



# Interactions between bone and immune systems: A focus on the role of inflammation in bone resorption and fracture healing

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## Abbreviations:

IL – interleukin  
LPS – lipopolysaccharide  
LTB<sub>4</sub> – leukotriene B<sub>4</sub>  
M-CSF – macrophage colony-stimulating factor  
PGE<sub>2</sub> – prostaglandin E<sub>2</sub>  
RANK – receptor activator of nuclear factor  $\kappa$ B  
RANKL – receptor activator of nuclear factor  $\kappa$ B  
ligand  
RAG1 – recombination activating gene 1  
TLR4 – Toll like receptor 4  
TNF – tumor necrosis factor

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## Abstract

*Functional interactions between the immune system and bone tissues are reflected in a number of cytokines, chemokines, hormones and other mediators regulating the functions of both bone and immune cells. Investigations of the mechanisms of those interactions have become important for the understanding of the pathogenesis of diseases like inflammatory arthritis, inflammatory bowel disease, periodontal disease and osteoporosis. This review first addresses the roles of the inflammatory mediators and mechanisms by which they cause inflammation-induced bone loss. In the second part of the review we stress the importance of proinflammatory mediators for normal fracture healing. Defective bone remodeling underlying different pathological processes may be caused by disturbed differentiation and function of either osteoclast or osteoblast lineage cells. Understanding of the mechanisms governing enhanced differentiation and activation of osteoclast progenitors in the inflammatory conditions on the one hand, and the role of inflammation in the recruitment and differentiation of multipotent progenitors into the skeletal lineage during the fracture healing on the other hand is a critical first step in developing interventions that modulate bone regeneration processes and in designing novel pharmacological strategies.*

## INTRODUCTION

Bone growth and remodeling are essential processes, which create the marrow space for hematopoiesis, shape the bones during development, adapt the bone tissue to changing biomechanical forces in the adult life, enable tooth eruption as well as regulate calcium and phosphorus homeostasis. On average, during normal bone remodeling in the adult human skeleton, 5–10% of the existing bone is replaced every year. Deregulated bone remodeling is a feature of a number of pathological states, including rheumatoid arthritis, hypercalcemia of malignancy, Paget's disease and osteopetrosis. A large number of cytokines, chemokines, hormones, tissue growth factors and other mediators are now known to influence bone cell functions and bone mass.

## Bone cells

Bone tissue constantly undergoes remodeling by bone-resorbing osteoclasts and bone-forming osteoblasts in a tightly coordinated sequence of events referred to as coupling (1).

Cells of the osteoblast lineage arise from the mesenchymal stem cells that have the ability to generate a diversity of lineages including osteoblasts, adipocytes, chondrocytes, myocytes and cells that support hematopoiesis (2-6). During the process of osteoblastic differentiation, an osteoprogenitor proliferates and undergoes a series of maturational steps before becoming a differentiated osteoblast responsible for bone formation (7). Commitment of mesenchymal stem cells to the osteoblast lineage requires the canonical Wnt/ $\beta$ -catenin pathway and associated proteins (8, 9).

Osteoclasts arise from hematopoietic progenitor cells of the monocyte/macrophage lineage that through a series of differentiation stages generate large multinucleated, tartrate-resistant acid phosphatase (TRAP)-positive cells capable of resorbing the mineralized matrix (10). Committed osteoclast progenitors express the receptor activator of nuclear factor  $\kappa$ B (RANK) and can be matured *in vitro* into fully functional osteoclasts in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor  $\kappa$ B ligand (RANKL) (11).

Defective bone remodeling underlying different pathological processes may be caused by disturbed differentiation and function of either osteoclast or osteoblast lineage cells. Cell culture techniques and mouse models with altered bone metabolism have advanced our understanding of molecular and cellular signals involved in the regulation of bone homeostasis.

## Osteoimmunology

It has been almost 40 years since the first observations that cells of the immune system affect the functions of bone cells. Since then, we have greatly expanded our understanding of the interactions between hematopoietic, immune, and bone cells, which is now known as the field of „osteimmunology” (12).

Immune cells and bone cells both originate from bone marrow environment and are closely functionally related. Crosstalk between the bone and the immune system is important for bone homeostasis and physiological bone remodeling. Members of the tumor necrosis factor (TNF)-related family of ligands and receptors (13,14), as well as other cytokines (15, 16), colony stimulating factors (17, 18), and signaling molecules (19, 20) are essential for normal development and function of both systems. This has been well documented in many experimental models and human diseases (13, 14, 17, 21). Moreover, it has been shown that bone and immune cells may share the same progenitors and that their differentiation may be driven by the same support cells (19). Multiple complex interactions between hematopoietic and mesenchymal lineage cells define the bone marrow microenvironment (6,12).

Various immune mediators, particularly proinflammatory cytokines and chemokines, may cause bone loss by stimulation of osteoclast formation and resorbing activity

as well as by inhibition of osteoblast activity and bone formation. Production of cytokines by immune cells has been associated to human diseases such as rheumatoid arthritis and osteoporosis, causing an inflammation-induced bone loss. On the other hand, some immune stimuli may support the osteoblast function and are important for the process of fracture healing and for the response of bone to infections.

In the first part of the review we will discuss the inflammatory mediators and mechanism by which they cause inflammation-induced bone loss, while in second we will stress the importance of proinflammatory mediators for normal fracture healing.

## Inflammation-induced bone loss

Irreversible bone destruction, resulting from imbalanced remodeling processes, is a serious complication associated with localized bacterial infections of bones or adjacent tissues, being a key feature of caries, periodontitis or osteomyelitis (22, 23). Bone loss is also observed in other inflammatory processes such as rheumatoid arthritis, certain malignancies, osteoporosis, chronic viral infections and inflammatory bowel diseases (22-24). Mechanisms underlying inflammation-induced bone loss have mainly been studied in various animal and *in vitro* models. Application of various Streptococcus strains or other Gram-positive bacteria, which are the most often cause of osteomyelitis and posttraumatic infections, is a typical model used in studies of these diseases (25). On the other hand, periodontitis and the inflammatory milieu that causes a systemic bone loss, which will be the main topic of our review, are most often induced by application of lipopolysaccharide (LPS), a major component of the cell wall of Gram-negative bacteria that increases the production of bone-resorptive factors and creates microenvironments that promote the differentiation of monocytes into bone-resorbing osteoclasts causing enhanced bone resorption (26).

LPS exerts its effects through the activation of Toll like receptor 4 (TLR4), which is expressed on both principal bone cell lineages: osteoblasts and osteoclasts (27-29). Studies *in vitro* suggest that LPS supports the survival of osteoclasts directly and, at least in part, independently of the production of pro-osteoclastogenic cytokines, such as interleukin (IL)-1 $\beta$ , TNF- $\alpha$ , and RANKL (28,30). However, Liu *et al.* demonstrated that LPS plays a bifunctional role in osteoclastogenesis by inhibiting osteoclast formation from naïve osteoclast precursors, but promoting osteoclastogenesis from precursors primed with RANKL (31). Studies on culture models supportive for osteoblast differentiation have shown that activation of TLR4 on osteoblast lineage cells induces gene expression for RANKL in differentiating osteoblasts (32) and, at the same time, has an inhibitory effect on late osteoblast differentiation (27, 33). *In vivo* conditions, LPS has an additional indirect effect in promoting bone destruction,

by activation of macrophages and other immune cells that contribute to the inflammatory environment.

In animals LPS can be applied either topically to cause local bone loss (in the gingival tissue as a model of periodontitis or in calvaria) or to cause systemic bone loss (by intraperitoneal injection). The significant bone loss occurs 7-20 days after the onset of inflammation and is characterized by enhanced osteoclast and suppressed osteoblast activity. Our preliminary results confirmed increased frequency and activity of circulating osteoclast progenitor cells after LPS treatment (unpublished data). These effects are associated with an increased production of osteoresorptive cytokines including IL-1, IL-6, IL-11, IL-17, TNF- $\alpha$ , RANKL, as well as several chemokines important for trafficking of cells mediating bone homeostasis (33-39). However, the specific mechanisms of the effects of inflammatory and immune cells on osteoclast lineage cell migration, homing and differentiation are not fully elucidated and are still the subject of the ongoing investigations.

In vivo effect of LPS administration is mainly mediated by induced production of various proinflammatory cytokines such as TNF- $\alpha$ , IL-1, IL-3, IL-6, IL-8, IL-12, IL-17 (40, 41), many of which have the potential to activate bone resorption and promote bone loss. Moreover, RANKL, expressed on osteoblasts and hypertrophying chondrocytes, as well as on activated T lymphocytes, may be up-regulated by pro-resorptive cytokines and mediators, such as prostaglandin E<sub>2</sub>, IL-1, IL-7, IL-11, IL-17 and IL-23 (42-44). Administration of TNF- $\alpha$  *in vivo* stimulates osteoclast formation and bone resorption (45, 46), possibly by increasing the number of circulating osteoclast precursors and by promoting their proliferation and differentiation in bone marrow through the up-regulation of c-Fms expression (receptor for M-CSF) (47). TNF- $\alpha$  can act in a RANKL-dependent and -independent manner, directly on osteoclast progenitors that express TNF-receptors (48-50). Inhibitory effects of TNF- $\alpha$  on osteoblast differentiation, through the suppression of RUNX2 and osterix, have also been reported (51, 52). Furthermore TNF- $\alpha$  stimulates the production of IL-1, which is a potent inducer of osteoclastogenesis and bone resorption by a RANKL-dependent mechanism (53, 54). Although it is generally accepted that IL-1 induces bone loss by stimulating the formation, survival and function of osteoclasts, its inhibitory effects on osteoblasts may contribute to decreased bone mineral density (55). Levels of IL-6 are also highly increased after the LPS treatment, but the reports on its influence on bone cells are inconsistent, as both stimulatory and inhibitory effects have been observed (56, 57, 58). Proinflammatory cytokines IL-12 and IL-18, up-regulated by LPS, are not likely to mediate inflammation-induced bone loss, as a majority of published reports suggest their inhibitory effect on osteoclast differentiation (59, 60, 61). On the other hand IL-17 stimulates osteoclast differentiation (62, 63) and induces

RANK expression on osteoclast progenitors (64). Zwerina *et al.* have reported that anti IL-17A therapy inhibits bone loss in TNF- $\alpha$ -mediated murine arthritis (65).

Products of arachidonic acid metabolism are also critically involved in LPS-induced bone loss. LPS treatment induces the production of PGE<sub>2</sub> in osteoblasts (66), which acts as a potent stimulator of bone resorption (67, 68). Hikiji *et al.* demonstrated that mice deficient in the high-affinity leukotriene B<sub>4</sub> (LTB<sub>4</sub>) receptor BLT<sub>1</sub> are protected from LPS-induced bone resorption (69). In line with this study Lee *et al.* have recently reported that inhibition of 5-lipoxygenase, an enzyme that catalyzes the formation of LTB<sub>4</sub> and cysteinyl leukotrienes, suppressed the LPS-induced osteoclast formation and bone loss (70).

### Pro-inflammatory mediators in the fracture healing process

Bone fractures are common injuries that generally heal by forming new bone in a rapid and efficient process. The fracture healing cascade involves the formation of a blood clot at the site of injury, an inflammatory phase, callus generation, primary bone induction, and secondary bone remodeling. However, in about 5-10% of patients fracture healing fails, and a delayed union or nonunion develops, associated with a significant cost and largely ineffective current therapies (71,72). Nonunion and segmental bone loss after fractures often require multiple surgical procedures associated with patient morbidity and reduced quality of life.

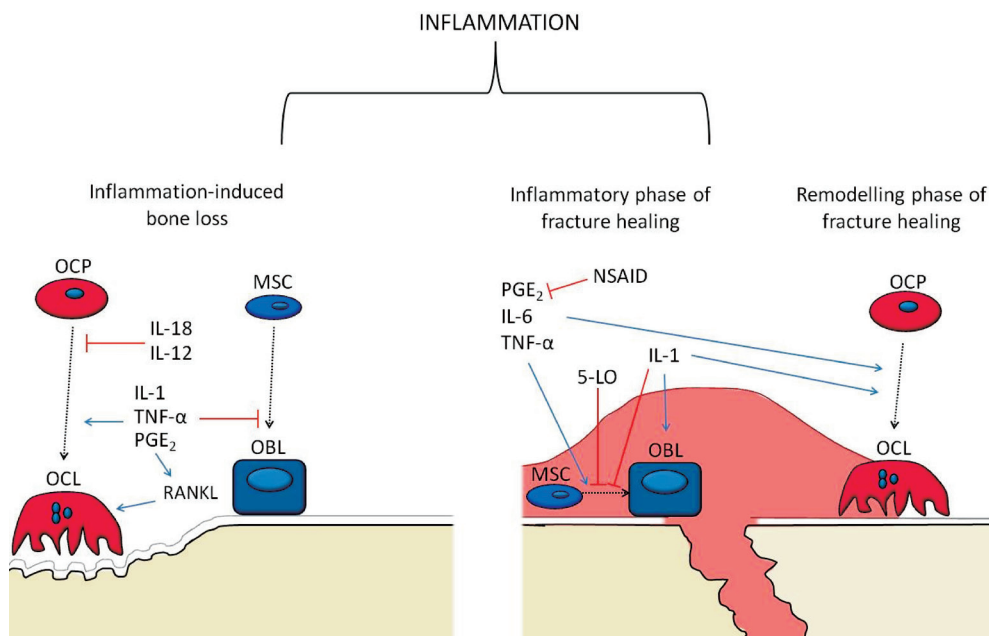
Bone formation during fracture healing is a complex process that involves multiple cell lineages and is still not fully understood. During bone regeneration, mesenchymal cells proliferate and differentiate along a cartilaginous or osteoblastic lineage in response to growth factors and cytokines (73,74). Regeneration of adult skeletal tissues requires a timely recruitment of skeletal progenitor cells to an injury site, differentiation of these cells into bone or cartilage, and re-establishment of a vascular network to maintain cell viability (71,72,75,76).

In contrast to fetal skeletal development, reparative skeletal healing involves an initial hematoma formation and an inflammatory response (77). Minimizing hematoma formation by removing bone marrow delays the periosteal response (78). In addition, macrophages appear to modulate bone formation during bone healing, whereas osteoclasts are required for the remodeling phase (79). Thus, it has been postulated that inflammation is an early physiological response to fracture injury and critically contributes to initiation of the tissue regeneration pathway. In line with this hypothesis it was shown that treatment with anti-inflammatory drugs such as non steroid anti-inflammatory drugs or corticosteroids impairs fracture healing (80-83). Moreover, the inhibition of osteal macrophages delays fracture healing, whereas the administration of M-CSF, a macrophage growth factor, had an opposite effect (79).

Up-regulation of TNF- $\alpha$ , IL-1, IL-6, IL-17 and IL-18 occurs in fracture calluses soon after injury (84-86). The post-fracture expression pattern of TNF- $\alpha$  and IL-1 has two peaks: the first peak occurs within the first 24 hours and a second peak after 3-4 weeks (87). Glass *et al.* have shown that local injections of TNF- $\alpha$  promote fracture healing in the mouse tibial fracture model by augmenting the recruitment and differentiation of muscle-derived stromal cells (88). Similarly, it was reported that mice deficient for TNF- $\alpha$  receptors (p55(-/-)/p75(-/-)) have impaired fracture healing (89). However, in a recent study Sandberg *et al.* found that blocking of TNF- $\alpha$  with etanercept had no substantial influence on the fracture healing process (90). This discrepancy might be explained by model differences and also by an incomplete inhibition of TNF- $\alpha$  receptors activation achieved by etanercept. Although etanercept inhibits both biological ligands for TNF- $\alpha$  receptors, i.e. TNF- $\alpha$  and lymphotoxin- $\alpha$  (91), the biological effectiveness of applied dose, as discussed by Sandberg *et al.*, was uncertain (90).

In the model of fracture repair IL- $\beta$  stimulated the proliferation of osteoblasts and the production of miner-

alized bone matrix, but suppressed the proliferation and inhibited the differentiation of bone marrow derived mesenchymal stem cells. The functional testing *in vivo* showed that its overall effect on fracture healing was minimal, including only slightly accelerated cartilage and bone formation (92). The studies on mice deficient for the IL-6 gene showed that these mice have an impaired early phase of the fracture healing process. However, no difference in histological and biomechanical parameters between the wild type and IL-6 knockout mice was found at the later time points of fracture healing (93, 94). In our preliminary studies we observed that inflammatory mediators induced by proinflammatory cytokines, such as pentraxin 3, may be important for bone healing (unpublished data). Less obvious is the importance of adaptive immune response in the bone regeneration processes. Toben *et al.* reported that mice deficient for the recombination activating gene 1 (RAG1), lacking the adaptive immune system, have an accelerated endochondral ossification after the closed femoral fracture (95). On the other hand, Nam *et al.* found an impaired tibial fracture healing and suggested RAG1 knockout mouse model as a suitable for studying impaired fracture healing (86).



**Figure 1.** Schematic presentation of the role of pro-inflammatory mediators in inflammation induced bone loss and in fracture healing. On the left side inflammation induced-bone loss is shown. Overproduction of IL-1, TNF- $\alpha$  and PGE<sub>2</sub> stimulates osteoclastogenesis through direct activation of osteoclast precursors and indirectly, by inducing RANKL expression in osteoblasts. Osteoclastogenesis is negatively controlled by IL-12 and IL-18. At the same time, proinflammatory mediators negatively regulate osteoblast differentiation and bone formation. On the right side, fracture healing process is shown. In the inflammatory phase, up-regulation of IL-6, TNF- $\alpha$  and PGE<sub>2</sub> stimulates the proliferation of mesenchymal stem cells and promotes their differentiation to mature osteoblasts. Treatment with NSAID in this phase suppresses PGE<sub>2</sub> production and subsequently delay the fracture healing. IL-1 stimulates the proliferation of osteoblasts and the production of mineralized bone matrix, but suppresses the proliferation and differentiation of mesenchymal stem cells. Osteoblast differentiation is negatively regulated by 5-lipoxygenase products. In the remodeling phase of fracture healing, IL-1 and TNF- $\alpha$  are important for osteoclast activation. For simplicity only selected regulators and interactions are shown. OCL - osteoclast, OCP - osteoclast progenitor, OBL - osteoblast, MSC - mesenchymal stem cell 5-LO - 5-lipoxygenase.

Further proofs for the involvement of the immune system in bone healing are investigations on the products of arachidonic acid metabolism, which influence bone regeneration as well as bone resorption. Treatments with cyclooxygenase inhibitors, such as indomethacin and celecoxib prolong the fracture healing process (82, 83). A positive effect of cyclooxygenase activation on fracture healing appears to be mediated by PGE<sub>2</sub>. Studies on animal models showed that administration of PGE<sub>2</sub>, or its agonists, stimulates bone induction and callus formation (96, 97). Studies investigating PGE<sub>2</sub> agonists selective for EP<sub>2</sub> or EP<sub>4</sub> receptors showed that they accelerate fracture healing (97, 98). Thus, we can propose that PGE<sub>2</sub> exerts its bone regenerative properties through the activation of these two receptors. Accordingly, Li et al. observed an impaired fracture healing in EP<sub>4</sub> knockout mice (99). On the other hand, signaling through the activation of EP<sub>1</sub> receptor negatively regulates the fracture healing process (100). In contrast to the cyclooxygenase activation, activation of 5-lipoxygenase appears to suppress fracture healing. Manigrasso et al reported on accelerated fracture healing in mice lacking the 5-lipoxygenase gene (101). Cottrell et al. recently found that a local inhibition of 5-lipoxygenase enhances bone formation in a unicortical femoral defect model in rats (102).

## CONCLUSION

The crosstalk between the bone and the immune systems is important for bone homeostasis and physiological bone remodeling. Sustained inflammation can stimulate osteoclast activity and their differentiation from osteoclast precursors, causing, eventually, a decreased bone mineral density. Inflammatory cytokines, particularly TNF- $\alpha$ , IL-1 and IL-17 play a key role in the pathogenesis of the inflammation-induced bone loss. Their effect is, at least partially, mediated through the stimulation of cyclooxygenase, mainly because of an increase in PGE<sub>2</sub> production. On the other hand, the inflammation is critically important for the early phase of normal fracture healing. Inflammatory mediators, particularly TNF- $\alpha$ , IL-6 and PGE<sub>2</sub>, are induced immediately after the fracture injury. They stimulate proliferation of mesenchymal stem cells and promote their differentiation to mature osteoblasts. The role of the most important pro-inflammatory mediators in inflammation-induced bone loss and in fracture healing is summarized schematically in Figure 1. Understanding of the mechanisms governing enhanced differentiation and activation of osteoclast progenitors in the inflammatory conditions on the one hand, and the role of inflammation in the recruitment and differentiation of multipotent progenitors into the skeletal lineage during the fracture healing on the other hand is a critical first step in developing interventions that modulate bone regeneration processes and in designing novel pharmacological strategies.

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