to exactly fit into the osteotomy gap (same height as gap width), but with a slightly larger diameter, to ensure that the osteotomy gap was spanned by the clot. No differences in clot quality/nature were observed at any point. It was previously shown, that bone formation after a regular healing time of 6 weeks in animals receiving an empty autologous blood clot without additional cell supplementation is comparable to the one seen in animals that received a PBMC supplemented blood

clot (24, 25) thus PBMC supplemented artificial hematoma was used as control in this study rather than the autologous blood clot alone.

## μCT

Bone healing was assessed *in vitro* with micro-computed tomography on formaldehyde-fixed left femurs extracted from euthanized animals 6 weeks after surgery. A region of interest (ROI) covering the 2 mm osteotomy gap plus 1 mm proximal and distal was scanned in a Viva CT 40 microCT (Scanco Medical AG) with application of a voxel size of 10.5 μm, 55 keVp, 145 μA. Bone microstructure trabecular number, trabecular space, trabecular thickness) and the key parameters of tissue and bone mineral content were assessed using the respective software from the device supplier (Scanco Software, Scanco Medical AG). 3D μCT reconstruction were done using CTvox (version 3.2.0.r1294). All analyses were performed in a blinded manner by two different observers with automatically assignment of μCT screen-numbers, to avoid bias to the treatment.

**TABLE 1 |** Animal numbers, weight, and group sizes for intraoperative cell transplantations.

| Animal ID | Weight (g) | Weight mean | Weight SD | Read-out | Group | Group size |
|---|---|---|---|---|---|---|
| 362 | 406 | 357 | 55.31 | Histomorphometry/αSMA | PBMC | $n = 5$ |
| 364 | 326 | | | | | |
| 365 | 345 | | | | | |
| 369 | 288 | | | | | |
| 371 | 420 | | | | | |
| 381 | 407 | 375.3 | 66.14 | Histomorphometry/αSMA | CD14+ | $n = 7$ |
| 382 | 343 | | | | | |
| 385 | 384 | | | | | |
| 386 | 294 | | | | | |
| 387 | 318 | | | | | |
| 388 | 387 | | | | | |
| 390 | 494 | | | | | |
| 362 | 406 | 349.6 | 59.62 | μCT | PBMC | $n = 5$ |
| 364 | 326 | | | | | |
| 366 | 308 | | | | | |
| 369 | 288 | | | | | |
| 371 | 420 | | | | | |
| 386 | 294 | 388.4 | 84.70 | μCT | CD14+ | $n = 5$ |
| 387 | 318 | | | | | |
| 388 | 387 | | | | | |
| 389 | 449 | | | | | |
| 390 | 494 | | | | | |

## Fracture Hematoma Extraction and Gene Expression Analysis

For gene expression analysis, femurs were excised from animals euthanized 3 and 7 days after osteotomy. Surrounding muscle tissue was dissected and tissue containing the fracture callus plus 1 mm proximal and distal to the osteotomy gap was extracted and immediately transferred to liquid nitrogen. Subsequently, tissue samples were pestled while frozen in liquid nitrogen and collected in TRIzol Reagent (LifeTechnologies) afterwards. RNA was isolated according to the manufacturer's protocol, followed by determination of RNA concentration using a Nano-Drop spectrophotometer. cDNA was transcribed from 25 ng/μl RNA with iScript reverse transcriptase as indicated by the manufacturer (Bio-Rad Laboratories GmbH) and gene expression was determined via quantitative real time PCR (iQ5 Cycler, Bio-Rad Laboratories GmbH). Primer sequences (**Table 2**) were generated using the primer 3 web based software (http://primer3.ut.ee/) and tested for specificity (ePCR, http://www.ncbi.nlm.nih.gov/tools/epcr/). Expression of each gene was calculated according to the ddCT method with adjustment for primer efficiency and normalization to TATA-box binding protein (Tbp) expression by utilizing the REST software (26). The housekeeping gene Tbp was tested against others (Gapdh,

**TABLE 2 |** Primer sequences.

| Gene | Gene name | Forward 5′-3′ | Reverse 5′-3′ |
|---|---|---|---|
| CD14 | Cluster of differentiation 14 | aactgaagcctttctcggagc | gcataagcttcatggtcggt |
| CD68 | Cluster of differentiation 68 | tccagcaattcacctggacc | aagagaagcatggcccgaag |
| CD80 | Cluster of differentiation 80 | gctgctggttggtcttttcc | ttcttgtactcgggccacac |
| CCR7 | C-C chemokine receptor type 7 | tacatcggcgagaacaccac | caggacttggcttcgctgta |
| CD163 | Cluster of differentiation 163 | ctggagcatgaacgaggtgt | ttcctgagcatcggttgtcc |
| CD206 | Cluster of differentiation 206 | cagtttgagggcagcaagag | acactcaggagctcagcatt |
| Tie-2 | TEK tyrosine kinase; angiopoetin receptor | tctgctctcaaggatggcaa | cacactgcagacccaaactc |
| Dectin | C-type lectin domain family 7 member A (CLEC7A)/Dectin | cgtcttttctggaccttgcc | acggcccttcactctgattg |
| PDGFα | Platelet derived growth factor alpha | ttgaacatgacccgagcaca | acacctctgtacgcgtcttg |
| PDGFRα | Platelet derived growth factor receptor alpha | agtgcttggtcggatcttgg | gagcatcttcacagccacct |
| PDGFβ | Platelet derived growth factor beta | ttgaacatgacccgagcaca | acacctctgtacgcgtcttg |
| PDGFRβ | Platelet derived growth factor receptor beta | cgttgcaggtggtgtttgag | acacggacagggacattgac |
| HIF-1α | Hypoxia-inducible factor 1, alpha subunit | tcatcagttgccacttcccc | actgggccatttctgtgtgt |
| VEGF | Vascular endothelial growth factor | aaagcccatgaagtggtgaa | tctgcatagtgacgttgctc |
| VEFGR | Vascular endothelial growth factor receptor | agaacagagctcaacgtggg | atctttgccacagtcccagg |

Actb, Eif4e, B2m) and was found to be the most stable gene in all investigated samples.

## Histological Analysis

Histological analyses were performed on frozen section according to the Kawamoto's film method (27). Excised femurs were fixed in a 4% PBS/PFA solution for 24 h at 4°C. For cryo-protection purposes, femurs were transferred into 10, 20, 30% sucrose solutions in ascending order for 24 h at 4°C at a time, subsequently embedded in SCEM-Medium and frozen by immersion into cold n-Hexan. Embedded and frozen samples were sectioned into 5 µm thick slices. All femurs were oriented in the same manner for the histological analyses, where the proximal part of the femur is placed on the left and the distal end of the femur on the right side of the image.

To distinguish between different calcified and soft tissues Movant Pentachrome staining was used. Prior to the staining procedure, slides were fixed with 4% PFA/PBS for 15 min. Five subsequent stainings are applied to stain mineralized bone (yellow/orange), collagen (yellow), cartilage (green/blue), osteoid (dark red), elastic fibers (orange/red), and nuclei (blue-black). After fixation samples were rehydrated for 5 min before alcian blue staining was applied for 30 min, which targets acid proteoglycans structures like chondroitin sulfate. One hour incubation in alkaline ethanol, stabilizing the blue-green pigment. Afterwards, slides were incubated in Weigert's Iron hematoxylin solution (15 min), to stain nuclei. Cell plasma was stained by brilliant crocein acid fuchsine (15 min), followed by differentiation in 0.5% acetic acid. As a last step, slides were incubated in phosphotungstic acid (20 min) and the connective tissue was stained by saffron du gatinais solution.

Vascularization analysis was performed on smooth muscle actin (αSMA) immunohistochemical staining. Slides were fixed with 4% PFA/PBS prior to the staining procedure. All subsequent staining steps were carried out in a humid chamber, at room temperature, unless stated otherwise. To block against unspecific background, samples were incubated with 2% normal horse serum before overnight application of the primary antibody (α-SMA, Dako M0851) at 4°C. Thirty minutes incubation with the secondary antibody (biotinylated anti-mouse IGG, rat-adsorbed, made in horse) and subsequent application of AB complex (ABC-AP Vectastain Kit—SP 5000) for 50 min. 2 × 5 min incubation with chromogen buffer before visualization of the vessels with AP- substrate (Red AP Substrate Kit, Vector—SK 5100). Color development was controlled under the microscope and ended by washing the slides with PBS. Nuclei were counter stained using Mayers Hämalaun for 1.5 min, unstained surrounding tissue is blued by tab water.

Pictures of the stained samples were taken with Zeiss Axioscope 40 Microscope, 10× objective (plus condenser) and the corresponding Imaging AxioVision LE Software (Carl Zeiss).

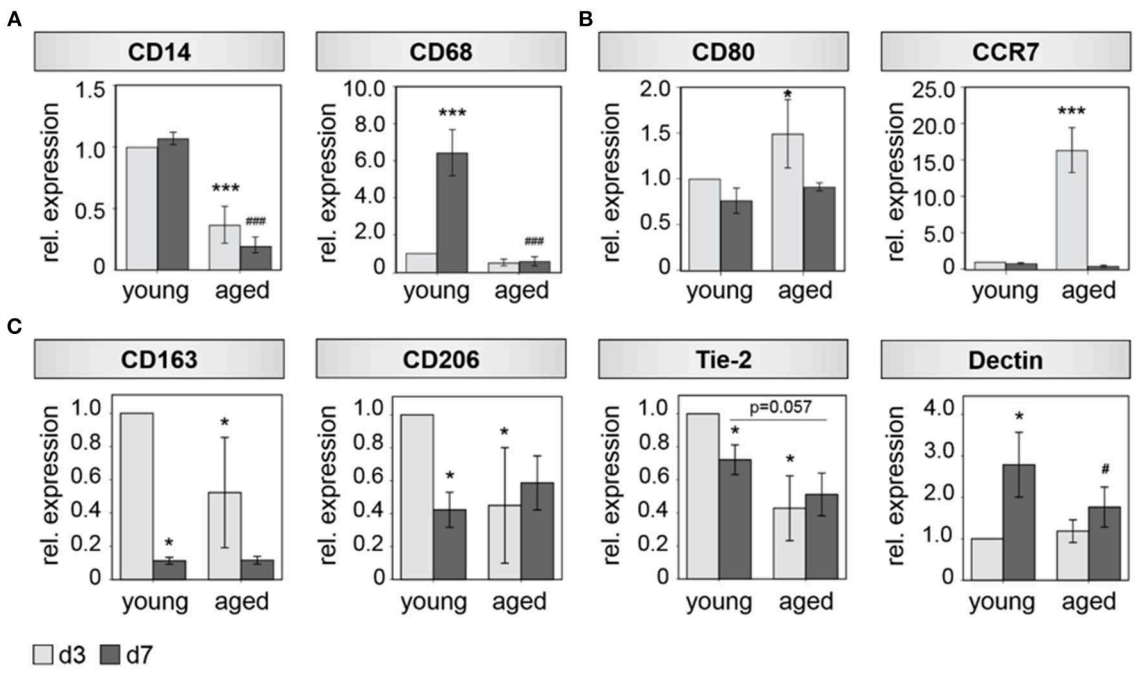


**FIGURE 1 |** M2 Macrophage function is impaired in bone hematoma of aged rats. **(A)** The general monocyte maker CD14 and the general mocrophage maker CD68 show a significant reduced expression 3 and 7 days after osteotomy in hematoma tissue extracted from aged animals compared to young ones. **(B)** The makers CD80 and CCR7 that are predominantly expressed by M1 macrophages show higher expression levels in fracture callus tissue from aged animals at day 3. **(C)** Marker genes that are mostly expressed by M2 macrophages (CD163, CD206, Tie-2, Dectin) show a significantly diminished expression in hematoma tissue from aged animals compared to the expression found in fracture hematoma extracted from young animals. $n = 4$–5, *significant to young d3, $^{*}p < 0.05$;$^{**}p < 0.001$; $^{\#}$significant to young d7, $^{\#}p < 0.05$; $^{\#\#\#}p < 0.001$, ANOVA.

Tissue quantification was done with ImageJ (Version 1.44p; http://rsbweb.nih.gov/ij/) using a semi-automated method on blinded sections. Vessels were counted manually in a blinded approach. Inclusion criteria included a clear endothelial cell border, a visible lumen and non-muscle association. A region of interest (ROI) including the osteotomy gap and 1 mm proximal and distal to it was investigated.

## Statistics

Determined values are depicted as bar charts showing mean ± standard deviation. For statistical analysis SigmaPlot 11.0 was used. Data were checked for normality distribution and analyzed with Student's *t*-test or ANOVA using a Bonferroni correction. If normality distribution could not be confirmed, data were analyzed using a non-parametric Man Whitney U Test or a multiple pairwise comparison according to Dunn's method. A $p \leq 0.05$ was considered as significant. Each analysis was performed with three technical replicates per biological samples. The applied statistical method and the amount of individual biological samples (n) that were analyzed are indicated in the respective figure legends.

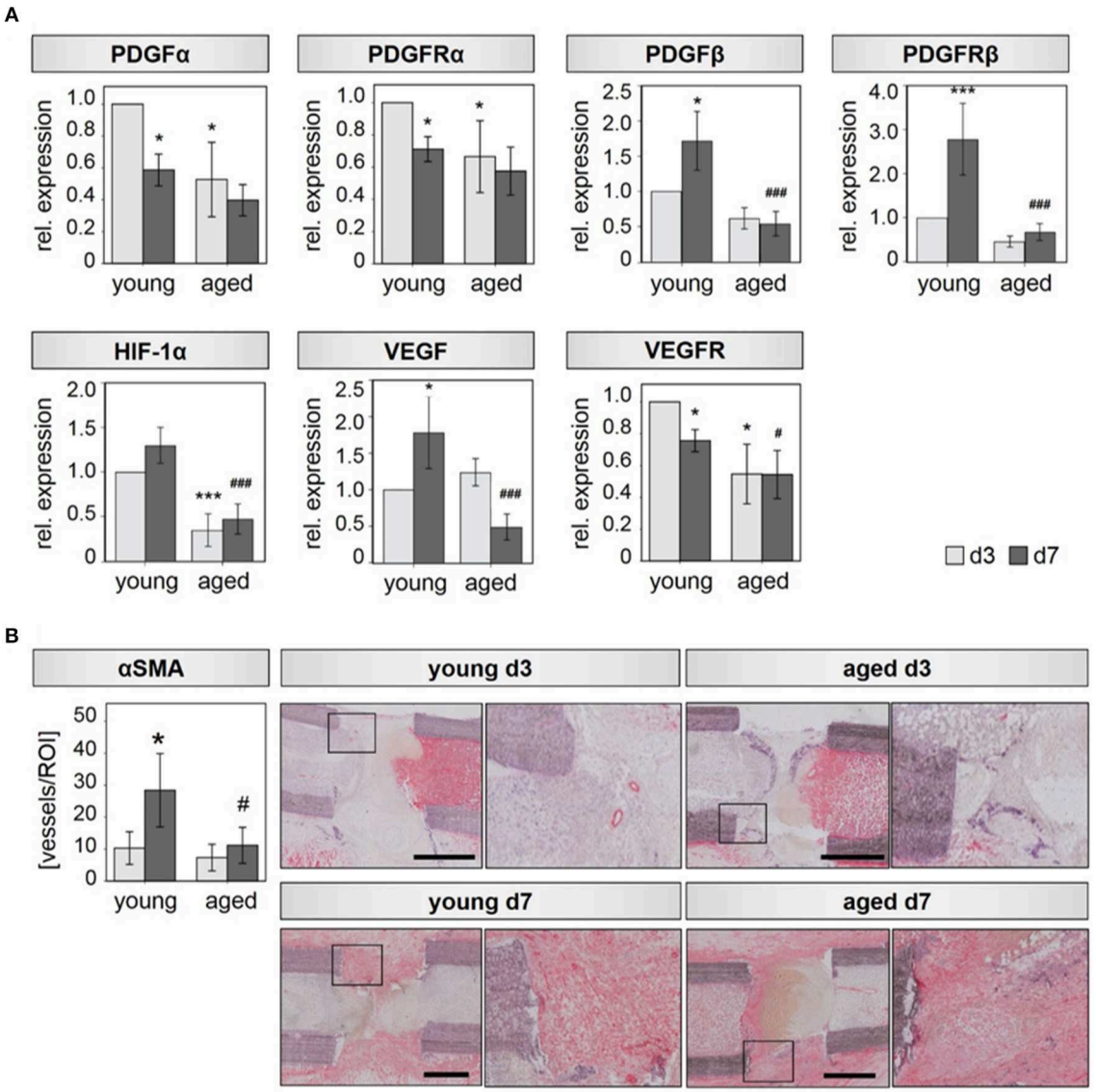


**FIGURE 2 |** Bone callus vascularization is compromised in aged rats. **(A)** Expression of the angiogenic transcription factor HIF-1α, the angiogenic growth factors PDGFα and VEGF and their respective receptors PDGFRα, PDGFRβ, and VEGFR is significantly reduced in callus tissue from aged rats extracted 3 and 7 days after osteotomy. $n = 4$–5, *significant to young d3; #significant to young d7, ANOVA on Ranks. **(B)** Vessel number evaluated in αSMA stained tissue sections. For each condition, the left picture shows the overview of the whole region of interest, the right picture shows the magnification of the region within the square–found in the respective overview mage. Femurs are placed in the same orientation, with the proximal side on the left and distal on the right side. y/d3, y/d7 $n = 5$, a/d3 $n = 3$, a/d7 $n = 6$, *significant to young d3, $^{*}p < 0.05$, $^{***}p < 0.001$; #significant to young d7, $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, ANOVA.

## RESULTS

### Diminished Macrophage Accumulation in Bone Callus of Aged Rats

To validate our hypothesis, we analyzed the expression of commonly applied macrophage markers at early fracture healing time-points (28–32). Therefore, we extracted hematoma/callus tissue 3 and 7 days after osteotomy from young (3 months) and aged (12 months), female rats. The aged animals served hereby as a model system for impaired bone regeneration that develop a non-union without additional treatment, as we have shown previously (4, 20–22).

Interestingly, a diminished expression of monocyte/macrophage related genes was detected in bone callus tissue derived from aged animals, which develop a non-union when no additional treatment is applied. The expression of the monocyte/macrophage precursor marker CD14 and the general macrophage marker CD68 were significantly downregulated in bone callus tissue of aged animals, when compared to their expression in callus tissue derived from young animals (**Figure 1A**). Next, we investigated the expression of specific M1 and M2 macrophage polarization markers in more detail, considering the various processes, in which macrophage subsets take part during healing cascades (10, 33). The M1 markers

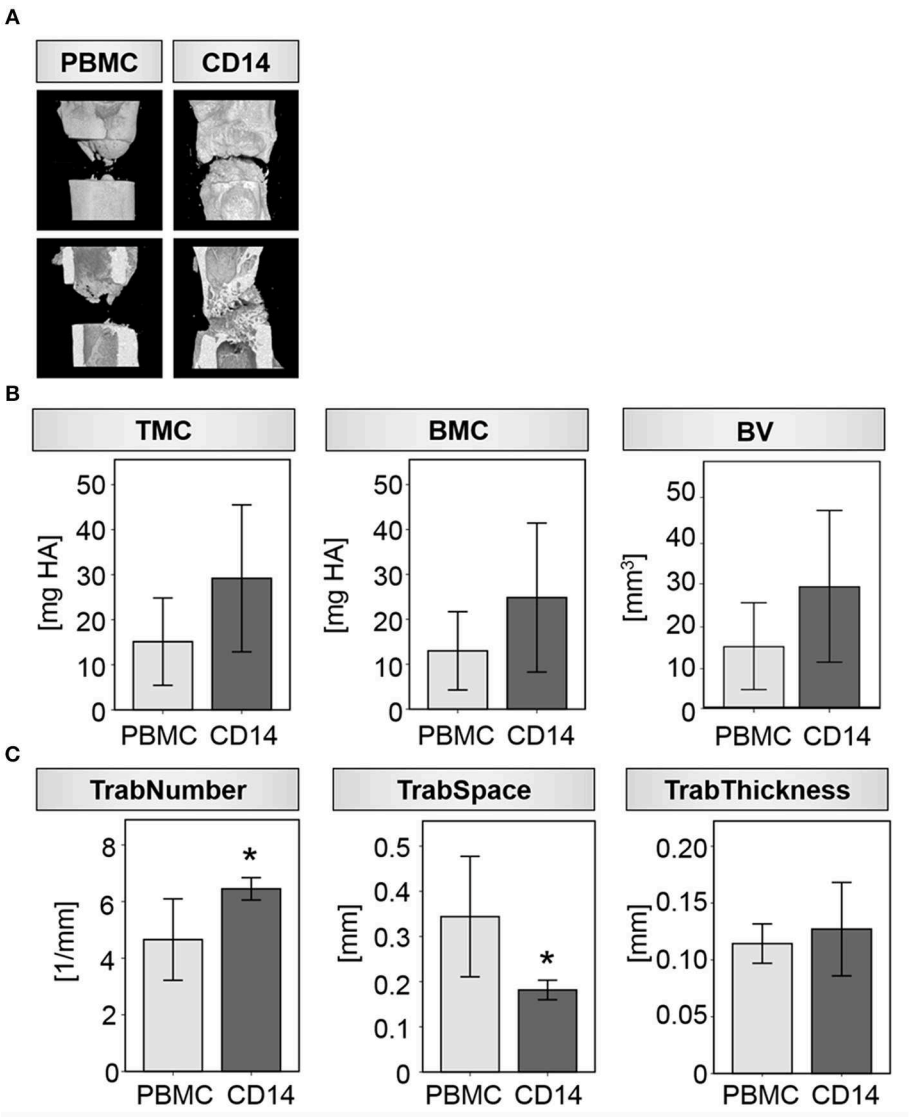


**FIGURE 3 |** Transplantation of CD14+ macrophage precursors partly rescues impaired bone regeneration in aged rats. **(A)** Exemplary 3D reconstructions from four individual animals receiving either PBMC or CD14+ cells locally at the osteotomy site. Bone healing was induced after CD14+ cell transplantation. **(B)** Mineral deposition was prone to be increased after CD14+ cell transplantation in the investigated ROI. **(C)** Formation of new trabeculae was significantly induced after CD14+ cell transplantation as indicated by an increased number and a reduced space between them. Thickness of the single trabeculae were unaffected by cell transplantation. $n = 5$, *significant to PBMC, $p < 0.05$, *t*-test.

CD80 and CCR7 showed a significant upregulation in fracture callus tissue of aged animals compared to young animals, at day 3 after osteotomy (**Figure 1B**). We further detected significant alterations, when we investigated the expression of M2 specific markers (**Figure 1C**). CD163, CD206, and Tie-2 showed lower expression levels in bone callus tissue derived from aged animals compared to younger ones (**Figure 1C**). These differences reached significance on day 3 after osteotomy. The M2 marker Dectin on the other hand, showed an upregulation from day 3 toward day 7 after osteotomy in young and aged animals. However, its expression was significantly lower in callus tissue from aged compared to young animals (**Figure 1C**). When investigating unfractured contralateral bone tissue no significant changes in marker gene expression levels could be detected (**Supplementary Figures 1A,B** and **Supplementary Table 1**).

## Disturbed Callus Revascularization in Aged Rats

M2 macrophages are known to be highly involved in the regulation of angiogenic responses (34, 35). We therefore hypothesized, that the altered M2 macrophage expression profile detected in bone callus of aged animals is further accompanied with impaired revascularization of the injured bone tissue. To this end, we investigated the expression of several pro-angiogenic (growth) factors and their corresponding receptors. Indeed, we found a significantly downregulated expression of PDGFα, PDGFRα, HIF1α, and VEGFRα in fracture callus tissue of aged animals 3 days after osteotomy compared to tissue harvested from young ones (**Figure 2A**). PDGFβ and its receptor PDGFRβ did not reach statistical significance but showed the same trend of lower expression levels in fracture calli from aged animals compared to young animals at day 3 (**Figure 2A**). Significantly lower expression of HIF1α and VEGFRα was still evident 7 days after osteotomy in aged rats. In addition, expression levels of PDGFβ, PDGFRβ, and VEGF were significantly reduced at day 7 when comparing aged animals to young ones (**Figure 2A**). The analyzed genes showed no significant regulation in expression levels when investigating control tissue (**Supplementary Figure 1C**).

The decreased expression of angiogenic growth factors in aged animals is also reflected by a diminished number of newly forming vessels, identified by immunohistochemical assessment. Aged rats displayed 1.4-fold reduced numbers of alpha smooth vessel actin (αSMA) positive vessels in the callus region compared to young animals at day 3. At day 7 αSMA positive vessel numbers were reduced by a factor of 2.5 in aged animals (**Figure 2B**). Vessel diameter was also significantly reduced in hematoma tissue derived from aged animals at day 7 (**Supplementary Figure 2**).

## Monocyte Transplantation Rescues Impaired Bone Healing in Aged Rats

Based on the findings discussed above, we assumed that a diminished monocyte number and possibly decreased M2 macrophage differentiation or the lack of a shift from M1 to M2 population may lead to the observed delayed healing. Enrichment of the naturally occurring monocyte/macrophage CD14+ precursor cells in the osteotomy gap region of aged animals may assist in steering a successful endogenous healing cascade. Thus, local transplantation of CD14+ cells, could improve the impaired bone regeneration observed in aged animals.

Indeed, upon local transplantation of an artificial blood clot containing CD14+ cells into the fracture gap of aged animals directly after osteotomy-induced trauma, induction of new bone formation was detected when compared to the control group (artificial blood clot containing PBMCS) (**Figure 3**). Reconstructions from μCT analysis showed a clear formation of new bone tissue within the osteotomy gap (**Figure 3A**), connected to a quantitatively increased mineral deposition in the CD14+ enriched transplantation group compared to the PBMC control group (Tissue Mineral Content-TMC, Bone Mineral Content-BMC and Bone Volume-BV) (**Table 3**; **Figure 3B**). When we investigated the microstructure of the newly formed bone more in detail, we detected a significant increase in trabecular number and a significantly diminished space between the trabeculae (**Table 3**; **Figures 3A,C**).

Histological evaluations confirmed the findings from the radiological analysis. A significant induction of new bone tissue mainly proximal to the bone trauma and partly within the gap region was detected in the CD14+ transplantation group (**Table 4**; **Figures 4A,B**). By trend, we also found a decreased formation of fibrous tissue, when CD14+ cells were transplanted in aged animals (**Table 4**; **Figures 4A,B**). Interestingly, the improved bone tissue regeneration after

**TABLE 3 |** μCT investigations 6 weeks after cell transplantation.

| | PBMC | CD14 |
|---|---|---|
| TMC [mg HA] | 15.13 ± 9.70 | 29.19 ± 16.32 |
| BMC [mg HA] | 12.99 ± 8.71 | 24.84 ± 16.58 |
| BV [$mm^3$] | 14.95 ± 10.60 | 29.51 ± 18.50 |
| Trabecular number [1/mm] | 4.66 ± 1.44 | 6.45 ± 0.40 |
| Trabecular space [mm] | 0.34 ± 0.13 | 0.18 ± 0.02 |
| Trabecular thickness [mm] | 0.11 ± 0.02 | 0.13 ± 0.04 |

**TABLE 4 |** Histomorphometric evaluations 6 weeks after cell transplantation.

| | PBMC | | | CD14 | | |
|---|---|---|---|---|---|---|
| | Proximal | Gap | Distal | Proximal | Gap | Distal |
| Mineralized tissue [% of total area] | 19.27 ± 8.81 | 18.96 ± 10.28 | 11.89 ± 3.26 | 34.54 ± 8.65 | 24.13 ± 14.38 | 13.39 ± 5.96 |
| Fibrotic tissue [% of total area] | n.d | 20.79 ± 21.10 | 14.29 ± 17.60 | 0.91 ± 1.12 | 4.18 ± 4.88 | 2.34 ± 3.46 |
| Cartilage [% of total area] | 0.01 ± 0.03 | 8.95 ± 10.88 | 0.21 ± 0.47 | 0.20 ± 0.38 | 13.18 ± 12.55 | 0.75 ± 0.92 |
| Vessel number [1/ROI] | 12 ± 3 | 23 ± 14 | 14 ± 9 | 23 ± 15 | 55 ± 16 | 21 ± 9 |

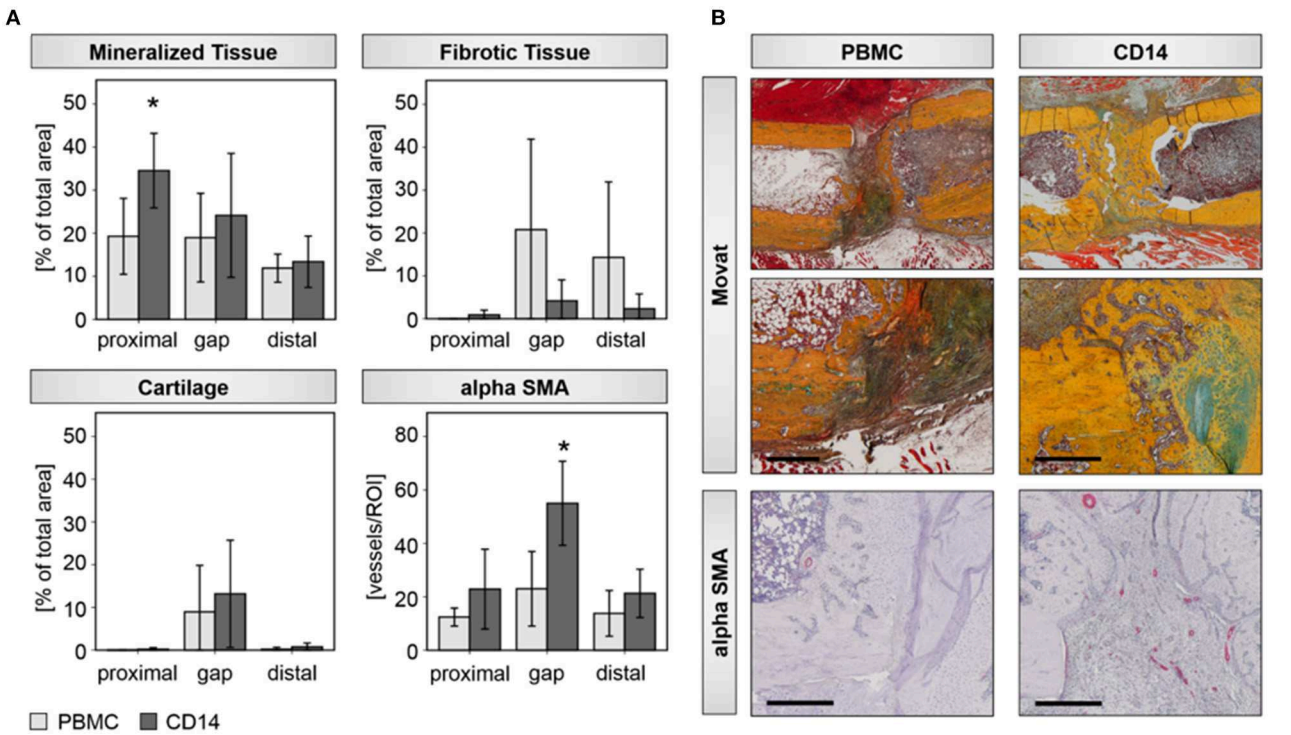


**FIGURE 4 |** Histological assessments verify improved bone healing after CD14+ cell transplantation aged rats. **(A)** Histomorphometric evaluations of mineralized, fibrotic, and cartilage tissue with the osteotomy gap, as well as distal and proximal to it, were done. The analysis revealed a significant induced deposition of mineralized bone matrix and by trend a diminished formation of fibrotic tissue, when aged animals received CD14+ cells locally at the osteotomy site. Number of αSMA positive vessel increased significantly in the region of interest after CD14+ cell transplantation. PBMCs $n = 5$, CD14+ $n = 7$, *significant to PBMC, $p < 0.05$. **(B)** Exemplary pictures of Movat-Pentachrom (mineralized bone, yellow/orange; collagen, yellow; cartilage, green/blue; osteoid, dark red; elastic fibers, orange/ref; nuclei, blue-black) and aSMA stained tissue sections. Femurs are placed in the same orientation, with the proximal side on the left and distal on the right side. Bar = 500 μm.

CD14+ cell transplantation was accompanied with a significant induction of new vessel formation (**Table 4**; **Figures 4A,B**).

## DISCUSSION AND CONCLUSION

Macrophages are essential for bone regeneration and they participate in all stages of healing, promote collagen I deposition and matrix mineralization by osteoblasts *in vitro* and *in vivo* (9, 36, 37). Additional evidence that macrophages play important roles during bone regeneration can be obtained from studies reporting on systemic depletions of macrophages in experimental mouse models. Independent of the time point of macrophage depletion, a diminished formation of new bone matrix and an altered endochondral ossification process is visible 7–28 days after injury (36, 38).

The M1 to M2 switch plays a vital role during healing progression as reported recently by co-workers from our institute and others (19, 38). We confirm here that macrophage activity plays a significant role in bone repair and that an imbalance in the M1/M2 macrophage differentiation is associated with disturbed bone regeneration in aged rats. Furthermore, we showed that local transplantation of macrophage precursors can enhance and potentially rescue bone repair under biologically impaired conditions, presumably by an induction of M2 macrophage differentiation. Our findings highlight the potential of local cell transplantations, here monocytes/macrophages to steer bone repair, especially under compromised conditions, since we used macrophage precursors derived from circulating blood of aged matched donors for local cell transplantation, which presumably shifted the endogenous cell balance and thus promoted healing. Ongoing research further highlights a close connection between macrophages and osteogenic differentiation. A recent study, reported on the osteogenic differentiation capacity of MC3T3 pre-osteoblasts in *in vitro* co-cultures with macrophages (19). When MC3T3 cells were cultivated together with M1 macrophages that underwent an IL-4 triggered M2 switch during MC3T3 osteoblast maturation, mineralized matrix deposition was significantly induced (19). This might be due to M2 macrophage-induced BMP-2 secretion, which is a major contributor to osteogenic differentiation (39). In addition, recent studies from Gibon et al. support our hypothesis of a disturbed M1/M2 phenotype balance in aged. They could show that bone marrow macrophages isolated from aged mice have a higher pre-activated resting state and increased expression of the pro-inflammatory cytokine TNFa after activation than macrophages isolated from young animals (40). Furthermore, they described an impaired M2 polarization of bone marrow macrophages derived from aged mice (40). Recently Vi et al.

also reported that the age of macrophages is crucial for fracture repair. Parabiosis and fractionated bone marrow transplantation experiments in mice showed that young macrophages can rejunvenate healing potential of old bone marrow stromal cells, while old macrophages impair healing of young bone marrow stromal cells (41).

There is also *in vivo* evidence that M2 rather than M1 macrophages regulate bone healing. Animals that were administered with CSF-1, which is required for macrophage differentiation, have an increased abundance of M2 macrophages within the fracture site and show improved healing outcomes (36, 42). In addition, M2 macrophages are highly pro-angiogenic and secrete various growth factors, as e.g., TGF-b, TGF-a, bFGF, PDGF, and VEGF (43, 44). Thereby they might regulate revascularization and matrix maturation of the callus tissue. The angiogenic capacity of monocytes and their descendent macrophages is also proven by investigations of $Ccr2^{-/-}$ mice. Beside compromised cartilage maturation, $Ccr2^{-/-}$ mice show an impaired formation of new blood vessels within the fracture site (45). Moldovan et al. reported that the angiogenic capacity of macrophages relates to their capability to degrade extracellular matrix. Using a transgenic mouse model of ischemic cardiomyopathy, where monocytes were attracted to the myocardium by the targeted overexpression of CCL2, they showed tunnel carving by macrophages, which provide growing vessels with a path for invading capillaries (12).

However, there is still an ongoing discussion on the state of macrophage polarization and activation and its effect on bone regeneration. M1 macrophages for instance can be beneficial or deleterious for bone formation, highly depending on the study design as recently reviewed by Pajarinen et al. (9). Another possibility is that all macrophage phenotypes can promote osteogenesis, but that their effectiveness is connected to different physiological and pathophysiological states (9).

Related to these recent reports and the current study, it is still a matter of discussion whether the pro-regenerative function of M2 macrophages in bone repair is related to their angiogenic properties and/or their ability for matrix degradation and how these characteristics may be effected by compromised biological conditions. While our work gives new insights concerning the beneficial effect of local macrophage enrichment on bone healing outcome, it is limited in showing M1/M2 dynamics early after transplantation. Additional research is needed to explore the exact role of M2 macrophages and other macrophage phenotypes in the early healing cascade.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committees, LaGeSo, G0120/14, G0172/15.

## AUTHOR CONTRIBUTIONS

AD has participated in conception and design of the study, acquisition, analysis and interpretation of data, and manuscript writing and editing. JL has contributed to data acquisition, analysis and interpretation of data, and manuscript writing and editing. AE, AR, FS, and SF have participated in acquisition, analysis and interpretation of data, and manuscript editing. GD contributed to conception and design of the study, interpretation of data, and manuscript editing. All co-authors approved the final version of the submitted manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02443/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# The Long Pentraxin PTX3 in Bone Homeostasis and Pathology

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The innate immune system is equipped with a number of germ-line encoded soluble pattern recognition molecules (PRMs) that collectively mediate the humoral host response to infection and damage in cooperation with cells and tissues of the immune and non-immune compartments. Despite the impressive diversity in structure, source, and regulation across PRMs, these all share remarkably similar functions inasmuch as they recognize microbes and damaged tissues, activate complement, exert opsono-phagocytic activities, and regulate inflammation. The long pentraxin 3 (PTX3) is a prototypic soluble PRM. Long known as a major player in innate immunity, inflammation and matrix remodeling, only recently has PTX3 emerged as a mediator of bone homeostasis in rodents and humans. *Ptx3*-targeted mice exhibit reduced trabecular volume during bone development, and impaired callus mineralization following experimental fracture. The murine gene is expressed *in vivo* by non-hematopoietic periosteal cells in the early phases of fracture healing, and *in vitro* by maturing osteoblasts. Human osteoblasts do express the PTX3 protein, whose levels positively correlate with bone density *in vivo* and osteoblast proliferation and maturation *in vitro*, thus pointing to a role in bone deposition. Contrasting evidence, however, suggest osteoclastogenesis-promoting effects of PTX3, where its expression has been associated with periodontitis, arthritis, and bone metastasis, conditions hallmarked by inflammation and bone resorption. Here, we review past and recent literature on the functions exerted by this long pentraxin in bone biology, with major emphasis on physiological skeletal remodeling, fracture healing, and chronic diseases of the bone.

**Keywords: pentraxins, PTX3, osteoblasts, osteoimmunology, periodontitis**

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## INTRODUCTION

The innate immune system holds an ancient place in evolution as a frontline mechanism of defense against exogenous and endogenous threats. The innate immune response initiates with recognition of pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively), by cell-borne and soluble mediators (i.e., PRMs), and progresses to pattern-tailored and microenvironment-dependent effector processes whose action extends far beyond pathogen disposal to embrace tissue homeostasis and cancer (1). Pentraxins are a superfamily of soluble PRMs with a multiplicity of functions in infection immunity, inflammation, and tissue remodeling.

C-reactive protein (CRP) and serum amyloid P component (SAP) are the "classical" or short pentraxins that share a typical quaternary structure with 5 or 10 identical subunits folding into pentameric rings. CRP and SAP are acute phase proteins in humans and mice, respectively, whose synthesis is primarily raised in the liver in response to the pro-inflammatory cytokine interleukin (IL)-6 (2, 3). Originally identified in the early 1990s as an IL-1β- and tumor necrosis factor (TNF)-α-inducible gene, PTX3 soon became the prototype of long pentraxins, a subfamily of proteins that contain a structurally unrelated N-terminal region in addition to the family-distinctive carboxy-terminal pentraxin domain (4). PTX3 is made by a number of both immune and non-immune cell types upon stimulation with primary pro-inflammatory mediators and microbial components, and exerts non-redundant roles in infection immunity (5), inflammation, and complement-mediated cancerogenesis (6).

An increasing body of evidence points to PTX3 as a key player in extracellular matrix (ECM) remodeling. In this regard, it is long known that *Ptx3*$^{-/-}$ female mice are sub-fertile, due to defective formation of the viscoelastic hyaluronic acid (HA)-rich matrix that surrounds the oocyte in the preovulatory follicle (i.e., the cumulus oophorous complex), where correct assembly of the cumulus matrix is required for fertilization *in vivo* (7, 8). PTX3 acts therein as a nodal molecule to crosslink HA in cooperation with tumor necrosis factor-inducible gene 6 (TNFAIP6, also known as TSG-6) and the heavy chains (HCs) of inter-alpha-trypsin inhibitor (IαI) (9–11). Also, in different mouse models of sterile tissue damage (skin wound, chemical injury of the liver and lung, arterial thrombosis), genetic ablation of *Ptx3* causes aberrant thrombotic responses, increased formation and prolonged duration of the fibrin clot, and enhanced collagen deposition (12–14). Within this frame, PTX3 expression and release is elicited in macrophages and mesenchymal cells by Toll-like receptors (TLRs) and IL-1β stimulation, and the locally released protein has fibrin remodeling and wound healing promoting effects (15). Furthermore, PTX3 has been shown to recognize selected fibroblast growth factors (FGFs), including FGF2 and FGF8b, through its N-terminal domain, and sequester them in the ECM, thus inhibiting their angiogenic and pro-tumorigenic effects *in vitro* and *in vivo* [see (16) for a review].

Bone remodeling is a peculiar instance of ECM turnover (17), where dynamic cell-cell and cell-matrix interactions set in place that involve a number of tissue growth factors, cytokines, and ECM components as well as increasingly acknowledged contributions from innate immune cells and soluble PRMs (18–20). The intimate crosstalk between immune and bone cells as well as soluble molecules is more apparent under conditions of extensive bone regeneration, e.g., after bone fracture or injury, where local acute inflammatory responses are required to initiate and propagate appropriate tissue healing and repair programs (21). Furthermore, infectious diseases of the bone, like periodontitis and osteomyelitis, set the scene for an even tighter cooperation between bone and immune components, as exemplified by the involvement of the complement system in the onset and progression of periodontitis (22).

As paradigmatic humoral PRM and key component of the ECM, PTX3 is emerging as a new mediator of bone physiopathology. Here, we present and discuss the current understanding of this long pentraxin in osteoimmunology, with an emphasis on recent evidence suggesting novel functions in physiological skeletal remodeling, bone healing, and chronic bone diseases (see **Tables 1** and **2**).

## GENE REGULATION AND PROTEIN STRUCTURE

Sequence and regulation of the *PTX3* gene are highly conserved in evolution, which has allowed assessing the pathophysiological roles of this long pentraxin in gene-targeted animals. The human and murine *PTX3* map on chromosome 3, and share a common structural organization with three exons coding for a leader peptide, the N- and C-terminal domains, respectively [see (4–6) and below].

Expression of the gene is promptly induced in a variety of immune and non-immune cell types by inflammatory cytokines (e.g., IL-1β, TNF-α), TLR agonists, microbial moieties (e.g., lipopolysaccharide, LPS, outer membrane protein A, OmpA, lipoarabinomannans), and intact microorganisms [see4 for a review on gene expression]. PTX3 production is also raised in granulosa cells by ovulation promoting hormones, whereby it participates in structuring the cumulus oophorous ECM, as discussed above (8, 9, 11). As opposed to this, transcription of the *PTX3* gene is inhibited by IFN-γ, IL-4, dexamethasone, 1α,25-dihydroxivitamin D3, and prostaglandin E2 (31, 32). Furthermore, PTX3 is constitutively stored as "pre-made" protein in the specific granules of polymorphonuclear cells (PMNs), is released in response to TLR stimulation, and localizes in the neutrophil extracellular traps (NETs) (33). Expression of the human *PTX3* gene is controlled by epigenetic mechanisms, including differential methylation of the promoter region and two enhancers in physiological and inflammatory conditions [see (34) for more details on epigenetic regulation]. We have recently reported that the murine *Ptx3* gene is expressed *in vitro* by maturing osteoblasts and *in vivo* by bone-encased osteocytes (23). Also, PTX3 expression has been documented in human osteoblasts, based on observations from both *in vivo* and *in vitro* studies (24, 26, 27, 35).

The human PTX3 protomer is a 381aa-long glycoprotein that contains a secretion-targeting signal peptide (1–17), an N-terminal region (18–178), and a C-terminal pentraxin domain (179–381). The N-terminal domain sequence is highly divergent from that of proteins with known structure, however, likely contains coiled-coils and intrinsically disordered regions, which are believed to contribute structural and functional versatility to the protein (36). The C-terminal pentraxin domain bears a single N-glycosylation site that is occupied by complex type oligosaccharides with tissue- and stimulus-dependent composition (37) and tuning effects on the protein's function in inflammation and innate immunity (38).

The mature PTX3 protein has a peculiar quaternary structure with eight identical protomer subunits folding into

**TABLE 1 |** PTX3 in bone homeostasis and experimental disease models.

| References | Species | Model | Cell lineage/Biological sample | Pathophysiological context/experimental condition | Biological effect |
|---|---|---|---|---|---|
| Grcevic et al. (23) | Mouse | *in vitro* | ob & oc | ob & oc differentiation | High PTX3 expression in early ob, but low in oc, differentiation |
| | | *in vivo* | Trabecular bone, fracture callus | Bone remodeling | Reduced BFR in *Ptx3*$^{-/-}$ mice |
| | | | | Fracture healing | Reduced callus mineralization in *Ptx3*$^{-/-}$ mice |
| Scimeca et al. (24) | Human | *in vivo* & *ex vivo* | Femoral head biopsy, ob | Osteoporosis | Reduced PTX3 expression in ob from osteoporotic patients |
| | | *in vitro* | ob | Treatment of primary ob from young healthy controls with anti-PTX3 antibody | Altered morphology, reduced RANKL and RUNX2 expression, and reduced mineralization |
| Zimmermann et al. (25) | Pig | *in vitro* | Bone-derived fibroblasts | Exposure to bone-conditioned medium w/o TGF-β receptor antagonist | Increased PTX3 expression, which is reversed by the TGF-β receptor antagonist |
| Chiellini et al. (26) | Human | *in vitro* | Multipotent adipose-derived stem cells | Osteogenic and adipogenic induction | Enhanced PTX3 expression as compared to undifferentiated cells, more pronounced during adipogenesis |
| Lee et al. (27) | Human | *in vitro* | BM-derived stromal cells | Osteogenic induction ± TNF-α | Increased PTX3 expression and secretion in early, but not late, steps of differentiation; further enhanced by TNF-α |
| | Mouse | *in vivo* | BM | LPS-induced bone loss | Higher PTX3 expression in LPS- versus vehicle-treated mice |
| | | *in vitro* | ob & oc | ob & oc differentiation & function in the presence of exogenous PTX3 | No effect on oc and ob differentiation; in early ob, increased RUNX2 and RANKL expression |
| Keles et al. (28) | Rat | *in vivo* | Gingival tissue & serum | Ligature-induced experimental periodontitis | PTX3 levels correlate with early, not late, phases of disease |
| Tsuge et al. (29) | Rat | *in vivo* | PDL | Orthodontic tooth movement | PTX3 levels increase at early time points |
| Garcia et al. (30) | Mouse | *in vivo* | Arthritic joint | STIA | PTX3 levels increase in *Mmp8*$^{-/-}$ mice |

*ob, osteoblast; oc, osteoclast; BFR, Bone Formation Rate; BM, Bone Marrow; TGF-β, Transforming Growth Factor-β; PDL, periodontal ligament; STIA, Serum Transfer-Induced Arthritis.*

an asymmetric and rather elongated molecule that is stabilized both by disulfide bonds and non-covalent interactions (36). This structural complexity is necessary for the long pentraxin to engage in a number of interactions with a variety of ligands, including microbes, complement and matrix proteins, and thereby accomplish its pleiotropic functions [reviewed in (39)].

## BONE HOMEOSTASIS AND FRACTURE HEALING

Excessive and uncontrolled inflammation has bone resorbing effects due to suppression of osteoblast and enhancement of osteoclast functions (17–21). On the other hand, proinflammatory mediators are required for physiological bone remodeling, a highly coordinated process that couples bone resorption and formation to maintain structural integrity and metabolic balance, and is regulated by mechanical loading, microdamage, hormonal signals, and local factors (17, 40, 41). Moreover, an inflammatory milieu is necessary to promote tissue regeneration after bone fracture or injury (21, 42, 43). Fracture healing proceeds through sequential steps of inflammation, induced angiogenesis, mesenchymal progenitors recruitment, cartilage and bone formation, extracellular matrix synthesis, and callus remodeling. During this process, a balanced local microenvironment, ensured both by immune and bone cells, is crucial for the beneficial effects of transient acute inflammation on bone regeneration. Amongst the bone-active inflammatory mediators, PTX3 has been shown to participate in bone homeostasis, based on *in vivo*, *ex vivo* and *in vitro* evidence, which is discussed in the following paragraphs and summarized in **Table 1**.

### *In vivo*

Our group has recently shown that genetic ablation of *Ptx3* in the mouse leads to reduced osteoblast function and bone

**TABLE 2 |** PTX3 in human chronic bone diseases.

| References | Model | Biological sample | Pathophysiological context/experimental condition | Biological effect |
|---|---|---|---|---|
| Pradeep et al. (46) and Fujita et al. (47) | *in vivo* | GCF & plasma | Gingivitis and periodontitis | PTX3 levels increase during disease progression |
| Gumus et al. (48) | *in vivo* | Saliva & serum | Periodontitis | PTX3 levels correlate with periodontal tissue inflammation |
| Lakshmanan et al. (49) | *in vivo* | Gingival tissue | Periodontitis | PTX3 concentration is higher in aggressive as compared to chronic periodontitis |
| Leira et al. (50) | *in vivo* | Serum | Periodontitis-Chronic Migraine (PD-CM) | Increased PTX3 levels as compared to CM without PD |
| Temelli et al. (58) | *in vivo* | Serum | Coronary Artery Disease (CAD) | PTX3 levels positively correlate with periodontal inflamed surface area (PISA) in CAD(-) groups |
| Leira et al. (59) | *in vivo* | Serum | Lacunar Infarct (LI) | PTX3 levels positively correlate with PISA in patients with poor prognosis |
| Surlin et al. (60) | *in vivo* | GCF | Orthodontic tooth movement | PTX3 levels increase at early time points |
| Luchetti et al. (51) | *in vitro* | Synoviocytes | RA & OA | PTX3 levels increase in OA cells upon TNF-α stimulation, while they are constitutively elevated in RA cells |
| | *ex vivo* | Synovial tissue | RA & OA | High PTX3 immunoreactivity in RA tissue as compared to controls |
| Yokota et al. (52) | *in vitro* | FLS | RA | PTX3 expression is inhibited by simvastatin treatment |
| Satomura et al. (53) | *in vitro* | Synoviocytes | RA | PTX3 expression is induced by serum amyloid A |
| Padeh et al. (54) | *in vivo* | SF | Juvenile idiopathic arthritis | Higher PTX3 levels associate with disease severity and prognosis |
| Choi et al. (56) | *in vitro* | GCC | Advanced gastric cancer | PTX3 expression is induced by TNF-α via NF-kB; PTX3 enhances tumor cell migration and macrophage recruitment |
| Choi et al. (57) | *ex vivo* | Metastatic tissues | Metastatic breast cancer | Elevated PTX3 expression correlates with poor survival |
| | *in vitro* | BM-BCCL | Metastatic breast cancer | High PTX3 levels. PTX3 silencing prevents BM-BCC migration, macrophage chemotaxis, and oc formation |

*GCF, gingival crevicular fluid; PDL, Periodontal Ligament; RA, Rheumatoid Arthritis; OA, Osteoarthritis; FLS, Fibroblast-Like Synoviocytes; SF, Synovial Fluid; GCC, Gastric Cancer Cells; BM-BCCL, Bone Metastatic-Breast Cancer Cell line; Oc, osteoclast.*

formation (23). Indeed, micro-computed tomography and bone histomorphometry indicated that *Ptx3*$^{-/-}$ mice on B6 background (2.5 months of age) had lower trabecular bone mass than their wild type littermates in long bones and axial skeleton. This phenotype was more obvious in female animals, known to have lower bone formation rate than males of the same age (44, 45). Similar observations were made in the long bones of young *Ptx3*$^{-/-}$ females on SV129 background and aged (6–8 months) *Ptx3*$^{-/-}$ females on B6 background (23).

Further histomorphometric investigations showed no alterations in osteoclast activity, however osteoblast functionality was defective, which resulted into decreased trabecular and endosteal bone formation rate in distal femora of both female and male *Ptx3*$^{-/-}$ mice. In this regard, additional data regarding the cortical compartment and mechanical testing would likely provide further insights into the bone phenotype associated to *Ptx3* deficiency.

The role of PTX3 in bone formation was further evaluated in a tibia mid-diaphyseal fracture model that showed the protein to be made by osteoprogenitor cells, hypertrophic chondrocytes, and active osteoblasts surrounding the fracture gap (23). Specifically, the *Ptx3* gene was found expressed in cells of the non-hematopoietic compartment including α-smooth muscle actin (α-SMA)$^{+}$ osteoprogenitors and CD51$^{+}$ preosteoblasts that populate the soft callus tissue early after bone fracture. These findings were corroborated by immunohistochemistry analyses showing high levels of the PTX3 protein and the osteoblast-specific transcription factors osterix (OSX) and runt-related transcription factor 2 (RUNX2) at the fracture site. Noticeably, proximal to the fracture gap FGF2-positive areas were observed that overlapped with those of osteoprogenitor cells' infiltration, suggesting that PTX3 and FGF2 co-localize and might engage in a complex with potential effects on the FGF2 suppressive activity on osteoblast differentiation (see the

"*Ex vivo* and *in vitro*" paragraph in this section). Furthermore, PTX3 was present in the callus during the mineralizing phase, and both percentage of mineralized callus and expression of type 1 collagen were lower in $Ptx3^{-/-}$ female mice than in $Ptx3^{+/+}$ controls (23).

These observations await confirmatory evidence from other mouse models of physiological bone remodeling and fracture healing, however they are in line with available information from human osteology. In this regard, Scimeca et al. have reported reduced PTX3 expression in the osteoblasts from femoral head biopsies of osteoporotic patients compared to age-matched osteoarthritic patients and young subjects who had undergone post-fracture hip arthroplasty (24). In the same clinical setting, biopsy specimens from osteoporotic patients had reduced trabecular volume (as assessed by histomorphometry) as well as lower expression of RUNX2 and vitamin D receptor (as analyzed by immunohistochemistry). Based on this, the authors proposed PTX3 as a positive regulator of the osteoblast function in physiological conditions, however their conclusions might suffer from the lack of aged-matched control groups without bone pathology in the study design.

### *Ex vivo* and *in vitro*

Human and animal mesenchymal and osteoblast lineage cells express PTX3 at various stages of differentiation (12, 23–27, 35). In a lineage tracing approach, PTX3 expression was documented in murine α-SMA$^+$ early osteoprogenitors with proliferative and multi-lineage potential (35). During *in vitro* differentiation of mouse bone marrow-derived stromal cells in osteogenic conditions, the *Ptx3* gene was highly expressed along with osteoblast differentiation markers (i.e., *Osx*, alkaline phosphatase and osteocalcin) (23). In similar osteogenic conditions, the PTX3 protein was found in cultured human bone marrow-derived stromal cells at the preosteoblast stage, and its expression was further increased by TNF-α (27). Moreover, a proteomic study on the human mesenchymal cell secretome indicated up-regulation of PTX3 in human multipotent adipose-tissue derived mesenchymal cells directed to adipogenesis or osteogenesis (26). Finally, human osteoblasts isolated from the trabecular bone of femoral head biopsies expressed high levels of both gene and protein when cultured *in vitro*, and the expression was downregulated in osteoporosis (24).

Despite relatively consistent findings on PTX3 expression in mouse and human osteoblast lineage cells, particularly at the early stages of differentiation, data on the protein's role in osteoblast differentiation are rather conflicting. Treatment of mouse calvarial osteoblasts with different doses of the recombinant protein (0.02–0.47 nM) had no effect on osteoblast proliferation, differentiation, and mineralization (as monitored by alkaline phosphatase, alizarin red, and Von Kossa staining) (27). Similarly, addition of higher concentrations of the exogenous protein (6.25–50 nM) did not change the area covered by colonies expressing alkaline phosphatase in differentiated mouse bone marrow-derived stromal cells. Furthermore, bone marrow-derived osteoprogenitors from $Ptx3^{+/+}$ and $Ptx3^{-/-}$ mice had similar differentiation potential. Nonetheless, PTX3 (and its N-terminal domain that binds FGF2) reversed the inhibitory effect of FGF2 on osteoblast differentiation, which suggests an indirect effect of the protein on these cells (23).

In contrast to the mouse studies, PTX3 (at 0.47 nM) was shown to accelerate proliferation and hydroxyapatite microcrystal formation in human osteoblasts derived from femoral head biopsies (24). However, these experiments were performed using osteoblasts from osteoporotic patients only at the first or second passage from confluence (∼4 weeks of culturing), in the absence of osteogenic stimuli. In addition, in a similar experimental setting, osteoblasts from control subjects (post-fracture hip arthroplasty) underwent significant functional and morphological changes upon treatment with an anti-PTX3 blocking antibody, in particular they acquired a fibroblast-like shape and downregulated the expression of RUNX2 and receptor activator of NF-κB ligand (RANKL) (24).

## CHRONIC DISEASES OF THE BONE

Several lines of evidence point to PTX3 as a key player in inflammatory diseases, however a few studies only have addressed its contribution to osteoclast activity and inflammation-induced bone loss. In this regard, data are available on periodontitis (28, 46–50), arthritis (30, 51–54), and tumor-associated osteolysis (55–57) (see **Table 2**). Periodontal infections initiate in the bacterial plaque attached to the tooth surface (mostly Gram-negative anaerobic bacteria), and progress to a chronic disease with irreversible periodontal tissue destruction and osteoclast activation, eventually leading to tooth loss (61, 62). Chronic joint diseases mostly develop as autoimmune processes (i.e., rheumatoid arthritis) or as a result of cartilage damage (i.e., osteoarthritis), and are characterized by joint inflammation and progressive destruction of cartilage and bone (30, 51–54). Malignancies commonly manifest in the skeleton in the form of focal osteolytic lesions associated to metastases, whereby bone resorption (as sustained by osteoclasts) is necessary for tumor cells to grow and invade the mineralized bone (63). Although clinically distinct, these pathological scenarios share a common hallmark, i.e., chronic uncontrolled inflammation with aggravated osteoresorption. In this regard, soluble factors, including acute phase proteins, proinflammatory cytokines and chemokines, antibodies, prostaglandins, tissue-destructive enzymes, and osteoclastogenic mediators collectively participate in the inflammatory process, contributing to bone tissue breakdown. Several pre-clinical and clinical studies point to an association between PTX3 expression and osteoclast activity, which are discussed in the following paragraphs.

### *In vivo*

Periodontal disease is often associated to elevation of inflammatory markers both in periodontal tissues and circulation. In a rat model of periodontitis, high levels of the PTX3 protein were found in the gingival tissue and serum, which correlated with alveolar bone resorption and inflammatory cells' infiltration (28). Among other acute phase proteins, PTX3 levels were elevated in the gingival crevicular fluid and plasma of patients with periodontitis, and correlated with the clinical

score of the disease (46, 47). Indeed, PTX3 concentration in the gingival tissue of patients with generalized aggressive periodontitis was higher than in those with chronic periodontitis (48, 49). Moreover, recent studies indicated that periodontitis may lead to systemic upregulation of both inflammatory and endothelial dysfunction markers, including PTX3, serum amyloid A (SAA), and amyloid-β peptide (50, 58, 59). In addition to periodontitis, PTX3 (and other inflammatory mediators) was found abundant in the periodontal ligament of the tension zone during orthodontic tooth movement, characterized by enhanced osteoclast activity and rapid bone remodeling (29, 60).

In a mouse model of LPS-induced inflammation, PTX3 expression was found up-regulated in the femoral bone-marrow in areas of increased osteoclast number and osteolytic phenotype (27). Also, PTX3 has been reported to accumulate in the arthritic joints, possibly contributing to the local inflammatory and osteodestructive milieu (30, 51). In a K/BxN serum-transfer arthritis model, PTX3 mRNA and protein levels were both elevated in the ankle joints of arthritic mice, and further increased in arthritic animals lacking matrix metallopeptidase 8, which had more severe disease (30). Expression of PTX3 has also been analyzed in human synovial fluids and tissues from total knee arthroplasty. Immunohistochemistry indicated that the protein co-localizes with endothelial cells and synoviocytes, and is particularly abundant in patients with rheumatoid arthritis, as compared to those with osteoarthritis and post-traumatic effusion (51). Increased levels of this pentraxin were found in the synovial fluid of patients with different clinical forms of juvenile idiopathic arthritis, with a positive correlation with disease severity and progression (54).

In the context of tumor-associated osteolysis, PTX3 expression (based on public genome-wide gene expression data) has been reported to be up-regulated in the distant bone metastases of breast cancer as compared to lung, liver and brain metastases (56, 57), and this has been linked to enhanced osteolysis (see below).

## *Ex vivo* and *in vitro*

*In vitro* expression of *Ptx3* has been documented in osteoclastogenic cultures of bone marrow cells stimulated with RANKL and monocyte/macrophage colony-stimulating factor (M-CSF) (23). In this setting, addition of the exogenous protein (0.02–50 nM) did not change the number of differentiated osteoclasts expressing tartrate-resistant acid phosphatase (23, 27). However, Lee et al. have reported that PTX3 had an indirect osteoclastogenic effect by increasing the

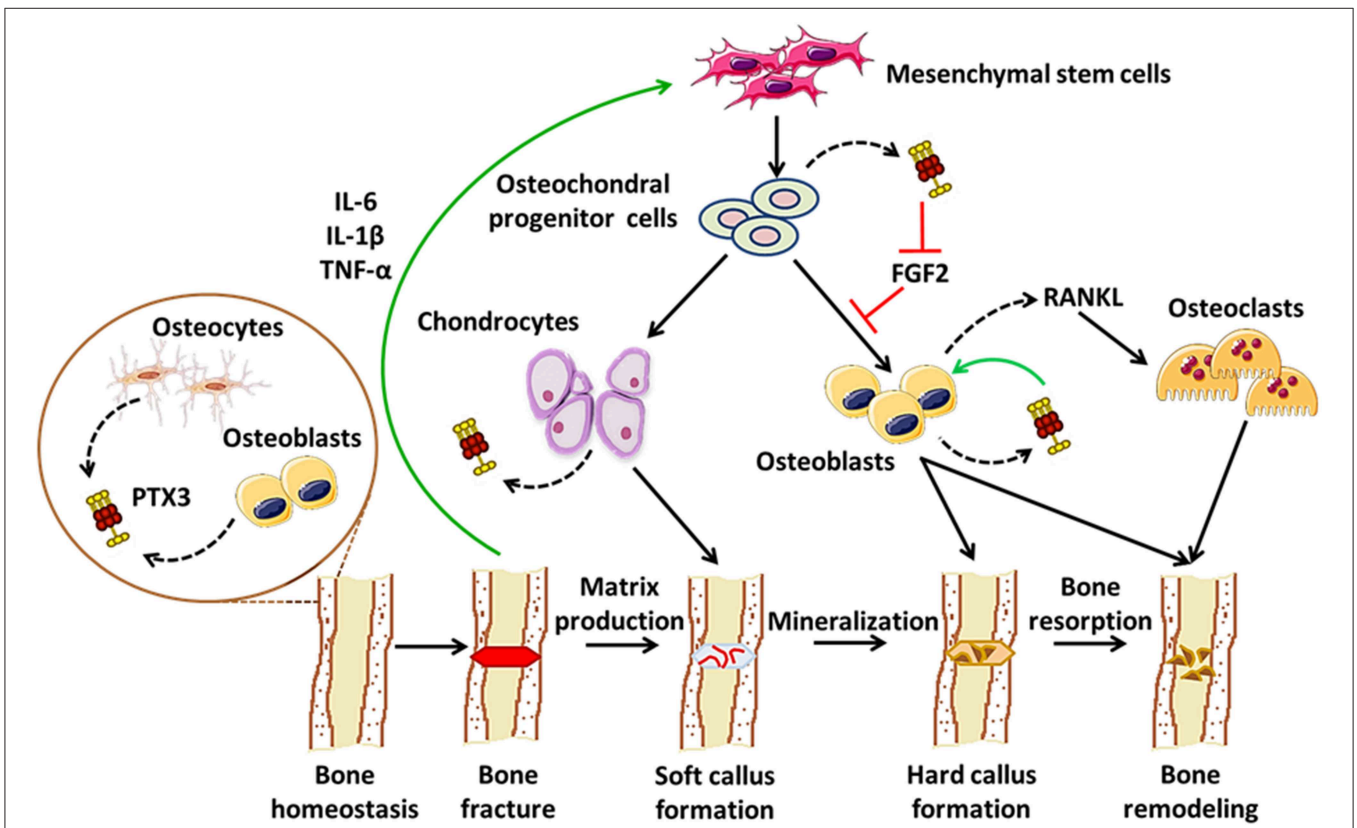


**FIGURE 1 |** Proposed roles of PTX3 in bone homeostasis and fracture healing. In conditions of physiological bone turn-over, PTX3 is expressed by osteoblasts and bone-encased osteocytes, likely contributing to bone deposition via yet unknown mechanisms (24). Following fracture, inflammatory mediators (e.g., IL-6, IL-1β, TNF-α), along with other factors, promote osteoblast development, and differentiation. In these conditions, PTX3 (made by osteochondral progenitor cells, chondrocytes, and osteoblasts) reverses the inhibitory effects exerted by FGF2 on osteoblast differentiation, thereby contributing to matrix mineralization (23). In the late stages of fracture healing, PTX3 likely participates in bone remodeling by stimulating RANKL production and osteoclastogenesis (27).

RANKL/osteoprotegerin (OPG) ratio in mouse calvarial preosteoblasts but not in mature osteoblasts (27). In co-cultures of mouse preosteoblasts and bone marrow cells (stimulated with vitamin $D_3$ and prostaglandin $E_2$), PTX3 (0.02–0.47 nM) enhanced osteoclast differentiation, and *Ptx3* silencing (by siRNA) in preosteoblasts had opposite effects. Addition of TNF-α further increased the number of differentiated osteoclasts, a process that was counteracted by *Ptx3* gene silencing (27). However, an active role of other bone marrow cells (that are present in the applied co-culture system) cannot be ruled out.

Inflammatory cytokines, particularly TNF-α, have been described to induce PTX3 expression in cultured human bone marrow-derived preosteoblasts (27), osteoarthritic synoviocytes (51), and synovial cell lines (54). Moreover, synoviocytes from patients with rheumatoid arthritis constitutively express high levels of PTX3 mRNA and protein *in vitro* (51, 52), and these were not affected by neutralization of TNF-α or IL-1β (51).

Breast and gastric cancer cell lines have been reported to express PTX3, and the exogenous protein promoted migration of breast cancer cells and macrophages (56, 57). In an *in vitro* system where a human breast cancer cell line and a mixture of mouse calvarial osteoblasts and bone marrow derived macrophages were co-cultured in the upper and lower chambers of a transwell, respectively, stimulation of PTX3 expression in the tumor cells by TNF-α enhanced *Rankl* expression and osteoclast formation in the lower compartment, suggesting a role for PTX3 in cancer-related osteolysis that however requires validation *in vivo* (57).

## CONCLUDING REMARKS

As a paradigmatic component of the humoral arm of innate immunity, PTX3 exerts a number of functions at the crossroad between host-pathogen interface, inflammation, and matrix remodeling (6). Recent evidence points to contrasting roles for PTX3 in bone pathophysiology: on one hand, it acts as a promoter of osteoblast differentiation and mineral matrix deposition (23); on the other, it supports osteoclastogenesis in inflammatory conditions, including arthritis and bone metastasis (27, 56, 57). Furthermore, PTX3 has been associated with periodontal tissue inflammation, a condition that precedes alveolar bone resorption (52–57). These pleiotropic effects possibly derive from inherent differences in the applied experimental models, and are likely amplified by the structural complexity of the PTX3 protein that supports a multiplicity of interactions, thereby context-dependent functions. In this regard, we propose a model that accounts for stage-specific expression and activity of this pentraxin in physiological bone remodeling and fracture healing, i.e., osteoblast-derived PTX3 likely contributes to bone growth, the inflammatory response that follows bone fracture leads to up-regulation of the gene in osteochondral progenitor cells, chondrocytes and osteoblasts, where the newly made protein has FGF2-dependent matrix mineralization promoting effects, in the late phases of fracture healing PTX3 participates in bone remodeling via stimulation of RANKL synthesis and osteoclastogenesis (see **Figure 1**). This information notwithstanding, little is known regarding bone-related effects of the interaction between PTX3 and its cognate ligands (besides FGF2) with established properties in bone physiopathology, for examples the complement system (64). Also, given the prominent protective role of PTX3 in the resistance to selected microbial pathogens, assessing its function in bone infections other than periodontitis (e.g., osteomyelitis) is relevant and deserves further investigations, owing to its potential application in human diseases.

## AUTHOR CONTRIBUTIONS

AI and DG wrote the manuscript. RP took care of the tables, figure, and legend. CS, BB, and AM contributed to critical revision.

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# Spatial Distribution of Macrophages During Callus Formation and Maturation Reveals Close Crosstalk Between Macrophages and Newly Forming Vessels

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Macrophages are essential players in the process of fracture healing, acting by remodeling of the extracellular matrix and enabling vascularization. Whilst activated macrophages of M1-like phenotype are present in the initial pro-inflammatory phase of hours to days of fracture healing, an anti-inflammatory M2-like macrophage phenotype is supposed to be crucial for the induction of downstream cascades of healing, especially the initiation of vascularization. In a mouse-osteotomy model, we provide a comprehensive characterization of vessel ($CD31^+$, $Emcn^+$) and macrophage phenotypes (F4/80, CD206, CD80, Mac-2) during the process of fracture healing. To this end, we phenotype the phases of vascular regeneration—the expansion phase (d1–d7 after injury) and the remodeling phase of the endothelial network, until tissue integrity is restored (d14–d21 after injury). Vessels which appear during the bone formation process resemble type H endothelium ($CD31^{hi}Emcn^{hi}$), and are closely connected to osteoprogenitors ($Runx2^+$, $Osx^+$) and $F4/80^+$ macrophages. M1-like macrophages are present in the initial phase of vascularization until day 3 post osteotomy, but they are rare during later regeneration phases. M2-like macrophages localize mainly extramedullary, and $CD206^+$ macrophages are found to express Mac-$2^+$ during the expansion phase. VEGFA expression is initiated by $CD80^+$ cells, including $F4/80^+$ macrophages, until day 3, while subsequently osteoblasts and chondrocytes are main contributors to VEGFA production at the fracture site. Using Longitudinal Intravital Microendoscopy of the Bone (LIMB) we observe changes in the motility and organization of $CX3CR1^+$ cells,

which infiltrate the injury site after an osteotomy. A transient accumulation, resulting in spatial polarization of both, endothelial cells and macrophages, in regions distal to the fracture site, is evident. Immunofluorescence histology followed by histocytometric analysis reveals that F4/80$^+$CX3CR1$^+$ myeloid cells precede vascularization.

**Keywords: bone regeneration, macrophage, endothelial cell, H-type vessel, intravital microscopy, LIMB, CX3CR1 myeloid cells**

## INTRODUCTION

Bone healing is a spatiotemporally regulated regeneration process, ideally leading to complete restoration of the broken bone without fibrous scar formation (1). Naturally, this regeneration process undergoes endochondral bone formation, if interfragmentary movements strain the fracture gap (2). In the majority of clinical cases, fracture healing follows the endochondral route and may be sub-divided into five phases, namely an (i) initial pro-inflammatory phase, (ii) anti-inflammatory phase, (iii) fibrocartilaginous or soft callus phase, (iv) mineralization or hard callus phase and (v) remodeling phase in which bone tissue regains its physiological shape with a restored bone marrow cavity. While fracture healing occurs in most cases without delay, still 5–10% of patients suffer from delayed healing or non-union. To avoid delayed healing and overcome non-unions, it is important to understand the finely orchestrated processes of bone regeneration (3, 4).

Upon a fracture, the vessels get disrupted and nutrient supply is lacking at the injury site. However, the vascular system is essential for healing, by supplying cells with oxygen and nutrients, removing debris and allowing the recruitment of circulating cells. Endothelial progenitors (CD31$^+$) migrate to the fracture site from the bone marrow or from pre-existing vessels of the periosteum (5–9). In earlier work, we could show that revascularization peaks during two phases of fracture healing: around day 7 (end of the inflammatory phase) and around day 21 (woven bone formation) in sheep (6). Drastic vascular structural plasticity has also been shown during bone marrow regeneration between 7 and 21 days by our group using a longitudinal microendoscopic method at cellular resolution (10). Angiogenic factors, such as vascular endothelial growth factor (VEGF) are of great importance in order to induce vascularization. Street et al. showed that the circulating plasma levels of VEGF are highly increased in patients with fractures and that the fibrin matrix within the fracture hematoma acts like a VEGF reservoir (11). In addition, we reported in a previous study that cells within the fracture hematoma exhibit upregulated VEGF expression and secretion (12, 13). Osteoprogenitor cells also produce VEGF as a consequence of the hypoxic environment, leading to enhanced transcriptional activity of hypoxia-inducible factor 1-alpha (HIF-1α) (14, 15). Buettmann et al. most recently showed that especially the release of VEGFA by Osterix (Osx)$^+$ osteoprogenitors/pre-osteoblasts is critical for vessel formation during fracture healing (16). It is well-known that the crosstalk between endothelium and bone cells is essential for the integrity and formation of bone. Osteoblasts support the vasculature by producing VEGF and basic fibroblast growth factor (bFGF) while endothelial cells (ECs) provide factors that further osteoblast differentiation and activity (17). Furthermore, pre-osteoclasts and non-bone-resorbing osteoclasts have been described to enhance vascularization and osteogenesis in the growth plate area by releasing platelet-derived growth factor-BB (PDGF-BB) or supporting vessel anastomosis (18, 19). While the production of angiogenic factors by osteoprogenitors is well-known, there is evidence accumulating that, conversely, endothelial cells can also impact on bone formation, at least during bone development (7). Bone development is initiated by blood vessel invasion, and the arrival of osteoprogenitors. Subsequently, specialized type H blood vessels secrete osteogenic factors, required for the induction of bone formation and growth (20). Although the presence of type H vessels has been reported at sites of bone regeneration (10, 21), early events inducing the formation of these vessels in those situations have not yet been investigated.

Next to the adaptive immunity, the importance of the innate immune system for regenerative processes has been shown by several studies (22–26). Macrophages have been identified as key players for the recovery of tissue integrity and function. Several different types of myeloid-lineage cells can be distinguished in bone regeneration (23). Tissue-resident macrophages (also termed osteomacs) which express F4/80, can be found closely to bone-lining cells, and support intramembranous bone formation as well as endochondral ossification (22, 25, 26). Recruited immune macrophages (M1-like/M2-like) are more pivotal in endochondral ossification, which has been shown by Schlundt et al., who deleted macrophages in osteotomized mice by treatment with clodronate liposomes, and Alexander et al. who examined macrophage subsets close to the periosteum during regeneration (24, 25). Furthermore, osteoclasts are multinucleated tartrate-resistant acid phosphatase (TRAP)$^+$F4/80$^-$ myeloid cells, which derive from fusion events (22).

In other tissues or scenarios, macrophages are essential for vascularization and angiogenesis. They are able to degrade extracellular matrix (ECM) and release pro-angiogenic factors. Degradation of the ECM enables the migration of endothelial progenitors and activates the angiogenic potential of some ECM molecules, as has been shown for fragments of hyaluronic acid (27). In addition, macrophages also release factors that attract, activate or even inhibit angiogenic cells depending on the phase of vascularization (28). Studies during mouse development revealed the tight association of macrophages with capillaries and the subsequent enhancement of angiogenesis (29, 30). Macrophages have also been shown to regulate vessel

permeability comparable to pericytes (31). *Vice versa,* endothelial cells (ECs) also promote the selective growth and differentiation of macrophages, especially the switch towards an M2-like phenotype, which requires direct contact with the endothelium and the regulation via macrophage colony-stimulating factor (M-CSF) signaling (32). However, a potential crosstalk between macrophages and ECs and the details of such interactions during bone regeneration have not been studied so far.

Within this study, we aim to analyze the initial phases of angiogenesis in bone healing with vascular regeneration and their dependence on the presence of macrophages. We focus on the early regeneration events, until the shift from pro- to anti-inflammatory phase, where a close crosstalk of blood vessels with macrophages is most prominent (d1–d7 after injury) and compare this to the bone remodeling phase driven by osteoprogenitors and mineralized tissue formation (d14–d21 after injury).

# METHODS

## Animal Husbandry, Housing, and Surgery

### Mouse-Osteotomy-Model

All animal experiments were approved by the local animal protection authority (LaGeSo; permit numbers: G0039/16 and G0111/13) following the German Animal Welfare Act.

Female C57BL/6N mice aged 10 weeks were ordered from Charles River Laboratories (Sulzfeld, Germany) and underwent surgery at the age of 12 weeks with an average body weight of 22 g. Housing took place in a conventional, semi-barrier (non-SPF) facility and randomly split in groups with at least 2 mice per cage housed in Eurostandard Type II clear-transparent plastic cages with a wire lid and filter top. Fine wood chips (Lignocel FS 14, J. Rettenmaier & Söhne GmbH + Co. KG, Germany) and nesting material (Envirodri®, Shepherd Specialty Papers, USA) was provided. Houses and pipes were removed after surgery to avoid injuries due to the external fixator. Food (Standard mouse diet, Ssniff Spezialdiäten, Germany) and tap water was provided *ad libitum*, and room temperature was between 20 and 22°C with a humidity of 45–50%. The light/dark cycle was a 12/12-h cycle. Animals were tail and cup handled. Anesthesia was induced at 2.5% isoflurane (CP-Pharma, Germany) and maintained at 1.5%. In order to cover pain after the surgery prior to surgery, all animals received Buprenorphine (0.03 mg/kg; Temgesic, Indivior Eu Ltd., UK) s.c. as analgesic, an eye ointment and clindamycin (0.02 ml; Ratiopharm, Germany). After shaving and disinfecting the left femur area animals were placed on a heating mat and osteotomy was performed under aseptic conditions as described earlier (21, 24). In short, the femur was prepared bluntly, after a lateral longitudinal incision of the skin between hip and knee. The external fixator (MouseExFix, RISystem, Switzerland) was placed parallel to the femur by serial drilling of the pins (0.45 mm diameter). With a Gigli wire saw (RISystem, Davos, Switzerland), a 0.70 mm osteotomy gap was created in the middle of the femur and flushed with NaCl. Following skin closure, mice received pre-warmed NaCl (0.2 ml) s.c., permeable wound dressing spray and could recover from anesthesia in their home cage under infrared light and close monitoring. Tramadol was applied via the drinking water (0.1 mg/ml; Grünenthal, Germany) for 3 days after osteotomy (33). Given the short time period of treatment, we expect no negative influence of the analgetics on the fracture healing outcome (33). Surgery was performed by two trained veterinarians. For general scoring and humane endpoints, optimized protocols were used which has been summarized in Lang et al. (34).

### Combined Osteotomy and Intravital Imaging Model

All animal experiments were approved by the local animal protection authority (LaGeSo; permit numbers: G0302/17) following the German Animal Welfare Act.

Cx3cr1$^{tm1Litt}$ (CX3CR1:GFP), a fractalkine receptor (CX3CR1) reporter mouse, and C57BL/6J animals were bred in our colony. Heterozygous female mice were 14 weeks of age when osteotomy was performed. Housing took place in a conventional SPF barrier facility. Prior to surgery, all animals received Buprenorphine (0.03 mg/kg; Temgesic, Indivior Eu Ltd., UK) s.c. as analgesic and eye ointment. After shaving and disinfecting the left femur area animals were placed on a heating mat and osteotomy was performed under aseptic conditions. Surgery was performed as previously described (10), using four bi-cortical screws, and combined with osteotomy. In short, the internal fixator's Gradient Refractive INdex (GRIN) lens tubing was modified to be screwed into the fixator plate after implantation and osteotomy. Osteotomy was performed using a 0.22 mm Gigli wire saw (RISystem, Switzerland) and two cuts for an osteotomy gap size of ∼816 µm (CI: 787–844 µm; $SD$ = 85 µm; $n$ = 37). After removing the generated bone piece, the lens tube was positioned into the osteotomy gap and screwed into the fixator plate. Analgetics (Tramadol, Buprenorphine) were applied as described above. For antibiotic treatment mice received one injection of 0.04% Enrofloxacin (Baytril, 10 mg/kg body weight Bayer AG, Germany) before surgery.

## Bone Sample Preparation

Femoral bones were explanted, muscles largely removed in a way that osteotomized bone parts maintained one entity. Tissue was fixed using 4% electron microscopy-grade PFA in PBS for 4 h at 4°C, washed in PBS, and ran through a sucrose gradient (10%, 20%, 30%; á 12–24 h). The fixators were removed from the fixed samples, underwent µCT measurement, bones were frozen in SCEM medium (Sectionlab, Japan), cut into slices of 7 µm using Kawamoto's film method (35), and stored at −80°C.

## Histology

Movat's Pentachrome staining was conducted as descried previously (21, 24). TRAP staining for quantification was performed using a kit following the manufacturer's instructions (Thermo Scientific, 386A-1KT, MA, US). Individual slides were stained using small volumes of staining solutions on a heating plate at 37°C. For immunofluorescence, individual sections were thawed, rehydrated in PBS, blocked with 10% donkey serum, and stained with antibodies in PBS/0.1% Tween 20/5% donkey serum containing DAPI for 1–2 h. Target

proteins were identified using antibodies against CD31/PECAM-1 (goat polyclonal unconjugated, AF2628, R&D Systems, 1:100), CD206/MMR (C068C2 conjugated to AF594, BLD-141726, 1:100), CD80 (goat polyclonal unconjugated, AF740-SP, 1:100), Endomucin (Emcn) (V.7C7 unconjugated, sc-65495, 1:100), F4/80 (Cl:A3-1 unconjugated, MCA497G, 1:400), GFP (goat polyclonal conjugated to AF488, 600-101-215, 1:100), Ly-6C (ER-MP20 biotinylated, MA5-16666, 1:20), Ly-6G (1A8 biotinylated, BLD-127603, 1:200), Mac-2/Galectin-3 (M3/38 unconjugated, BLD-125401, 1:100), Osx (rabbit polyclonal, sc-22536-R, 1:200), Runx2 (EPR14334 conjugated AF647, ab215955, 1:100), Sox9 (EPR14335 unconjugated, ab185230, 1:200), VEGFA (rabbit polyclonal unconjugated, ab46154, 1:100). Primary antibodies were stained with secondary antibodies when unconjugated (1:500, Thermo Fisher, anti-rat conjugated AF488, A21208; anti-rat conjugated AF546, A11081; anti-rat conjugated AF594, A21209; anti-rabbit conjugated AF488, A21206; anti-rabbit conjugated AF546, A10040; anti-rabbit conjugated AF647, A31573; anti-goat conjugated AF647, A21447; or streptavidin conjugated AF546, S11225). Samples were washed between steps and after staining with PBS/0.1 % Tween 20 for 3 × 5 min. Stained samples were kept in PBS for 5 min and embedded using aqueous mounting medium (Fluoromount, Thermo Fisher, MA, US) and analyzed microscopically within 6 days. Simultaneous detection of Ly6C and Ly6G was considered to indicate presence of the Gr-1 protein.

Movat's Pentachrome images were taken with a light microscope in a 2.5 × magnification and the program AxioVision (both Carl Zeiss Microscopy GmbH, Germany). For **Figure 2**, images (CD31 & Emcn) were taken with a Keyence microscope (BZ 9000) using a 10x or 4x objective. All other images were acquired at a Zeiss LSM880 in tile scan mode at a resolution of 2048 × 2048 using a 20x objective, unless specified otherwise. For display, pictures were background subtracted and contrast adjusted using ImageJ 1.52i.

## Image Analysis

Image analysis of cell and tissue distribution (**Figure 2**) was performed with ImageJ and an own developed pipeline which has been published and described in detail previously (21). Mean intensity was determined with ImageJ within the marked ROIs distinguishing between the gap, the adjacent to the gap and the bone marrow area. Mean intensities were normalized to the maximum intensity of the image.

Quantification was performed in *CellProfiler 3.1.8.* (36) (**Supplementary Figure 1**). Macrophage subsets were described via the co-localization of identified CX3CR1$^+$, Gr-1$^+$, and F4/80$^+$ objects. CX3CR1$^+$F4/80$^+$Gr1$^+$ objects, representing cells were divided by roundness based on object shape features (FormFactor, Perimeter, Min Feret Diameter, **Supplementary Figure 5**). Objects were only considered cells when they overlapped with a nucleus (DAPI) signal. In a second pipeline localization of F4/80$^+$ macrophages towards the Emcn$^+$ endothelium was analyzed by examination of the direct ($\leq$3.5 μm) and distant (>7 μm) neighborhood of the subset. Localization of the identified subsets was analyzed by counting the identified objects in regions of interests (ROIs). ROIs for the osteotomy gap were determined as shown in **Figure 5E**. For neighborhood analysis and frequency determination (**Figures 3E,F**) either Emcn$^{hi}$ or Emcn$^{lo}$ areas within the osteotomy gap or distant thereof were encircled freehand using ImageJ 1.52i, respectively (**Figure 3D**). Areas close to Sox9$^+$ chondrocytes were excluded as they did contain few, if any macrophages. Emcn$^{hi}$ areas largely overlapped with F4/80$^{hi}$ areas.

### Object Identification and Neighborhood Analysis

For the segmentation, the model-based approach was applied (37). *Otsu* was used as a segmentation algorithm calculating the thresholds for the object edge identification. Mid-level pixels were assigned to the background. The threshold calculation was performed adaptively, which allows adaptation of the threshold to different image sub-regions. For each channel, the parameters were manually optimized by visually inspecting the segmentation results. To aid the segmentation, channel specific object ranges were estimated (e.g., nuclei = 3.1–12.5 μm). Clumped cells were separated based on their intensity distribution. Only partly visible cells touching the border of the image were removed from the segmentation process. Nuclei, CX3CR1, F4/80, and Emcn primary objects were identified based exclusively on the image. Due to the dense packing of Gr-1$^+$ cells, segmentation was performed via secondary object identification with prior segmented nuclei as cellular reference. The segmentation was channel dependently refined based on various object features (**Supplementary Figure 1B**). Objects features were extracted on the boundaries of the objects for shape (e.g., perimeter, area, radius; **Supplementary Figure 5**), and intensity, computed from the signal intensity values of the object area within a defined image channel. Filter and thresholds were determined by visual evaluation of specific phenotypic measurements of individual objects within the image and the global distribution of the phenotypic measurement within the whole image.

### Spatial Polarization Scoring

Bone orientation was determined using Movat's Pentachrome overview images. Scores were determined for individual channels (DAPI, F4/80, or CD31). Scores were determined considering the staining intensity and abundance of the signal in parts of the bone marrow not affected by the injury (homeostatic control), on the same slide in at least 400 μm distance from the injury site. Scores were: −2 for the absence of staining in the area or reduced signal abundance and intensity throughout the entire area.; −1 for signal either reduced in intensity but displaying comparable abundance, or reduced abundance and comparable intensity; 0 for appearance that resembles homeostasis (unaffected bone marrow in the same section); 1 for higher intensity at comparable or increased abundance that localized only partially along the line between the gap and the adjacent tissue. Adjacent tissue is tissue along the contour between bone marrow and hematoma, bone fragments, and callus; 2 for higher intensity at comparable or increased abundance along the entire length of the line between the gap and the adjacent tissue or in large areas extending >400 μm distance from the gap in the bone marrow tissue. Regions often

showed enlarged and unstructured vessel organization, with very bright F4/80 cells between the endothelial lines. Samples were considered spatially polarized when the scored values for F4/80 and vessel marker of proximal and distal to the fracture gap resulted in a difference >2 favoring one side (proximal, distal). Samples which scored a difference of <2 were considered neutral. In total $n = 20$ samples were analyzed, 9 samples scored values other than 0 of which 2 samples were considered neutral, 1 polarized proximally and 6 polarized distally (**Supplementary Figure 4A**).

## Longitudinal Intravital Microendoscopy of Murine Osteotomy

As a GRIN lens system, we used custom singlet GRIN needle microendoscope (length ca. 5.07 mm, diameter = 0.60 mm; NEM-060-10-10-850-S-1.0p, GRINTECH Jena, Germany). The GRIN lens was glued into the lens tubing to a final penetration depth of 650 µm when screwed into the fixator plate. CX3CR1:GFP mice were anesthetized and mounted to the microscope as previously described (10). Qtracker 655 Vascular Label (Thermo Fisher, MA, US) were injected and images were acquired (505 × 505 px, 500 × 500 µm, unidirectional, line average 4, step size 4.5–6.5 µm, ca. 18 steps, stack time 60 sec). Image stacks were loaded into Imaris 9.3.0 software (Bitplane Zürich, Switzerland), median filter (3 × 3 × 3) was applied. Videos were exported (1024 × 1024, 4 fps) and images were angled maximum intensity projections. Individual mice were measured at 940–950 nm on day 2, 3, 4, 5 after osteotomy.

## Statistical Analysis

Statistical analysis was carried out with GraphPad Prism V.5 or V.8 software. All values are expressed as the mean ± SD if not stated otherwise. Mann-Whitney *U*-test, Wilcoxon-signed rank test and Friedman test with Dunn's *post-hoc* test were mainly used, since Gaussian distribution was not expected due to inter-individual variations. A $p < 0.05$ was considered statistically significant. Image analysis was blinded for time points.

# RESULTS

## Emcn$^{hi}$CD31$^{hi}$ Endothelium Is Present During Endochondral Bone Formation in the Osteotomy Gap

Fracture healing consists of consecutive phases. Progression into each phase depends on the undisturbed and error-free course of the respective previous phase (38). In the mouse-osteotomy model used in this study, residuals of cells in the fracture hematoma are visible in Movat's Pentachrome staining at day 3, while the osteotomy gap is filled with a mixture of bone marrow cells including hematopoietic cells at day 7, and endochondral bone formation occurs between day 14 and day 21 (**Figure 1A**). Using immunofluorescence histology, a positive signal for CD31 (Platelet endothelial cell adhesion molecule 1) is seen in elongated cells inside the fracture hematoma, which include no detectable nuclei (**Supplementary Figures 2A–C**), probably indicating that these residuals represent endothelial cells that may have lost their integrity. In order to further characterize the cellular composition within the fracture area, immunofluorescence staining for key transcription factors of mesenchymal differentiation was performed on serial sections of the same bones (**Figure 1B**). Already at day 3, osteoblast progenitors characterized by nuclear expression of Runt-related transcription factor 2 (Runx2) are dispersed in the fracture gap. By day 7, their number has increased, leading to a dense population of the gap, whilst sparing a region in the center. The peripheral borders of the gap and adjacent periosteal regions are populated by cells co-expressing Runx2 and SRY-box transcription factor 9 (Sox9) in the nucleus, indicating their potential for either osteogenic or chondrogenic differentiation (**Figure 1B**). This is in accordance with the blue-greenish color in corresponding regions of Movat's Pentachrome staining, indicative of cartilaginous tissue (Alcian blue positive). At day 14, Runx2$^+$ cells are found to localize on both sides of the gap. Single Runx2$^+$ cells are surrounded by areas characterized by the presence of only a few nuclei, in line with the presence of mineralized bone in those areas, as visualized by Movat's Pentachrome staining (compare: **Figures 1A,B**). Sox9$^+$ cells are exclusively present in the center of the gap at this time point. Notably, the majority of these cells do not co-express Runx2. In contrast, at day 21, a time point when mineralization of the gap is complete, the progenitors identified in this area are almost exclusively Runx2$^+$. In a next step, Osterix (Osx) and CD31 stainings were performed on serial slides from the same individuals. Direct comparison reveals that at day 3, only some Runx2$^+$ cells are also Osx$^+$, indicating various degrees of osteogenic differentiation. From day 7 on, Runx2$^+$ cells are also positive for Osx$^+$, consistent with ongoing maturation of osteoblasts. Osx$^+$ osteoblasts localize in the fracture gap alongside existing and newly formed bone, as indicated by differential interference contrast (DIC) signal. Sox9$^+$ cells can be found at day 14 and 21 in areas with cartilage and close to Osx$^+$ cells, indicating ongoing further differentiation and mineralization, which is accompanied by vascularization. For further quantitative analysis of the vasculature sprouting into the gap, we defined the gap as a rectangular region between the cortical ends and analyzed CD31$^+$ vessels and Osx$^+$ bone cells in this region (**Figure 1C**). At day 3, CD31$^+$ matrix (residuals of the fracture hematoma) and some CD31$^+$ cells are present (compare to **Supplementary Figures 2A–C**), while Osx$^+$ cells enter the osteotomy gap at later time points, between day 7–21 (**Figures 1C,E**). At later phases of fracture healing, endothelial cells displaying the phenotype of type H vessels (Emcn$^{hi}$CD31$^{hi}$) closely associate with Osx$^+$ osteoprogenitors/pre-osteoblasts (**Figure 1C**). Investigating the co-localization of Runx2$^+$ and Sox9$^+$ cells with respect to CD31$^+$ endothelium reveals a close localization of Runx2$^+$ cells around vascularized areas, while Sox9$^+$ cells are only found in less vascularized areas (**Figure 1D**). Our results confirm a close relationship between vessel formation and osteoprogenitors/osteoblasts and extend previous reports of this phenomenon, which focused on bone development (7), to a regenerative scenario.

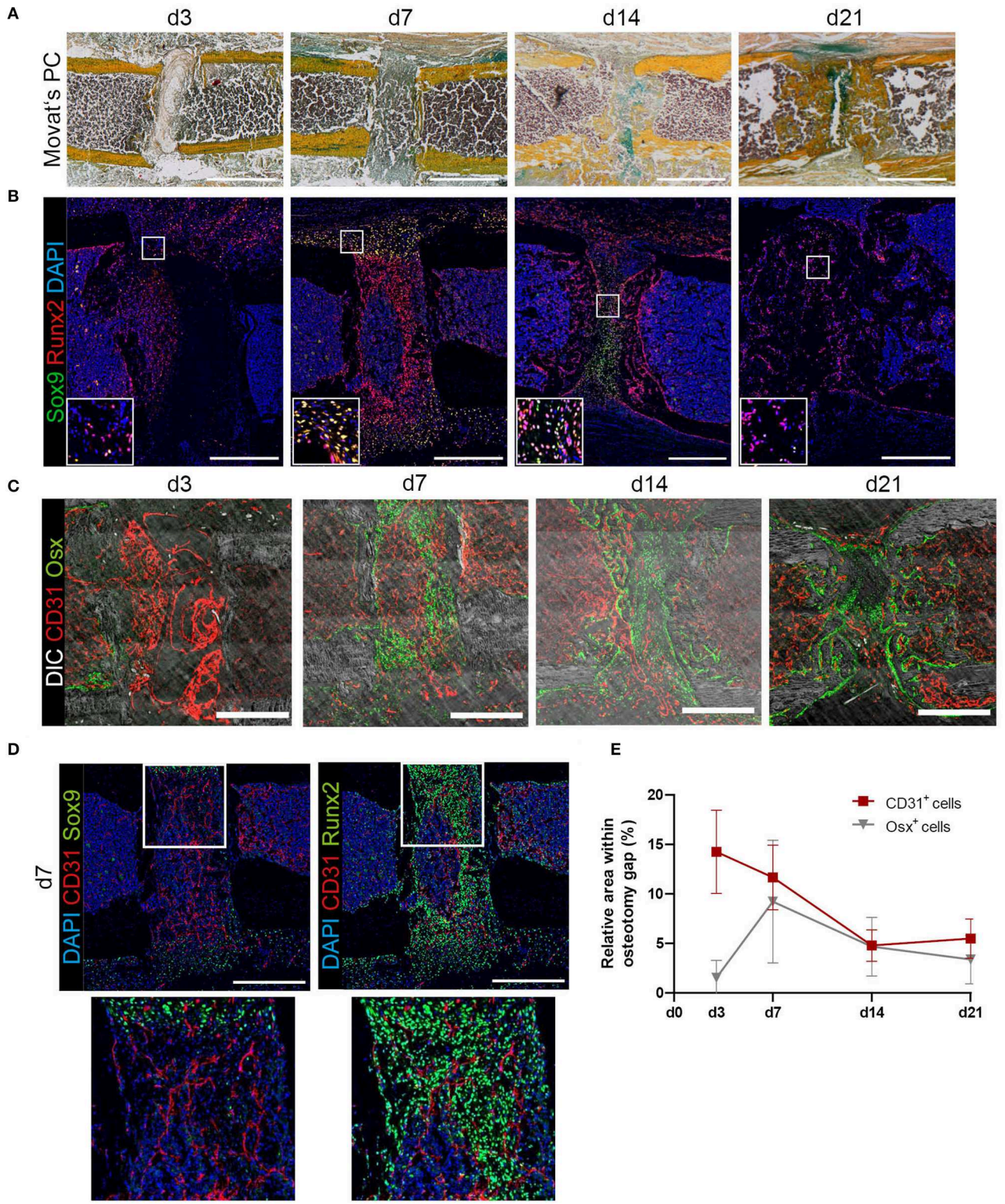


**FIGURE 1 |** Histological characterization of tissue formation and distribution of mesenchymal progenitor cells, osteoblasts and endothelial cells during bone regeneration in a mouse-osteotomy model. **(A)** Movat's Pentachrome staining indicates the phases of bone regeneration over 21 days. Scale bars = 500 μm. **(B)** Sox9 and Runx2 immunofluorescence staining for progenitor cells undergoing differentiation at day 3, 7, 14, and 21 indicating double-positive cells at day 7 and high numbers of Runx2$^{+}$ cells within the fracture gap at day 14 and 21. Exemplary images of $n = 3$–6 per time point. Scale bars = 200 μm. Insets show enlarged

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**FIGURE 1 |** representative areas marked by white frames. **(C)** Exemplary images at day 3, 7, 14, and 21 displaying the distribution of $CD31^+$ ECs and $Osx^+$ osteoprogenitors/pre-osteoblasts revealing close proximity between $CD31^+$ endothelium and $Osx^+$ cells. Scale bars = 200 μm. **(D)** Exemplary images for Sox9 or Runx2 and CD31 staining at day 7. Insets show enlarged representative areas marked by white frames. While $Runx2^+$ cells can be found closed to endothelial cells, $Sox9^+$ cells can be found in not yet vascularized areas. **(E)** Quantification of cellular compartments present in the fracture gap during regeneration. Endothelial cells (ECs; $CD31^+$) and osteoprogenitors/pre-osteoblasts ($Osx^+$) were quantified based on the relative presence of positive pixels with the respective markers in immunofluorescence images supporting the descriptive analysis on the spatiotemporal distribution of progenitor cells, osteoblasts and endothelial cells over time. Data show mean ± SD for $n = 3$–6.

## Localization and Morphologic Characterization of $Emcn^{hi}CD31^{hi}$ Type H Endothelium

While $Emcn^{hi}CD31^{hi}$ endothelium can be found directly in the osteotomy gap and in the adjacent tissue, $Emcn^{lo}CD31^{lo}$ endothelium is prominent in bone marrow regions not affected by the injury. Pixel intensity analysis confirms the differences between the $Emcn^+$ and/or $CD31^+$ cells in and adjacent to the osteotomy gap, compared to the bone marrow (**Figures 2A,B**).

We further characterized the type H vessels in the fracture gap (**Figures 2C,D**). During vascularization of cartilaginous tissue, invading vessel buds (distal loops), previously described as a morphological criterion for type H endothelium (7), can be identified (**Figure 2E**, first row marked by arrows). Furthermore, $CD31^+$ arterioles, which are negative for Emcn, are found within the osteotomy gap. However, a second morphological criterion described to be typical for type H vessels in the growth plate areas, namely their columnar structure, does not appear during regeneration, suggesting that this ordered arrangement is a specific feature of bones undergoing longitudinal growth. Taken together, our data reveal the appearance of $Emcn^{hi}CD31^{hi}$ in the fracture gap, which share additional distinct morphological features with the previously described type H vessels, although they are not organized in columnar structures. This finding suggests an important role of this osteogenic vessel type not only during bone development, but also during regenerative processes of the bone.

## M2-Like Macrophages Localize Preferentially Extramedullary

In order to determine the role of macrophages in the context of vascularization during bone regeneration, we analyzed macrophage subsets and their spatial localization relative to type H vessels. Based on the pan-macrophage marker F4/80, which includes also osteomacs, we defined M1- and M2-like macrophages labeled by CD80 and CD206 (Mannose receptor), respectively, as well as Mac-2 (Galectin-3), a marker associated with pro-inflammatory macrophages (22). Over the course of endochondral bone regeneration, $F4/80^+$ cells localize throughout the bone marrow, as well as on the border to bone surfaces, accumulating in various areas (**Figure 3A**). At day 3 post-osteotomy, high numbers of $F4/80^{hi}$ cells localize in periosteal regions adjacent to the osteotomy gap. They can be found periosteal in varying numbers throughout the regeneration process, until the bone remodeling phase at day 21. At day 14, $F4/80^{hi}$ cells are found exclusively in periosteal regions, whereas they are found in both the medulla and the periost at day 21. Extra-medullar $F4/80^+$ macrophages are positive for the anti-inflammatory M2(a) macrophage marker CD206 (**Figure 3A**, blue framed inset). Those M2-like cells are not abundant in the marrow or at sites of vascularization, where expression of CD206 is generally lower and macrophage morphology rather ramified (**Figure 3A**, yellow and orange framed insets). CD206 expression is also found in cells resembling the morphology of endothelia ($Emcn^+$), which are $F4/80^-$ throughout the regeneration process as well as under homeostatic conditions (**Figure 3A**, yellow framed inset; **Supplementary Figures 3C,D**). M1-like macrophages, defined by expression of CD80, are extremely rare and only few are found at day 3, with their abundance comparable to control tissue (**Supplementary Figures 3B,D**). Using the marker Mac-2 in addition, almost all $CD206^+$ macrophages are found to be positive for Mac-2 at day 3, however, not all $Mac\text{-}2^+$ cells are $CD206^+$ (**Figure 3B**). Mac-2 positive cells localize in the fracture gap and in proximity to the bone surfaces. Over the course of regeneration, double-positive $Mac\text{-}2^+$ and $CD206^+$ cells vanish and cells which are single positive for each of the two markers are detected at day 21, a time point when remodeling is ongoing (**Figure 3B**). Quantitative pixel-based area analysis of F4/80 macrophages, CD31 and TRAP shows that $CD31^+$ and $F4/80^+$ cells are reduced over time, while the number of $TRAP^+$ cells (osteoclasts or activated macrophages) increases (**Figure 3C**; **Supplementary Figure 3A**).

$CD206^+$ macrophages are present throughout the healing process with no apparent preferential localization towards the vasculature, and they are mainly $Mac\text{-}2^+$ in the early phase of regeneration. Over the course of regeneration, F4/80-expression decreases in regions adjacent to the callus, and at later time points only few areas, which contain cells expressing F4/80 at intermediate levels, are observed.

## $F4/80^+$ and $CD80^+$ Cells Produce VEGFA During Early Regeneration and Osteoblasts and Chondrocytes Are the Main Producers at Later Time Points

Since we could not find substantial amounts of $CD80^+$ cells at day 3 post-osteotomy, we analyzed the regeneration at day 1 and day 2. Some $CD80^+$ cells were found at day 1, and they were abundant in higher numbers at day 2 post-osteotomy (**Figure 4A**). Some of those are $F4/80^+CD80^+$ macrophages, which localize in areas adjacent to the osteotomy gap. $CD80^+$ cells are located directly at the injury site (**Figure 4A**, day 2). Macrophages are known to support vascularization via VEGFA during tissue regeneration, which is why next, we analyzed VEGFA using immunofluorescence histology over the time

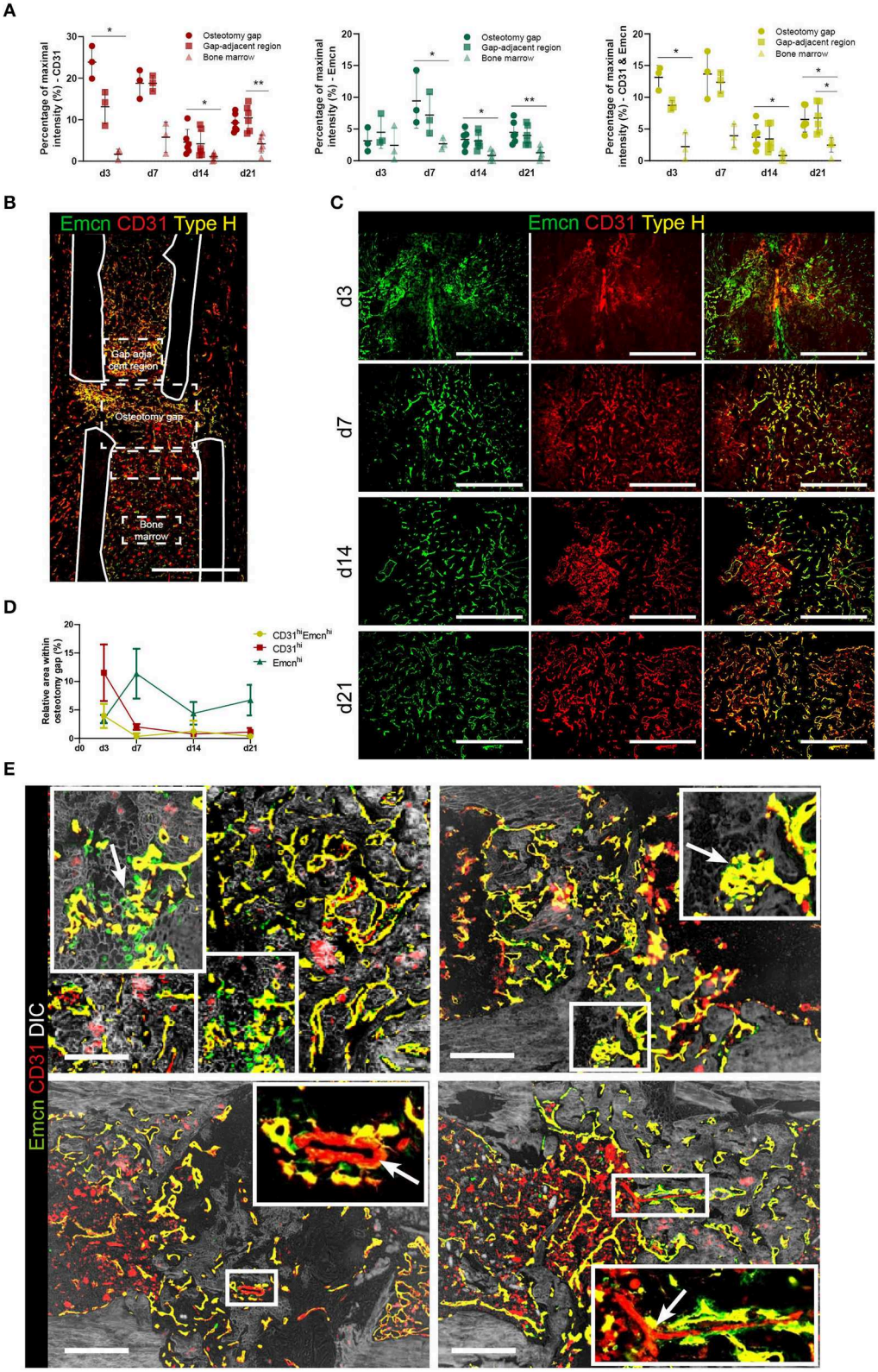


**FIGURE 2 |** Vessels formed during fracture healing show characteristics of type H endothelium and are embedded in mineralized tissue. **(A)** Intensity analysis of Emcn and/or CD31 in the fracture gap. Mean intensities were normalized to the maximum intensity present in each image. Mean intensities were normalized to 780 the maximum intensity present in each image. Data are shown as scatter dot plot with mean ± SD while one dot is representative for one slide of one individual animal.

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**FIGURE 2 |** Friedman test with Dunn's *post-hoc* test were performed to determine statistical differences; *p*-values are indicated with $^{*}p < 0.05$, $^{**}p < 0.01$. **(B)** Emcn$^{hi}$ CD31$^{hi}$ endothelium shows spatial differences in abundance throughout the bone. Exemplary image showing the definition for the osteotomy gap, the gap adjacent regions and the bone marrow area, as used in our analyses. Scale bar = 200 μm. **(C)** Images of Emcn$^{hi}$ CD31$^{hi}$ endothelium at day 3, 7, 14, and 21. Data are representative for $n = 3$ (d3/7) and $n = 6$ (d14/21). **(D)** Quantification of areas occupied by Emcn$^{hi}$ CD31$^{hi}$ type H endothelium during the time course of bone healing. Data are shown as Mean ± SD. **(E)** Emcn and CD31 staining combined with phase contrast images (DIC) in the soft to hard callus transition of fracture healing highlight morphological characteristics also present in growth plate bone growth. Upper panels: invasion of vessels in cartilaginous tissue and type H vessel-like budding is indicated by arrows. Lower panels: CD31$^{+}$ only vessels are assumed to be arterioles (arrows) (7). Scale bars = 200 μm.

course of bone regeneration (**Figures 4B–D**). VEGFA is not expressed by F4/80$^{+}$CD80$^{-}$ macrophages throughout the entire process, but CD80$^{+}$F4/80$^{-}$ and a fraction of CD80$^{+}$F4/80$^{+}$ cells express VEGFA. This expression was restricted to very early time points, namely day 1 and day 2 (**Figures 4B,C**). From day 3 on, VEGFA is found to be expressed in the fracture gap and along bone surfaces (endosteal, periosteal) and in the bone forming areas (**Figure 4D**). The signal for VEGFA at day 14 is pronounced in areas, which also contain Sox9$^{+}$ and Runx2$^{+}$ cells (**Figures 1B**, **4D**). Taken together, VEGFA expression is observed in CD80$^{+}$ cells, including a fraction of F4/80$^{+}$ cells, in the early phase until day 3, after that, VEGFA is expressed predominantly by osteoblasts and chondrocytes in bone forming areas.

## Cells Within the Osteotomy Gap Localize in Proximity to the Endothelium

High abundance and expression of the vascular markers CD31 and Emcn is observed in both, the damaged tissue in the early regeneration, and in bone forming tissue over the subsequent course of regeneration in the osteotomy gap (**Figure 2C**). These areas rich in type H vessels, simultaneously contain F4/80$^{hi}$ cells (**Figures 3A,D**). In order to evaluate the proximity in localization between macrophages and type H endothelium, we defined two regions for each sample, the Emcn$^{hi}$ region and the Emcn$^{lo}$ region (**Figure 3E**). Objects in these regions were segmented based on marker expression of Emcn, F4/80 and DAPI. Over the course of regeneration, similar frequencies of 25.0 ± 11.6 % F4/80$^{+}$ cells are present in both, the Emcn$^{hi}$ and Emcn$^{lo}$ regions. The frequencies of F4/80$^{+}$ cells decrease significantly from day 3 until day 21 post-injury (**Figure 3E**), confirming the qualitative results from **Figure 3C**. Next, we analyzed the position of macrophages relative to the endothelium, and found that, a two- to three-fold higher number of objects (F4/80$^{+}$ objects or identified nuclei, which serve as a control for all cells) localize in proximity to the endothelium in the Emcn$^{hi}$ region as compared to the Emcn$^{lo}$ region (**Figure 3F**). Cells are defined as "in proximity" when they are located less than half a nuclear diameter in distance (<3.5 um) away from the endothelium.

During analysis of immunofluorescence staining in samples of osteotomized, LIMB-implanted bones, we noticed a transient, strongly polarized distribution of newly formed vessels to the distal area of the osteotomy gap (**Figure 5A**; **Supplementary Figure 4A**). A similar polarization occurs in samples of the osteotomy-model (**Supplementary Figure 4B**). In some cases, this phenomenon is accompanied by a variation in cell density in the respective area, as shown by differences in the abundance of the DAPI signal. Scoring according to the criteria described in the methods section for the abundance and intensity of the markers in the gap-adjacent regions reveals that CD31$^{+}$ vessels are significantly brighter and more abundant adjacent distally to the gap, as compared to the proximal site (**Figure 5B**). This spatial polarization with respect to bright CD31$^{+}$ vessels is pronounced at day 4 and decreases quickly afterwards (**Figure 5B**). A similar trend is observed for F4/80$^{+}$ cells in the same regions (**Figure 5B**). Taken together, these analyses reveal a transient, directional spatial polarization (regarding the localization from the fracture gap) of CD31$^{hi}$ vessels, adjacent to the fracture gap.

## CX3CR1$^{+}$ F4/80$^{+}$ Macrophages Precede Vascularization

In order to understand the dynamics of macrophages during bone regeneration, we took advantage of the fractalkine receptor (CX3CR1) reporter mouse strain CX3CR1:GFP. These mice were implanted with a Gradient Refractive INdex (GRIN) lens, enabling longitudinal imaging of the bone marrow during regeneration (10). At the same time, these mice underwent osteotomy surgery. Since the first wave of vascularization occurs during resolution of the fracture hematoma, bones that had been treated using the procedure described were fixed and analyzed at day 4, 6, and 8 using immunofluorescence histology. In order to be able to exclude monocytes and granulocytes from the macrophage analysis, we stained sections using antibodies against Ly-6C/Ly-6G (Gr-1). We find that almost all F4/80$^{+}$ macrophages in the osteotomy gap are also CX3CR1$^{+}$ (**Figures 5C,D**, yellow), confirming that the reporter mice are suitable for tracking macrophage dynamics during bone regeneration. GFP$^{+}$ cells are present within the osteotomy gap at early time points and become less abundant over time. They locate in close proximity to CD31$^{hi}$ endothelium, do not express Gr-1 (**Figure 5D**), excluding the possibility that some of them are granulocytes, which are also described to be CX3CR1$^{+}$. We analyzed the osteotomy gap between the cortices and the bone marrow as described in **Figure 5E**. At day 6, of all identified cells in the osteotomy gap, the CX3CR1$^{+}$ cells display the highest increase in cell number, as compared to homeostasis. A minor increase is detected in F4/80$^{+}$ cells and a strong reduction of Gr-1$^{+}$ cells is observed compared to homeostasis (**Figure 5F**). The space is largely occupied by F4/80$^{+}$ myeloid cells, which are not monocytes (Gr-1$^{-}$). The strongest increase in cell number is observed in the CX3CR1$^{+}$Gr-1-F4/80$^{+}$ subset, which make up to 12% in ratio to all nuclei at day 6 (**Figure 5G**). Almost 6% of CX3CR1$^{+}$ objects are F4/80$^{-}$ and only few are Gr-1$^{+}$. Morphologically, the cell phenotype is of non-round shape (**Figure 5E**, **Supplementary Figure 5**). We then longitudinally sampled time-lapse videos with two-photon microscopy from individual mice over the course of the early regeneration process. On the first and second day, almost no signal is detected inside

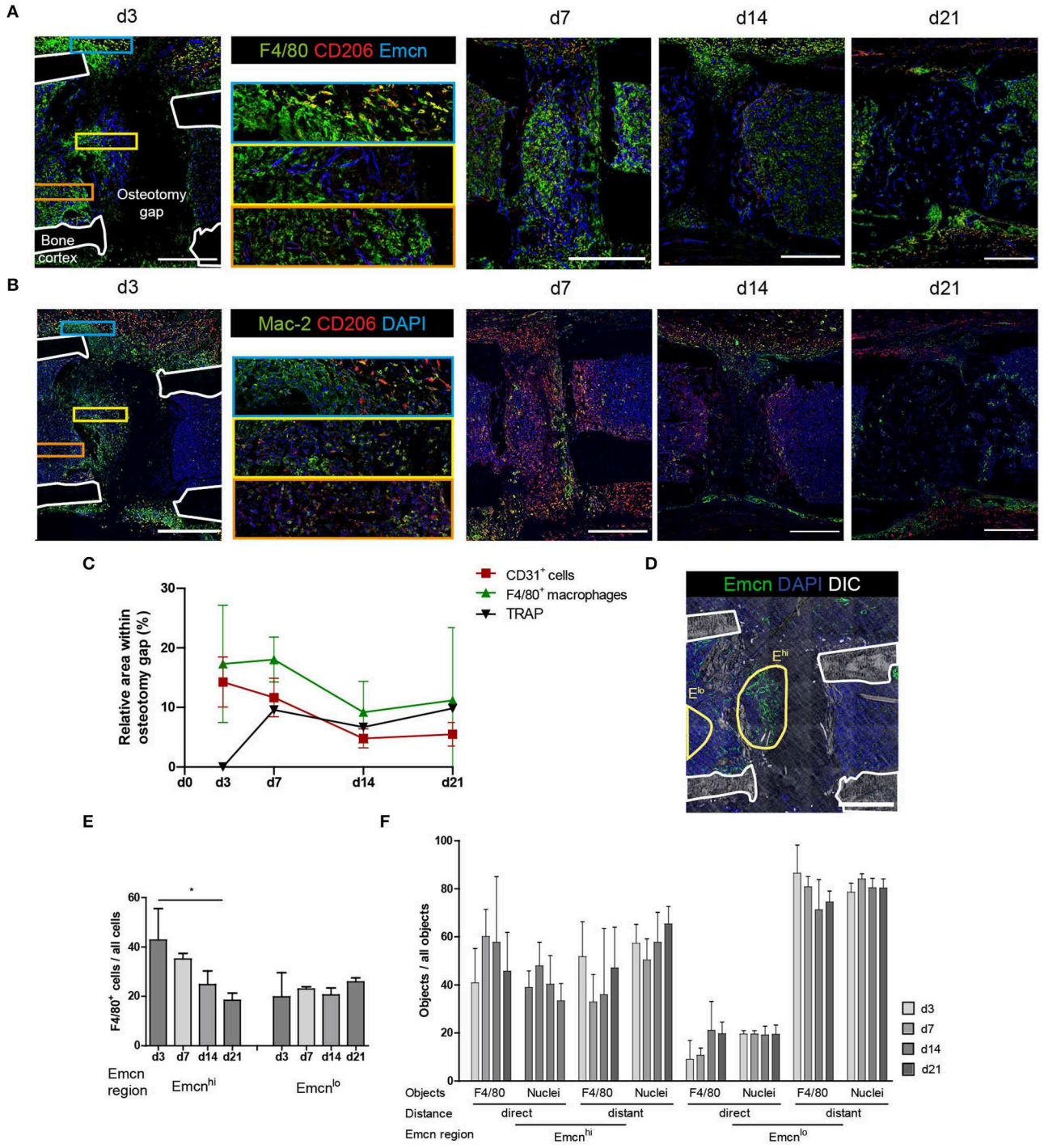


**FIGURE 3 |** Distinct localization of macrophage subsets during bone regeneration indicates location-dependence of their functions. **(A)** Immunofluorescence staining of F4/80, CD206, and Emcn shows the abundance of F4/80$^{+}$ cells in the osteotomy gap. They localize close to Emcn$^{hi}$ endothelium. F4/80$^{+}$CD206$^{+}$ M2-like macrophages primarily localize in extramedullar areas (blue inset). F4/80$^{hi}$ cells localize in proximity to Emcn$^{hi}$ endothelium (yellow inset). Endothelium shows CD206-positivity in proximity to the gap (orange inset). Scale bars = 500 μm. **(B)** Immunofluorescence staining reveals spatiotemporal distribution of CD206$^{+}$ and Mac-2$^{+}$ cells. CD206$^{+}$ M2-like macrophages were Mac-2$^{+}$ at d3 and single-positive by d21. Scale bars = 500 μm. **(C)** Pixel-based area analysis of immunofluorescence images of CD31, F4/80, and TRAP show a reduction of CD31 and F4/80 signals over time and an increase in osteoclasts. **(D)** Object-based analysis for **(E,F)**, based on regions within the osteotomy gap which show high or low expression of Emcn (Emcn$^{hi}$ vs. Emcn$^{lo}$). Scale bar = 500 μm. **(E)** F4/80$^{+}$ cell frequency in the Emcn$^{hi}$ region decreases between d3 and d21 to levels of the Emcn$^{lo}$ region. Mann Whitney test, two-tailed, $^{*}p = 0.0476$. **(F)** Proximity analysis

*(Continued)*

**FIGURE 3 |** performed by object-based quantification in two distances from the endothelium. Data was normalized to the overall number of the object population in the respective region. Compared to the $Emcn^{lo}$ region, more objects ($F4/80^+$ or nuclei) localize in proximity to the endothelium in the $Emcn^{hi}$ region. Cells were considered in proximity to each other when their distance amounted to that equivalent to less than half of a nucleus diameter (<3.5 μm), in order to include cells which are either in contact with or in the direct vicinity of vessels. Cells were considered distant were located further than one cell layer (>7 μm) apart from each other. Data are representative for $n = 3$ (d3/7) and $n = 6$ (d14/21).

the osteotomy gap (data not shown). On day 3, individual $CX3CR1^+$ cells enter the hematoma (**Figure 5H**d3). Those cells display a non-round, but non-ramified shape and move through the tissue (**Supplementary Movie 1**). A front of $CX3CR1^+$ cells forms at the edge of the field of view at day 4, containing both round and non-round cellular phenotypes, forming a dense, partially resident population which expands into the entire field of view (**Figure 5H**, **Supplementary Movies 2, 3**). This invasion is accompanied by the occurrence of perfused vessels that expand, following the $CX3CR1^+$ cell front until the field of view appears vascularized (**Supplementary Movie 4**). Under full vascularization, sessile $CX3CR1^+$ cells localize towards the vasculature and motile cells can be observed to move along the endothelium (**Supplementary Movie 3**). Generally, the abundance of $CX3CR1^+$ in the gap increases with progression of the regeneration process (**Figure 5F**, **Supplementary Movies 3, 4**).

These results indicate that most cells in the fracture gap are myeloid, non-monocytic, non-granulocytic, non-round $CX3CR1^+$ which invade the osteotomy hematoma starting day 2–3, become gradually sessile in the fracture gap at day 3–4, where they precede the vasculature until it becomes fully perfused. After vascularization, $CX3CR1^+F4/80^+$ cells persist until the onset of the remodeling phase.

## DISCUSSION

Vascularization is pivotal to the success of complete, scar-free bone regeneration. Here, we show that after bone injury, type H endothelium evolves in the bone formation region and persists throughout the entire bone regeneration process. Our findings show that the phenotype of type H endothelial cell structures described in endochondral long bone growth, is also reflected in bone regeneration. $Emcn^{hi}CD31^{hi}$ endothelium shows association with $Osx^+$ osteoblasts and displays typical features that can be observed in the growth plate during longitudinal growth, such as invading vessel buds and arch-like structures described before (7). Other than their counterparts in the growth plate, type H vessels generated during regeneration are not columnar. However, this feature is probably not inherent to type H vessels, but merely the result of the highly ordered structure of chondrocytes in the metaphyseal areas. These chondrocytes are organized into columns that are produced in the growth plate, and probably impose their structure on the type H vessels in these areas. In contrast, the tissue structure surrounding type H vessels in a fracture gap appears more disorganized. We have previously shown that the amount of type H vessels can be used as a measure of fracture healing progression (21). Ramasamy et al. reported the effect of shear stress in the vascular formation during bone development, therefore it would be interesting to analyze whether interfragmentary, compressional mechanical cues also act on the formation and structure of type H vessels in fracture healing (39).

We found an extensive staining of CD31 in the gap, which dominates the initial hematoma phase (until day 3–4). This staining seems not to be associated with intact cells, since no nuclei are present. It is possible that $CD31^+$ cells are present in the early fracture hematoma during the first hours after injury and undergo cell death due the hypoxic microenvironment, including a low pH and high lactate level in the tissue, and that the staining pattern observed represents dead endothelial cells.

Based on our results, and similar to a previously reported model in sheep (6), we propose that two phases of vascularization occur during bone regeneration also in mice. The first wave of vascularization occurs during disintegration of the fracture hematoma, where vascular sprouts from existing endothelial cells enter into a hypoxic environment. It occurs between the initial injury and day 6, until the entire volume is fully vascularized for soft callus formation. We observe this phase histologically and by intravital microscopy. After the first wave, and once the callus becomes calcified, we assume that the vascular network within hard callus and bone is remodeled in a second phase, based on the changes that happen during tissue reorganization. It has been described that most of the progenitor cells that contribute to fracture repair immigrate from the periosteum (40). In addition, these progenitor cells either express Sox9 or Runx2, which are regulated by direct repression of the opposing pathway (via β-catenin expression) and define further differentiation into chondrocytes or osteoblasts, respectively (41). However, several studies show the importance of Sox9 expressing progenitor cells that support mineralization and osteogenesis within the fracture gap (42–44). At day 7, we observe a co-localization of Sox9 and Runx2 (**Figure 1B**), it can be speculated that co-expression of both transcription factors marks a switch in the genetic program, from uncommitted pre-osteoblasts to chondrocyte differentiation, similar to what has been shown in bone development (45). Alternatively, Sox9 expressing cells might differentiate into osteoblasts, as described previously (42–44). Our results show close proximity between osteoprogenitors ($Runx2^+$) or osteoblasts ($Osx^+$) and $CD31^+$ endothelium (**Figures 1C,D**). Endothelial cells, which display the phenotype of type H vessels ($Emcn^{hi}CD31^{hi}$) closely associate with $Osx^+$ cells in this osteotomy (= cortical defect) model. Type H endothelia have been previously described to be present in the metaphyseal areas of young animals, where they promote bone growth (7). The results presented here indicate a similar crosstalk to occur in endochondral osteogenesis during regeneration.

The large extent of angiogenesis, which occurs in the fracture gap, raises the question for factors and cell types which trigger this event. In the growth plate, hypertrophic chondrocytes

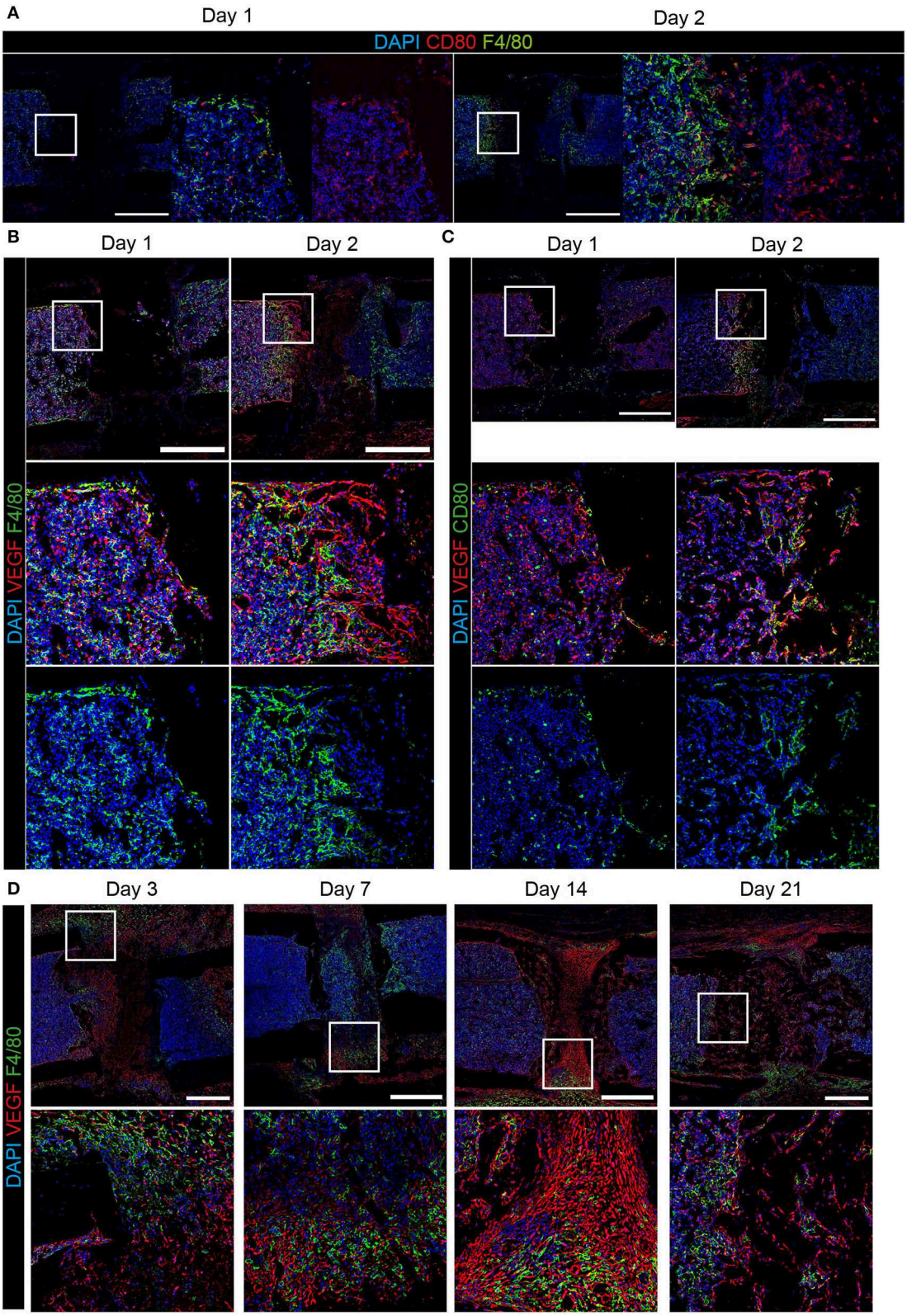


**FIGURE 4 |** Expression of VEGFA in CD80$^+$ cells and bone forming areas at the osteotomy site. Immunofluorescence staining over the course of regeneration shows **(A)** pro-inflammatory CD80$^+$ cells adjacent to the osteotomy gap, which are in part F4/80$^+$ in the early phase (at day 1 and 2). **(B)** F4/80$^+$ cells were predominantly VEGFA$^-$. **(C)** CD80$^+$VEGFA$^+$ cells localize at the interface between the hematoma and surrounding tissue. **(D)** In areas of bone formation VEGFA expression is high, but F4/80$^+$ cells re VEGFA$^-$ during progressing bone regeneration until day 21. Scale bars = 500 μm. *n*(day 1) = 3, *n*(day 2) = 1.

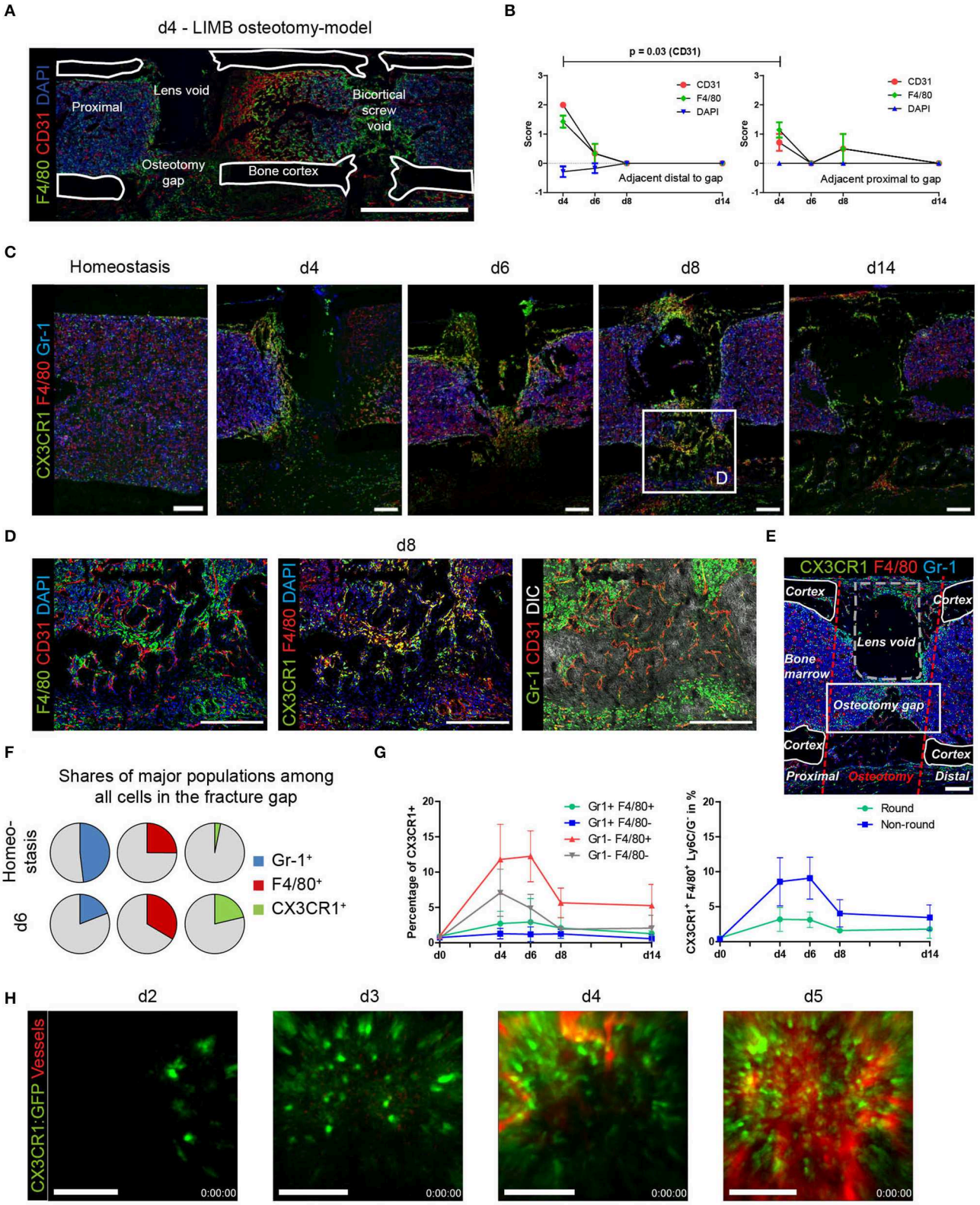


**FIGURE 5 |** Quantitative analysis and LIMB imaging demonstrate high abundance of the myeloid CX3CR1$^{+}$ F4/80$^{+}$ cell subset preceding vascularization. **(A)** Overview image of a section from a whole bone taken at day 4 after LIMB osteotomy. The LIMB osteotomy-model model uses a gradient refractive index lens which is removed before bone sectioning thus a *lens void* remains. Vessels and macrophages polarize distally in this example. **(B)** Scoring of CD31 and F4/80 allows

*(Continued)*

**FIGURE 5 |** quantification of polarization in proximal or distal orientation with respect to the fracture gap. Scoring of DAPI staining was used as internal control. Wilcoxon matched-pairs signed rank test. Data are representative for $n = 7$ (d4), $n = 6$ (d6), $n = 4$ (d8), and $n = 3$ (d14). **(C)** Immunofluorescence images of sections from bones showing the presence of myeloid subsets at various time points after LIMB osteotomy. CX3CR1:GFP$^+$ and F4/80$^+$ cells negative for Ly6C/Ly6G (Gr-1), accumulate at and invade into the fracture gap. Scale bars = 200 μm. **(D)** Inset of the area of d8. The majority of myeloid cells are CX3CR1$^+$F4/80$^+$ and localize in distance from Gr-1$^+$ cells. Scale bars = 200 μm. **(E)** Quantitative analysis is performed in the rectangular region of the fracture gap between corticalis, osteotomy cuts, and lens void (osteotomy gap). Scale bar = 100 μm. **(F)** Quantitative, object-based analysis of the osteotomy gap for Gr-1, F4/80, CX3CR1:GFP show a decrease of Gr-1$^+$, an increase of F4/80$^+$ and massive increase of CX3CR1$^+$ cells. Results of 20 pooled samples. **(G)** Object-analysis among the CX3CR1$^+$ cells reveals that Gr-1$^-$F4/80$^+$ account for the majority of cells present in the gap over the whole time course of regeneration (left panel). Of those, the majority were non-round cells (right panel). **(H)** Intravital two-photon LIMB-microscopy of the osteotomy gap in an individual CX3CR1:GFP mouse. CX3CR1:GFP$^+$ cells invade the osteotomy gap in the displayed field of view (250 × 250 μm) at day 2–3 and fully populate the region by day 3–4. Simultaneously, numerous CX3CR1$^+$ cells precede the vascularization in space and time. Vasculature was made visible using intravenous injection of Qtracker 655. Scale bars = 100 μm. Data are representative of 5 mice analyzed.

produce high amounts of VEGFA, due to the hypoxic microenvironment in those areas, in which HIF stimulates VEGFA expression (7). In a recent publication, Buettmann et al. deleted VEGFA from early osteolineage (Osx$^+$) cells, mature osteoblasts and osteocytes (Dmp1$^+$) as well as ubiquitously in models of cortical fractures (full and stress fractures) and a cortical defect model (drilling). Bone regeneration and periosteal angiogenesis after a cortical defect was impaired only when VEGFA was deleted either ubiquitously or from Osx$^+$ cells, indicating a predominant role of osteolineage cells. After drill hole injury, however, the deletion did not lead to delayed healing, indicating that another cell type than Osx$^+$ is responsible for VEGFA production and the progression of vascularization.

Macrophages have been identified to play a role in bone regeneration, as their deletion either via clodronate (24, 25) or genetically delays healing. Data available in the literature indicate multiple functions for macrophages during bone regeneration. Recently, the supportive function of F4/80$^+$ macrophages during bone regeneration via enhanced osteogenesis when transplanted to aged individuals was demonstrated (46). In addition, it is known that F4/80$^+$ osteomacs support osteoblast function (47). Osteoblast differentiation and mineralization have also been shown to be modulated by macrophages (48, 49). However, the role of macrophages in the vascularization of the fracture is largely unknown. Here, for the first time, we quantitatively and qualitatively map out the vascular network, relate it to the presence of macrophages at relevant time points during bone regeneration in mice and analyze presence and proximity with different approaches. We find that F4/80$^+$ macrophages and type H endothelium localize at the front of damaged tissue in the first phase until soft callus formation, when their presence is reduced. In detail, our proximity analysis reveals that all cells, including F4/80$^+$ macrophages in the regenerating tissue, localize much closer to the endothelium than in unaffected areas in both, the first (d3–d7 after injury) and second (d14–d21 after injury) phase of revascularization. We find CX3CR1$^+$F4/80$^+$ macrophages to be a predominant subset in this process. Phenotypically, these cells are for the most part non-round and ramified. They localize predominantly in a one-cell layer around the endothelium. Future work is needed to study the fate of this subset. It is possible that those cells differentiate or merge with osteoclasts and therefore promote remodeling of the bone.

Using longitudinal intravital microendoscopy of the osteotomized region, we find that CX3CR1$^+$ macrophages precede the occurrence of perfused vessels into the hematoma in the first phase and remain associated to the endothelium. We show the type H endothelium to be closely associated with CX3CR1$^+$F4/80$^+$ macrophages, suggesting that this myeloid population is responsible for vascularization and progression of bone regeneration.

In the fracture hematoma, no chondrocytes are present, so macrophages may be a source of VEGFA in that situation. Here, we identify two major cell populations, which produce VEGFA over the course of regeneration. In the earliest phase, at day 1 and 2 post injury, CD80$^+$ cells are found to be positive for VEFGA by immunofluorescence. From day 3 on, cells inside the area of bone formation and on bone surfaces show a strong VEGFA signal. Based on their localization, the proximity to bone surfaces, we assume that these cells are precursors of, and committed osteoblasts, as well as chondrocytes in cartilage (16; compare **Figures 1A–C**). Until day 3, among the CD80$^+$ cells, we find F4/80$^+$, which we consider M1-like cells, as well as F4/80$^-$, which could either be a subpopulation of mature F4/80$^-$ macrophages or precursors of macrophages, such as F4/80$^{low}$ monocytes (50). In addition, other antigen-presenting cells such as B cells and dendritic cells can be CD80$^+$ (51, 52). It has been previously described that VEGFA-expression of macrophages depends on the stimulation state (53, 54). Additionally, tissue resident F4/80$^+$ macrophages are not known to express VEGFA constantly, but rather they interact with VEGFA-producing cells on promoting vascularization and supporting sprout fusion (29, 55). Our results support previous literature by showing VEGFA-expression from immune cells (56). Here, CD80$^+$ cells were expressing VEGFA in the early phase until day 3, while the majority was F4/80$^-$. CD80$^+$ M1-like macrophages have not been reported to express VEGFA. It can be speculated from our data that CD80$^+$ cells contribute to the presence of VEGFA within the fracture gap until bone cells differentiate and become the dominant producers of VEGFA during the bone formation and remodeling phase.

The presence of M1-like macrophages until day 3, the subsequent decrease, and the absence at later time points in our study indicates that the transition from the pro-inflammatory phase to the anti-inflammatory phase in the myeloid compartment has already taken place at day 3 in the osteotomy models analyzed here. It has been described that a switch from M1-like macrophages to an M2-like phenotype is essential for successful healing. Under chronic inflammatory conditions

it is impaired and accompanied by the prolonged presence of M1-like macrophages (57). Consequently, recent research focuses on this switch to improve healing (58, 59). Interestingly, M1- and M2-like macrophages do not localize inside the fracture gap, and rather CX3CR1$^+$F4/80$^+$ cells participate in the initial and crucial vascularization process. Our spatial analyses reveal that CD206$^+$ macrophages localize in extramedullary areas and, moreover, that they remain positive for the pro-inflammatory maker Mac-2 until d14. After that, cells that are positive for either CD206 or Mac-2 remain present until the remodeling phase. The extramedullary localization of CD206$^+$ macrophages may indicate that M2-like macrophages serve functions beyond bone regeneration, for example skeletal muscle regeneration (60).

Of note, during the regeneration process, the immunofluorescence signal for Emcn$^+$ in the endothelium partially co-localizes with the signal for the mannose receptor CD206. CD206 expression in endothelial cells has been previously reported to be linked to the phagocytic activity of this cell type (61). In addition, Awert et al. describe CD206 as a marker for perivascular macrophages and show that they locate closely to Emcn$^+$ cells in tumors (62). CD206$^+$ endothelial cells can be found in different tissues such as the liver (63, 64) or placenta (61). Future experiments need to be performed to investigate the cellular origin of the CD206 signal we observe.

In tissue areas adjacent of the fracture gap, an inhomogeneous distribution of macrophages, as well as endothelia toward the distal end of the femur (spatial polarization), is evident. We suspect that the osteotomy, which interrupts all vessels in the tissue, in combination with placement of the neighboring, distal screw (one of four screws) creates damage to the blood supply occurring from both sides of the fracture (**Supplementary Figure 6**). Large veins and the main sinus exit the bone at few points. However, trans-cortical vessels are predominantly responsible for the arterial blood supply in long bones, as described recently (65). Since the cortical integrity is disrupted by the osteotomy and the screw placement, the blood supply is disturbed to a high degree in a complete osteotomy model. We assume that this results in extensive tissue damage, which in turn initiates the recruitment of macrophages followed by vascularization, leading to the observed phenomenon of transient distal polarization.

Taken together, we demonstrate here that type H endothelium is present throughout the regeneration in standardized osteotomy models in mice. Osx$^+$ osteoblasts as well as macrophages are present in close proximity to the vasculature, indicating an important crosstalk. M2-like macrophages are mainly found in extramedullar regions, with no obvious interconnection to the vasculature. In addition, we describe CX3CR1$^+$F4/80$^+$ cells to be the predominant macrophage population, which progressively infiltrates the hematoma. These macrophages precede perfused vascularization in the first phase of vascularization and reside there until remodeling takes place. A strong polarization of type H endothelium as well as of macrophages distally to the fracture gap is found in the fracture models. Our findings underline the importance of the innate immune system within the bone regeneration process by linking myeloid cells present in the fracture gap to local angiogenesis.

## DATA AVAILABILITY STATEMENT

The authors declare that all data supporting the findings of this study are available within the paper and its supplementary information file. Further information is made available by the authors upon request.

## ETHICS STATEMENT

The animal study was reviewed and approved by the local animal protection authority (LaGeSo; permit numbers: G0039/16, G0111/13, and G0302/17), and were performed in accordance with the German Animal Welfare Act.

## AUTHOR CONTRIBUTIONS

JS, AL, TG, RN, and AH: study design. JS, AL, AR, LA, MK, KS-B, and AF: data collection and analysis. JS, AL, and AH: data interpretation and writing of the manuscript. KS-B, FB, RN, TG, and GD: revising manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02588/full#supplementary-material

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# Loss of Dkk-1 in Osteocytes Mitigates Alveolar Bone Loss in Mice With Periodontitis

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**Background:** Periodontitis is a highly prevalent infection-triggered inflammatory disease that results in bone loss. Inflammation causes bone resorption by osteoclasts, and also by suppression of bone formation via increase of Dickkopf-1 (Dkk-1), an inhibitor of Wnt signaling. Here, we tested the hypothesis that osteocytic Dkk-1 is a key factor in the pathogenesis of periodontitis-induced alveolar bone loss (ABL).

**Methods:** Twelve-week-old female mice with a constitutive deletion of Dkk-1 specifically in osteocytes (Dkk-1$^{fl/fl}$;Dmp1:Cre) were subjected to experimental periodontitis (EP). Cre-negative littermates served as controls. EP was induced by placing a ligature around the upper 2nd left molar, the contralateral side was used as control. Mice were killed after 11 days and maxillae removed for micro-CT and histological analyses. The mRNA expression of Dkk-1, Runx2, Osteocalcin, OPG, RANKL, RANKL/OPG ratio, LEF-1, and TCF-7 were assessed in maxillae, while mRNA expressions of TNF and IL-1 were evaluated on gingiva using real-time PCR. Blood samples were collected for Dkk-1, CTX, and P1NP measurement by ELISA.

**Results:** The deletion of Dkk-1 in osteocytes prevented ABL in mice with EP, compared to Cre-negative control mice with EP. Micro-CT analysis showed a significant reduction of bone loss (−28.5%) in EP Dkk-1$^{fl/fl}$;Dmp1:Cre-positive mice compared to their littermate controls. These mice showed a greater alveolar bone volume, bone mineral density, trabecular number, and trabecular thickness after EP when compared to the Cre-negative controls. The local expression in maxillae as well as the serum levels of Dkk-1 were reduced in Dkk-1$^{fl/fl}$;Dmp1:Cre-positive mice with EP. The transgenic mice submitted to EP showed increase of P1NP and reduction of CTX-I serum levels, and increase of TCF-7 expression. Histological analysis displayed less inflammatory infiltrates, a reduction of TNF and IL-1 expressions in the gingiva and fewer osteoclasts in Cre-positive animals with EP. Moreover, in mice with EP, the osteocytic deletion of Dkk-1 enhanced bone formation due to increased expressions of Runx2 and Osteocalcin and decreased expression of RANKL in maxillae.

**Conclusion:** In summary, Dkk-1 derived from osteocytes plays a crucial role in ABL in periodontitis.

**Keywords: periodontitis, Dkk-1, osteocyte, inflammation, bone loss, osteoimmunology**

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## INTRODUCTION

Alveolar bone loss and connective tissue destruction are the characteristic clinical hallmarks of periodontitis, which is a highly prevalent and infectious-inflammatory disease, the second major cause of tooth loss worldwide (1). Periodontitis is mainly initiated by an oral biofilm. However, its development and progression is closely related to the exacerbated host response, which plays an important role on tissue breakdown (2).

It is known that inflammatory cytokines for example, TNF and IL-1beta, play an important role in periodontitis, inducing bone loss by promoting the expression of receptor activator of nuclear factor kappa-B ligand (RANKL) in other cells such as T cells and fibroblasts, favoring osteoclastogenesis (3). Recently, it was reported that osteocytes are also major producers of RANKL (4).

Despite the well-known RANK-RANKL axis, other pathways including the Wnt signaling have also been implicated in the process of bone loss (5). Wnt signaling is a crucial developmental pathway, and Dickkopf-1 (Dkk-1) is an important secreted inhibitor of Wnt signaling. Dkk-1 is expressed in various organs and by several cell types, although osteoprogenitors seems to contribute mostly to systemic Dkk-1 levels (6). It binds to lipoprotein receptor-related protein (LRP) 5/6 receptor blocking the interaction with Wnt proteins and leading to beta-catenin degradation. In bone tissue the lack of translocation of beta-catenin into the nucleus impairs the activation of osteoblast-related genes (Runx2, osteocalcin, and osteoprotegerin), leading to reduced osteoblastogenesis and low bone mass (7).

Besides bone homeostasis, Dkk-1 may play an important role in pathological bone loss (8) since inflammatory mediators induce Dkk-1 production (9, 10). It has been previously reported by our group and others, that during periodontitis, there is an increase of Dkk-1 in the periodontal tissue (11–13). However, to date, there is no substantial evidence regarding the actual origin and role of Dkk-1 in the bone loss related to periodontitis. Therefore, this study shows for the first time that Dkk-1 derived from osteocytes plays an essential role on periodontal bone loss.

## MATERIALS AND METHODS

### Animal Selection

The experiments were performed on female young adult mice (12 weeks old) osteocyte-specific deletion of Dkk-1 (Dkk-1$^{fl/fl}$;Dmp1:Cre) in a transgenic mouse line (C57BL/6 background), which were previously described (6). Respective Cre-negative littermates were used as controls. All mice were genotyped using standard PCR protocols.

Mice were maintained in groups of up to four animals per cage, weighing 20–25 g, and were kept in a dark cycle of 12:12 h at room temperature in filter top cages with cardboard houses as enrichment. Mice were randomly assigned to treatment groups and the subsequent analyses were performed in a blinded-fashion. All invasive procedures were approved by the Medical Faculty of the Technische Universität Dresden and the Landesdirektion Sachsen.

A power calculation was performed to determine the sample size. The animal was considered the study unit. The sample size was determined to provide 80% power to recognize a significant difference of 20% among groups and the standard deviation of 15% with a 95% confidence interval ($p = 0.05$), considering the change in alveolar bone loss (ABL) as the primary outcome variable. Therefore, a sample size of at least six mice per group was required.

### Experimental Periodontitis

After 2 weeks of acclimation to the laboratory environment, the mice were subjected to experimental periodontitis (EP). For that, the mice were anesthetized with ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) intraperitoneally. Following, all the animals received a sterile polyacrylamide ligature (6–0) around the cervical area of their maxillary left second molars (14, 15). After 11 days, all mice were euthanized. The contralateral right side was used as the unligated control.

### Assessment of Alveolar Bone Microarchitecture

For μCT measurements the maxillae were analyzed *ex vivo* (vivaCT40, ScancoMedical, Switzerland) with an isotropic voxel size of 10.5 μm (70 kVp, 114 μA, 200 ms integration time). Initially, the 3D reconstruction was performed and the measurement of ABL, in the buccal side, was performed using ImageJ software (National Institutes of Health, Washington, DC, USA). For that, the area between the cementum-enamel junction until the reminiscent bone border from left and right sides of the maxillae were used. For volumetric analyses 20 slices from the second molar were selected. Bone volume (BV/TV), bone mineral density (BMD), trabecular number (Tb.N), and trabecular thickness (Tb.Th) were assessed (16). All micro-CT analyses were performed by one blinded and calibrated examiner.

### Bone Histology and Histomorphometry

The maxillae were removed and fixed in 4% PBS-buffered paraformaldehyde for 48 h. Thereafter samples were demineralized using EDTA solution (Osteosoft®, Merck, Darmstadt, Germany). After that, the specimens were dehydrated and embedded in paraffin. Serial sections of 2 μm thickness were obtained in a mesio-distal direction. The sections were stained with hematoxylin and eosin and tartrate-resistant acid phosphatase (TRAP). Hematoxylin and eosin slides were performed to evaluate periodontal architecture and inflammatory status in the area between the first and second molars, using scores varying from 0 to 3 according to the intensity of findings, as follows: Score 0: absence or only discrete cellular infiltration, few osteoclasts, preserved alveolar process, and cementum; Score 1: moderate cellular infiltration, presence of some osteoclasts, some but minor alveolar process resorption and intact cementum; Score 2: severe cellular infiltration, large number of osteoclasts, accentuated degradation of the alveolar process, and partial destruction of cementum; Score 3: severe cellular infiltrate, total destruction of alveolar process, and cementum (17). Hematoxylin and eosin staining was also used to assess the number of osteoblasts per bone perimeter (N.Ob/B.Pm). Osteoblasts were characterized by its well-known cuboidal morphology and location over bone surface. The TRAP staining was performed to assess the number of osteoclasts per

bone perimeter (N.Oc/B.Pm) using the Osteomeasure® software (OsteoMetrics, Atlanta, Georgia, USA) (18).

## RNA Isolation and Quantitative PCR

RNA was extracted from maxillae as well as gingiva of Dkk-$1^{fl/fl}$;Dmp1:Cre mice by crushing them in liquid nitrogen and collecting the powder in Trifast (Peqlab, Germany). RNA isolation was performed according to the manufacturer's protocol. Five hundred nanograms of RNA were reverse transcribed using Superscript II (Invitrogen) and subsequently used for SYBR green-based real-time PCR (ABI 7500 Fast; Applied Biosystems). The primer sequences were: ß-actin s: ATCTGGCACCACACCTTCTT, ß-actin as: GGGGTGTTG AAGGTCTCAAA; Dkk1 s: GAGGGGAAATTGAGGAAAGC, Dkk1 as: AGCCTTCTTGTCCTTTGGTG, Runx2 s: CCCAGC CACCTTTACCTACA, Runx2 as: TATGGAGTGCTGCTGG TCTG, OCN s: GCGCTCTGTCTCTCTGACCT,OCN as: ACCT TATTGCCCTCCTGCTT, OPG s: CCTTGCCCTGACCACT CTTA, OPG as: ACACTGGGCTGCAATACACA, RANKL s: CCGAGACTACGGCAAGTACC, RANKL as: GCGCTCGA AAGTACAGGAAC, IL-1ß s: ACAAGGAGAACCAAGCAACG, IL-1ß as: GCCGTCTTTCATTACACAGG, TNF s: CCTCTTCT CATTCCTGCTTGTG, TNF as: CACTTGGTGGTTTGCT ACGAC, LEF1 s: CAAATAAAGTGCCCGTGGTG, LEF1 as: TCGTCGCTGTAGGTGATGAG, TCF7 s: GGACATCAGCCA GAAGCAAG, TCF7 as: GGACAGGGGGTAGAGAGGAG. PCR conditions used were: 50°C for 2 min and 95°C for 10 min followed by 40 cycles with 95°C for 15 s and 60°C for 1 min. The melting curve was assessed by the 95°C for 15 s, 60°C for 1 min, and 95°C for 30 s. The results were calculated using the ΔΔCT method and are presented as x-fold increase relative to beta-actin (18).

## Serum Analysis

Blood was taken via heart punctuation and serum was collected after 10 min centrifugation at 400 × g. Dkk-1, C-terminal telopeptide (CTX) and type 1 procollagen amino-terminal-propeptide (P1NP) were measured using an immunoassay kit (Dkk1: R&D Systems, USA; CTX and P1NP: Immundiagnostik Systems, Germany) according to the manufacturer's protocols.

## Statistical Analysis

The data are presented as means ± standard errors of the mean or as median (range), when appropriate. Normality and homoscedasticity of the data were verified. ANOVA followed by the Bonferroni test were used to compare the means, and Kruskal–Wallis and Dunn's tests were used to compare the medians. The significance level was set at 5% in all tests. All calculations were performed using Prism 5 (GraphPad Software Inc., San Diego, CA, USA). All protocols and analyses were performed by blinded and calibrated examiners.

# RESULTS

## Osteocytic Deletion of Dkk-1 Prevents Periodontal Bone Loss

In Cre-negative mice, EP caused significant alveolar bone loss (**Figures 1A,B**) as well as reduction of bone volume (−40.6%) (**Figure 1C**), bone mineral density (−46.7%) (**Figure 1D**) and trabecular number (−67.6%) (**Figure 1E**), when compared to the control site. Loss of Dkk-1 derived from osteocytes, however, resulted in less alveolar bone loss (−28.5%) in mice subjected to EP compared to the EP Cre-negative group (**Figures 1A,B**). In line with these results, also bone volume (−24.6%) (**Figure 1C**), bone mineral density (−23.3%) (**Figure 1D**), trabecular number (−58.8%) (**Figure 1E**), and trabecular thickness (−3.1%) (**Figure 1F**) showed milder reductions compared to the Cre-positive mice.

We also evaluated the serum bone turnover markers and found high levels of CTX after EP induction in Dkk-$1^{fl/fl}$;Dmp1:Cre-negative mice ($p < 0.05$) (**Figure 1H**). In Dkk-$1^{fl/fl}$;Dmp1:Cre-positive animals, CTX levels were significantly reduced together with increased P1NP serum levels (**Figure 1G**).

## Osteocytic Deletion of Dkk-1 Activated Wnt Signaling During Periodontitis

Dkk-1 gene expression in maxillae (**Figure 2A**) as well as Dkk-1 serum levels (**Figure 2B**) were also investigated. EP significantly increased Dkk-1 gene expression in maxillae only in Cre-negative, but not Cre-positive mice. No differences were seen in the serum levels of Dkk-1 between Cre-negative and Cre-positive mice submitted to periodontitis. The expression of Wnt target genes LEF-1 (**Figure 2C**) and TCF-7 (**Figure 2D**) were evaluated. Periodontitis tended to reduce the expression of LEF-1 and drastically reduced TCF-7 expression in wildtype littermates. However, in Dkk-1 conditional knock-out mice with periodontitis, TCF-7 expression remained at control level (**Figure 2D**). There was no change on the expression of LEF-1 (**Figure 2C**).

## Osteocytic Deletion of Dkk-1 Modulates Inflammation and Increases Osteoblast Activity During Periodontitis

The osteocytic deletion of Dkk-1 maintained the periodontium architecture in mice submitted to EP when compared to the Cre-negative group (**Figure 3A**). In the normal maxillae of either Cre-negative or Cre-positive mice, it is possible to observe the normal organization of the periodontal tissue [0 (0–0)]. However, EP in Cre-negative animals provoked the great amount of inflammatory infiltrate on the gingival tissue, as well as bone and cementum resorption, marked by an increase of osteoclasts [3 (2–3)] (**Figure 3C**), which was statistically significant when compared to the Cre-negative control. All these histological findings were mitigated in the mice with osteocytic deletion of Dkk-1 submitted to EP [1 (1–2)] (**Figure 3A**) ($p < 0.05$). Furthermore, the number of osteoblasts significantly increased (**Figure 3B**) in these animals.

The effect of osteocytic deletion of Dkk-1 in the periodontal inflammation was confirmed by the downregulation of TNF (**Figure 3D**) and IL-1β (**Figure 3E**) gene expression in the gingiva when compared to the Cre-negative group, both submitted to EP ($p < 0.05$).

The analysis of gene expression in maxillae showed that periodontitis caused a significant decrease of Runx2 (**Figure 3F**) and OCN (**Figure 3G**), as well as an increase of RANKL

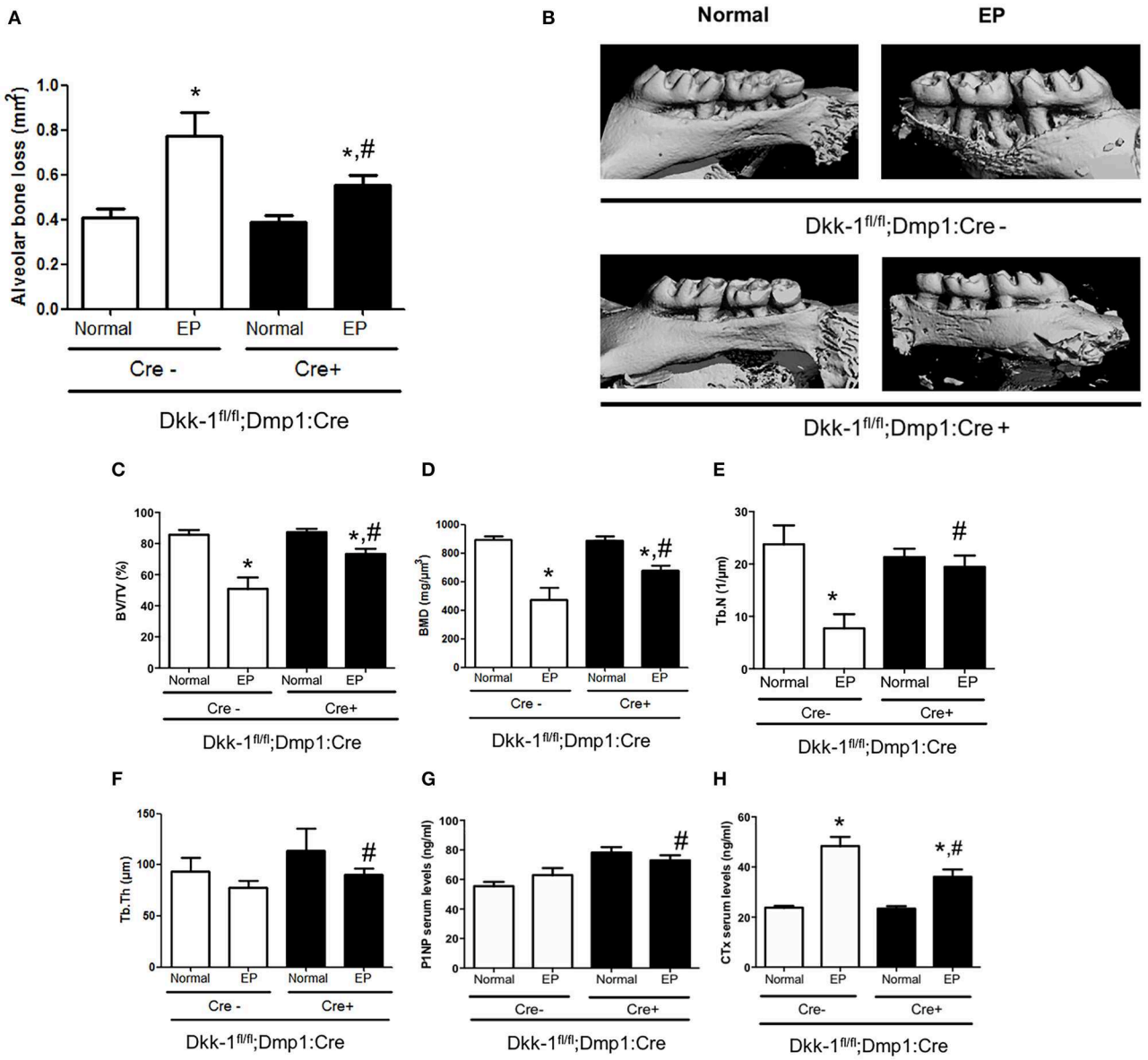


**FIGURE 1 |** Lack of Dkk-1 in osteocytes prevents periodontal bone loss. Maxillae of 12-week-old female Dkk1fl/fl;Dmp1:Cre-positive and -negative mice were analyzed by μCT. **(A)** Alveolar bone loss, **(B)** representative 3D reconstruction of hemimaxillae with and without ligature in buccal view, **(C)** trabecular bone volume per total volume (BV/TV), **(D)** bone mineral density, **(E)** trabecular number (Tb.N), and **(F)** trabecular thickness (Tb.Th) of hemimaxillae. **(G)** Serum levels of procollagen type 1 aminoterminal propeptide (P1NP) and **(H)** carboxy-terminal collagen cross-links (CTX-I) were assessed using commercially available ELISAs. Data represent the mean ± SEM of at least six animals per group. Statistical analyses were performed by ANOVA followed by the Bonferroni test. $^{*}P < 0.05$ vs. respective normal hemimaxillae; $^{\#}P < 0.05$ vs. EP Cre-negative control.

(**Figure 3I**) in Cre-negative control mice. However, when Dkk-1 derived from osteocytes was deleted, an increase of Runx2 and OCN expression was observed ($p<0.05$). While OPG expression was not affected (**Figure 3H**), expression of RANKL in the Dkk-1$^{fl/fl}$;Dmp1:Cre-positive mice with EP was significantly decreased compared to the Cre-negative control mice. Thus, the osteocytic deletion of Dkk-1 reduced the RANKL/OPG ratio (**Figure 3J**), which may explain the reduced activation of osteoclasts.

## DISCUSSION

In this study, we show that the deletion of Dkk-1 derived from osteocytes plays an important role in the pathogenesis of periodontal bone loss. The osteocytic Dkk-1 deletion reduced bone loss, mitigated inflammation, and enhanced bone formation in mice submitted to ligature-induced periodontitis.

Importantly, the osteocytic-specific deletion of Dkk-1 prevented EP-induced bone loss. The Dmp1 promoter has

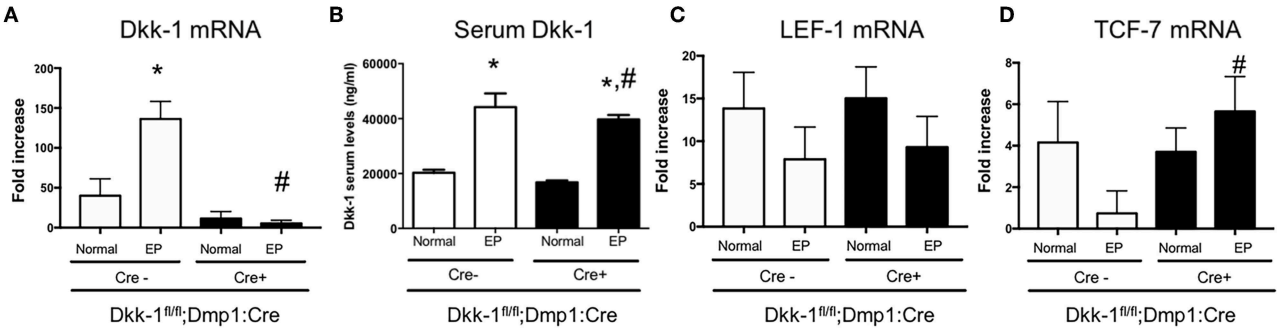


**FIGURE 2 |** Lack of Dkk-1 in osteocytes activated Wnt signaling during periodontitis. **(A)** Real-time PCR analysis was performed for Dickkopf-1 (Dkk-1) in hemimaxillary bone tissue. **(B)** Serum levels of Dkk-1 were assessed using commercially available ELISA. **(A)** Real-time PCR analysis was performed for **(C)** LEF-1 and **(D)** TCF-1 were also performed in hemimaxillary bone tissue. Data represent the mean ± SEM of at least six animals per group. Statistical analyses were performed by ANOVA followed by the Bonferroni test. $^{*}P < 0.05$ vs. respective normal hemimaxillae; $^{\#}P < 0.05$ vs. EP Cre-negative control.

been shown to target osteocytes, mature osteoblasts, and occasional bone lining cells (4, 19), even though also non-specific deletions have been observed in muscle, intestine, and brain (18). Nonetheless, these mice have been characterized well and show a significant reduction of Dkk-1 in cortical bone tissue, which mostly contains osteocytes, while no decrease is observed systemically (6). This study now further shows that Dkk-1 mRNA levels are reduced in the maxillae of Dkk-$1^{fl/fl}$;Dmp1:Cre-positive mice and that osteocytes mostly contribute to EP-induced Dkk-1 levels in the maxilla, as this increase was absent in Dkk-$1^{fl/fl}$;Dmp1:Cre-positive mice. Thus, this study supports the previous observation from our group that local, but not systemic Dkk-1 levels are critical to determine bone loss.

TCF/LEF are transcription factors for β-catenin expressed in the nucleus, that mediate the canonical Wnt signaling in several cell types (20). An increase on TCF expression was observed in mice submitted to EP with osteocytic deletion of Dkk-1. Different from LEF, TCF was detected in prechondrocytes in the palate, nasal bone, occipital bone, vertebrae, ribs, and jaws during mouse embryo (21). Moreover, it has been reported that $Tcf^{-/-}$ mice have an increased osteoclast number and function without any change in osteoblast number of function (22), and also showed accelerated bone resorption (23). Consistent with our results, Shin et al. (24) reported that the activation of beta-catenin/TCF decreased the expression of RANKL while mRNA level of OPG was unchanged. However, whether RANKL is a direct target of the beta-catenin/TCF pathway is not clear and requires further work.

Osteocytes are now considered master regulators of osteoblast and osteoclast function by connecting with them via their dendritic processes (25). In fact, osteocytic deletion of Dkk-1 increased osteoblast numbers and reduced osteoclast parameters in the maxillae of mice with EP, suggesting that Dkk-1 derived from osteocytes is a critical factor in the communication with osteoblasts and osteoclasts during EP. Moreover, P1NP levels were increased, while CTX levels were decreased in osteocyte-specific Dkk-1 knock-out mice with EP. Mechanistically, this may be derived from increased Wnt signaling in osteoblasts, which may activate bone-related target genes. Our results showed an increase of Runx2 expression, which is a key transcriptional modulator of osteoblast differentiation (26, 27). Moreover, osteocalcin expression was increased, a major non-collagenous protein that is important for both, the biological and mechanical functions of bone (28). Finally, there was a significant reduction of RANKL expression in the maxillae, leading to a reduced RANKL/OPG ratio, which may account for the reduced number of osteoclasts. Similar reductions of the RANKL/OPG ratio were also found in other studies using Dkk-1-deficient mice (18). Taken together, these findings indicate the osteocyte-derived Dkk-1 plays an important role in modulating osteoblast and osteoclast function in EP.

Inflammation is a major trigger for bone loss, and our data show that the lack of Dkk-1 in osteocytes during periodontitis resulted in less inflammatory infiltrates. Therefore, the immunomodulation seen in this study may indicate that osteocyte-derived Dkk-1 is necessary for the initiation of the inflammatory process. This was confirmed by the reduced expression of TNF and IL-1β in the gingiva of animals with specific-deletion of Dkk-1 in osteocytes submitted to periodontitis. TNF and IL-1β are well-known key cytokines for periodontal disease (29). Furthermore, Dkk1 produced by platelets has been shown to control neutrophil invasion in acute lung inflammation via modulating ICAM expression (30) and the inflammatory interaction with endothelial cells during atherosclerosis (31). This, it could be envisaged that also osteocyte-produced Dkk1 alters the alveolar bone microenvironment in such a way to promote immune cell attraction and subsequent inflammatory reactions. Further, studies have shown an immunomodulation role of Dkk-1 in cancer immune surveillance (32) or promoting pathological chronic type

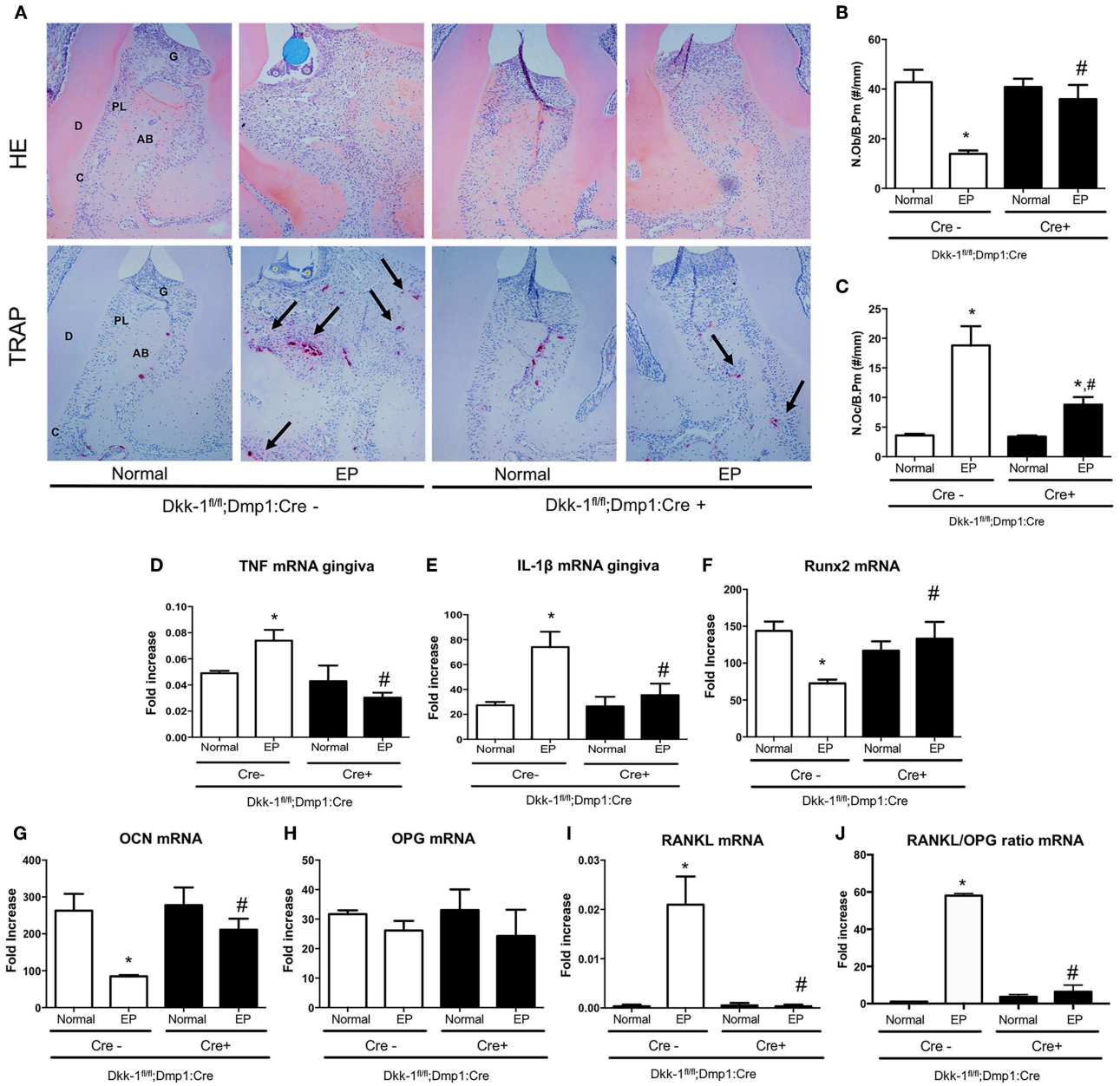


**FIGURE 3 |** Lack of Dkk-1 in osteocytes modulates inflammation and enhances bone formation. **(A)** H&E and TRAP staining of hemimaxillae 11 days after periodontitis induction were analyzed to assess **(B)** number of osteoblasts (N.Ob./B.Pm.) and **(C)** number of osteoclasts (N.Oc./B.Pm.). Real-time PCR analysis was performed for **(D)** TNF and **(E)** IL-1β in gingiva as well as for **(F)** runt-related transcription factor 2 (Runx2), **(G)** osteocalcin (OCN), **(H)** osteoprotegerin (OPG), **(I)** receptor activator of nuclear factor-kB ligand (RANKL), and **(J)** RANKL/OPG ratio, in hemimaxillary bone tissue. Data represent the mean ± SEM of at least six animals per group. Statistical analysis was performed by ANOVA followed by the Bonferroni test. $^{*}P < 0.05$ vs. respective normal hemimaxillae; $^{\#}P < 0.05$ vs. EP Cre-negative control.

2 inflammation (33). Recently, it was demonstrated that Dkk-1 is uniquely expressed in Foxp3$^{+}$ Treg cells to inhibit T-cell-mediated autoimmune colitis as a membrane-bound form (34). However, despite the eminent role of osteocyte derived Dkk-1 on inflammation, the exact mechanism of the immunomodulatory property of Dkk-1 deserves further investigations.

In summary, within the limits of this study, our findings emphasize the role of osteocytes in periodontitis, demonstrating for the first time that Dkk-1 secreted by osteocytes is essential for periodontal bone loss. These findings may contribute to a better understanding how osteocytes act on inflammatory bone loss and may also be important for the view of Dkk-1 as a mechanism that could be targeted in the future in bone diseases.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care Committee and the Landesdirektion Sachsen.

## AUTHOR CONTRIBUTIONS

PG, MR, and ST designed the study. PG and ST induced periodontitis and performed all the assays. CD and LL performed the molecular biology assay. LH and MR supervised the study. All authors contributed to the interpretation of the results.

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REVIEW
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# The Role of Mast Cells in Bone Metabolism and Bone Disorders

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Mast cells (MCs) are important sensor and effector cells of the immune system that are involved in many physiological and pathological conditions. Increasing evidence suggests that they also play an important role in bone metabolism and bone disorders. MCs are located in the bone marrow and secrete a wide spectrum of mediators, which can be rapidly released upon activation of mature MCs following their differentiation in mucosal or connective tissues. Many of these mediators can exert osteocatabolic effects by promoting osteoclast formation [e.g., histamine, tumor necrosis factor (TNF), interleukin-6 (IL-6)] and/or by inhibiting osteoblast activity (e.g., IL-1, TNF). By contrast, MCs could potentially act in an osteoprotective manner by stimulating osteoblasts (e.g., transforming growth factor-β) or reducing osteoclastogenesis (e.g., IL-12, interferon-γ). Experimental studies investigating MC functions in physiological bone turnover using MC-deficient mouse lines give contradictory results, reporting delayed or increased bone turnover or no influence depending on the mouse model used. By contrast, the involvement of MCs in various pathological conditions affecting bone is evident. MCs may contribute to the pathogenesis of primary and secondary osteoporosis as well as inflammatory disorders, including rheumatoid arthritis and osteoarthritis, because increased numbers of MCs were found in patients suffering from these diseases. The clinical observations could be largely confirmed in experimental studies using MC-deficient mouse models, which also provide mechanistic insights. MCs also regulate bone healing after fracture by influencing the inflammatory response toward the fracture, vascularization, bone formation, and callus remodeling by osteoclasts. This review summarizes the current view and understanding of the role of MCs on bone in both physiological and pathological conditions.

**Keywords: mast cells, inflammation, bone disorders, osteoporosis, fracture healing**

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## INTRODUCTION

Mast cells (MCs) are tissue-resident immune cells and are best known for promoting allergic reactions (1). However, research over recent decades has revealed important functions of MCs in numerous physiological conditions, including the regulation of angiogenesis and tissue homeostasis, but also in pathological conditions, such as gastrointestinal and cardiovascular diseases. MCs are distributed throughout several tissues, including the skeletal system (2). They are suitable candidates to be involved in bone metabolism and bone disorders, because MCs store and

de novo synthesize many mediators, including cytokines and enzymes (3), which have been shown to regulate bone homeostasis and to be involved in the pathogenesis of several skeletal diseases (4). Indeed, increased numbers of MCs have been found in patients with reduced bone mass observed in mastocytosis or postmenopausal osteoporosis (5, 6). Furthermore, it has been shown that the synovial fluids of patients suffering from rheumatoid arthritis (RA) or osteoarthritis (OA) contain increased MC numbers and elevated concentrations of certain MC mediators including tryptase or histamine (7, 8). Importantly, numerous experimental studies using MC-deficient mouse models confirmed the involvement of MCs in the pathologies of osteoporosis and arthritis (9, 10). Interestingly, several groups using MC-deficient mouse models discovered that MCs also play an important role in the process of bone fracture healing and might be involved in the regulation of osteoclastogenesis (10, 11). However, further research needs to elucidate the molecular mechanisms of MC actions in these various physiological and pathological conditions.

The scope of this review is to provide an overview of the physiological role of MCs in bone homeostasis based on the current state of knowledge. Moreover, the role of MCs in bone disorders will be discussed, focusing on osteoporosis and bone fracture healing, including both current clinical and experimental data. The involvement of MCs in RA and OA will be discussed only briefly, because there are several comprehensive reviews from other authors, which summarize the important function of MCs in these bone disorders (12–14).

## MAST CELLS AND THEIR PHYSIOLOGICAL ROLES

MCs are tissue-resident hematopoietic cells and are identified by their large number of secretory granules, which contain a broad variety of preformed mediators, including biogenic amines (e.g., histamine), heparin, cytokines [e.g., tumor necrosis factor (TNF), interleukin-6 (IL-6)], enzymes (e.g., chymases, tryptases), and various growth factors [e.g., vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)] (3). Unlike most other hematopoietic cells, mature MCs are not found in the circulation under physiological conditions. They are released from the bone marrow as MC progenitors (MCps). MCps are characterized by their expression of CD34, as are other early hematopoietic cells, and by MC-related surface markers, including CD117 (c-Kit), also known as stem cell factor (SCF) receptor (15). c-Kit is highly expressed on hematopoietic stem cells and its activity is crucial for hematopoiesis. Interestingly, only MCs retain c-Kit expression throughout their lifetime, whereas it is lost in other hematopoietic lineages during differentiation. SCF/c-Kit signaling is essential for MC growth, differentiation, and survival. Late MCps also express the high-affinity immunoglobulin E (IgE) receptor (FcεRI), as do mature MCs, however, MCps are less or non-granulated in contrast to mature MCs, which have many metachromatic granules (15–17). Committed MCps enter the target tissues and complete their maturation based on the local microenvironment (18). That is why their types and amounts of mediators can vary during MC maturation depending on the respective tissue (19). While MCs are located in almost all tissues, high numbers are found in tissues facing the external environment, including the skin, lungs, and intestines, where pathogen exposure is most likely. Thereby, MCs serve as immunological sentinels in the first line of defense. Their long lifespan of up to several months as well as their perivascular, perilymphatic, and perineuronal locations potentiate MCs to respond rapidly to pathogens. Moreover, they can also react to humoral and neuronal stimuli as well as tissue damage (e.g., physical injury inducing damage-associated molecular patterns) or environmental insults (20, 21).

Mature MCs are mainly divided into two subsets in both humans and rodents, which differ in their anatomical distribution and the types of proteases produced (22). In humans, so-called $MC_T$ express only tryptases and are located predominantly in the lungs and small intestinal mucosa, whereas $MC_{TC}$ produce both tryptases and chymases as well as carboxypeptidase A3 (Cpa3). $MC_{TC}$ predominate in the skin and the submucosa of the small intestine (23). In rodents, MCs are classified into connective tissue MCs (CTMCs) and mucosal MCs (MMCs). In terms of tissue localization and protease content, CTMCs are thought to resemble human $MC_{TC}$, whereas MMCs closely correspond to human $MC_T$. CTMCs are particularly located in the skin, peritoneal cavity, and submucosa of the intestine, while MMCs occupy the mucosal epithelium of the lungs and the gastrointestinal tract (24). Both MC subtypes are mainly identified via their protease content. While MMCs predominantly express the chymases MC protease-1 (Mcpt-1) and Mcpt-2, CTMCs express the chymases Mcpt-4 and Mcpt-5 as well as the tryptases Mcpt-6 and Mcpt-7, and additionally Cpa3. Furthermore, both subclasses react differently in response to stimulation and inhibition by drugs and interactions with T cells. MMCs expand remarkably during T cell-dependent immune responses, whereas CTMCs do not require T cells for expansion (22–24).

MCs can be activated by numerous factors, including immunoglobulins, cytokines, neuropeptides, complement proteins, and pathogen-associated molecular patterns (e.g., by

**Abbreviations:** CIA, Collagen-Induced Arthritis; Cpa3, Carboxypeptidase A3; CTMCs, Connective Tissue Mast Cells; CXCL, Chemokine (C-X-C Motif) Ligand; DT, Diphtheria Toxin; DTR, Diphtheria Toxin Receptor; ER, Estrogen Receptor; f, female; FcεRI, High Affinity Immunoglobulin E (IgE) Receptor; FGF, Fibroblast Growth Factor; GM-CSF, Granulocyte-Macrophage Colony-Stimulating Factor; IL, Interleukin; INF-γ, Interferon-γ; m, male; Mas-TRECK, Mast Cell-Specific Enhancer Mediated Toxin Receptor Mediated Conditional Cell Knockout; MCs, Mast Cells; Mcl-1, Myeloid Cell Leukemia Sequence-1; MCP-1, Monocyte Chemoattractant Protein-1; MCps, Mast Cell Progenitors; Mcpt, Mast Cell Protease; M-CSF, Macrophage Colony-Stimulating Factor; MIP-1α, Macrophage Inflammatory Protein-1α; MMCs, Mucosal Mast Cells; MMPs, Matrix Metalloproteinases; MSCs, Mesenchymal Stem Cells; NO, Nitric Oxide; NSAID, Non-Steroidal Anti-Inflammatory Drugs; OA, Osteoarthritis; OVX, Ovariectomy; PAF, Platelet Activating Factor; RA, Rheumatoid Arthritis; RANKL, Receptor Activator Of Nuclear Factor Kappa B Ligand; RFP, Red Fluorescent Protein; RMB, Red Mast Cell And Basophil Mouse; rPTH, Teriparatide; SC, Sodium Cromolyn; SCF, Stem Cell Factor; SM, Systemic Mastocytosis; tdT, td-Tomato; TGF-β, Transforming Growth Factor-β; TLR, Toll-Like Receptor; TNF, Tumor Necrosis Factor; TRAP, Tartrate Resistant Acid Phosphatase; VEGF, Vascular Endothelial Growth Factor; YFP, Yellow Fluorescent Protein; ↓, Decreased; ↑, Increased.

alarmins). Activation results in the release of preformed and newly synthesized mediators via degranulation. Furthermore, depending on the stimulus, MCs can also release the mediators selectively without degranulation (25). The most important and well known mechanism of MC activation is the crosslinking of the FcεRI via IgE and multivalent antigen complexes (26). FcεRI crosslinking triggers a cascade of intracellular signaling events, comprising protein phosphorylation, intracellular calcium mobilization, and transcription factor activation, and culminates in MC degranulation (27). Because MCs are present at the tissue boundaries, they are the first immune cells encountering invading endogenous and exogenous pathogens. Thereby, MCs can be activated directly by pathogens as well as by many pathogen-derived soluble products, including lipopolysaccharide (derived from gram-negative bacteria) and peptidoglycan (derived from gram-positive bacteria). They directly activate MCs via toll-like receptors (TLRs) or indirectly by activating the complement system through its receptors on MCs. Activation through TLRs induces selective cytokine synthesis and release depending on the stimuli, allowing specific responses to certain immunological insults (21, 25, 28, 29). For example, whereas TLR1 stimulation results in degranulation and additional IL-1 production, TLR2 activation induces the synthesis of cytokines and leukotrienes without degranulation (30). MCs can directly kill the pathogens by phagocytosis or extracellular traps similar to neutrophils. Additionally, they enhance the mucus production of epithelial cells to immobilize pathogens and modulate vascular permeability and blood flow to initiate rapid immune cell recruitment of effector cells, including neutrophils, eosinophils, and natural killer cells. Therefore, MCs play an important role in initiating the immune response. MCs and their products are also involved in the regulation of adaptive immune responses. For example, they modulate the migration, maturation, and activation of dendritic cells, present antigens to cytotoxic T cells, and attract effector T cells through their mediators (2, 21, 27, 31).

Beyond the host defense, MCs have many physiological functions. Several studies demonstrated that MCs enhance angiogenesis by secreting pro-angiogenic factors, including VEGF, basic FGF, TNF-α, heparin, histamine, IL-8, and various proteases (32, 33). Furthermore, MCs are considered important for tissue homeostasis, because many of their mediators, including FGF, histamine, and tryptase, induce epithelial cell and fibroblast proliferation. In addition, MCs are the main source of proteases, including tryptases, chymases, and cathepsin G, which activate matrix metalloproteinases (MMPs), thus initiating tissue remodeling (28, 34). MCs also appear to be critical for wound healing. They are present in the connective tissue of the skin in large numbers and are activated by injuries caused by trauma, heat, irradiation, or chemical agents. Thereby, MCs influence the inflammatory response, revascularization, and tissue formation and remodeling (35, 36). However, experimental studies are contradictory as to whether or not MCs promote skin wound healing. These contradictions might depend on the type and size of the wound and the mouse models used (37–39).

## THE ROLE OF MAST CELLS IN PHYSIOLOGICAL BONE TURNOVER

A role for MCs in bone metabolism was long suspected (40). Whereas MCs are located in low numbers in the bone marrow at the epiphysis and diaphysis, they are numerous in the metaphyseal bone marrow, where bone remodeling mainly occurs (41). They are preferentially located adjacent to bone surfaces undergoing bone growth or turnover (**Figure 1A**). MCs at the endocortical bone surface are more flattened compared to those at a distance from the bone surface, which are typically round shaped (42). Their close proximity to the bone remodeling surface and the wide spectrum of their mediators, including histamine, heparin, proteases, and various cytokines, raise the question of a potential role for MCs in bone physiology. Many of the MC mediators are able to induce or modulate osteocatabolic effects by promoting osteoclastogenesis (e.g., histamine, TNF, IL-6) and/or inhibiting osteoblast activity (e.g., IL-1, TNF) (4, 43). By contrast, other mediators could act in an osteoprotective manner by stimulating osteoblasts [e.g., transforming growth factor-β (TGF-β)] or reducing osteoclastogenesis [e.g., IL-12, interferon-γ (IFN-γ)] under certain circumstances (4). **Table 1** summarizes the proposed or proven roles of MC mediators in bone formation and resorption.

To investigate the role of MCs and MC-derived products, ideally MCs would be selectively inhibited with a compound or depleted by genetic modification. Because there are no human conditions with reduced numbers or a complete absence of MCs, most data concerning the physiological role of MCs in bone development and turnover were gained either *in vitro* or in MC-deficient mouse models. To date, MC-deficient mice with mutations in the c-Kit receptor ($Kit^{W/W-v}$ and $Kit^{W-sh/W-sh}$ mice) or its ligand SCF ($Kitl^{Sl/Sl-d}$ mice) have been widely used. Whereas the point mutation KitW prevents cell surface c-Kit expression, the KitW-v mutation reduces the receptor kinase activity. KitW-sh is an inversion mutation and affects the transcriptional regulatory elements at the c-Kit transcription site. Furthermore, several mutations of the SCF ligand, including KitlSl and KitlSl-d, lead to a complete or partial deletion of the SCF gene. Because SCF/c-Kit signaling is essential for MC growth and survival, mice with alterations in this signaling lack MCs (109, 110).

Silberstein et al. using $Kit^{W/W-v}$ mice were the first to suggest that MCs might play a role in physiological bone turnover. They found that bone remodeling is delayed in this mouse model because of reduced osteoclast recruitment and osteoblast activity (111). Other studies in $Kit^{W-sh/W-sh}$ mice reported an osteopenic bone phenotype with high bone turnover because osteoclast activity exceeds osteoblast activity (112–114). However, c-Kit-dependent MC-deficient mouse models have many other abnormalities, because c-Kit is expressed not only by MCs but also by numerous other cells, including hematopoietic progenitor cells. Importantly, c-Kit is essential for osteoclast development and also regulates osteoblast activity (113, 115). Therefore, it is difficult to distinguish the effect of MCs on bone physiology from pleiotropic c-Kit effects in these mouse models.

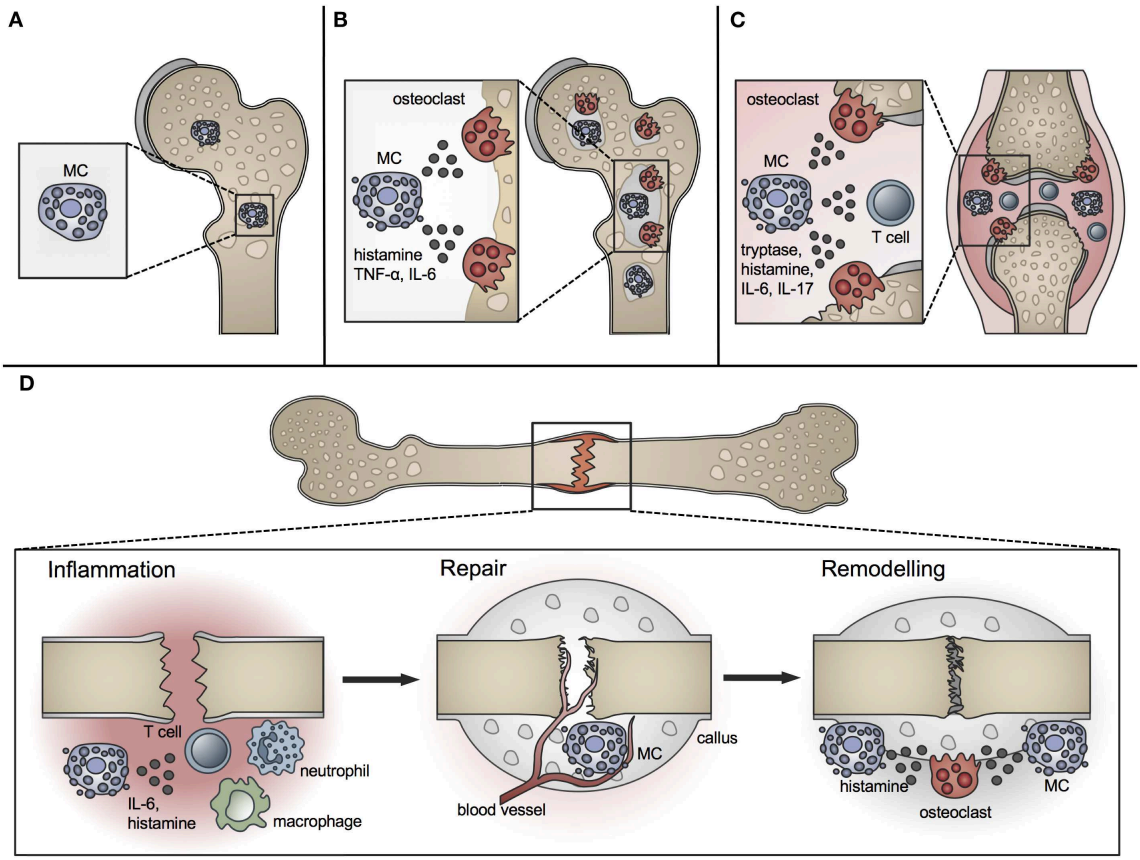


**FIGURE 1 |** Role of MCs in physiological bone turnover and bone disease. **(A)** In physiological bone turnover, few MCs are located in the bone marrow of the metaphysis, preferentially adjacent to bone surfaces. **(B)** In osteoporotic bone, more MCs are found in the bone marrow which are frequently co-localized with osteoclasts and influence their resorption activity by releasing mediators including histamine, TNF-α and IL-6. **(C)** In rheumatoid arthritis, increased MC numbers and concentrations of MC-mediators including histamine, tryptase, IL-6, and IL-17 are found in the inflamed joint, inducing osteoclastic bone resorption and T-cell driven inflammation. **(D)** In fracture healing, MCs regulate bone-fracture induced inflammation by releasing inflammatory cytokines including IL-6, and influence innate immune cell recruitment. During the repair phase, few MCs are located in the fracture callus mainly near blood vessels; MC numbers increase during callus remodeling, where MCs are found in close proximity to osteoclasts and regulate bone resorption by releasing osteocatabolic mediators including histamine.

In contrast to the above-mentioned studies, our group proposed that MCs do not affect bone formation and turnover under physiological conditions (10). We used the Mcpt-5 Cre R-DTA mouse line, a c-Kit-independent model of MC deficiency expressing diphtheria toxin (DT) under the control of the Mcpt-5 chymase promoter, which is specific for CTMCs and drives Cre-specific ablation of these cells (116). It has been previously proven that these mice specifically lack CTMCs, whereas other immune cell populations are unaffected. Mcpt-5 Cre R-DTA mice have been demonstrated to be useful in elucidating the function of MCs in immune disorders, including contact allergy and RA (9, 116, 117). Therefore, our study may more reliably reflect the role of MCs in bone metabolism than previous investigations (10). We analyzed the bone phenotype in young and adult female and male Mcpt-5 Cre R-DTA mice and compared it to MC-competent mice. The size and shape of the skeleton were not different. We could not detect significant alterations in bone microstructure or osteoblast and osteoclast numbers or activities in MC-deficient mice compared to age- and sex-matched wildtype mice. Bone mass decreased with aging, particularly in the trabecular compartment, both in MC-competent and -deficient mice. These results suggest that MCs might be redundant for physiological bone turnover as well as in age-induced bone loss (**Figure 1A**) (10). However, further studies are needed to elucidate the function of MCs in bone homeostasis. Thereby, other c-Kit-independent mouse models could be used, which were developed to overcome the abnormalities related to c-Kit structure or expression. Feyerabend et al. generated $Cpa3^{Cre/+}$ "Cre-Master" mice by using a knock-in strategy to induce Cre expression under the control of the MC Cpa3 promoter, which yielded deletion of CTMCs and MMCs by a genotoxic Trp53-dependent mechanism. However, Cpa3 is also expressed in basophils, therefore, Cre-Master mice had slightly altered basophil numbers (118). Likewise, another constitutive MC-deficient mouse model Cpa3-Cre; $Mcl\text{-}1^{fl/fl}$ was generated by crossing Cpa3-Cre transgenic mice with mice having a floxed allele of myeloid cell leukemia sequence 1 (Mcl-1). Cpa3-Cre; $Mcl\text{-}1^{fl/fl}$ mice are deficient in both CTMCs and MMCs,

**TABLE 1 |** Selected MC mediators with effects on bone formation and bone resorption.

| Mediators | Bone formation by osteoblasts | Bone resorption by osteoclasts |
|---|---|---|
| **PRE-FORMED** | | |
| Amines | | |
| Histamine | ↑ Bone formation after depleting the histamine-producing enzyme (44) | ↑ Osteoclast formation and bone resorption (43, 45)<br>↓ Osteoclast activity and recruitment after histamine blocking (46, 47) |
| Serotonin | ↓ Osteoblast formation (48) | |
| Enzymes | | |
| Chymase | ↑ Bone formation in Mcpt4-deficient mice (49) | |
| Proteoglycans | | |
| Heparin | ↓ Osteoblast formation (50, 51) | ↑ Osteoclast formation and bone resorption (52) |
| Chemokines | | |
| IL-8 | ↑ Bone formation (53) | ↑ Osteoclast formation and bone resorption (54, 55) |
| MCP-1 | ↓ Bone formation in MCP-1-deficient mice (56) | ↓ Osteoclast formation in MCP-1-deficient mice (57) |
| Polypeptide | | |
| Renin | | ↓ Bone resorption after renin inhibition (58) |
| Substance P | ↑ Osteoblast and bone formation (59–61)<br>↓ Bone formation in the absence of substance P (62) | |
| Glycoproteins | | |
| Osteopontin | | ↓ Osteoclast formation and bone resorption in osteopontin-deficient mice (63, 64) |
| ***De novo*** | | |
| Cytokines | | |
| IL-1 | | ↑ Osteoclast formation and bone resorption (65, 66) |
| IL-1β | ↑ Osteoblast formation (67) | ↓ Osteoclast formation (68) |
| IL-4 | | ↓ Osteoclast formation and bone resorption (69–71) |
| IL-6 | ↓ Bone formation in IL-6-deficient mice (72)<br>↑ Bone formation (73, 74) | ↑ Osteoclast formation and bone resorption (75, 76) |
| IL-10 | ↓ Bone formation in IL-10-deficient mice (77) | ↓ Bone resorption (78) |
| IL-11 | | ↑ Osteoclast formation (79, 80) |
| IL-13 | | ↓ Osteoclast formation and bone resorption (69) |
| IL-15 | ↓ Osteoblast apoptosis (81) | ↑ Osteoclast formation (82) |
| IL-18 | ↑ Bone formation (83) | ↑ Osteoclast formation (84) ↓ Bone resorption (85) |
| IFN-γ | | ↑ Osteoclast formation and bone resorption (86)<br>↓ Osteoclast formation (87–89) |
| MIP-1α | | ↑ Osteoclast formation (90) |
| TNF-α | ↓ Osteoblast formation (91) | ↑ osteoclast formation and bone resorption (92–94) |
| TGF-β | ↑ Osteoblast and bone formation (95, 96) | ↓ Osteoclast formation (97) |
| Phospholipid metabolites | | |
| Prostaglandin E2 | ↑ Osteoclast formation and bone resorption (98) | |
| PAF | | ↓ Bone resorption in PAF receptor-deficient mice (99) |
| Growth factors | | |
| FGF | ↓ Bone formation in FGF-2-deficient and -overexpressing mice (100, 101) | |
| GM-CSF | ↑ Osteoblast formation (102) | ↑ Osteoclast formation (103)<br>↓ Osteoclast formation (104) |
| M-CSF | | ↑ Osteoclast formation (105, 106) |
| SCF | | ↑ Osteoclast formation (107) |
| Nitric oxide | | |
| NO | ↓ Bone formation in NO synthase-deficient mice (108) | |

but also exhibit reduced basophil numbers (119). In addition to constitutive MC-deficient mouse models, some inducible models are also available. Transgenic Mas-TRECK mice (for Mast cell-specific enhancer mediated Toxin Receptor mediated Conditional cell Knock out) express the human DT receptor (DTR) under the control of an intronic enhancer element of the IL-4 gene, which is essential for IL-4 expression in MCs but not in other immune cells. Repeated intraperitoneal DT injection results in complete MC deletion accompanied by transient blood basophil depletion (120, 121). Recently, Dahdah et al. generated a knock-in mouse model called RMB (Red Mast Cell and Basophil) mice (122). The FcεRI β chain of these mice includes a cassette composed of a sequence coding for the bright red td-Tomato (tdT) fluorescent protein and human DTR, allowing both visualization and conditional ablation of MCs and basophils. Although both basophils and MCs were deleted after DT injections, the authors reported that basophils were fully reconstituted 12 days after DT treatment, whereas MCs remained absent (122). However, to the best of our knowledge, in none of these mouse models have to date the skeletal phenotype or bone turnover been analyzed.

In conclusion, the experimental data regarding a possible regulatory role for MCs in physiological bone turnover are contradictory and dependent on the mouse model used. Therefore, further translational studies on human MCs with respect to their role in bone metabolism are needed. There are no human MC-deficient conditions. However, patients with mastocytosis who display abnormal high MC numbers often suffer from bone disorders (see section Osteoporosis). Analyses of the bone turnover of these patients over their

course of treatment (mainly anti-histamine treatment) could provide further insights into MC functions in human bone. Complementary, *in vitro* studies including co-culture models of human MCs derived from healthy individuals and mastocytosis patients and human osteoblasts and osteoclasts could provide deeper mechanistic insights into the interaction of MCs and bone cells in humans.

# THE ROLE OF MAST CELLS IN BONE DISORDERS

## Osteoporosis

Osteoporosis is a major bone disorder, which is characterized by the deterioration of bone microarchitecture and bone mass reduction. This results from an imbalanced activity of osteoblasts and osteoclasts and leads to an increased fracture risk (123). In Europe, ∼20 million people suffer from osteoporosis with an annual incidence of 2.7 million fragility fractures (124). Osteoporosis is categorized into primary and secondary forms. Primary osteoporosis is the most common form, including postmenopausal osteoporosis, which results from a decline in sex hormone levels, and age-related osteoporosis, which gradually develops during aging. Secondary osteoporosis is caused, for example, by drugs (e.g., corticosteroids, barbiturates), comorbidities (e.g., kidney diseases, diabetes, hyperparathyroidism, mastocytosis), and adverse lifestyle and nutrition (e.g., cigarette smoking, alcohol abuse, immobilization, malnutrition) (123, 125). Despite extensive research during recent decades, the pathomechanisms of osteoporosis are still not completely understood. There is evidence that MCs contribute to this multifactorial disease, because increased MC numbers have been found in individuals with bone loss (**Figure 1B**) (5, 6, 40, 126, 127).

Postmenopausal osteoporosis is driven by the decline of estrogen after menopause, which induces increased bone resorption by osteoclasts (128). In 1983, Fallon et al. reported increased numbers of MCs in iliac crest biopsies of females with postmenopausal osteoporosis compared to non-osteoporotic males and females, indicating an involvement of MCs in bone loss (5). Confirming this, other authors similarly observed MC accumulation in bone biopsies of osteoporotic patients compared to healthy controls (6). Interestingly, treatment of postmenopausal females with calcium and promethazine, a blocker of the histamine H1 receptor, significantly increased bone mineral density compared to calcium treatment alone (129). This indicates that histamine, one of the main preformed components in MC granules, could be involved in osteoporotic bone loss (**Figure 1B**). Experimental studies in ovariectomized (OVX) rodents, a common experimental model for postmenopausal bone loss, confirmed the clinical observations. Lesclous and Saffar demonstrated that after OVX-induced estrogen decline the MC numbers in the rat bone marrow were significantly increased (130). The authors further showed that the accumulation of MCs started early and was associated with the increase in osteoclast numbers induced by OVX (131). Confirming this, our group showed that Mcpt-5 Cre R-DTA mice, which lack CTMCs, were protected from OVX-induced bone loss and no increase in osteoclast numbers or activity occurred after OVX (10). Using Mcpt-5 Cre tdRFP MC reporter mice, we further found that after OVX the MCs, and osteoclasts were not only enhanced in number but also frequently co-localized (10). These results are strong indications that MCs may promote osteoclast formation under estrogen-deficient conditions (**Figure 1B**). Confirming this, further *in vitro* studies investigating osteoclast formation under the influence of MC supernatants revealed that estrogen strongly affects MCs and their mediator release (10). When estrogen was present, supernatants derived from MCs that were stimulated with the complement anaphylatoxin C5a, an inducer of MC degranulation, did not enhance osteoclast formation *in vitro* in a preosteoclastic cell line (RAW 264.7 cells) nor in primary bone marrow-derived osteoclast precursors. By contrast, when estrogen was absent, osteoclast formation was induced, suggesting that estrogen has an inhibitory effect on the osteoclast-inducing potential of MCs (10). Indeed, estrogen receptors (ER) are expressed in MCs of various tissues (132, 133) and several groups reported that estrogen influences MC migration, degranulation, and cytokine release (133–138). However, the observed effects are not always consistent. Some authors found that estrogen induces MC degranulation (134, 135), whereas others reported inhibitory effects on the mediator release (136, 137). For example, estrogen did not stimulate the degranulation of MCs derived from ERα knockout mice (134), indicating that estrogen is involved in MC activation via ERα signaling. In agreement with this, estrogen treatment of the human MC line HMC-1 induced the de novo production of tryptase β1 and MC degranulation (135). By contrast, OVX-induced estrogen deficiency reduced MC degranulation in the rat mammary gland (137), and Kim et al. showed that estrogen treatment diminished the *in vitro* release of MC cytokines, including TNF-α and IL-6 (136). Therefore, the effects of estrogen on MC degranulation and mediator release appear to depend on the tissue investigated and the experimental model used.

MCs may also play a role in the development of age-related osteoporosis, the second type of primary osteoporosis, because Frame and Nixon already in 1968 described that MC numbers were increased in bone marrow aspirates of aged female and male patients with reduced bone mineral density compared to healthy controls (126). Because both males and females are affected by age-related osteoporosis, MCs appear to also provoke estrogen-independent osteoclastogenic effects. This is supported by the observation that MCs also play a role in secondary forms of osteoporosis. Most of these indications arise from mastocytosis, a disease characterized by abnormally high MC numbers in one or more organ (139). The clinical picture is categorized into cutaneous mastocytosis, which is restricted to the skin, and systemic mastocytosis (SM), where high MC numbers infiltrate the skin and/or one internal organ, for example, the gastrointestinal tract, bone marrow, lymph nodes, liver, and spleen (139). Mastocytosis is caused by gain-of-function point mutations within the SCF/c-Kit signaling axis, most prominently D816V (140), resulting in a constitutively active c-Kit receptor. This leads to increased MC proliferation,

maturation, survival, and activity (141). The boost of released mediators, mainly of histamine and pro-inflammatory cytokines, and excessive MC infiltration cause mild to severe organ-specific symptoms, including flushing, syncope, anaphylactic shock, diarrhea, vomiting, ascites, and hypertension. The clinical picture of SM is very heterogeneous, ranging from indolent to aggressive forms with severe organ dysfunctions (139). Of note, ∼50% of patients display a skeletal involvement (142). Several case studies and small clinical trials described a reduced bone mass and an increased fracture occurrence in patients with MC accumulation in the bone marrow (143–146). Larger cohort studies confirmed the high prevalence of osteoporosis (up to ∼60%), and fragility fractures (up to ∼40%) in SM patients (147–150), as recently reviewed in more detail by Greene et al. (151). The pathomechanisms of MC-induced bone loss are not yet fully understood. Seitz et al. found increased osteoblast and osteoclast numbers in patients with indolent SM, indicating a high bone turnover status (152). Confirming this, bone formation and resorption markers were found to be increased in SM patients (153). However, other authors reported increased serum levels of dickkopf 1 and sclerostin, both inhibitors of the osteoanabolic Wnt signaling pathway, indicating reduced bone formation (154, 155). IL-6 levels are also increased in SM, and correlate with the severity of the symptoms and bone loss (155, 156). The existing data on tryptase levels, a marker for MC activity, in SM are inconsistent. Many authors describe increased concentrations, which correlate with reduced bone mass, whereas others report normal levels despite bone loss (148, 150, 155).

MCs appear also to be involved in secondary bone loss induced by malnutrition or immobilization. Urist et al. observed an accumulation of MCs in osteoporotic bones of rats fed a calcium-deficient diet (40). Additionally, in bone loss caused by the unloading of the hind-limbs in rats, MC numbers were significantly increased (127). These results indicate that MCs may regulate osteoclast activity independently from endocrine dysregulation or inflammatory stimuli. Confirming this, male patients suffering from idiopathic osteoporosis also displayed higher numbers of MCs that were highly organized in clusters in biopsies of the bone marrow. In these patients, the urine N-methylhistamine concentration, a marker for increased MC activity, correlated with the reduced bone mineral density (157).

The above-mentioned clinical and experimental studies suggest that MCs may be involved in osteoporosis development by promoting osteoclast formation (**Figure 1B**). The question arises as to which MC mediators are mainly responsible for the observed osteoclast-stimulating effects. There are many possible candidates, including histamine, heparin, TNF, IL-6, and receptor activator of nuclear factor kappa B ligand (RANKL), as listed in **Table 1**. One of the main components in preformed MC granules is histamine, which was already shown to be associated with bone resorption in RA (158). Furthermore, in patients with SM, histamine levels were reported to predict osteoporotic manifestations (144, 147). Confirming this, histamine-deficient mice displayed an increased bone mass because of reduced osteoclast numbers and were also completely protected from OVX-induced bone loss (44). Lesclous et al. injected histamine receptor blockers in OVX rats, which prevented the OVX-induced bone loss by reducing osteoclast numbers (45, 159). In the above-mentioned study of our group (10), osteoclast formation and activity were studied *in vitro* in the presence of supernatants harvested from MC cultures stimulated with C5a (induces the release of preformed granule-stored mediators). Notably, the blockade of the histamine H1 receptor abolished osteoclast formation by MC supernatants, indicating that histamine may play a crucial role in MC-mediated osteoclast activity (10). This is confirmed by the already above-mentioned clinical study, which demonstrated that the blockade of the histamine H1 receptor with promethazine significantly increased bone mineral density in postmenopausal women (129). However, our experimental data also showed that histamine alone supported osteoclast formation but not their resorption activity (10). This indicates that histamine is not the only MC-derived factor involved in osteoclast activation and that MC-osteoclast interaction might be much more complex.

## Rheumatoid Arthritis

RA is a systemic autoimmune disease affecting around 1% of the population, which is associated with a chronic joint inflammation (160, 161). The inflamed joint is characterized by a massive infiltration of immune cells, extensive hyperplasia of synovial macrophages and fibroblasts and thickening of the synovial membrane. The unrestrained inflammatory response leads to the formation of an invasive structure, calles synovial pannus, which finally causes cartilage destruction and bone erosions. The clinical picture is characterized by swelling, pain, and stiffness of the affected joints (160). The pathogenesis of RA is complex and still not entirely known. In addition to other immune cell populations, MCs have been suggested to play a crucial role, because MCs are abundant in inflamed synovial joints of RA patients, especially around blood vessels in the synovial sub-lining, at the cartilage-pannus junction at sites of cartilage erosions, and in joint fluid (**Figure 1C**) [comprehensively reviewed by Rivellese et al. (162)]. Importantly, some of the clinical studies observed a correlation of MC numbers with joint inflammation and disease activity (163–167). In addition, the levels of MC mediators, including histamine and tryptase, were significantly increased in the synovial tissue of RA patients (**Figure 1C**) (8, 168, 169). Therefore, these studies support MC involvement in the pathogenesis of RA.

In agreement with this, MCs are highly responsive to the inflammatory milieu in the synovial joint. For example, they are stimulated by IL-33 and IL-6 (170, 171). Moreover, it has been shown that synovial MCs can be activated by immune complexes, auto-antibodies and complement factors as well as by direct cross-linking of Fc-receptors (172–175). MCs might contribute to the pathogenesis of RA by different mechanisms, which were reviewed in detail by other authors (12–14). Briefly, synovial MCs can rapidly release and produce inflammatory cytokines and chemokines and thus contribute to joint inflammation and immune cell recruitment. For example, MC-derived IL-1 is involved in the initiation of autoantibody-mediated arthritis (176), and activated MCs in human synovial tissue produce TNF-α, IL-1β, and IL-1 receptor antagonist (177). Additionally, MC-derived proteases may play important roles in cartilage and bone breakdown. Histological analysis of inflamed joint specimens showed abundant MC tryptase present in areas of

cartilage destruction (178). Supporting these findings, mice deficient in Mcpt-6 or Mcpt-7 displayed an attenuated disease activity and reduced bone and cartilage destruction (179). Similarly, mice lacking the MC chymase Mcpt-4, showed a reduced joint inflammation and pannus formation, diminished cartilage destruction probably due to a reduction in MMP-2 and MMP-9 (180). Of note, the joints of Mcpt-4 deficient mice displayed less infiltrates of MCs and mononuclear cells implicating a crucial role of this MC chymase in disease progression (180). Importantly, some MC mediators, including histamine, TNF-α, IL-6, IL-11, and IFN-γ, have the capacity to increase osteoclast activity (see **Table 1**), and thus may contribute to bone erosion in RA. Indeed, increased levels of RANKL, which is also secreted by MCs, were found in the synovial tissue of RA patients (181, 182).

The specific role of MCs in RA was investigated in different mouse models of MC deficiency. However, these studies revealed contradictory results, depending on the MC-deficient mouse strain and the respective model of RA induction. Rivellese et al. recently reviewed these animal studies in detail (183). Briefly, $Kit^{W/W-v}$ mice were protected from K/BxN serum-induced arthritis (serum from K/BxN mice contains autoantibodies against glucose-6-phosphate isomerase) (184, 185). However, $Kit^{W/Wv}$ mice are fully susceptible to collagen-induced arthritis (CIA), which is induced by the injection of type II collagen in Freund's adjuvant (186). By contrast, another MC-deficient $Kit^{W-sh/W-sh}$ mouse line developed arthritis induced by K/BxN serum as well as CIA (187, 188). However, as already mentioned, Kit-mutant mice exhibit severe alterations of the immune system beyond the MC-deficiency, which may possibly account for the inconsistent outcomes of these studies. Furthermore, the arthritis models used differ in their mechanisms of disease induction. In the CIA model, joint inflammation is induced by autoreactive effector T cells, while in the K/BxN model, immune cell infiltrations and activation is stimulated by the transferred autoantibodies thereby bypassing the T cell response (183).

c-Kit-independent MC-deficient $Cpa3^{Cre/+}$ and Mcpt-5 Cre iDTR mice were not protected from K/BxN serum-induced arthritis (9, 118). However, Mcpt-5 Cre iDTR mice displayed reduced arthritis severity in CIA, indicating that MCs contribute to arthritis induction or progression by affecting the T cell arm of adaptive immunity (9, 118). Interestingly, in CIA, Mcpt-5 Cre iDTR mice showed reduced CD4+ and CD8+ T cell numbers in the lymph nodes draining the site of immunization accompanied by reduced IFN-γ and IL-17 production (**Figure 1C**). These results indicate that MCs may regulate T cell expansion and polarization to Th1 and Th17 effector cells in T cell-driven RA (9). Supporting these findings, the depletion of MCs during the early preclinical phase of CIA decreased joint inflammation in another model of c-Kit-independent inducible MC-deficiency, the RMB mouse. Similarly, numbers of CD4+ T cells, in particular IL-17 producing T cells, and serum levels of IL-6 and IL-17 were reduced also here (189). Additionally, in a pharmacological approach in wildtype mice, in which MCs were inhibited using salbutamol and cromolyn, RA development was diminished as indicated by reduced ankle swelling, joint inflammation, and bone destruction (185). Collectively, data of the CIA model demonstrate an important pro-inflammatory role of MCs in the onset of RA by promoting the expansion of autoreactive T cells and the T cell-driven inflammation (**Figure 1C**), whereas in the later disease phase, MCs may have redundant functions as implicated by most of the K/BxN studies. However, more studies are required to further decipher the specific role of MCs in RA-associated joint inflammation and bone resorption.

Interestingly, it has been shown that MCs may also play a role in OA, in which joint destruction is mainly caused by degeneration, abnormal high loads, or traumatic injuries (190), and driven by an increased inflammatory response (191). Several clinical studies reported increased MC numbers in the synovial tissue of OA patients and/or elevated histamine or tryptase levels in the synovial fluid (7, 192–196). Gene cluster analysis revealed increased expression of genes involved in MC differentiation and activity (*c-KIT*, tryptase genes *TPSAB1,* and *TPSAB2)* in the synovial membranes of OA patients (196). Interestingly, two different MC-deficient mouse lines, the c-Kit-dependent $Kit^{W-sh/W-sh}$ line and the Kit-independent $Cpa3^{Cre}$; $Mcl\text{-}1^{fl/fl}$ mice, were protected from OA as demonstrated by reduced inflammation and cartilage destruction, while MC engraftment reversed the protective effects in both mouse lines (196). Furthermore, the inhibition of tryptase activity in wildtype mice prevented OA and reduced the concentrations of the pro-inflammatory and proteolytic mediators, e.g., IL-6, IL-1β, IL-8, and MMP-3. The authors further showed that in OA, MCs are activated via the IgE/FcεRI receptor axis (196). Another study showed that synovial MCs from OA patients produce TNF-α upon stimulation via the high-affinity receptor for IgG (174). These results indicate an important role of MCs in OA development. In support of these findings, a cross-sectional cohort study showed that the usage of H1anti-histamine treatment correlated with decreased OA prevalence (197), suggesting that MCs could potentially be a therapeutic target in OA, but this needs to be clarified in further studies.

## Bone Fracture Healing

The immune system plays a major role in bone repair, because the healing process begins with an acute immune response locally at the fracture site (207, 208). In addition, conditions of acute orresults indicate that MCs may regul chronic inflammation, including poly-trauma, osteoporosis, and RA, negatively impact the fracture healing outcome (207). Bone fracture leads to the rupture of blood vessels and to tissue and cell damage, resulting in the formation of a hematoma, which is characterized by hypoxia, low pH, high lactate levels, as well as high concentrations of inflammatory mediators that attract cells of the innate immune response. First, neutrophils invade the fracture hematoma. They secrete further cytokines, including chemokine (C-X-C motif) ligand 1 (CXCL1) and IL-1β, which attract other immune cells, mainly macrophages. These cells further phagocytize cell and tissue debris and pathogens. Subsequently, T and B cells arrive and initiate adaptive immune responses. Consequently, angiogenesis starts, ensuring debris removal, nutrient and oxygen supply, and the recruitment of mesenchymal stem cells (MSCs). Recruited MCSs initiate the repair phase, where in the process of endochondral healing, first a cartilaginous soft callus is generated that is converted into a hard bony trabecular callus. The bony

**TABLE 2 |** Experimental studies investigating MC appearance and function in fracture healing.

| References | Model | Treatment | Main results |
|---|---|---|---|
| Lindholm et al. (198) | White rats (m/f), tibia fracture | – | Progressive MC accumulation in the periosteal callus; MCs decreased during callus remodeling |
| Lindholm et al. (199) | White rats (m/f), tibia fracture | 17-hydroxy-corticosterone | Progressive MC accumulation in the periosteal callus; delayed healing due to treatment affected MC morphology in size, granulation, and staining |
| Lindholm et al. (200) | White rats (m/f), tibia fracture | Somatotropin and thyrotropin | Progressive MC accumulation in the periostal callus; improved healing due to treatment led to earlier MC accumulation |
| Lindholm and Lindholm (201) | Rabbits (m/f), forearm fracture | – | MCs more abundant in the periosteal callus compared to the endosteal callus |
| Taniguchi (41) | Wistar rats (m), bilateral tibia fracture | – | Few MCs near blood vessels and in the marrow of the early endosteal callus;<br>MCs increase in the late periosteal callus and peak during remodeling |
| Banovac et al. (202) | Sprague-Dawley rats (f), femur fracture | NSAIDs | Few MCs near blood vessels and cartilage of the early endosteal callus;<br>MC accumulation near osteoclastic bone resorption during late remodeling;<br>NSAIDs delayed healing and MC appearance |
| Meyer et al. (203) | Sprague-Dawley rats (f), femur fracture | – | Microarray analysis revealed increased MC marker activity from weeks 2 to 4 after fracture in all age groups |
| Behrends et al. (11) | MC-deficient $Kit^{W-sh/W-sh}$, C57BL/6J mice, femoral cortical window defect | – | Disturbed healing in MC-deficient mice:<br>↓ Cortical bridging, bone content, endothelial cells, macrophages; ↑ TRAP+ cells |
| Ramirez-GarciaLuna et al. (204) | MC-deficient $Cpa3^{Cre/+}$ and C57BL/6J mice (m/f), femoral cortical window defect | – | MCs appeared in the connective tissue and marrow of the defect;<br>Disturbed healing in MC-deficient mice:<br>↓ Cortical bridging, bone content, vascularization, bone mineralization, osteoclasts |
| Kroner et al. (10) | MC-deficient Mcpt-5 Cre+/– R-DTA mice (m), femur fracture | – | MCs increase in the periostal callus near newly formed bony trabeculae and osteoclastic bone resorption sites;<br>Disturbed healing in MC-deficient mice:<br>↓ Local and systemic inflammation (cytokine release, immune cell recruitment); ↑ bone content; ↓ osteoclastic remodeling |
| Zhang et al. (205) | C57BL/6J, Mcpt-5 Cre YFP, Mcpt-5 Cre iDTR mice (m/f), cranial window defect | rPTH, SC | MC inhibition (SC) and deficiency (Mcpt-5 Cre iDTR):<br>↑ Healing, ↓ arteriogenesis; rPTH: ↓ MCs in the inflammatory phase by acting on osteoblasts releasing anti-MC factors |
| Hebb et al. (206) | C57BL/6 (m/f), bilateral tibia fracture | – | Microarray analysis revealed increased MC prevalence in younger compared to older mice |

callus is finally remodeled to the original bone shape (207, 209). Studies have shown that certain immune cell populations, including neutrophils, macrophages, but also B and T cells, essentially contribute to successful bone repair, because their absence or disturbed function resulted in disrupted fracture healing (210–212). This might also be true for MCs. Indeed, some older phenomenological studies described MC appearance in bone repair, while more recent studies using different MC-deficient models also revealed some specific MC functions. The few existing studies that explored MCs in fracture healing are summarized in **Table 2**.

The presence of MCs during fracture healing was already described in 1967 by the group of Lindholm using a rat tibial fracture model. The authors showed that MC numbers progressively increase in the periostal fracture callus, followed by a decline during callus remodeling (198). In further investigations of experimentally delayed or accelerated fracture healing, the same group observed alterations in MC accumulation, morphology, and degranulation (199, 200). On the basis of these results, the authors concluded that MC invasion and degranulation are essential for endochondral bone formation and mineralization. The presence of MCs in the periostal callus was further confirmed in a rabbit fracture model (201). Two later studies confirmed MC accumulation during fracture healing and both described only a few MCs during the early healing phase, mainly around blood vessels and in the bone marrow cavity of the endosteal callus (41, 202). In the later healing phases, increasing MC numbers were observed in the marrow of the newly formed periostal callus, particularly next to newly formed bony trabeculae. Both studies found the highest numbers of MCs in close proximity to osteoclasts and bone resorption sites during the callus-remodeling phase and suggested that MCs might contribute to callus remodeling by influencing osteoclast activity (41, 202). Furthermore, microarray analysis of a rat femoral fracture callus found increased MC marker gene expression, including the MC *tryptase β1* and *Cpa3*

from weeks 2 to 4 after fracture (203). In addition, a recent microarray analysis of a tibial fracture callus of young and old C57Bl/6 mice found a higher MC occurrence in younger mice compared to old mice evaluated by cell type enrichment analysis, showing higher MC *IgE* gene expression in young mice (206). On the basis of the above-mentioned studies, the presence and accumulation of MC is the fracture callus is clear (**Figure 1D**).

To elucidate MC functions, Behrends et al. investigated bone repair in an uni-cortical window defect of MC-deficient $Kit^{W-sh/W-sh}$ mice (11). Interestingly, MC-deficient $Kit^{W-sh/W-sh}$ mice displayed a delayed healing with reduced bone quality because of an impaired transformation of woven into lamellar bone. The authors further observed diminished endothelial cell numbers, but increased numbers of osteoclasts, and suggested that healing was impaired because of disturbed revascularization and increased osteocatabolic activity (11). However, these results were obtained in a c-Kit-dependent mouse model, from which it is known that also osteoclasts and other immune cells are affected (113, 115). This could have influenced the outcome. Investigations of bone repair in a cortical window defect in a c-Kit-independent $Cpa3^{Cre/+}$ mouse model found impaired bone regeneration, as confirmed by reduced cortical bridging, vascularization, and bone mineralization. The authors further observed diminished osteoclast activity at earlier stages, but increased osteoclast activity in the late healing phase (204). Therefore, MC functions in bone repair may comprise blood vessel formation as well as anabolic and catabolic processes during fracture repair and remodeling (**Figure 1D**). However, it was shown that Cpa3 is also expressed in basophils and some T cells (109), which needs to be considered when interpreting these results. Overcoming these drawbacks, our group recently investigated the functions of MCs in bone repair in MC-deficient Mcpt-5 Cre R-DTA mice, which lack CTMCs without affecting other immune cell populations (116, 213). Interestingly, we found reduced levels of pro-inflammatory cytokines, including IL-6, IL-1β, and CXCL1, locally in the early fracture callus, but also systemically, and a reduced recruitment of neutrophils and macrophages to the fracture site in the absence of MCs (10). These results indicate a strong contribution of MCs to fracture-induced systemic inflammation and to the inflammatory mediator and cell milieu at the fracture site (**Figure 1D**). MC-mediated neutrophil recruitment was already described during acute inflammation, including bacteria-induced pneumonia and ischemic-induced gut injury, as well as in inflammatory diseases such as meningitis and periodontitis, contributing to the disease onset and progression (214, 215). During the later healing stage, we found an increased bone content of the fracture callus in MC-deficient Mcpt-5 Cre R-DTA mice (10). Further histomorphometric analysis revealed no changes in osteoblast parameters, however, osteoclast numbers and activity were significantly reduced in the fracture callus of Mcpt-5 Cre R-DTA mice (10). These results indicate that MCs may mediate callus remodeling by regulating osteoclast activity (**Figure 1D**). As indicated earlier in the osteoporosis chapter, MC-derived histamine might be one mechanism contributing to increased osteoclastic bone resorption. Supporting our experimental outcomes, Zhang et al. investigated bone repair in a cranial window defect model in MC-deficient Mcpt-5 Cre iDTA mice, and found accelerated defect closure and impaired angiogenesis in the absence of MCs (205). They observed the same effects by inhibiting MCs in wildtype mice using cromolyn, and suggested that MCs may be negative regulators of bone repair (205).

Concluding, several studies demonstrated MC accumulation in the periostal fracture callus during the healing process. More recent experimental studies also revealed possible functions of MCs in fracture healing, including the regulation of the immune response toward fracture and of angiogenesis as well as anabolic and catabolic effects during the repair and remodeling processes (**Figure 1D**).

## CONCLUSION

The important role of MCs in allergic reactions has been known for several decades. However, the involvement of MCs in physiological bone turnover and bone disorders has been described only recently in more detail. As reviewed here, MCs secrete several mediators that are known to regulate bone formation and resorption, including histamine, IL-6, and TNF. Experimental data on the role of MCs in physiological bone turnover are contradictory and depend on the mouse model used. However, the involvement of MCs in various pathological skeletal conditions is clear, particularly in osteoporosis and RA. MCs may also regulate the fracture healing process by influencing the inflammatory response, angiogenesis, bone formation, and osteoclastogenesis (**Figure 1**). Osteoclastogenesis might be mainly, but not solely, regulated by MC-derived histamine. Further mechanistic investigations are required to elucidate MC functions in physiological and pathological conditions in bone. In consequence of the involvement of MCs in bone disorders, MC targeting drugs such as histamine H1 receptor blockers should be further tested for their therapeutic potential to treat osteoporosis, inflammatory bone disorders or disturbed bone repair.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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