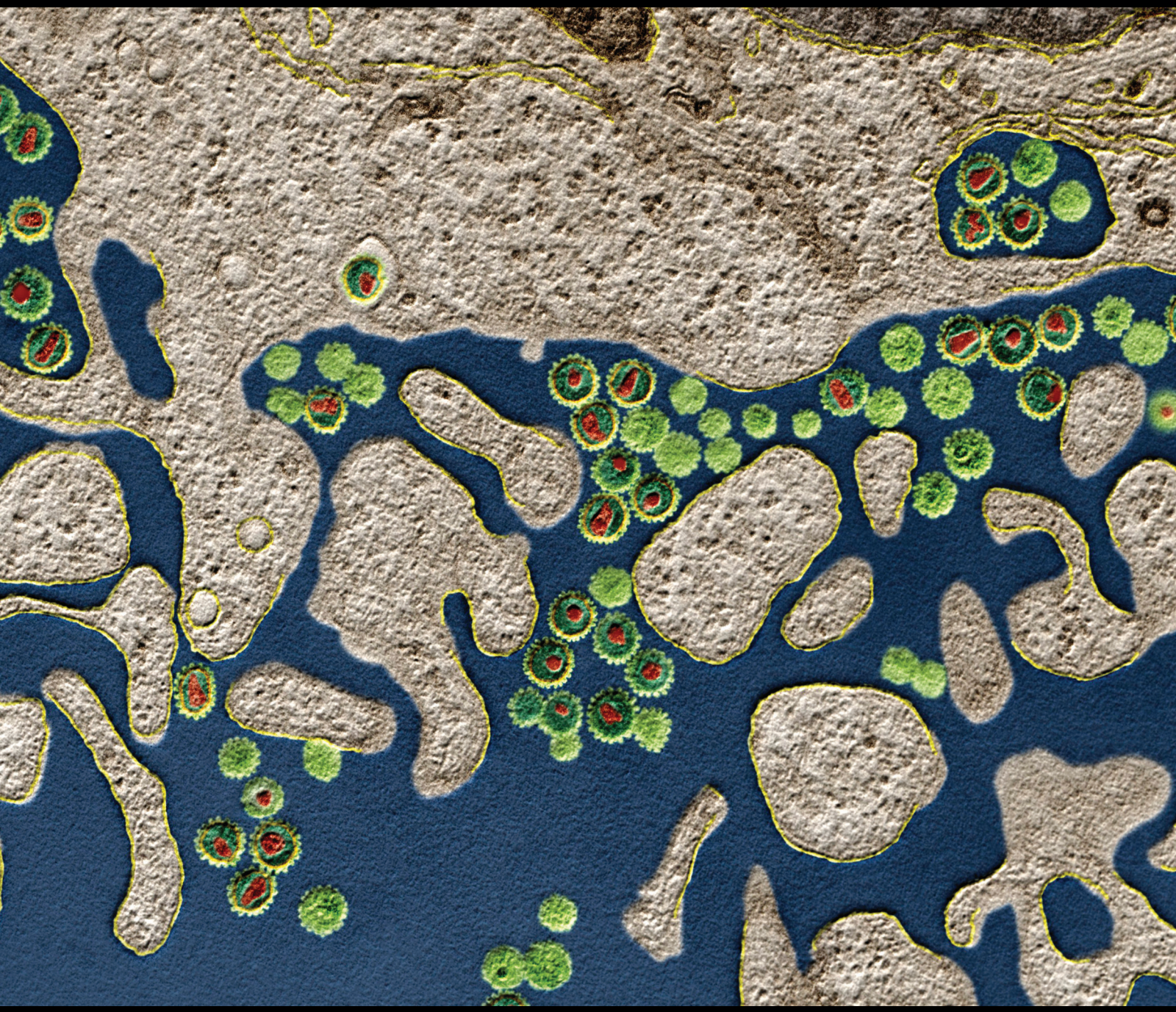
Journal of Immunology Research

# Pathogenesis of Bone Diseases: The Role of Immune System

Guest Editors: Giacomina Brunetti, Giorgio Mori, Patrizia D'Amelio, and Roberta Faccio



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# Editorial Board

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*Editorial*

# Pathogenesis of Bone Diseases: The Role of Immune System

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Bone is a metabolically active tissue that undergoes continuous remodeling by two sequential events, bone formation and resorption. These events are strongly linked and tightly regulated to maintain skeletal homeostasis. The bone cells responsible for the dual events include the bone-resorbing cells, the osteoclasts, arising from monocyte-macrophage precursors, and the bone forming cells, the osteoblasts, having a mesenchymal origin. Immune and bone cell activities are linked by several pathways [1] and the former can promote bone building or destruction. Further, immune cells can be involved in the mineralization process occurring in extra-skeletal sites. In this special issue different authors highlighted these items both through research articles and reviews.

In detail, interaction between osteoblast precursors, the mesenchymal stem cells (MSCs) [2], and immune cells during fracture repair acts as one of the key factors governing successful bone healing. Additionally, bone damage following immune deregulation may be local as in arthritis and periodontal disease (PD) or systemic as in osteoporosis [3] and osteotropic cancers [4, 5]. It could be multifactorial and thus due to genetic modifications (i.e., Gaucher disease) as well as to lipopolysaccharide- (LPS-) mediated release of inflammatory cytokines (i.e., PD, osteomyelitis, and arthritis), and so forth. New insights suggest that, in immune-mediated bone diseases, bone resorption active phases are characterized by increased levels of immunoreceptor tyrosine-based activation motifs (ITAMs); these molecules together with OSCAR could be indicative of disease progression. Further, osteotropic cancer-related immune alterations showed distinct immune cell phenotype as observed in chronic myeloid leukemia, multiple myeloma, and bone metastatic solid tumors.

State-of-the-art and new mechanisms are clearly described in this special issue; they can be useful for the identification of new therapeutic targets and bone disease markers.

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## References

[1] G. Mori, P. D'Amelio, R. Faccio, and G. Brunetti, "The interplay between the bone and the immune system," *Clinical and Developmental Immunology*, vol. 2013, Article ID 720504, 16 pages, 2013.

[2] G. Mori, M. Centonze, G. Brunetti et al., "Osteogenic properties of human dental pulp stem cells," *Journal of Biological Regulators and Homeostatic Agents*, vol. 24, no. 2, pp. 167–175, 2010.

[3] P. D'Amelio, A. Grimaldi, S. di Bella et al., "Estrogen deficiency increases osteoclastogenesis up-regulating T cells activity: a key mechanism in osteoporosis," *Bone*, vol. 43, no. 1, pp. 92–100, 2008.

[4] G. Brunetti, G. Colaianni, M. F. Faienza, S. Colucci, and M. Grano, "Osteotropic cancers: from primary tumor to bone," *Clinical Reviews in Bone and Mineral Metabolism*, vol. 11, no. 3-4, pp. 94–102, 2013.

[5] I. Roato, "Interaction among cells of bone, immune system, and solid tumors leads to bone metastases," *Clinical and Developmental Immunology*, vol. 2013, Article ID 315024, 7 pages, 2013.

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*Review Article*

# Bone Loss Triggered by the Cytokine Network in Inflammatory Autoimmune Diseases

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Bone remodeling is a lifelong process in vertebrates that relies on the correct balance between bone resorption by osteoclasts and bone formation by osteoblasts. Bone loss and fracture risk are implicated in inflammatory autoimmune diseases such as rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel disease, and systemic lupus erythematosus. The network of inflammatory cytokines produced during chronic inflammation induces an uncoupling of bone formation and resorption, resulting in significant bone loss in patients with inflammatory autoimmune diseases. Here, we review and discuss the involvement of the inflammatory cytokine network in the pathophysiological aspects and the therapeutic advances in inflammatory autoimmune diseases.

## 1. Introduction

Bone is the main calcified tissue of vertebrates and serves multiple functions including mechanical support, protection, and storage [1]. The composition of bone is approximately 10% cells, 60% mineral crystals (crystalline hydroxyapatite), and 30% organic matrix [2]. Bone is continuously maintained by the process of bone remodeling through clusters of bone-resorbing osteoclasts and bone-forming osteoblasts [1, 3]. During bone remodeling, old or damaged bone is removed by osteoclasts and replaced by new bone formed by osteoblasts over several weeks [1, 3].

Osteoblasts are of mesenchymal origin and function primarily as bone-forming cells [1, 4]. Osteoblasts secrete the organic matrix, which predominantly contains collagen, and induce calcification during the process of new bone formation [5]. During bone remodeling, osteoblasts rebuild the bone matrix in regions where the bone has been resorbed by osteoclasts [1, 4]. The differentiation and function of osteoblasts are regulated by the activation of transcription factors (i.e., Runx-2/Cbfa-1, osterix (Osx), TAZ, and Atf4) [6–9], growth factors (i.e., tumor growth factor-$\beta$ (TGF-$\beta$), bone morphogenetic proteins (BMPs), Wnt, and vascular endothelial growth factor) [10–13], cytokines (i.e., interleukin-1 (IL-1), IL-6, and tumor necrosis factor-$\alpha$ (TNF-$\alpha$)), and interactions with various matrix proteins (i.e., collagen type I, biglycan, laminin, and fibronectin) [14, 15]. At the end of the bone-forming phase during bone remodeling, osteoblasts incorporate into the bone as osteocytes and the rest either remain on the bone surface as lining cells or undergo apoptosis [5, 16].

Osteocytes are former osteoblasts that become trapped during the process of bone deposition and remain regularly distributed throughout the mineralized bone matrix. These cells comprise more than 90% of bone cells within the matrix or on bone surfaces [17]. Osteocytes are the primary mechanosensory cells that act as regulators of mineral metabolism during bone remodeling [17]. Studies have revealed that osteocytes can send signals of bone resorption to osteoclasts during bone remodeling [17, 18]. Osteoclasts, the sole bone-resorbing cells, are multinucleated giant cells that are derived from mononuclear cells of the monocyte/macrophage lineage following stimulation by two essential factors: the macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor-kappa B (RANK) ligand (RANKL) [1, 3, 4].

The process of bone remodeling depends on the tight coupling of bone formation and bone resorption to ensure that there is no net change in the bone mass and to

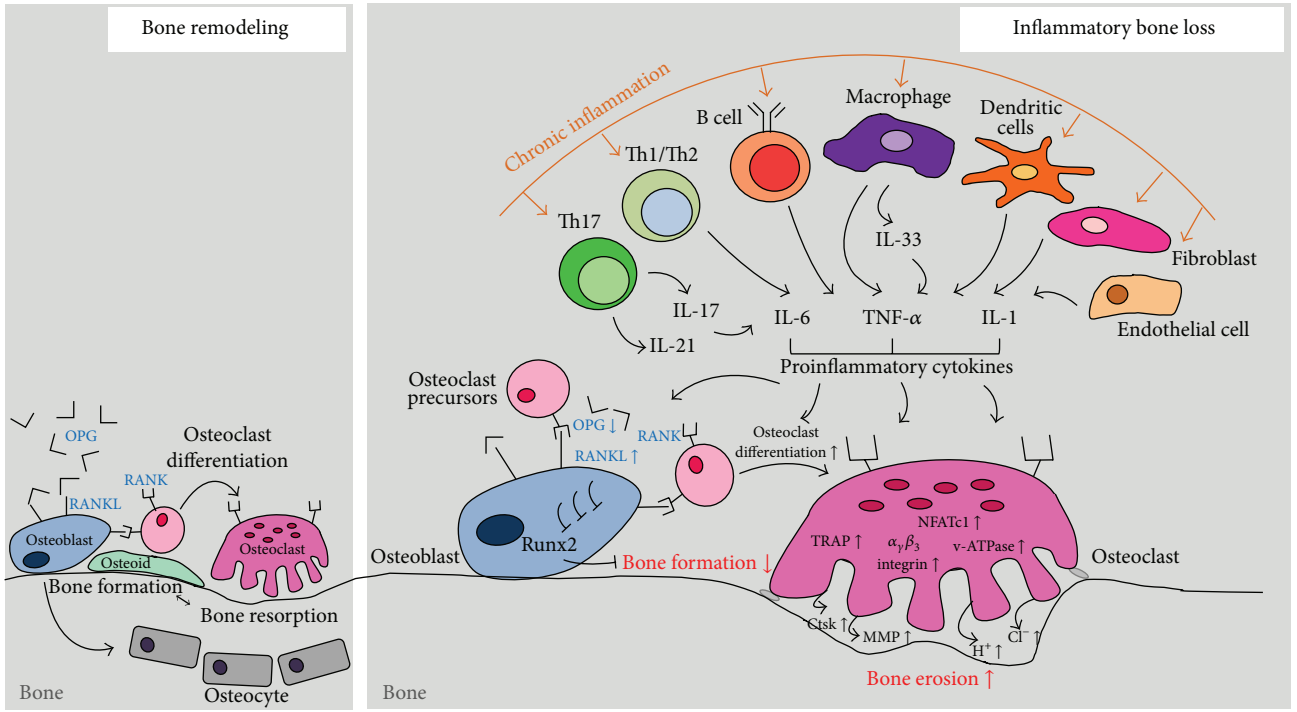


FIGURE 1: The role of inflammatory cytokine network in inflammatory bone loss. Bone remodeling is tightly regulated by the balanced action between bone-forming osteoblasts and bone-resorbing osteoclasts. In chronic inflammatory condition, inflammatory cytokine networks induce an uncoupling of bone formation and resorption that result in significant inflammatory bone loss. RANK: receptor activator of nuclear factor κB. RANKL: RANK ligand. OPG: osteoprotegerin. Runx2: runt-related transcription factor 2. TRAP: tartrate-resistant acid phosphatase. NFATc1: nuclear factor of activated T cells cytoplasmic 1. v-ATPase: vacuolar-type $H^+$-ATPase. MMP: matrix metalloproteinase. Ctsk: cathepsin K.

maintain the quality after each remodeling cycle [1, 3, 4]. An imbalance in this process is closely linked to various types of bone diseases, such as osteoporosis, osteopetrosis, periodontitis, and rheumatoid arthritis (RA) [19]. Osteoporosis is a skeletal disorder characterized by compromised bone strength, predisposing patients to an increased risk of fracture [20]. Osteoporosis was first considered to be an age-related disorder characterized by low bone mass and increased bone fragility, thereby putting the patient at risk of fractures. However, over time, it has come to be viewed as a heterogeneous condition that can occur at any age and its etiology is attributed to various endocrine, metabolic, and mechanical factors [19]. Studies have reported an increased risk of developing osteoporosis in patients with various inflammatory conditions [1–4]. Inflammation is characterized by the activation of several cell populations of the innate and adaptive immune system that produce inflammatory cytokines [21]. Inflammation perturbs normal bone homeostasis and is known to induce bone loss because it promotes both local cartilage degradation and local and systemic bone destruction by osteoclasts and inhibits bone formation by osteoblasts (Figure 1).

Inflammatory joint diseases share in common the presence of an inflammatory process that targets the joints, with adverse effects on structure and function [22]. RA is one of the most common autoimmune *diseases* that results in chronic *inflammation* of the *joints* [23]. Autoimmune diseases are characterized by impaired function and destruction of tissues caused by the presence of autoantibodies due to abnormally activated lymphocytes and nonlymphoid cells, such as macrophages, dendritic cells, and fibroblasts [24, 25]. Dysregulation of inflammatory or anti-inflammatory cytokine production or action is reported to play a central role in the pathogenesis of autoimmune diseases such as RA, ankylosing spondylitis (AS), inflammatory bowel disease (IBD), and systemic lupus erythematosus (SLE) [26–32]. Studies have revealed that therapeutic approaches using inflammatory/anti-inflammatory cytokines, including neutralizing antibodies (i.e., anti-TNF-α, anti-IL-6, and anti-IL-17), soluble receptors/inhibitors (i.e., TNF receptor, IL-1 receptor, IL-17 receptor, and IL-6 receptor inhibitor), and anti-inflammatory cytokines (i.e., IL-10 and IL-27), have been successful in controlling the progression of autoimmune diseases [33–37]. These studies have demonstrated a possible link between chronic inflammation and the pathogenesis of autoimmune diseases. Moreover, chronic inflammatory autoimmune diseases are frequently associated with bone destruction [38]. Bone loss is commonly observed in inflammatory joint diseases such as RA and AS [22]. Studies have also found an increase in bone loss and fractures with low BMD in individuals with SLE and IBD [38].

Although a large number of studies have focused on inflammatory autoimmune diseases over the past 10 years, the role of the inflammatory cytokine network involved in bone

loss in patients with inflammatory autoimmune diseases has not been well addressed. Therefore, in this review, we will provide an overview of the interaction between inflammatory autoimmune diseases and bone destruction through the regulation of the inflammatory cytokine network.

## 2. Methodology

We performed an extensive internet search for scientific articles indexed in the PubMed/Medline database over the past 15 years using the following keywords: bone loss, osteoporosis, autoimmunity, rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel disease, and systemic lupus erythematosus. We specifically focused on how bone loss and fracture risk are implicated in inflammatory autoimmune diseases.

## 3. Rheumatoid Arthritis (RA)

RA is a chronic autoimmune inflammatory disease characterized by the production of two main autoantibodies, rheumatoid factor and anticitrullinated peptide antibody, against common autoantigens that are widely expressed outside the joints, thereby resulting in local bone erosion, joint space narrowing, and extra-articular manifestations [23, 39]. In severe cases, RA can lead to periarticular osteopenia, systemic osteoporosis, and systemic bone erosion [40]. Disturbance of bone homeostasis in RA patients is driven by the cellular action of osteoclasts [41]. The enhanced osteoclast formation and activation is due to the increased accumulation of osteoclastogenic factors in the inflamed synovium [42–45]. In RA, elevated inflammatory cytokines have been implicated in bone destruction through recruitment of osteoclast precursors to the bone environment, where they differentiate into mature osteoclasts [46–48]. These inflammatory cytokines, such as TNF-$\alpha$, IL-1, IL-6, IL-7, and IL-17, are responsible for the overexpression of RANKL and decreased levels of osteoprotegerin (OPG), a decoy receptor of RANK. This perturbation leads to an imbalance in the RANKL/OPG ratio, thereby increasing osteoclast differentiation (also known as osteoclastogenesis) [42, 49–52]. However, levels of anti-inflammatory cytokines such as IL-10, IL-13, and TGF-$\beta$ have been reported to be present in significant amounts in RA joints [53, 54]. These anti-inflammatory cytokines have a negative effect on the joint destruction and inflammation associated with RA [55].

The role of TNF-$\alpha$ in arthritic bone destruction has been demonstrated in several experimental models and confirmed by clinical trials [56]. TNF-$\alpha$ enhances osteoclastogenesis through elevated expression of RANKL in the osteoblast [57]. Moreover, TNF-$\alpha$ induces the expression of the osteoclast-associated receptor (OSCAR), a key costimulatory molecule in osteoclastogenesis, on monocytes in RA patients [58]. TNF-$\alpha$ is also involved in osteoclastogenesis through modulation of the Wnt signaling pathway, although Wnt signaling is considered to be a key regulatory pathway for bone formation by osteoblasts [59]. In RA, TNF-$\alpha$ is a strong inducer of the Wnt antagonist Dickkopf-1 (Dkk-1) expression [60]. Dkk-1 impairs local bone formation through the inhibition of Wnt signaling by binding to low density lipoprotein-coupled receptor related protein-5/6 [61]. The blockade of Dkk-1 inhibits local bone resorption by reducing osteoclast numbers through the downregulation of OPG expression in the joints; this is further compounded because OPG regulates Dkk-1 expression through a feedback loop [60]. Consequently, the enhanced levels of Dkk-1 induced by TNF-$\alpha$ promote bone resorption by increasing the RANKL/OPG ratio but also block bone formation and repair in the diseased joint [62]. Furthermore, TNF-$\alpha$ is reported to directly inhibit osteoblast differentiation and bone nodule formation [63]. The transcription factors Runx-2/Cbfa-1 and Osx, which are critical regulators of osteoblast differentiation, are reported to be inhibited by TNF-$\alpha$, thereby decreasing osteoblast differentiation and inhibiting bone formation [13]. Because TNF-$\alpha$ is the most important cytokine involved in both pathogenesis and joint inflammation associated with RA, TNF-$\alpha$ blockers were the first class of biologics used in RA [41]. A study by Smolen et al. showed that TNF-$\alpha$ blockers had a beneficial effect on inflammatory disease activity and joint degradation, achieving high rates of sustained clinical remission by preventing radiographic damage in RA [64]. Moreover, studies have reported that TNF-$\alpha$ antibodies can decrease systemic bone loss and increase bone mineral density indicating that anti-TNF-$\alpha$ can be used against systemic osteoporosis and osteopenia [65, 66].

IL-1 is a key regulatory cytokine in mouse models of inflammatory arthritis. Overexpression of IL-1$\alpha$ or IL-1$\beta$ or deletion of the IL-1 receptor antagonist (IL-1Ra) leads to the development of arthritis with cartilage and bone destruction [48, 67]. IL-1 upregulates the production of RANKL, resulting in an imbalance in the synovial RANKL/OPG ratio [51, 68, 69]. In TNF-transgenic mice lacking IL-1 signaling, cartilage destruction is completely blocked and bone destruction partly reduced despite the presence of synovial inflammation, indicating that TNF-induced local bone destruction and systemic inflammatory bone loss are largely dependent on IL-1 [48]. Moreover, it is evident that TNF-induced synthesis of RANKL is inhibited by IL-1Ra [51]. In addition to IL-1 and TNF, IL-6 is another key proinflammatory cytokine involved in the pathogenesis of RA [70]. IL-6 stimulates the synthesis of RANKL by osteoblasts and promotes the development of T helper 17 (Th17) cells together with TGF-$\beta$ and IL-1 [71]. Studies have shown that the IL-6 antagonist tocilizumab has a beneficial effect on joint destruction and disease progression in RA patients [72, 73]. In mouse RA models, inflammatory cytokines such as IL-1$\beta$, TNF-$\alpha$, and IL-6 activate the signal transducer and activator of transcription 3 (STAT3) either directly or indirectly in murine osteoblasts and fibroblasts [68]. Studies have shown that STAT3 is the key mediator of both chronic inflammation and joint destruction in RA [68]. STAT3 activation induces the expression of RANKL [68, 74]. Therefore, STAT3 inhibition is also considered to be effective in treating RA.

IL-17 is the most recently described subclass of inflammatory cytokines. IL-17 induces the secretion of proinflammatory cytokines (i.e., TNF-$\alpha$, IL-1$\beta$, and IL-6) and chemokines (i.e., CXCL1/KC/GRO$\alpha$, CXCL2/MIP2$\alpha$/GRO$\beta$, CXCL8/IL-8, CCL2/MCP1, and CCL20/MIP-3$\alpha$) from

cartilage, synoviocytes, macrophages, and bone cells [75–81]. These elevated inflammatory cytokines and chemokines serve to activate and recruit neutrophils, macrophages, and lymphocytes to the inflamed synovium, thereby enhancing synovial inflammation [82]. Intra-articular injection of recombinant IL-17 also results in joint inflammation and damage [79, 83]. Interestingly, IL-17 activity is synergistically increased when combined with proinflammatory cytokines such as TNF-$\alpha$, IL-1$\beta$, and IL-6 [84, 85]. Moreover, IL-17 contributes to extensive cartilage and bone erosion in the advanced stages of RA by inducing the expression of RANKL, matrix metalloproteinases (MMPs), prostaglandin E2, and cyclooxygenase-2 [83, 86, 87]. The role of IL-17 as a potent stimulator of osteoclastogenesis in RA patients was first demonstrated by Kotake et al. [46]. IL-17 regulates osteoclastogenesis both directly and indirectly through osteoblasts/stromal cells, although the direct effect of IL-17 on osteoclast precursors is still controversial [87–89]. IL-17 induces RANKL expression from osteoblasts, synovial cells, and mesenchymal cells, and the increased RANKL/OPG ratio results in local or systemic bone destruction through enhancement of osteoclastogenesis [42, 46, 90]. Moreover, IL-17-producing Th17 cells, a subset of RANKL-expressing $CD4^+$ T cells, are involved in bone destruction through the function of osteoclastogenic helper T cells [87, 91]. In animal model studies, therapeutic approaches using IL-17 antibodies or a soluble IL-17 receptor have resulted in significant suppression of joint inflammation and bone erosion through downregulation of synovial RANKL and inflammatory cytokine expression [92–94]. Therefore, blocking IL-17, the IL-17 receptor (IL-17R), or its inducers (i.e., IL-23 and IL-6) can be used as a putative treatment method for RA.

In conclusion, bone destruction in RA is caused by a complex network of inflammatory cytokines, resulting in the *chronic inflammation* of the synovium. These studies have revealed several promising targets for the treatment of inflammatory bone loss in RA. In this respect, the initiation of biological therapies targeting inflammatory cytokines and/or lymphocyte activation has modified RA therapy not only by blocking local and systemic inflammatory cascades but also by providing beneficial effects against bone and joint destruction.

## 4. Ankylosing Spondylitis (AS)

AS is a systemic rheumatic disease characterized by chronic inflammation that chiefly affects the sacroiliac joints and the spine, whereas RA primarily affects the synovial membrane [95, 96]. One of the main features of structural damage in AS is bony ankyloses characterized by excessive bone formation that leads to the formation of bone spurs, such as syndesmophytes and enthesophytes, that contribute to ankylosis of the joints and poor physical function [96]. Moreover, the excessive loss of trabecular bone in the center of the vertebral body causing osteopenia or osteoporosis and leading to vertebral fractures with increased spinal deformity has been documented in AS patients [97].

TNF-$\alpha$ is a pivotal cytokine fueling inflammation in AS [96, 98]. TNF-$\alpha$-targeted therapies have influenced short-term control of the disease by limiting the symptoms caused by inflammation, which translates into better physical function and quality of life [96]. However, little or no effect on structural remodeling is achieved [99]. The elevated levels of IL-1 and IL-6 in the serum and in the sacroiliac joints of AS patients are also implicated in AS [32, 100]. However, antibody therapies blocking IL-6R signaling with tocilizumab or sarilumab failed to show clinical efficacy in a phase II clinical trial with AS patients, suggesting that IL-6 is not a pivotal inflammatory cytokine in the pathogenesis of AS [101, 102].

The involvement of Th17 cells in the promotion of the inflammatory process in AS patients is shown by the significantly elevated levels of Th17 cells in the peripheral blood of patients with AS [103, 104]. IL-17 and IL-23 are also high in the serum of AS patients [30]. Moreover, antibody therapies such as blocking IL-17 with secukinumab were shown to significantly downregulate the signs, symptoms, and objective parameters of inflammation in a phase II clinical trial in AS patients [105]. Currently, phase III clinical trials consisting of antibody therapy with secukinumab in AS patients are ongoing [106].

Previous studies have documented that the serum level of RANKL is higher in AS patients and that the expression of RANKL is increased on CD4 and CD8 T cells in AS patients [107]. Inflammatory cytokines including IL-1, IL-6, TNF-$\alpha$, and IL-17 can stimulate the expression of the soluble form of RANKL, which imbalances the RANKL/OPG ratio in AS patients [38]. The increased RANKL/OPG ratio thus promotes osteoclast differentiation, resulting in the bone destruction that is characteristic of AS [108, 109].

## 5. Inflammatory Bowel Disease (IBD)

IBD primarily refers to Crohn's disease and ulcerative colitis [110]. Crohn's disease can affect any part of the gastrointestinal tract, and classically presents with fatigue, prolonged diarrhea with or without gross bleeding, abdominal pain, weight loss, and fever [111]. Ulcerative colitis is limited to the colon area; common symptoms include rectal bleeding, frequent stools, mucus discharge from the rectum, tenesmus, and lower abdominal pain [111]. Crohn's disease is reported to be associated with Th1 cytokines IL-2, IL-17, interferon-$\gamma$ (IFN-$\gamma$), and TNF-$\alpha$, while ulcerative colitis is associated with Th2 cytokines, such as IL-4, IL-5, and IL-13 [112]. Therefore, Th1, Th2, and Th17 cells seem to be broadly involved in the pathogenesis of IBD through the regulation of inflammatory cytokine network. Interestingly, low bone matrix density (BMD) (defined as osteopenia or osteoporosis) is a known *chronic* complication of IBD [113]. Although IBD is not the sole risk factor for developing osteoporotic bone loss, it appears to be related to other known osteoporosis risk factors such as age, sex, body mass index, and medication [113]. Thus, the acceleration of the development of new biological drugs for IBD requires expanded insights into understanding the physiology, mechanism, and pathogenesis of IBD.

The principal mechanisms behind reduced BMD in IBD patients are still not completely understood, but a complex network of inflammatory cytokines that influence bone destruction has been reported [110, 113]. Mucosal and systemic concentrations of many pro- and anti-inflammatory cytokines are elevated in IBD patients [114]. In particular, the enhanced production of proinflammatory cytokines such as TNF-$\alpha$, IL-1$\beta$, and IL-6 is well documented in IBD patients [115, 116]. These proinflammatory cytokines stimulate bone resorption by osteoclasts through the induction of RANKL expression [1, 4]. Interestingly, anti-TNF-$\alpha$ therapy has been shown to improve markers of bone metabolism and BMD (i.e., osteocalcin, alkaline phosphatase, and P1NP) by decreasing serum OPG levels in IBD patients [117–120]. The increased RANKL/OPG ratio is known to promote osteoclast differentiation and bone destruction in IBD patients [121].

IL-17-producing Th17 cells are considered to be a new subset of cells that is critical for the reduced BMD in chronic IBD patients [122]. Th17 cells produce IL-17, IL-17F, IL-21, and IL-22; IL-17, IL-21, and IL-22 levels were reported to be markedly *elevated* in IBD patients [122]. IL-21 secreted by Th17 cells is one of the crucial cytokines involved in the pathogenesis of IBD via the induction of Th1 and Th17 immune responses in the gut [110]. Studies have shown that IL-21-deficient mice were resistant to Th1/Th17 cell-driven colitis [123, 124]. Correspondingly, IL-17 and IFN-$\gamma$ production by activated lamina propria mononuclear cells from IBD patients were inhibited by an IL-21 blocking antibody [123, 124].

IL-33, a new member of the IL-1 family, is a ligand for the IL-1 receptor-related protein (ST2) that is anticipated to be essential for the induction of Th2 immune responses [125]. Enhanced IL-33 levels are closely associated with IBD, particularly in ulcerative colitis patients [126]. Correspondingly, the inhibition of IL-33 signaling through anti-ST2 antibody treatment attenuates the severity of arthritis in an animal RA model [127]. Furthermore, IL-33 stimulates human osteoclast differentiation through the activation of ST2 receptor signaling [128]. Thus, it may be possible that IL-33 directly or indirectly regulates RANKL- or Th2 response-mediated bone loss in IBD.

Therapeutic anti-TNF-$\alpha$ antibodies such as infliximab and adalimumab are used for the treatment of severe cases of IBD [129, 130]. However, approximately one-third of the patients benefit minimally or not at all from this treatment [129, 130]. This could indicate that, among patients with IBD, nonresponders to anti-TNF therapy are more likely to have an inflammatory response mediated by other proinflammatory cytokines, such as IL-1$\beta$, IL-6, IL-17, and IFN-$\gamma$. Therefore, new drugs targeting other inflammatory cytokines could potentially be useful for treating IBD patients who do not respond to anti-TNF therapy [131].

## 6. Systemic Lupus Erythematosus (SLE)

SLE is an autoimmune disease that predominantly affects young women and is characterized by immunological hyperactivity and multiorgan damage. The exact causative factors of SLE are still unknown [132]. Unrestricted hyperactivation of the immune system may lead to the overproduction of autoantibodies, immune complex deposition, and inflammatory cytokine release, eventually resulting in the SLE phenotype [132]. In particular, the dysregulation of T/B cell activation leads to the production of autoantibodies such as anti-double-stranded DNA, anti-Ro (SS-A), anti-La (SS-B), anti-Smith (Sm), and anti-ribonucleoprotein (RNP) in SLE patients [133]. Autoantibodies bound with antigens are deposited in organs, thereby causing chronic inflammation and tissue damage [132].

The abnormal expression of various inflammatory cytokines due to chronic inflammation induces an imbalance among different immune cell subsets, such as Th1/Th2 and Th17/regulatory T (Treg) cells; this imbalance plays a crucial pathogenic role in SLE [132]. TNF-$\alpha$ has been implicated in SLE murine models [26], and elevated serum TNF-$\alpha$ levels are observed in SLE patients, similar to the other inflammatory autoimmune diseases discussed here [134]. However, the therapeutic effects of TNF-$\alpha$ blockers in SLE patients are still controversial [135]. Abnormal IL-6 levels were also observed in both serum and local tissues in patients with SLE [136]. The dominant role of IL-6 in SLE pathogenesis is to accelerate autoantibody production by promoting the proliferation of autoreactive B cells [132]. The autoantibody production induced by IL-6 is indirectly mediated by IL-21 produced by $CD4^+$ T cells [137]. Interestingly, it has been reported that IL-6 produced by dendritic cells inhibits Treg cell function in mouse SLE models [138]. Thus, IL-6 is implicated as the most important inflammatory cytokine in the pathogenesis of SLE, and antibody therapies blocking IL-6 receptor signaling with tocilizumab are reported to be effective in treating SLE [139].

IL-17 is a proinflammatory cytokine with multiple functions in the regulation of tissue inflammation [132]. An increased number of Th17 cells and elevated serum IL-17 levels are reported in SLE patients [140, 141]. In SLE patients, IL-17 seems to facilitate both T cell activation and infiltration into tissues via the expression of intercellular adhesion molecule-1 (ICAM-1) and B cell activation and antibody production in combination with B-cell-activating factor (BAFF) [140, 142]. A strong correlation between IL-17 and IL-23 levels in SLE patients suggests that IL-23 contributes to SLE severity by activating Th17 cells [143]. Moreover, the IL-23/IL-17 activation pathway is closely associated with increased immunoglobulin deposition and complement activation in the kidney in mouse SLE models [144]. IL-17 has not been therapeutically targeted in SLE patients to date, but data from recent clinical trials in other inflammatory autoimmune diseases such as and Crohn's disease can partially inform us about the efficacy and safety of blocking IL-17 either directly or indirectly by targeting IL-23 in SLE patients [145].

Since the first reported association between type I IFN and SLE in 1979 [146], many reports have implicated elevated levels of serum IFN-$\alpha$ in SLE [147]. Plasmacytoid dendritic cells (pDCs), which are abundant in the skin and lymph nodes, are reported to be the primary sources of IFN-$\alpha$ in SLE patients. The IFN signature produced by pDCs can

promote the pathogenesis of SLE by enhancing autoantibody production and activating Th17 cells to secrete cytokines [148–150]. Considering the essential role of type I IFN in SLE, more than five monoclonal antibodies specific for different IFN-$\alpha$ isoforms or their receptors are in different clinical phases of testing [151].

There seems to be a high prevalence of osteoporosis in SLE patients, but the prevalence frequencies differ widely as a consequence of differences in body mass, age, sex, ethnicity, disease severity, and medication use [152]. Glucocorticoid use, longer disease duration due to chronic inflammation, neuropsychiatric disease complications, and previous fractures were identified as associated factors for SLE-related osteoporotic fractures [152]. Although the direct correlation between inflammatory cytokine levels and bone defects in SLE patients remains unclear, bone destruction in SLE patients is thought to be the result of accelerated osteoclastogenesis induced by proinflammatory cytokines [153]. The increased level of proinflammatory cytokines such as TNF-$\alpha$, IL-1, IL-6, and IL-17 in SLE patients might result in an RANKL/OPG imbalance by enhancing RANKL induction, leading to accelerated osteoclastogenesis. Interestingly, increased levels of oxidized low density lipoproteins (LDL) have been reported in SLE patients [154]. The enhanced oxidized LDL can induce T cell activation, thereby sequentially inducing RANKL expression and TNF-$\alpha$ production [154–156]. Furthermore, a recent study by Tang et al. has shown that impaired osteoblast differentiation through the inhibition of the BMP/Smad pathway by activated NF-$\kappa$B signaling plays a role in the pathology of osteoporosis in SLE patients [153]. The number of Th17 cells and IL-17 levels are elevated in the serum of many SLE patients [140, 141]. Although the exact role of IL-17 in bone destruction in SLE patients remains unclear, IL-17 may affect bone remodeling through its effects on both osteoblasts and osteoclasts as discussed above; IL-17 can induce bone loss by mediating an imbalance in RANKL/OPG via the expression of RANKL in osteoblasts or activated T cells and can act in synergy with TNF-$\alpha$ or chemokines to influence osteoclast resorption [46, 75, 84, 85, 157].

## 7. Discussion

Bone remodeling is a highly coordinated process that involves bone resorption and formation, which are essential for repairing damaged bones and maintaining mineral homeostasis. However, in chronic inflammatory conditions, the inflammatory cytokine network induces an uncoupling of bone formation and resorption that results in significant inflammatory bone loss. In particular, inflammatory cytokines such as IL-1, IL-6, IL-17, and TNF-$\alpha$ are involved in the pathogenesis of inflammatory autoimmune diseases of interest. However, the effects of inflammatory cytokines on inflammatory bone loss and in the pathogenesis of inflammatory autoimmune diseases are more complicated. As discussed in this review, bone loss in inflammatory autoimmune diseases may be caused by direct or indirect effects with complicated mechanisms by inflammatory cytokines or the inflammatory cytokine network in chronically inflamed tissues. Therefore, drugs targeting multiple cytokines could be an effective strategy for disease prevention and reducing disease progression. Because most inflammatory cytokines are involved in bone damage though inducing an imbalance in RANKL/OPG, focusing on OPG or RANKL management may be a better strategy than focusing on inhibiting a single cytokine.

Inflammatory autoimmune diseases continue to be a mounting public health concern worldwide. The cost and the social burden associated with these diseases, while being difficult to pin down accurately, are increasing. To stick with the saying "easing the burden: solutions for the future," it is imperative to accelerate the development of new treatment options for these diseases. A better understanding of the mechanisms by which the inflammatory cytokine network elicits chronic inflammation in autoimmunity will provide new therapeutic approaches to reduce bone destruction in inflammatory autoimmune diseases.

## Abbreviations

RA: Rheumatoid arthritis
AS: Ankylosing spondylitis
IBD: Inflammatory bowel disease
SLE: Systemic lupus erythematosus
BMD: Bone mineral density
RANKL: Receptor activator of nuclear factor-kappa B ligand
M-CSF: Macrophage colony-stimulating factor
OPG: Osteoprotegerin
TNF-$\alpha$: Tumor necrosis factor-$\alpha$
IL: Interleukin
Dkk-1: Dickkopf-1
IL-1Ra: IL-1 receptor antagonist
STAT3: Signal transducer and activator of transcription 3
MMPs: Matrix metalloproteinases
IL-17R: IL-17 receptor
IFN-$\gamma$: Interferon-$\gamma$
Treg cells: Regulatory T cells
ICAM-1: Intercellular adhesion molecule-1
BAFF: B-cell-activating factor
pDCs: Plasmacytoid DCs
OSCAR: Osteoclast-associated receptors
Osx: Osterix
BMPs: Bone morphogenetic proteins.

## Disclosure

Dulshara Sachini Amarasekara and Jiyeon Yu are co-first authors.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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## References

[1] M. C. Walsh, N. Kim, Y. Kadono et al., "Osteoimmunology: interplay between the immune system and bone metabolism," *Annual Review of Immunology*, vol. 24, pp. 33–63, 2006.

[2] X. Feng and J. M. McDonald, "Disorders of bone remodeling," *Annual Review of Pathology: Mechanisms of Disease*, vol. 6, pp. 121–145, 2011.

[3] J. Rho, M. Takami, and Y. Choi, "Osteoimmunology: interactions of the immune and skeletal systems," *Molecules and Cells*, vol. 17, no. 1, pp. 1–9, 2004.

[4] H. Takayanagi, "Osteoimmunology and the effects of the immune system on bone," *Nature Reviews Rheumatology*, vol. 5, no. 12, pp. 667–676, 2009.

[5] S.-I. Harada and G. A. Rodan, "Control of osteoblast function and regulation of bone mass," *Nature*, vol. 423, no. 6937, pp. 349–355, 2003.

[6] P. Ducy, R. Zhang, V. Geoffroy, A. L. Ridall, and G. Karsenty, "Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation," *Cell*, vol. 89, no. 5, pp. 747–754, 1997.

[7] K. Nakashima, X. Zhou, G. Kunkel et al., "The novel zinc finger-containing transcription factor Osterix is required for osteoblast differentiation and bone formation," *Cell*, vol. 108, no. 1, pp. 17–29, 2002.

[8] J.-H. Hong, E. S. Hwang, M. T. McManus et al., "TAZ, a transcriptional modulator of mesenchymal stem cell differentiation," *Science*, vol. 309, no. 5737, pp. 1074–1078, 2005.

[9] W. Wang, N. Lian, Y. Ma et al., "Chondrocytic Atf4 regulates osteoblast differentiation and function via Ihh," *Development*, vol. 139, no. 3, pp. 601–611, 2012.

[10] M. Centrella, M. C. Horowitz, J. M. Wozney, and T. L. Mccarthy, "Transforming growth factor-$\beta$ gene family members and bone," *Endocrine Reviews*, vol. 15, no. 1, pp. 27–39, 1994.

[11] D. Chen, X. Ji, M. A. Harris et al., "Differential roles for bone morphogenetic protein (BMP) receptor type IB and IA in differentiation and specification of mesenchymal precursor cells to osteoblast and adipocyte lineages," *The Journal of Cell Biology*, vol. 142, no. 1, pp. 295–305, 1998.

[12] T. Gaur, C. J. Lengner, H. Hovhannisyan et al., "Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression," *Journal of Biological Chemistry*, vol. 280, no. 39, pp. 33132–33140, 2005.

[13] Y. Liu, A. D. Berendsen, S. Jia et al., "Intracellular VEGF regulates the balance between osteoblast and adipocyte differentiation," *Journal of Clinical Investigation*, vol. 122, no. 9, pp. 3101–3113, 2012.

[14] S. Mathews, R. Bhonde, P. K. Gupta, and S. Totey, "Extracellular matrix protein mediated regulation of the osteoblast differentiation of bone marrow derived human mesenchymal stem cells," *Differentiation*, vol. 84, no. 2, pp. 185–192, 2012.

[15] X. Wang, K. Harimoto, S. Xie, H. Cheng, J. Liu, and Z. Wang, "Matrix protein biglycan induces osteoblast differentiation through extracellular signal-regulated kinase and smad pathways," *Biological and Pharmaceutical Bulletin*, vol. 33, no. 11, pp. 1891–1897, 2010.

[16] R. L. Jilka, R. S. Weinstein, T. Bellido, A. M. Parfitt, and S. C. Manolagas, "Osteoblast programmed cell death (apoptosis): modulation by growth factors and cytokines," *Journal of Bone and Mineral Research*, vol. 13, no. 5, pp. 793–802, 1998.

[17] T. Bellido, "Osteocyte-driven bone remodeling," *Calcified Tissue International*, vol. 94, no. 1, pp. 25–34, 2014.

[18] A. R. Wijenayaka, M. Kogawa, H. P. Lim, L. F. Bonewald, D. M. Findlay, and G. J. Atkins, "Sclerostin stimulates osteocyte support of osteoclast activity by a RANKL-dependent pathway," *PLoS ONE*, vol. 6, no. 10, Article ID e25900, 2011.

[19] S. L. Teitelbaum and F. P. Ross, "Genetic regulation of osteoclast development and function," *Nature Reviews Genetics*, vol. 4, no. 8, pp. 638–649, 2003.

[20] N. E. Lane, "Epidemiology, etiology, and diagnosis of osteoporosis," *The American Journal of Obstetrics and Gynecology*, vol. 194, no. 2, pp. S3–S11, 2006.

[21] K. Redlich and J. S. Smolen, "Inflammatory bone loss: pathogenesis and therapeutic intervention," *Nature Reviews Drug Discovery*, vol. 11, no. 3, pp. 234–250, 2012.

[22] C. Roux, "Osteoporosis in inflammatory joint diseases," *Osteoporosis International*, vol. 22, no. 2, pp. 421–433, 2011.

[23] M.-C. Boissier, "Cell and cytokine imbalances in rheumatoid synovitis," *Joint Bone Spine*, vol. 78, no. 3, pp. 230–234, 2011.

[24] J. J. O'Shea, A. Ma, and P. Lipsky, "Cytokines and autoimmunity," *Nature Reviews Immunology*, vol. 2, no. 1, pp. 37–45, 2002.

[25] H.-F. Pan, X.-P. Li, S. G. Zheng, and D.-Q. Ye, "Emerging role of interleukin-22 in autoimmune diseases," *Cytokine and Growth Factor Reviews*, vol. 24, no. 1, pp. 51–57, 2013.

[26] M. Aringer and J. S. Smolen, "The role of tumor necrosis factor-alpha in systemic lupus erythematosus," *Arthritis Research and Therapy*, vol. 10, article 202, 2008.

[27] T. Ciucci, L. Ibáñez, A. Boucoiran et al., "Bone marrow Th17 TNF$\alpha$ cells induce osteoclast differentiation, and link bone destruction to IBD," *Gut*, 2014.

[28] G. Eilertsen, C. Nikolaisen, A. Becker-Merok, and J. C. Nossent, "Interleukin-6 promotes arthritis and joint deformation in patients with systemic lupus erythematosus," *Lupus*, vol. 20, no. 6, pp. 607–613, 2011.

[29] M. Linker-Israeli, R. J. Deans, D. J. Wallace, J. Prehn, T. Ozeri-Chen, and J. R. Klinenberg, "Elevated levels of endogenous IL-6 in systemic lupus erythematosus: a putative role in pathogenesis," *Journal of Immunology*, vol. 147, no. 1, pp. 117–123, 1991.

[30] Y. Mei, F. Pan, J. Gao et al., "Increased serum IL-17 and IL-23 in the patient with ankylosing spondylitis," *Clinical Rheumatology*, vol. 30, no. 2, pp. 269–273, 2011.

[31] K. D. Moudgil and D. Choubey, "Cytokines in autoimmunity: role in induction, regulation, and treatment," *Journal of Interferon and Cytokine Research*, vol. 31, no. 10, pp. 695–703, 2011.

[32] S. K. Sharma, S. Ahmad, and S. K. Sharma, "Serum IL-6 level as a marker of disease activity in ankylosing spondylitis patients with pure axial involvement," *Indian Journal of Rheumatology*, vol. 9, no. 3, pp. 115–119, 2014.

[33] J. A. Fischer, A. J. Hueber, S. Wilson et al., "Combined inhibition of tumor necrosis factor $\alpha$ and interleukin-17 as a therapeutic opportunity in rheumatoid arthritis: development and characterization of a novel bispecific antibody," *Arthritis & Rheumatology*, vol. 67, no. 1, pp. 51–62, 2015.

[34] C.-C. Chao, S.-J. Chen, I. E. Adamopoulos et al., "Anti-IL-17A therapy protects against bone erosion in experimental models of rheumatoid arthritis," *Autoimmunity*, vol. 44, no. 3, pp. 243–252, 2011.

[35] D. Hürlimann, A. Forster, G. Noll et al., "Anti-tumor necrosis factor-$\alpha$ treatment improves endothelial function in patients with rheumatoid arthritis," *Circulation*, vol. 106, no. 17, pp. 2184–2187, 2002.

[36] Y. Shirota, C. Yarboro, R. Fischer, T.-H. Pham, P. Lipsky, and G. G. Illei, "Impact of anti-interleukin-6 receptor blockade on circulating T and B cell subsets in patients with systemic lupus erythematosus," *Annals of the Rheumatic Diseases*, vol. 72, no. 1, pp. 118–128, 2013.

[37] J. D. Whalen, E. L. Lechman, C. A. Carlos et al., "Adenoviral transfer of the viral IL-10 gene periarticularly to mouse paws suppresses development of collagen-induced arthritis in both injected and uninjected paws," *Journal of Immunology*, vol. 162, no. 6, pp. 3625–3632, 1999.

[38] R. Hardy and M. S. Cooper, "Bone loss in inflammatory disorders," *Journal of Endocrinology*, vol. 201, no. 3, pp. 309–320, 2009.

[39] M. Vis, M. Güler-Yüksel, and W. F. Lems, "Can bone loss in rheumatoid arthritis be prevented?" *Osteoporosis International*, vol. 24, no. 10, pp. 2541–2553, 2013.

[40] D. Vosse and K. de Vlam, "Osteoporosis in rheumatoid arthritis and ankylosing spondylitis," *Clinical and Experimental Rheumatology*, vol. 27, no. 4, pp. S62–S67, 2009.

[41] T. Dimitroulas, S. N. Nikas, P. Trontzas, and G. D. Kitas, "Biologic therapies and systemic bone loss in rheumatoid arthritis," *Autoimmunity Reviews*, vol. 12, no. 10, pp. 958–966, 2013.

[42] E. Lubberts, L. van den Bersselaar, B. Oppers-Walgreen et al., "IL-17 promotes bone erosion in murine collagen-induced arthritis through loss of the receptor activator of NF-$\kappa$B ligand/osteoprotegerin balance1," *The Journal of Immunology*, vol. 170, no. 5, pp. 2655–2662, 2003.

[43] E. M. Gravallese, C. Manning, A. Tsay et al., "Synovial tissue in rheumatoid arthritis is a source of osteoclast differentiation factor," *Arthritis & Rheumatism*, vol. 43, no. 2, pp. 250–258, 2000.

[44] Y. Shigeyama, T. Pap, P. Kunzler, B. R. Simmen, R. E. Gay, and S. Gay, "Expression of osteoclast differentiation factor in rheumatoid arthritis," *Arthritis & Rheumatism*, vol. 43, no. 11, pp. 2523–2530, 2000.

[45] M. Stolina, S. Adamu, M. Ominsky et al., "RANKL is a marker and mediator of local and systemic bone loss in two rat models of inflammatory arthritis," *Journal of Bone and Mineral Research*, vol. 20, no. 10, pp. 1756–1765, 2005.

[46] S. Kotake, N. Udagawa, N. Takahashi et al., "IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis," *The Journal of Clinical Investigation*, vol. 103, no. 9, pp. 1345–1352, 1999.

[47] K. Redlich, S. Hayer, A. Maier et al., "Tumor necrosis factor $\alpha$-mediated joint destruction is inhibited by targeting osteoclasts with osteoprotegerin," *Arthritis and Rheumatism*, vol. 46, no. 3, pp. 785–792, 2002.

[48] J. Zwerina, K. Redlich, K. Polzer et al., "TNF-induced structural joint damage is mediated by IL-1," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 28, pp. 11742–11747, 2007.

[49] J. Lam, S. Takeshita, J. E. Barker, O. Kanagawa, F. P. Ross, and S. L. Teitelbaum, "TNF-$\alpha$ induces osteoclastogenesis by direct stimulation of macrophages exposed to permissive levels of RANK ligand," *The Journal of Clinical Investigation*, vol. 106, no. 12, pp. 1481–1488, 2000.

[50] G. Toraldo, C. Roggia, W.-P. Qian, R. Pacific, and M. N. Weitzmann, "IL-7 induces bone loss in vivo by induction of receptor activator of nuclear factor $\kappa$B ligand and tumor necrosis factor $\alpha$ from T cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 1, pp. 125–130, 2003.

[51] S. Wei, H. Kitaura, P. Zhou, F. P. Ross, and S. L. Teitelbaum, "IL-1 mediates TNF-induced osteoclastogenesis," *The Journal of Clinical Investigation*, vol. 115, no. 2, pp. 282–290, 2005.

[52] M. Hashizume, N. Hayakawa, and M. Mihara, "IL-6 trans-signalling directly induces RANKL on fibroblast-like synovial cells and is involved in RANKL induction by TNF-$\alpha$ and IL-17," *Rheumatology*, vol. 47, no. 11, pp. 1635–1640, 2008.

[53] P. D. Katsikis, C.-Q. Chu, F. M. Brennan, R. N. Maini, and M. Feldmann, "Immunoregulatory role of interleukin 10 in rheumatoid arthritis," *The Journal of Experimental Medicine*, vol. 179, no. 5, pp. 1517–1527, 1994.

[54] P. Miossec, "Interleukin-17 in rheumatoid arthritis: if T cells were to contribute to inflammation and destruction through synergy," *Arthritis & Rheumatism*, vol. 48, no. 3, pp. 594–601, 2003.

[55] J. A. G. van Roon, F. P. J. G. Lafeber, and J. W. J. Bijlsma, "Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis," *Arthritis & Rheumatism*, vol. 44, no. 1, pp. 3–12, 2001.

[56] H. Matsuno, K. Yudoh, R. Katayama et al., "The role of TNF-$\alpha$ in the pathogenesis of inflammation and joint destruction in rheumatoid arthritis (RA): a study using a human RA/SCID mouse chimera," *Rheumatology*, vol. 41, no. 3, pp. 329–337, 2002.

[57] Y.-H. Zhang, A. Heulsmann, M. M. Tondravi, A. Mukherjee, and Y. Abu-Amer, "Tumor necrosis factor-alpha (TNF) stimulates RANKL-induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways," *The Journal of Biological Chemistry*, vol. 276, no. 1, pp. 563–568, 2001.

[58] S. Herman, G. Kroenke, R. Mueller, J. Zerina, K. Redlich, and G. Schett, "OSCAR, a key co-stimulation molecule for osteoclasts, is induced in patients with rheumatoid arthritis," *Bone*, vol. 43, supplement 1, p. S80, 2008.

[59] D. A. Glass II, P. Bialek, J. D. Ahn et al., "Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation," *Developmental Cell*, vol. 8, no. 5, pp. 751–764, 2005.

[60] D. Diarra, M. Stolina, K. Polzer et al., "Dickkopf-1 is a master regulator of joint remodeling," *Nature Medicine*, vol. 13, no. 2, pp. 156–163, 2007.

[61] B. Mao, W. Wu, Y. Li et al., "LDL-receptor-related protein 6 is a receptor for Dickkopf proteins," *Nature*, vol. 411, no. 6835, pp. 321–325, 2001.

[62] D. Daoussis and A. P. Andonopoulos, "The emerging role of Dickkopf-1 in bone biology: is it the main switch controlling bone and joint remodeling?" *Seminars in Arthritis and Rheumatism*, vol. 41, no. 2, pp. 170–177, 2011.

[63] M. Tsukasaki, A. Yamada, D. Suzuki et al., "Expression of POEM, a positive regulator of osteoblast differentiation, is suppressed by TNF-$\alpha$," *Biochemical and Biophysical Research Communications*, vol. 410, no. 4, pp. 766–770, 2011.

[64] J. S. Smolen, D. M. F. M. van Der Heijde, E. W. St.Clair et al., "Predictors of joint damage in patients with early rheumatoid arthritis treated with high-dose methotrexate with or without concomitant infliximab: results from the ASPIRE trial," *Arthritis & Rheumatism*, vol. 54, no. 3, pp. 702–710, 2006.

[65] N. Saidenberg-Kermanac'h, A. Corrado, D. Lemeiter, M. C. Devernejoul, M. C. Boissier, and M. E. Cohen-Solal, "TNF-alpha antibodies and osteoprotegerin decrease systemic bone loss associated with inflammation through distinct mechanisms in collagen-induced arthritis," *Bone*, vol. 35, no. 5, pp. 1200–1207, 2004.

[66] U. Lange, J. Teichmann, U. Müller-Ladner, and J. Strunk, "Increase in bone mineral density of patients with rheumatoid arthritis treated with anti-TNF-$\alpha$ antibody: a prospective open-label pilot study," *Rheumatology*, vol. 44, no. 12, pp. 1546–1548, 2005.

[67] R. Horai, S. Saijo, H. Tanioka et al., "Development of chronic inflammatory arthropathy resembling rheumatoid arthritis in interleukin 1 receptor antagonist-deficient mice," *The Journal of Experimental Medicine*, vol. 191, no. 2, pp. 313–320, 2000.

[68] T. Mori, T. Miyamoto, H. Yoshida et al., "IL-1$\beta$ and TNF$\alpha$-initiated IL-6-STAT3 pathway is critical in mediating inflammatory cytokines and RANKL expression in inflammatory arthritis," *International Immunology*, vol. 23, no. 11, pp. 701–712, 2011.

[69] D. R. Haynes, T. N. Crotti, M. Loric, G. I. Bain, G. J. Atkins, and D. M. Findlay, "Osteoprotegerin and receptor activator of nuclear factor kappaB ligand (RANKL) regulate osteoclast formation by cells in the human rheumatoid arthritis joint," *Rheumatology*, vol. 40, no. 6, pp. 623–630, 2001.

[70] B. Le Goff, F. Blanchard, J.-M. Berthelot, D. Heymann, and Y. Maugars, "Role for interleukin-6 in structural joint damage and systemic bone loss in rheumatoid arthritis," *Joint Bone Spine*, vol. 77, no. 3, pp. 201–205, 2010.

[71] P. K. K. Wong, J. M. W. Quinn, N. A. Sims, A. Van Nieuwenhuijze, I. K. Campbell, and I. P. Wicks, "Interleukin-6 modulates production of T lymphocyte-derived cytokines in antigen-induced arthritis and drives inflammation-induced osteoclastogenesis," *Arthritis and Rheumatism*, vol. 54, no. 1, pp. 158–168, 2006.

[72] J. S. Smolen, A. Beaulieu, A. Rubbert-Roth et al., "Effect of interleukin-6 receptor inhibition with tocilizumab in patients with rheumatoid arthritis (OPTION Study): a double-blind, placebo-controlled, randomised trial," *The Lancet*, vol. 371, no. 9617, pp. 987–997, 2008.

[73] Y. Tanaka, T. Takeuchi, K. Amano et al., "Effect of interleukin-6 receptor inhibitor, tocilizumab, in preventing joint destruction in patients with rheumatoid arthritis showing inadequate response to TNF inhibitors," *Modern Rheumatology*, vol. 24, no. 3, pp. 399–404, 2014.

[74] T. Miyamoto, T. Mori, A. Yoshimura, and T. Toyama, "STAT3 is critical to promote inflammatory cytokines and RANKL expression in inflammatory arthritis," *Arthritis Research & Therapy*, vol. 14, supplement 1, p. P43, 2012.

[75] M. J. Ruddy, F. Shen, J. B. Smith, A. Sharma, and S. L. Gaffen, "Interleukin-17 regulates expression of the CXC chemokine LIX/CXCL5 in osteoblasts: implications for inflammation and neutrophil recruitment," *Journal of Leukocyte Biology*, vol. 76, no. 1, pp. 135–144, 2004.

[76] F. Fossiez, O. Djossou, P. Chomarat et al., "T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines," *The Journal of Experimental Medicine*, vol. 183, no. 6, pp. 2593–2603, 1996.

[77] S. Shahrara, S. R. Pickens, A. Dorfleutner, and R. M. Pope, "IL-17 induces monocyte migration in rheumatoid arthritis," *Journal of Immunology*, vol. 182, no. 6, pp. 3884–3891, 2009.

[78] M. Chabaud, F. Fossiez, J.-L. Taupin, and P. Miossec, "Enhancing effect of IL-17 on IL-1-induced IL-6 and leukemia inhibitory factor production by rheumatoid arthritis synoviocytes and its regulation by Th2 cytokines," *Journal of Immunology*, vol. 161, no. 1, pp. 409–414, 1998.

[79] M. Chabaud, G. Page, and P. Miossec, "Enhancing effect of IL-1, IL-17, and TNF-alpha on macrophage inflammatory protein-3alpha production in rheumatoid arthritis: regulation by soluble receptors and Th2 cytokines," *Journal of Immunology*, vol. 167, no. 10, pp. 6015–6020, 2001.

[80] Y. Katz, O. Nadiv, and Y. Beer, "Interleukin-17 enhances tumor necrosis factor $\alpha$-induced synthesis of interleukins 1,6, and 8 in skin and synovial fibroblasts: a possible role as a 'fine-tuning cytokine' in inflammation processes," *Arthritis & Rheumatism*, vol. 44, no. 9, pp. 2176–2184, 2001.

[81] S.-Y. Hwang, J.-Y. Kim, K.-W. Kim et al., "IL-17 induces production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF-$\kappa$B- and PI3-kinase/Akt-dependent pathways," *Arthritis Research & Therapy*, vol. 6, no. 2, pp. R120–R128, 2004.

[82] F. Shen and S. L. Gaffen, "Structure-function relationships in the IL-17 receptor: implications for signal transduction and therapy," *Cytokine*, vol. 41, no. 2, pp. 92–104, 2008.

[83] M. Chabaud, P. Garnero, J.-M. Dayer, P.-A. Guerne, F. Fossiez, and P. Miossec, "Contribution of interleukin 17 to synovium matrix destruction in rheumatoid arthritis," *Cytokine*, vol. 12, no. 7, pp. 1092–1099, 2000.

[84] P. J. Koshy, N. Henderson, C. Logan, P. F. Life, T. E. Cawston, and A. D. Rowan, "Interleukin 17 induces cartilage collagen breakdown: novel synergistic effects in combination with proinflammatory cytokines," *Annals of the Rheumatic Diseases*, vol. 61, no. 8, pp. 704–713, 2002.

[85] A. LeGrand, B. Fermor, C. Fink et al., "Interleukin-1, tumor necrosis factor $\alpha$, and interleukin-17 synergistically up-regulate nitric oxide and prostaglandin $E_2$ production in explants of human osteoarthritic knee menisci," *Arthritis & Rheumatism*, vol. 44, no. 9, pp. 2078–2083, 2001.

[86] D. V. Jovanovic, J. Martel-Pelletier, J. A. Di Battista et al., "Stimulation of 92-kd gelatinase (matrix metalloproteinase 9) production by interleukin-17 in human monocyte/macrophages: a possible role in rheumatoid arthritis," *Arthritis & Rheumatology*, vol. 43, pp. 1134–1144, 2000.

[87] K. S. A. Sato, A. Suematsu, K. Okamoto et al., "Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction," *The Journal of Experimental Medicine*, vol. 203, no. 12, pp. 2673–2682, 2006.

[88] T. Yago, Y. Nanke, N. Ichikawa et al., "IL-17 induces osteoclastogenesis from human monocytes alone in the absence of osteoblasts, which is potently inhibited by anti-TNF-$\alpha$ antibody: a novel mechanism of osteoclastogenesis by IL-17," *Journal of Cellular Biochemistry*, vol. 108, no. 4, pp. 947–955, 2009.

[89] S. Kitami, H. Tanaka, T. Kawato et al., "IL-17A suppresses the expression of bone resorption-related proteinases and osteoclast differentiation via IL-17RA or IL-17RC receptors in RAW264.7 cells," *Biochimie*, vol. 92, no. 4, pp. 398–404, 2010.

[90] H. Huang, H. J. Kim, E.-J. Chang et al., "IL-17 stimulates the proliferation and differentiation of human mesenchymal stem cells: implications for bone remodeling," *Cell Death and Differentiation*, vol. 16, no. 10, pp. 1332–1343, 2009.

[91] J. Kikuta, Y. Wada, T. Kowada et al., "Dynamic visualization of RANKL and Th17-mediated osteoclast function," *The Journal of Clinical Investigation*, vol. 123, no. 2, pp. 866–873, 2013.

[92] M. I. Koenders, E. Lubberts, B. Oppers-Walgreen et al., "Blocking of interleukin-17 during reactivation of experimental arthritis prevents joint inflammation and bone erosion by decreasing RANKL and interleukin-1," *The American Journal of Pathology*, vol. 167, no. 1, pp. 141–149, 2005.

[93] K. A. Bush, K. M. Farmer, J. S. Walker, and B. W. Kirkham, "Reduction of joint inflammation and bone erosion in rat adjuvant arthritis by treatment with interleukin-17 receptor IgG1 Fc fusion protein," *Arthritis and Rheumatism*, vol. 46, no. 3, pp. 802–805, 2002.

[94] M. Chabaud and P. Miossec, "The combination of tumor necrosis factor alpha blockade with interleukin-1 and interleukin-17 blockade is more effective for controlling synovial inflammation and bone resorption in an ex vivo model," *Arthritis & Rheumatism*, vol. 44, no. 6, pp. 1293–1303, 2001.

[95] T. Pham, "Pathophysiology of ankylosing spondylitis: what's new?" *Joint Bone Spine*, vol. 75, no. 6, pp. 656–660, 2008.

[96] G. Schett and J.-P. David, "The multiple faces of autoimmune-mediated bone loss," *Nature Reviews Endocrinology*, vol. 6, no. 12, pp. 698–706, 2010.

[97] N. Davey-Ranasinghe and A. Deodhar, "Osteoporosis and vertebral fractures in ankylosing spondylitis," *Current Opinion in Rheumatology*, vol. 25, no. 4, pp. 509–516, 2013.

[98] B. Osta, G. Benedetti, and P. Miossec, "Classical and paradoxical effects of TNF-$\alpha$ on bone homeostasis," *Frontiers in Immunology*, vol. 5, article 48, 2014.

[99] X. Baraliakos, H. Haibel, J. Listing, J. Sieper, and J. Braun, "Continuous long-term anti-TNF therapy does not lead to an increase in the rate of new bone formation over 8 years in patients with ankylosing spondylitis," *Annals of the Rheumatic Diseases*, vol. 73, no. 4, pp. 710–715, 2014.

[100] R. J. François, L. Neure, J. Sieper, and J. Braun, "Immunohistological examination of open sacroiliac biopsies of patients with ankylosing spondylitis: detection of tumour necrosis factor $\alpha$alpha in two patients with early disease and transforming growth factor beta in three more advanced cases," *Annals of the Rheumatic Diseases*, vol. 65, no. 6, pp. 713–720, 2006.

[101] J. Sieper, J. Braun, J. Kay et al., "Sarilumab for the treatment of ankylosing spondylitis: results of a Phase II, randomised, double-blind, placebo-controlled study (ALIGN)," *Annals of the Rheumatic Diseases*, 2014.

[102] J. Sieper, B. Porter-Brown, L. Thompson, O. Harari, and M. Dougados, "Assessment of short-term symptomatic efficacy of tocilizumab in ankylosing spondylitis: results of randomised, placebo-controlled trials," *Annals of the Rheumatic Diseases*, vol. 73, no. 1, pp. 95–100, 2014.

[103] H. Shen, J. C. Goodall, and J. S. Hill Gaston, "Frequency and phenotype of peripheral blood Th17 cells in ankylosing spondylitis and rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 60, no. 6, pp. 1647–1656, 2009.

[104] L. Zhang, Y. G. Li, Y. H. Li et al., "Increased frequencies of th22 cells as well as th17 cells in the peripheral blood of patients with ankylosing spondylitis and rheumatoid arthritis," *PLoS ONE*, vol. 7, Article ID e31000, 2012.

[105] D. Baeten, X. Baraliakos, J. Braun et al., "Anti-interleukin-17A monoclonal antibody secukinumab in treatment of ankylosing spondylitis: a randomised, double-blind, placebo-controlled trial," *The Lancet*, vol. 382, no. 9906, pp. 1705–1713, 2013.

[106] J. F. Zambrano-Zaragoza, J. M. Agraz-Cibrian, C. González-Reyes, M. D. J. Durán-Avelar, and N. Vibanco-Pérez, "Ankylosing spondylitis: from cells to genes," *International Journal of Inflammation*, vol. 2013, Article ID 501653, 16 pages, 2013.

[107] D. Stupphann, M. Rauner, D. Krenbek et al., "Intracellular and surface RANKL are differentially regulated in patients with ankylosing spondylitis," *Rheumatology International*, vol. 28, no. 10, pp. 987–993, 2008.

[108] C. H. Im, E. H. Kang, J. Y. Ki et al., "Receptor activator of nuclear factor kappa B ligand-mediated osteoclastogenesis is elevated in ankylosing spondylitis," *Clinical and Experimental Rheumatology*, vol. 27, no. 4, pp. 620–625, 2009.

[109] A. Leibbrandt and J. M. Penninger, "RANK/RANKL: regulators of immune responses and bone physiology," *Annals of the New York Academy of Sciences*, vol. 1143, pp. 123–150, 2008.

[110] A. Kaser, S. Zeissig, and R. S. Blumberg, "Inflammatory bowel disease," *Annual Review of Immunology*, vol. 28, pp. 573–621, 2010.

[111] K. L. Wallace, L.-B. Zheng, Y. Kanazawa, and D. Q. Shih, "Immunopathology of inflammatory bowel disease," *World Journal of Gastroenterology*, vol. 20, no. 1, pp. 6–21, 2014.

[112] D. Q. Shih, S. R. Targan, and D. McGovern, "Recent advances in IBD pathogenesis: genetics and immunobiology," *Current Gastroenterology Reports*, vol. 10, no. 6, pp. 568–575, 2008.

[113] L. E. Targownik, C. N. Bernstein, and W. D. Leslie, "Inflammatory bowel disease and the risk of osteoporosis and fracture," *Maturitas*, vol. 76, no. 4, pp. 315–319, 2013.

[114] G. Rogler and T. Andus, "Cytokines in inflammatory bowel disease," *World Journal of Surgery*, vol. 22, no. 4, pp. 382–389, 1998.

[115] M. F. Neurath and S. Finotto, "IL-6 signaling in autoimmunity, chronic inflammation and inflammation-associated cancer," *Cytokine and Growth Factor Reviews*, vol. 22, no. 2, pp. 83–89, 2011.

[116] H. C. Reinecker, M. Steffen, T. Witthoeft et al., "Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease," *Clinical and Experimental Immunology*, vol. 94, no. 1, pp. 174–181, 1993.

[117] S. G. Veerappan, C. A. O'Morain, J. S. Daly, and B. M. Ryan, "Review article: the effects of antitumour necrosis factor-$\alpha$ on bone metabolism in inflammatory bowel disease," *Alimentary Pharmacology and Therapeutics*, vol. 33, no. 12, pp. 1261–1272, 2011.

[118] P. Miheller, G. Muzes, K. Rácz et al., "Changes of OPG and RANKL concentrations in Crohn's disease after infliximab therapy," *Inflammatory Bowel Diseases*, vol. 13, no. 11, pp. 1379–1384, 2007.

[119] N. Franchimont, V. Putzeys, J. Collette et al., "Rapid improvement of bone metabolism after infliximab treatment in Crohn's disease," *Alimentary Pharmacology and Therapeutics*, vol. 20, no. 6, pp. 607–614, 2004.

[120] M. Pazianas, A. D. Rhim, A. M. Weinberg, C. Su, and G. R. Lichtenstein, "The effect of anti-TNF-alpha therapy on spinal bone mineral density in patients with Crohn's disease," *Annals of the New York Academy of Sciences*, vol. 1068, no. 1, pp. 543–556, 2006.

[121] A. R. Moschen, A. Kaser, B. Enrich et al., "The RANKL/OPG system is activated in inflammatory bowel diseases and relates to the state or bone loss," *Gut*, vol. 54, no. 4, pp. 479–487, 2005.

[122] W. Jiang, J. Su, X. Zhang et al., "Elevated levels of Th17 cells and Th17-related cytokines are associated with disease activity in patients with inflammatory bowel disease," *Inflammation Research*, vol. 63, no. 11, pp. 943–950, 2014.

[123] D. Fina, M. Sarra, M. C. Fantini et al., "Regulation of gut inflammation and Th17 cell response by interleukin-21," *Gastroenterology*, vol. 134, no. 4, pp. 1038.e2–1048.e2, 2008.

[124] G. Monteleone, I. Monteleone, D. Fina et al., "Interleukin-21 enhances T-helper cell type I signaling and interferon-$\gamma$ production in Crohn's disease," *Gastroenterology*, vol. 128, no. 3, pp. 687–694, 2005.

[125] J. B. Seidelin, G. Rogler, and O. H. Nielsen, "A role for interleukin-33 in TH2-polarized intestinal inflammation," *Mucosal Immunology*, vol. 4, no. 5, pp. 496–502, 2011.

[126] L. R. Lopetuso, S. Chowdhry, and T. T. Pizarro, "Opposing functions of classic and novel IL-1 family members in gut health and disease," *Frontiers in Immunology*, vol. 4, article 181, 2013.

[127] G. Palmer, D. Talabot-Ayer, C. Lamacchia et al., "Inhibition of interleukin-33 signaling attenuates the severity of experimental arthritis," *Arthritis and Rheumatism*, vol. 60, no. 3, pp. 738–749, 2009.

[128] S. H. Mun, N. Y. Ko, H. S. Kim et al., "Interleukin-33 stimulates formation of functional osteoclasts from human $CD14^+$ monocytes," *Cellular and Molecular Life Sciences*, vol. 67, no. 22, pp. 3883–3892, 2010.

[129] S. Mascheretti, J. Hampe, T. Kühbacher et al., "Pharmacogenetic investigation of the TNF/TNF-receptor system in patients with chronic active Crohn's disease treated with infliximab," *Pharmacogenomics Journal*, vol. 2, no. 2, pp. 127–136, 2002.

[130] P. Rutgeerts, W. J. Sandborn, B. G. Feagan et al., "Infliximab for induction and maintenance therapy for ulcerative colitis," *The New England Journal of Medicine*, vol. 353, no. 23, pp. 2462–2476, 2005.

[131] S. Bank, P. S. Andersen, J. Burisch et al., "Associations between functional polymorphisms in the NFkappaB signaling pathway and response to anti-TNF treatment in Danish patients with inflammatory bowel disease," *The Pharmacogenomics Journal*, vol. 14, pp. 526–534, 2014.

[132] D.-L. Su, Z.-M. Lu, M.-N. Shen, X. Li, and L.-Y. Sun, "Roles of pro- and anti-inflammatory cytokines in the pathogenesis of SLE," *Journal of Biomedicine and Biotechnology*, vol. 2012, Article ID 347141, 15 pages, 2012.

[133] O. P. Rekvig, C. Putterman, C. Casu et al., "Autoantibodies in lupus: culprits or passive bystanders?" *Autoimmunity Reviews*, vol. 11, no. 8, pp. 596–603, 2012.

[134] C. E. Weckerle, D. Mangale, B. S. Franek et al., "Large-scale analysis of tumor necrosis factor $\alpha$ levels in systemic lupus erythematosus," *Arthritis & Rheumatism*, vol. 64, no. 9, pp. 2947–2952, 2012.

[135] M. Aringer and J. S. Smolen, "Therapeutic blockade of TNF in patients with SLE—Promising or crazy?" *Autoimmunity Reviews*, vol. 11, no. 5, pp. 321–325, 2012.

[136] H.-Y. Chun, J.-W. Chung, H.-A. Kim et al., "Cytokine IL-6 and IL-10 as biomarkers in systemic lupus erythematosus," *Journal of Clinical Immunology*, vol. 27, no. 5, pp. 461–466, 2007.

[137] O. Dienz, S. M. Eaton, J. P. Bond et al., "The induction of antibody production by IL-6 is indirectly mediated by IL-21 produced by $CD4^+$ T cells," *Journal of Experimental Medicine*, vol. 206, no. 1, pp. 69–78, 2009.

[138] S. Wan, C. Xia, and L. Morel, "IL-6 produced by dendritic cells from lupus-prone mice inhibits $CD4^+CD25^+$ T cell regulatory functions," *The Journal of Immunology*, vol. 178, no. 1, pp. 271–279, 2007.

[139] T. Tanaka, M. Narazaki, and T. Kishimoto, "Anti-interleukin-6 receptor antibody, tocilizumab, for the treatment of autoimmune diseases," *FEBS Letters*, vol. 585, no. 23, pp. 3699–3709, 2011.

[140] A. Doreau, A. Belot, J. Bastid et al., "Interleukin 17 acts in synergy with B cell-activating factor to influence B cell biology and the pathophysiology of systemic lupus erythematosus," *Nature Immunology*, vol. 10, no. 7, pp. 778–785, 2009.

[141] H.-C. Hsu, P. Yang, J. Wang et al., "Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice," *Nature Immunology*, vol. 9, no. 2, pp. 166–175, 2008.

[142] C. Albanesi, A. Cavani, and G. Girolomoni, "IL-17 is produced by nickel-specific T lymphocytes and regulates ICAM-1 expression and chemokine production in human keratinocytes: synergistic or antagonist effects with IFN-$\gamma$ and TNF-$\alpha$," *Journal of Immunology*, vol. 162, no. 1, pp. 494–502, 1999.

[143] C. K. Wong, L. C. W. Lit, L. S. Tam, E. K. M. Li, P. T. Y. Wong, and C. W. K. Lam, "Hyperproduction of IL-23 and IL-17 in patients with systemic lupus erythematosus: implications for Th17-mediated inflammation in auto-immunity," *Clinical Immunology*, vol. 127, no. 3, pp. 385–393, 2008.

[144] K. Shah, W.-W. Lee, S.-H. Lee et al., "Dysregulated balance of Th17 and Th1 cells in systemic lupus erythematosus," *Arthritis Research and Therapy*, vol. 12, no. 2, article R53, 2010.

[145] J. C. Martin, D. L. Baeten, and R. Josien, "Emerging role of IL-17 and Th17 cells in systemic lupus erythematosus," *Clinical Immunology*, vol. 154, no. 1, pp. 1–12, 2014.

[146] J. J. Hooks, H. M. Moutsopoulos, S. A. Geis, N. I. Stahl, J. L. Decker, and A. L. Notkins, "Immune interferon in the circulation of patients with autoimmune disease," *New England Journal of Medicine*, vol. 301, no. 1, pp. 5–8, 1979.

[147] M. K. Crow and K. A. Kirou, "Interferon-alpha in systemic lupus erythematosus," *Current Opinion in Rheumatology*, vol. 16, no. 5, pp. 541–547, 2004.

[148] J. Banchereau and V. Pascual, "Type I interferon in systemic lupus erythematosus and other autoimmune diseases," *Immunity*, vol. 25, no. 3, pp. 383–392, 2006.

[149] Z. Brkic, O. B. Corneth, C. G. van Helden-Meeuwsen et al., "T-helper 17 cell cytokines and interferon type I: partners in crime in systemic lupus erythematosus?" *Arthritis Research & Therapy*, vol. 16, article R62, 2014.

[150] Z. Liu, R. Bethunaickan, W. Huang et al., "Interferon-$\alpha$ accelerates murine systemic lupus erythematosus in a T cell-dependent manner," *Arthritis and Rheumatism*, vol. 63, no. 1, pp. 219–229, 2011.

[151] K. A. Kirou and E. Gkrouzman, "Anti-interferon alpha treatment in SLE," *Clinical Immunology*, vol. 148, no. 3, pp. 303–312, 2013.

[152] I. E. M. Bultink, "Osteoporosis and fractures in systemic lupus erythematosus," *Arthritis Care and Research*, vol. 64, no. 1, pp. 2–8, 2012.

[153] Y. Tang, H. Xie, J. Chen et al., "Activated NF-$\kappa$B in bone marrow mesenchymal stem cells from systemic lupus erythematosus patients inhibits osteogenic differentiation through downregulating smad signaling," *Stem Cells and Development*, vol. 22, no. 4, pp. 668–678, 2013.

[154] E. Svenungsson, I. Gunnarsson, G.-Z. Fei, I. E. Lundberg, L. Klareskog, and J. Frostegård, "Elevated triglycerides and low levels of high-density lipoprotein as markers of disease activity in association with up-regulation of the tumor necrosis factor

$\alpha$/tumor necrosis factor receptor system in systemic lupus erythematosus," *Arthritis & Rheumatism*, vol. 48, no. 9, pp. 2533–2540, 2003.

[155] E. Svenungsson, G.-Z. Fei, K. Jensen-Urstad, U. de Faire, A. Hamsten, and J. Frostegård, "TNF-$\alpha$: A link between hypertriglyceridaemia and inflammation in SLE patients with cardiovascular disease," *Lupus*, vol. 12, no. 6, pp. 454–461, 2003.

[156] L. S. Graham, F. Parhami, Y. Tintut, C. M. R. Kitchen, L. L. Demer, and R. B. Effros, "Oxidized lipids enhance RANKL production by T lymphocytes: implications for lipid-induced bone loss," *Clinical Immunology*, vol. 133, no. 2, pp. 265–275, 2009.

[157] F. Shen, M. J. Ruddy, P. Plamondon, and S. L. Gaffen, "Cytokines link osteoblasts and inflammation: microarray analysis of interleukin-17- and TNF-$\alpha$-induced genes in bone cells," *Journal of Leukocyte Biology*, vol. 77, no. 3, pp. 388–399, 2005.

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*Review Article*

# Pathogenesis of Bone Alterations in Gaucher Disease: The Role of Immune System

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Gaucher, the most prevalent lysosomal disorder, is an autosomal recessive inherited disorder due to a deficiency of glucocerebrosidase. Glucocerebrosidase deficiency leads to the accumulation of glucosylceramide primarily in cells of mononuclear-macrophage lineage. Clinical alterations are visceral, hematological, and skeletal. Bone disorder in Gaucher disease produces defects on bone metabolism and structure and patients suffer from bone pain and crisis. Skeletal problems include osteopenia, osteoporosis, osteolytic lesions, and osteonecrosis. On the other hand a chronic stimulation of the immune system is a well-accepted hallmark in this disease. In this review we summarize the latest findings in the mechanisms leading to the bone pathology in Gaucher disease in relationship with the proinflammatory state.

## 1. Osteoimmunology

A diverse number of interactions between bone and immune cells occur within the bone microenvironment. Bone and immune cells share the same progenitors residing in the bone marrow and these progenitors are under the effect of the same molecules including cytokines; these molecules can have a high influence in the hematopoiesis process, local immune responses, and bone cell development.

There is evidence that several immune cells can influence bone cell development and activity. However, the key players in this regulation are activated T-cells. After successful antigen-specific activation, T-cells produce a number of proinflammatory cytokines [1] that can act directly or indirectly on cells involved in bone turnover shifting bone balance towards bone resorption or bone generation.

The bone turnover process involves bone removal by resorbing osteoclasts and bone formation by osteoblasts. These processes are strictly regulated in physiological conditions, and this regulation implies the participation of osteocytes, which are the final step of osteoblast differentiation [2].

Osteoclasts are bone resorbing cells that derive from the same progenitors as macrophages and dendritic cells (monocyte/macrophage lineage) [3]. RANKL and macrophage colony stimulating factor (M-CSF) are essential for commitment of the common precursor to the osteoclast lineage and survival of differentiated osteoclasts. In addition numerous cytokines are also able to influence osteoclast differentiation and/or function [4].

Osteoblasts are the bone forming cells that originate from bone marrow-residing multipotent mesenchymal stem cells. Osteoblasts are one of the major sources of RANKL and in this manner they control bone resorption. These cells can influence immune cells and are critical regulators of the hematopoietic stem cells (HSC) from where immune and other blood cells derive [5].

RANKL is a transmembrane protein of the TNF superfamily encoded by the *Tnfsf11* gene. It is expressed on the surface of osteoblasts (at different stages of differentiation), osteocytes, stromal cells of undefined origin, B- and T-cells, synovial fibroblasts, hypertrophic chondrocytes, and even osteoclasts themselves. The receptor of RANKL is RANK,

which is encoded by the *Tnfrsf11a* gene. Upon stimulation of RANK by RANKL under costimulatory signals such as M-CSF, the process of osteoclast differentiation and maturation begins [6]. The third protein member of the osteoclastogenesis axis is called osteoprotegerin (OPG) and is encoded by the *Tnfrsf11b* gene. OPG functions as a soluble decoy receptor for RANKL, inhibiting RANKL interaction with RANK, thus acting like an antiosteoclastogenic molecule [7]. OPG is expressed by osteoblasts and other mesenchymal cells [8].

The RANK/RANKL/OPG axis is essential in osteoclast differentiation *in vivo* as mutations in genes encoding RANKL, RANK, or OPG lead to disorders with high bone pathology [9]. RANKL is presented in two different forms as a membrane-anchored molecule or as a soluble protein released by the action of matrix metalloproteinases [10]. Both forms of the protein have osteoclastogenesis activity; however, the membrane-anchored form functions more efficiently [11].

The expression of RANKL on mesenchymal cells, such as osteoblasts, is upregulated by osteoclastogenic factors such as vitamin D3, prostaglandin E2, parathyroid hormone, and several cytokines including IL-1, IL-6, IL-11, IL-17, and TNF-$\alpha$ [12].

## 2. Osteoimmunology in Pathological Conditions

The activation of immune cells is a requisite for defense of the host against pathogens; however, a persistent overactivation of effector cells under certain pathological conditions can result in tissue damage.

In the early 1980s, osteoclasts were identified throughout the synovium and at the synovium/bone interface in joints of rheumatoid arthritis (RA) patients [13]. These observations led to the determination that osteoclasts play an important role in certain pathological conditions [14, 15].

Inflammatory cytokines such as IL-1, IL-6, and TNF-$\alpha$ are present at high levels in the synovial fluid and synovium of RA patients. These cytokines have a potent capacity to induce the expression of RANKL on synovial fibroblasts and bone derived stromal cells and to affect osteoclast differentiation, thus directly contributing to the bone destruction process [13].

Osteoporosis (OP) has been traditionally considered as an endocrine disease resulting mainly from the estrogens decline after menopause. This change affects bone remodeling, leading to higher risk of fractures. Since the endocrine point of view by itself does not completely explain the pathogenesis of OP, the osteoimmunological approach raised and suggested that the production of proinflammatory cytokines such as TNF-$\alpha$, IL-1, IL-6, IL-7, and IFN-$\gamma$ by activated T lymphocytes could contribute to menopausal changes in bone dynamics [16, 17].

Phenylketonuria (PKU) is an inborn error of amino acid metabolism resulting from deficiency of phenylalanine hydroxylase, the key enzyme for phenylalanine metabolism. Bone impairment has been widely documented in PKU, using both radiological and ultrasound methods [18, 19], and it is typically associated with increasing age. In 2010 it was shown that PKU patients present increased numbers of circulating osteoclast precursors with higher differentiation potential compared to healthy controls. TNF-$\alpha$ levels and the RANKL/OPG ratio were increased in supernatants of PBMC cultures from patients and it was shown that the increased osteoclast differentiation from PBMC was RANKL dependent [20].

## 3. Gaucher Disease

Gaucher disease is the most prevalent lysosomal disorder [21], of around 1 : 13,000–60,000, and with a higher frequency in the Ashkenazi Jewish population [22]. Gaucher disease (GD; MIM #230800) is an autosomal recessive inherited lysosomal storage disorder that is due to a deficiency of glucocerebrosidase (acid beta glucosidase; GCase; EC 3.2.1.45). GCase deficiency results in progressive, intralysosomal accumulation of glucosylceramide in different tissues, primarily in cells of mononuclear-macrophage lineage. Lipid accumulation in macrophages results in engorged cells called "Gaucher cells." Rarely, a variant GD may be secondarily caused by deficiency of the saposin C, the activator of the enzyme [23].

The first specific treatment for a lysosomal disorder was introduced for Gaucher disease, the enzyme replacement therapy (ERT) [24].

Clinical phenotype of GD reflects a continuum ranging from neuronopathic forms (GD types II and III) to the more frequent visceral form (GD type I) and from early onset to late onset [25].

Type I is observed in 90% of cases and is characterized by the lack of CNS manifestations. Clinical alterations are visceral (hepatosplenomegaly without organ disfunction), hematological (anemia and thrombopenia), and skeletal [26].

Bone pathology remains the main problem for GD I patients after the introduction of enzyme replacement therapy. Bone disease is a common and often painful and disabling manifestation of GD. Multiple compartments of bone that are affected are caused by alterations in bone metabolism (turnover, remodeling, and mineralization).

Almost all GD patients develop skeletal complications, consisting mainly of remodeling failure, osteopenia, osteoporosis, marrow infiltration, avascular necrosis, and osteolysis [27]. It may be suggested that patients with early onset GD I are at risk of skeletal disease. One of the early signs is the typical "Erlenmeyer flask" deformity of the distal femur. These changes predominantly affect long bones and the vertebrae. Patients could be asymptomatic with or without radiological signs or present symptoms including bone pain involving one limb or joint, avascular necrosis, or pathological fractures. An international registry of Gaucher patients worldwide revealed that 62% of them had some form of radiologic bone disease and 43% experienced bone pain [28]. The M$\phi$ are prominent in the bone marrow and contribute to acute episodes of osteonecrosis, particularly during growth. Necrosis of the marrow leads to impaired

function of joints. Other effects on the skeleton include local swellings known as Gaucheromas.

Imaging methodologies for the evaluation of skeletal involvement, such as conventional (plain) radiography and scintigraphy, MRI, computed tomography, or dual energy X-ray absorptiometry, are currently employed and provide accurate evaluation and staging of bone lesions in GD [29].

Much evidence demonstrates substantial improvement of hematological and visceral parameters upon introduction of specific ERT for Gaucher patients [30]. However, bone tissue does not respond equally; it is, in some degree, refractory to therapy. Patients at risk may benefit from early intervention with ERT, although many lesions and osteonecrosis are irreversible. Enzyme therapy cannot reverse established osseous injury [31]. Several prospective studies have been performed to evaluate the effectiveness of ERT in treating skeletal pathology. Bone pain is present at baseline in around two-thirds of the patients. Some patients improve in this aspect, but 40% of patients remain with this symptom after 18 months of treatment. In a recent study of patients treated with imiglucerase for 10 years, a positive effect was observed in skeletal symptoms, as well as a reduction of bone pain and crises in patients who suffer from them at baseline. Moreover, most of the patients who did not report bone symptoms at baseline continued to be pain-free after 10 years of ERT [32].

Bone mineral density tends to increase during therapy, but the response is slow [31]. Patients with preexistent skeletal complications tend to suffer incidents during ERT, such as medullary infarctions, avascular necrosis, or fractures, but the frequency of these events is reduced [33]. Low bone density manifests early in children with GD, and mineral density deficit is maximal in the adolescent period. Moreover, this group is most responsive to ERT, underscoring the importance of early diagnosis and intervention to achieve optimal peak bone mass [34]. In the largest study with treated pediatric patients bone mineral health was impaired in a large proportion of the group before ERT and improved considerably with treatment [35].

## 4. Inflammation in Gaucher Disease

A chronic stimulation of the immune system is a well-accepted hallmark in GD. Studies of the proinflammatory state in patients were mainly focused on analyzing cytokine levels in sera [36–38]. Although there is a high variation among patients, increased levels of IL-1$\alpha$, IL-1$\beta$, IL-1Ra, sIL-2R, IL-6, IL-8, IL-10, IL-18, TNF-$\alpha$, TGF-$\beta$, M-CSF, MIP-1, and CCL18 have been reported in sera [39–41].

Macrophages (M$\phi$) are the principal cell type compromised in patients with GD. M$\phi$ have several different functions including tissue remodeling and host defense; on the other hand they play central roles in many disease processes. They can secrete both anti- or proinflammatory cytokines depending on the activation signals. Upon activation, two main phenotypes of M$\phi$ could be produced: classical or alternative, depending on environment present at the time of the stages of activation [42]. Gaucher cells resemble alternative activated M$\phi$ [43], characterized by the expression of chitotriosidase and CCL18.

Several immune cells have been shown to be impaired in GD including monocytes, M$\phi$, dendritic cells, and T- and B-cells [44–46]. It has been shown that monocytes in GD patients expressed higher levels of CD1d and MHCII on their surface, which could lead to an increased T-cell activation [47]. Abnormalities in the B-cell subset are mainly IgG and IgM hypergammaglobulinemia and plasmacytosis [48]. An increased incidence of gammopathies and multiple myeloma has been reported, further showing the interplay between Gaucher cells and the immune system [49].

A GD murine model was generated, in which the *GBA* gene was conditionally deleted on hematopoietic cells [50]. This model presented all the hallmark characteristics of GD I, including organomegaly, and it was the only murine model so far to show bone involvement. In this model an alteration of immune cell compartment was observed. This alteration included thymic maturation impairment with higher levels of $CD4^+$ and antigen-presenting cells. What is more, activated B-cells on the thymus were also increased, which could explain the alteration of normal T-cell maturation [51].

In another murine model of GD, higher levels of $CD4^+$ cells were found on the lungs, spleen, and liver as well as an increased expression of costimulatory molecules [52]. Higher levels of proinflammatory cytokines including IFN-$\gamma$, IL-12p40, TNF-$\alpha$, IL-17A/F, IL-6, and TGF-$\beta$ were found in sera of these mice. When T-cells were cocultured with dendritic cells in the presence of glucosylceramide, higher levels of Th1/Th17 cytokines were secreted.

More recently, using a different approach, Panicker et al. differentiated M$\phi$ from patient induced pluripotent stem cells (hiPSC); with this model they showed increased production of IL-1$\beta$, TNF-$\alpha$, and IL-6 by GD derived M$\phi$ and an exacerbated response to LPS treatment [53].

This deregulation of immune system cells is tightly related to the increased levels of cytokines and chemokines. These molecules are secreted by the immune cells, which, in turn, are recruited and activated by chemokines and cytokines, respectively. This could create a loop in which immune cells from Gaucher patients are being continuously activated, leading to systemic and focal activation of the immune system.

## 5. Osteoclast-Osteoblast Uncoupling in Gaucher Disease

The molecular and cellular bases of GD bone physiopathology are not well understood and opposing studies have emerged in the last few years. As mentioned before in 2010, Mistry et al. [50] generated a conditional KO mouse model of GD I which presented the main GD clinical hallmarks. The most striking feature about this model is the presence of bone involvement as previous mouse models of GD did not present bone involvement. Bone manifestations included medullar infarctions with associated avascular necrosis and osteopenia at all sites. The bone formation rate presented

a significant impairment in these mice while the quantification of TRAP-labeled surfaces did not present differences.

A significant impairment in osteoblast proliferation and differentiation was present in the model, while osteoclast differentiation and activity did not seem to be altered. The impairment on osteoblast proliferation was shown to be dependent on a decrease in PKC activity due to the accumulation of glucosylsphingosine and, to a lesser extent, glucosylceramide. More recent studies present sphingosine as the most probable candidate for osteoblast impairment in the mouse model [54]. These findings suggest that bone complications in GD would result from an osteoblast source without osteoclast involvement [50, 54].

Different reports have shown the involvement of osteoclasts on GD bone pathophysiology. Using an *in vitro* model of GD in which mesenchymal stem cells and monocytes were exposed to conduritol-$\beta$-epoxide (CBE), a specific glucocerebrosidase inhibitor, Lecourt et al. showed that although direct CBE treatment had no effect on osteoclast differentiation if mesenchymal stem cells were cultured in the presence of conditioned media from CBE-exposed monocytes, an increased osteoclastogenesis and resorption activity was detected [55].

Our group showed, using a similar approach, that treatment of osteoclast precursors with conditioned media from peripheral blood mononuclear cells (PBMCs) exposed to CBE resulted in an increased level of osteoclast differentiation when compared to control conditioned media.

What is more, we showed that one of the central molecules involved in the increased osteoclast differentiation was the proinflammatory cytokine TNF-$\alpha$ and that T-cells also played an important role in this process [56]. The same results were obtained using a mice model in which conditioned media were obtained from peritoneal M$\phi$ or splenocytes exposed to CBE; in this model involvement of TNF-$\alpha$ was also shown using osteoclast precursors derived from TNF-$\alpha$ receptor deficient mice [57]. In addition to this we could show that treatment of the osteoblastic cell line MC3T3 with conditioned media from CBE treated M$\phi$ reduced mineralization and collagen deposit [57]. These results would indicate an impairment of both osteoclast and osteoblast activity in GD leading to bone loss as the involvement of immune cells and molecules in this process.

The group of Reed et al. isolated PBMC from patients with GD and showed that patients' monocytes, when exposed to osteoclastogenic mediators, presented a higher differentiation towards active osteoclasts. What is more, osteoclasts differentiated from patients had bigger diameter and a greater number of nuclei when compared with osteoclasts differentiated from healthy controls' PBMCs. They showed that the higher osteoclastogenic potential presented a clinical correlation with patient's bone involvement [58].

## 6. Future Perspectives

GD is the most common lysosomal disorder and the first for which specific treatment has been developed. Bone disease in Gaucher patients is one of the most disabling features of the disease, so the possibility of knowing the mechanisms underlying the bone pathology is a main challenge to ameliorate the quality of life of patients.

Studies are based on the explanation of the cellular and molecular pathways that result upon glucosylceramide accumulation in M$\phi$ and the possible relationship with different bone cells.

The bases of osteoimmunology are being applied to bring light in this aspect. In this regard, there are several questions to be answered. T-cell involvement and a better understanding of the effects and importance of proinflammatory cytokines such as TNF-$\alpha$ on bone pathology in GD are necessary.

On the other hand, crosstalk between osteoblasts and osteoclasts in GD could provide new mechanisms involved in the process of bone loss.

Finally the effect of ERT and substrate reduction therapy on bone involvement is a central aspect to be studied, especially, how these treatments affect different bone cells and their function.

The results of basic research will be of utility in order to identify new targets for coadjuvant therapies to treat skeletal pathology in GD.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## References

[1] P. J. Delves and I. M. Roitt, "The immune system. First of two parts," *The New England Journal of Medicine*, vol. 343, no. 1, pp. 37–49, 2000.

[2] J. Xiong and C. A. O'Brien, "Osteocyte RANKL: new insights into the control of bone remodeling," *Journal of Bone and Mineral Research*, vol. 27, no. 3, pp. 499–505, 2012.

[3] T. J. de Vries, T. Schoenmaker, B. Hooibrink, P. J. M. Leenen, and V. Everts, "Myeloid blasts are the mouse bone marrow cells prone to differentiate into osteoclasts," *Journal of Leukocyte Biology*, vol. 85, no. 6, pp. 919–927, 2009.

[4] H. Takayanagi, K. Sato, A. Takaoka, and T. Taniguchi, "Interplay between interferon and other cytokine systems in bone metabolism," *Immunological Reviews*, vol. 208, pp. 181–193, 2005.

[5] T. Yin and L. Li, "The stem cell niches in bone," *The Journal of Clinical Investigation*, vol. 116, no. 5, pp. 1195–1201, 2006.

[6] W. J. Boyle, W. S. Simonet, and D. L. Lacey, "Osteoclast differentiation and activation," *Nature*, vol. 423, no. 6937, pp. 337–342, 2003.

[7] W. S. Simonet, D. L. Lacey, C. R. Dunstan et al., "Osteoprotegerin: a novel secreted protein involved in the regulation of bone density," *Cell*, vol. 89, no. 2, pp. 309–319, 1997.

[8] R. Siddappa, H. Fernandes, J. Liu, C. van Blitterswijk, and J. de Boer, "The response of human mesenchymal stem cells to osteogenic signals and its impact on bone tissue engineering," *Current Stem Cell Research and Therapy*, vol. 2, no. 3, pp. 209–220, 2007.

[9] A. Leibbrandt and J. M. Penninger, "RANK/RANKL: regulators of immune responses and bone physiology," *Annals of the New York Academy of Sciences*, vol. 1143, pp. 123–150, 2008.

[10] F. Kanamaru, H. Iwai, T. Ikeda, A. Nakajima, I. Ishikawa, and M. Azuma, "Expression of membrane-bound and soluble receptor activator of NF-kappaB ligand (RANKL) in human T cells," *Immunology Letters*, vol. 94, no. 3, pp. 239–246, 2004.

[11] T. Nakashima, M. Hayashi, T. Fukunaga et al., "Evidence for osteocyte regulation of bone homeostasis through RANKL expression," *Nature Medicine*, vol. 17, no. 10, pp. 1231–1234, 2011.

[12] T. Nakashima, Y. Kobayashi, S. Yamasaki et al., "Protein expression and functional difference of membrane-bound and soluble receptor activator of NF-$\kappa$B ligand: modulation of the expression by osteotropic factors and cytokines," *Biochemical and Biophysical Research Communications*, vol. 275, no. 3, pp. 768–775, 2000.

[13] H. Takayanagi, "Osteoimmunology: shared mechanisms and crosstalk between the immune and bone systems," *Nature Reviews Immunology*, vol. 7, no. 4, pp. 292–304, 2007.

[14] E. M. Gravallese, Y. Harada, J.-T. Wang, A. H. Gorn, T. S. Thornhill, and S. R. Goldring, "Identification of cell types responsible for bone resorption in rheumatoid arthritis and juvenile rheumatoid arthritis," *The American Journal of Pathology*, vol. 152, no. 4, pp. 943–951, 1998.

[15] H. Takayanagi, H. Oda, S. Yamamoto et al., "A new mechanism of bone destruction in rheumatoid arthritis: synovial fibroblasts induce osteoclastogenesis," *Biochemical and Biophysical Research Communications*, vol. 240, no. 2, pp. 279–286, 1997.

[16] R. L. Jilka, G. Hangoc, G. Girasole et al., "Increased osteoclast development after estrogen loss: mediation by interleukin-6," *Science*, vol. 257, no. 5066, pp. 88–91, 1992.

[17] R. Pacifici, "Estrogen deficiency, T cells and bone loss," *Cellular Immunology*, vol. 252, no. 1-2, pp. 68–80, 2008.

[18] L. Hillman, C. Schlotzhauer, D. Lee et al., "Decreased bone mineralization in children with phenylketonuria under treatment," *European Journal of Pediatrics*, vol. 155, supplement 1, pp. S148–S152, 1996.

[19] M. P. A. Hoeks, M. den Heijer, and M. C. H. Janssen, "Adult issues in phenylketonuria," *The Netherlands Journal of Medicine*, vol. 67, no. 1, pp. 2–7, 2009.

[20] I. Roato, F. Porta, A. Mussa et al., "Bone impairment in phenylketonuria is characterized by circulating osteoclast precursors and activated T cell increase," *PLoS ONE*, vol. 5, no. 11, Article ID e14167, 2010.

[21] P. J. Meikle, J. J. Hopwood, A. E. Clague, and W. F. Carey, "Prevalence of lysosomal storage disorders," *The Journal of the American Medical Association*, vol. 281, no. 3, pp. 249–254, 1999.

[22] J. Wittmann, E. Karg, S. Turi et al., "Newborn screening for lysosomal storage disorders in Hungary," *JIMD Reports*, vol. 6, pp. 117–125, 2012.

[23] G. A. Grabowski, "Gaucher disease and other storage disorders," *Hematology*, vol. 2012, no. 1, pp. 13–18, 2012.

[24] N. W. Barton, R. O. Brady, J. M. Dambrosia et al., "Replacement therapy for inherited enzyme deficiency—macrophage-targeted glucocerebrosidase for Gaucher's disease," *The New England Journal of Medicine*, vol. 324, no. 21, pp. 1464–1470, 1991.

[25] P. Mistry and D. P. Germain, "Phenotype variations in Gaucher disease," *La Revue de Médecine Interne*, vol. 27, supplement 1, pp. S3–S10, 2006.

[26] A. Zimran, G. Altarescu, B. Rudensky, A. Abrahamov, and D. Elstein, "Survey of hematological aspects of Gaucher disease," *Hematology*, vol. 10, no. 2, pp. 151–156, 2005.

[27] M. Itzchaki, E. Lebel, A. Dweck et al., "Orthopedic considerations in Gaucher disease since the advent of enzyme replacement therapy," *Acta Orthopaedica Scandinavica*, vol. 75, no. 6, pp. 641–653, 2004.

[28] R. J. Wenstrup, M. Roca-Espiau, N. J. Weinreb, and B. Bembi, "Skeletal aspects of Gaucher disease: a review," *The British Journal of Radiology*, vol. 75, supplement 1, pp. A2–A12, 2002.

[29] R. Katz, T. Booth, R. Hargunani, P. Wylie, and B. Holloway, "Radiological aspects of Gaucher disease," *Skeletal Radiology*, vol. 40, no. 12, pp. 1505–1513, 2011.

[30] D. Elstein, A. J. Foldes, D. Zahrieh et al., "Significant and continuous improvement in bone mineral density among type 1 Gaucher disease patients treated with velaglucerase alfa: 69-month experience, including dose reduction," *Blood Cells, Molecules, & Diseases*, vol. 47, no. 1, pp. 56–61, 2011.

[31] K. B. Sims, G. M. Pastores, N. J. Weinreb et al., "Improvement of bone disease by imiglucerase (Cerezyme) therapy in patients with skeletal manifestations of type 1 Gaucher disease: results of a 48-month longitudinal cohort study," *Clinical Genetics*, vol. 73, no. 5, pp. 430–440, 2008.

[32] N. J. Weinreb, J. Goldblatt, J. Villalobos et al., "Long-term clinical outcomes in type 1 Gaucher disease following 10 years of imiglucerase treatment," *Journal of Inherited Metabolic Disease*, vol. 36, no. 3, pp. 543–553, 2013.

[33] J. Stirnemann, N. Belmatoug, C. Vincent, O. Fain, B. Fantin, and F. Mentré, "Bone events and evolution of biologic markers in Gaucher disease before and during treatment," *Arthritis Research and Therapy*, vol. 12, no. 4, article R156, 2010.

[34] P. K. Mistry, N. J. Weinreb, P. Kaplan, J. A. Cole, A. R. Gwosdow, and T. Hangartner, "Osteopenia in Gaucher disease develops early in life: response to imiglucerase enzyme therapy in children, adolescents and adults," *Blood Cells, Molecules, and Diseases*, vol. 46, no. 1, pp. 66–72, 2011.

[35] H. Andersson, P. Kaplan, K. Kacena, and J. Yee, "Eight-year clinical outcomes of long-term enzyme replacement therapy for 884 children with gaucher disease type 1," *Pediatrics*, vol. 122, no. 6, pp. 1182–1190, 2008.

[36] V. Barak, M. Acker, B. Nisman et al., "Cytokines in Gaucher's disease," *European Cytokine Network*, vol. 10, no. 2, pp. 205–210, 1999.

[37] C. E. M. Hollak, L. Evers, J. M. F. G. Aerts, and M. H. J. van Oers, "Elevated levels of M-CSF, sCD14 and IL8 in type 1 Gaucher disease," *Blood Cells, Molecules and Diseases*, vol. 23, no. 2, pp. 201–212, 1997.

[38] H. Michelakakis, C. Spanou, A. Kondyli et al., "Plasma tumor necrosis factor-a (TNF-a) levels in Gaucher disease," *Biochimica et Biophysica Acta*, vol. 1317, no. 3, pp. 219–222, 1996.

[39] R. G. Boot, M. Verhoek, M. de Fost et al., "Marked elevation of the chemokine CCL18/PARC in Gaucher disease: a novel surrogate marker for assessing therapeutic intervention," *Blood*, vol. 103, no. 1, pp. 33–39, 2004.

[40] M. J. van Breemen, M. de Fost, J. S. A. Voerman et al., "Increased plasma macrophage inflammatory protein (MIP)-1alpha and MIP-1beta levels in type 1 Gaucher disease," *Biochimica et Biophysica Acta—Molecular Basis of Disease*, vol. 1772, no. 7, pp. 788–796, 2007.

[41] M. Yoshino, Y. Watanabe, Y. Tokunaga et al., "Roles of specific cytokines in bone remodeling and hematopoiesis in Gaucher disease," *Pediatrics International*, vol. 49, no. 6, pp. 959–965, 2007.

[42] S. Gordon and F. O. Martinez, "Alternative activation of macrophages: mechanism and functions," *Immunity*, vol. 32, no. 5, pp. 593–604, 2010.

[43] L. A. Boven, M. van Meurs, R. G. Boot et al., "Gaucher cells demonstrate a distinct macrophage phenotype and resemble alternatively activated macrophages," *The American Journal of Clinical Pathology*, vol. 122, no. 3, pp. 359–369, 2004.

[44] C. Braudeau, J. Graveleau, M. Rimbert et al., "Altered innate function of plasmacytoid dendritic cells restored by enzyme replacement therapy in Gaucher disease," *Blood Cells, Molecules, and Diseases*, vol. 50, no. 4, pp. 281–288, 2013.

[45] F. Camou and J.-F. Viallard, "Extended remission of B-cell lymphoma with monoclonal gammopathy in a patient with type 1 Gaucher disease treated with enzyme replacement therapy," *Blood Cells, Molecules, and Diseases*, vol. 48, no. 1, pp. 51–52, 2012.

[46] L. Lacerda, F. A. Arosa, R. Lacerda et al., "T cell numbers relate to bone involvement in Gaucher disease," *Blood Cells, Molecules, and Diseases*, vol. 25, no. 2, pp. 130–138, 1999.

[47] A. Balreira, L. Lacerda, C. Sá Miranda, and F. A. Arosa, "Evidence for a link between sphingolipid metabolism and expression of CD1d and MHC-class II: monocytes from Gaucher disease patients as a model," *British Journal of Haematology*, vol. 129, no. 5, pp. 667–676, 2005.

[48] G. E. Marti, E. T. Ryan, N. M. Papadopoulos et al., "Polyclonal B-cell lymphocytosis and hypergammaglobulinemia in patients with Gaucher disease," *American Journal of Hematology*, vol. 29, no. 4, pp. 189–194, 1988.

[49] T. H. Taddei, K. A. Kacena, M. Yang et al., "The underrecognized progressive nature of N370S Gaucher disease and assessment of cancer risk in 403 patients," *American Journal of Hematology*, vol. 84, no. 4, pp. 208–214, 2009.

[50] P. K. Mistry, J. Liu, M. Yang et al., "Glucocerebrosidase gene-deficient mouse recapitulates Gaucher disease displaying cellular and molecular dysregulation beyond the macrophage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 45, pp. 19473–19478, 2010.

[51] J. Liu, S. Halene, M. Yang et al., "Gaucher disease gene GBA functions in immune regulation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 25, pp. 10018–10023, 2012.

[52] M. K. Pandey, R. Rani, W. Zhang, K. Setchell, and G. A. Grabowski, "Immunological cell type characterization and Th1-Th17 cytokine production in a mouse model of Gaucher disease," *Molecular Genetics and Metabolism*, vol. 106, no. 3, pp. 310–322, 2012.

[53] L. M. Panicker, D. Miller, O. Awad et al., "Gaucher iPSC-derived macrophages produce elevated levels of inflammatory mediators and serve as a new platform for therapeutic development," *Stem Cells (Dayton, Ohio)*, vol. 32, no. 9, pp. 2338–2349, 2014.

[54] P. K. Mistry, J. Liu, L. Sun et al., "Glucocerebrosidase 2 gene deletion rescues type 1 Gaucher disease," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 13, pp. 4934–4939, 2014.

[55] S. Lecourt, V. Vanneaux, A. Cras et al., "Bone marrow microenvironment in an in vitro model of gaucher disease: consequences of glucocerebrosidase deficiency," *Stem Cells and Development*, vol. 21, no. 2, pp. 239–248, 2012.

[56] J. M. Mucci, R. Scian, P. N. De Francesco et al., "Induction of osteoclastogenesis in an in vitro model of Gaucher disease is mediated by T cells via TNF-$\alpha$," *Gene*, vol. 509, no. 1, pp. 51–59, 2012.

[57] J. M. Mucci, F. Suqueli García, P. N. de Francesco et al., "Uncoupling of osteoblast-osteoclast regulation in a chemical murine model of gaucher disease," *Gene*, vol. 532, no. 2, pp. 186–191, 2013.

[58] M. Reed, R. J. Baker, A. B. Mehta, and D. A. Hughes, "Enhanced differentiation of osteoclasts from mononuclear precursors in patients with Gaucher disease," *Blood Cells, Molecules, and Diseases*, vol. 51, no. 3, pp. 185–194, 2013.

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*Research Article*

# Death Receptor 3 (TNFRSF25) Increases Mineral Apposition by Osteoblasts and Region Specific New Bone Formation in the Axial Skeleton of Male DBA/1 Mice

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*Objectives*. Genome wide association studies identified TNFSF member TNF-like protein 1A (TL1A, TNFSF15) as a potential modulator of ankylosing spondylitis (AS). TL1A is the only confirmed TNFSF ligand of death receptor 3 (DR3, TNFRSF25); however, its role in disease pathology is not characterised. We evaluated DR3's role in controlling osteoblast- (OB-) dependent bone formation *in vitro* and *in vivo*. *Methods*. Osteoprogenitor cells and OB were cultured from male DR3-deficient ($DR3^{ko}$) and wild-type ($DR3^{wt}$) DBA/1 mice. DR3 and RANKL expression were tested by flow cytometry. Alkaline phosphatase and mineralization were quantified. Osteopontin, osteoprotegerin, and pro MMP-9 were measured by ELISA. A fluorescent probe (BoneTag) was used to measure *in vivo* mineralization in 10-month-old mice. *Results*. DR3 was expressed on osteoprogenitors and OB from $DR3^{wt}$ mice. Alkaline phosphatase, osteopontin, and mineral apposition were significantly elevated in $DR3^{wt}$ cultures. Levels of RANKL were comparable whilst osteoprotegerin was significantly increased in $DR3^{wt}$ cultures. *In vivo* incorporation of BoneTag was significantly lower in the thoracic vertebrae of 10-month-old $DR3^{ko}$ mice. *Conclusions*. These data identify new roles for DR3 in regulating OB-dependent bone mineral apposition. They potentially begin to explain the atypical pattern of new bone formation observed in the axial skeleton of grouped, aging DBA/1 mice.

## 1. Introduction

In a normal physiological state the process of bone remodeling is accomplished through the coupled activities of the osteoclast (OC) and the osteoblast (OB). The OC is responsible for the degradation of bone while the OB produces the organic matrix and aids in its mineralization [1, 2]. In certain pathological conditions, however, OC and OB activity can become uncoupled leading to an increase or decrease in bone resorption and bone formation. Increased bone formation is observed in the spondyloarthritides; delicate marginal syndesmophytes are common in ankylosing spondylitis (AS) whilst nonmarginal syndesmophytes are frequently observed in reactive arthritis and psoriatic arthritis (PsA) [3–5]. The cause of this excessive focal bone formation by OB is currently not clear. The immune system and increased expression of proinflammatory cytokines such as members of the tumour necrosis factor super family (TNFSF) and their receptors have been shown to be implicated [3]. Our study assesses the impact of the TNF receptor superfamily (TNFRSF) member death receptor 3 (DR3) upon OB-directed mineral apposition with a view to understanding how it modulates bone remodelling in the axial skeleton.

Identification of a link between the DR3/TL1A pathway and the spondyloarthritides was first demonstrated when single-nucleotide polymorphisms (SNPs) located in the direct vicinity of the *TNFSF15* gene were shown to be strongly associated with predisposition to spondyloarthropathy [6]. Further evidence for a role of the DR3/TL1A pathway in AS was provided by Konsta et al., who identified that serum

levels of TL1A are significantly elevated in AS patients over healthy controls [7]. The mechanism through which the DR3/TL1A pathway potentially drives the pathogenesis of the spondyloarthritides, however, is not clear. Expanded numbers of circulating $CD8^+$ $CD28^-$ T cells have been identified in AS patients, which correlated with a more severe course of disease [8]. A study by Twohig et al., demonstrated that TL1A signalling on TCR stimulated $CD8^+$ T cells resulted in increased proliferation [9]. The indirect effect of DR3/TL1A signalling in $CD8^+$ T cells upon the expansion of OBs or mineral apposition by OB, however, has not been resolved. Studies by Bu et al. [10] and Borysenko et al. [11] demonstrated the expression of DR3 on the surface of a human OB cell line and primary human OB. These findings imply that the DR3/TL1A signaling pathway could directly modulate apposition of bone matrix by OB, a notion that has not been investigated previously.

This study is the first to specifically investigate the functional role of DR3 on mineral apposition by OB *in vitro* and *in vivo* using male DBA/1 mice lacking the DR3 gene ($DR3^{ko}$). The spontaneous development of ankylosing enthesopathy has been reported previously in aging male DBA/1 mice and our study provides the first insight to DR3's role in controlling new bone formation by OB in the axial skeleton [12]. Here we report that OB-derived DR3 increases expression of the early OB differentiation marker alkaline phosphatase, increases expression of the transcription factors Runx2 and OSX, and regulates mineral apposition. MMP-9 and OPN production by OB were measured as surrogate markers of increased OB activation. OB and OC cross talk is an important factor in bone homeostasis. We measured OB-derived RANKL and OPG production to determine the role of DR3 in regulating OC differentiation by OB. These findings examine potentially important, hitherto unknown, mechanisms whereby DR3 regulates OB-dependent bone homeostasis.

## 2. Materials and Methods

*2.1. Animals.* All experiments were undertaken in male DBA/1 $DR3^{wt}$ and $DR3^{ko}$ mice generated in a $DR3^{het} \times DR3^{het}$ colony. The DBA/1 $DR3^{ko}$ strain was generated through backcrossing C57BL/6 $DR3^{het}$ mice [13] with DBA/1 wild-type mice for 7 generations. All DBA/1 $DR3^{wt}$ and $DR3^{ko}$ animals were generated in-house. Animals used for *in vitro* experiments were 8–12 weeks of age. Animals for the *in vivo* BoneTag experiments were 10 months of age. Animals were housed in filter top cages at a constant temperature and humidity on a 12-hour light/dark cycle. Food and water were available *ad libitum*. All procedures were approved by the Local Research Ethics Committee and performed in strict accordance with Home Office approved license PPL 30/2361.

*2.2. OPC and OB Cell Culture.* To obtain osteoprogenitors (OPCs) bone marrow was isolated from the femora of $DR3^{wt}$ ($n = 5$) and $DR3^{ko}$ ($n = 5$) DBA/1 mice and cultured in $\alpha$MEM supplemented with 20% foetal calf serum, 50 $\mu$g/mL Penicillin-Streptomycin (control medium) and 5% $CO_2$ at 37°C. At confluence OPCs were detached from the culture vessel by scraping and were replated in a 12-well plate at a density of $4 \times 10^4$ cells/well in control medium. Osteoblasts were subsequently differentiated from OPCs by addition of $\alpha$MEM supplemented with 10% foetal calf serum, 50 $\mu$g/mL Penicillin-Streptomycin, 10 mM $\beta$-glycerophosphate, 50 $\mu$g/mL ascorbic acid, and 10 nM dexamethasone (mineralisation medium). Osteoblasts were maintained for up to 26 days (5% $CO_2$ at 37°C) with medium changes every 3-4 days.

*2.3. Cellular Staining and Flow Cytometry.* OPCs ($DR3^{wt}$ $n = 6$, $DR3^{ko}$ $n = 4$) and OB ($DR3^{wt}$ $n = 3$, $DR3^{ko}$ $n = 2$) were cultured for 15 days using the method described above. Cells were removed from the culture vessel and incubated with Fc block (BD Pharmingen, CA, USA) for 15 min followed by a 30 min block with 2% normal goat serum. Cells were stained with either polyclonal $\alpha$-mouse DR3-biotin (BAF 2437; R&D Systems, MN, USA) followed by SA-APC (Molecular Probes, OR, USA) or PE-conjugated $\alpha$-mouse RANKL mAb (IK22/5; Santa Cruz Biotechnology Inc., TX, USA). Data were acquired on a BD Accuri C6 flow cytometer and analysed with CFlow software (BD Biosciences).

*2.4. TL1A RT-PCR.* RNA was extracted from OPCs and mineralising OB ($DR3^{wt}$ $n = 1$, $DR3^{ko}$ $n = 1$) using RNeasy (QIAGEN) following manufacturer's instructions and converted to cDNA using SuperScript II Reverse Transcriptase (Life Technologies). RT-PCR was performed according to standard Life Technologies protocols. PCR primers were as follows: TL1A, forward 5′-CAG CAG AAG GAT GGC AGA-3′ and reverse 5′-CTC TGG CCT GTG TCT ACA-3′, giving a 91-bp product; and $\beta$-actin, forward 5′-CGG CCA GGT CAT CAC TAT TG-3′ and reverse 5′-CTC AGT AAC CCG CCT AG-3′, giving a 450-bp product. The PCR comprised 33 cycles with an annealing temperature of 59°C. The PCR products were size fractionated by agarose gel electrophoresis and analysed using a UVP BioDoc It imaging system (Cambridge, UK).

*2.5. Quantitative PCR.* RNA was extracted from OPCs and OB and converted to cDNA as described above ($DR3^{wt}$ $n = 1$, $DR3^{ko}$ $n = 1$). Quantitative PCR was performed using the iCycler iQ system (Bio-Rad) with RT2 Real Time SYBR green PCR master mix (SA Biosciences) following the recommended protocol for SYBR green (Bio-Rad). Assays were run in duplicate. mRNA levels were determined using the comparative $C_t$ method and normalized to $\beta$-actin mRNA levels. Primers used for quantitative PCR were as follows: RUNX2, forward 5′-CCCTGAACTCTGCACCAAGT-3′ and reverse 5′-TGGCTCAGATAGGAGGGGTA-3′; OSX, forward 5′-TCTCCATCTGCCTGACTCCT-3′ and reverse 5′-CAGGGGACTGGAGCCATAGT-3′; $\beta$-actin as above.

*2.6. Alkaline Phosphatase and Mineralisation Staining.* Alkaline phosphatase (ALP) activity was determined in OB cultures ($DR3^{wt}$ $n = 5$, $DR3^{ko}$ $n = 5$) using SigmaFast BCIP/NBT stain (Sigma). The mineralized matrix was stained for calcium deposition by Alizarin-red staining. Briefly, at time points indicated in results, cultures were fixed with a 4%

formaldehyde/PBS solution for 15 min. Cultures were then incubated with either SigmaFast BCIP/NBT solution or Alizarin-red (1% solution in water) for 30 minutes. Cultures stained for mineral apposition were washed with 50% methanol to remove nonspecific background. Plates were air-dried and scanned and the percentage of the well was covered by ALP positive cells or mineral calculated with Image J (NIH).

*2.7. Measurement of Soluble Mediators in Cell Culture Supernatant.* Expression of soluble mediators in cell culture supernatants was analysed by ELISA using mouse osteoprotegerin (OPG), pro MMP-9, receptor activator of nuclear factor kappa-B ligand (RANKL) and osteopontin (OPN) DuoSet kits (all R&D Systems).

*2.8. In Vivo Imaging.* DBA/1 male mice were housed together as previously described [12]. Animals at 10 months ($DR3^{wt}$ $n = 3$, $DR3^{ko}$ $n = 3$) of age were subjected to either an intraperitoneal injection with IRDye 680 BoneTag optical probe (0.08 nmol/g) or equal volume of PBS as control. After 24 hours animals were sacrificed by $CO_2$ asphyxiation. Images were taken using the Xenogen IVIS 200 (excitation wavelength 676 nm, emission wavelength 698 nm, f-stop 1) and rectangular regions of interest of fixed dimensions were selected at the thoracic spine, knee, and tail. Total efficiency of the marker was analyzed using the Live Image software, version 2.50.2 (Caliper). The efficiency values obtained from the PBS control animals were subtracted from the values obtained with BoneTag, to account for background autofluorescence.

*2.9. Statistical Analysis.* Data are expressed as mean ± SEM and statistical analysis performed using the Mann-Whitney $U$ test for nonparametric data, unpaired $t$-test for parametric data, or 2-way analysis of variance (ANOVA) using GraphPad Prism software (GraphPad, San Diego, CA, USA). $P$ values of $\leq 0.05$ were considered significant and values of $\leq 0.01$ were considered highly significant.

## 3. Results

*3.1. Expression of DR3 and TL1A by OPCs and OB.* Expression of DR3 on the human MG63 osteosarcoma cell line has previously been demonstrated by Borysenko et al. [11]. Here we investigated DR3 expression on primary, murine OPCs and OB using flow cytometry. The signal from the surface of $DR3^{wt}$ OPCs was 1.8 ± 0.2-fold higher than the isotype control (Figure 1(a)) and was significantly higher than from $DR3^{ko}$ OPCs ($P \leq 0.05$). Levels of DR3 signal were reduced on $DR3^{wt}$ mineralising OB (1.4 ± 0.8-fold increase) compared to the OPCs. RT-PCR analysis of $DR3^{wt}$- and $DR3^{ko}$-derived OPCs and OB showed constitutive expression of *TL1A* mRNA across a 15 day time course (Figure 1(b)).

*3.2. DR3/TL1A Signalling Promotes RUNX2 and OSX Expression.* To identify whether DR3 signalling in OPCs and OB modulates Runx2 and OSX expression qPCR was performed on differentiating OB across a 14-day time course (Figure 1(c)). No difference in *RUNX2* and *OSX* levels was detected between the $DR3^{wt}$ and $DR3^{ko}$ cultures after the first 3 days of differentiation. At day 7 an increase in Runx2 gene expression was observed in the $DR3^{wt}$ cultures. At day 14 both Runx2 and OSX expression levels were elevated in the $DR3^{wt}$ cultures compared to the $DR3^{ko}$ cultures.

*3.3. Effect of DR3 on OB Differentiation, OPN and Pro MMP-9 Expression and Mineral Apposition.* To assess the effect of DR3 on OB differentiation and mineral apposition, mineralization assays were performed and expression of the early OB differentiation marker ALP was studied. At the earliest time point assessed (day 17), the percentage area of the wells demonstrating ALP-positive cells was elevated in the $DR3^{wt}$ (41 ± 5%) versus $DR3^{ko}$ (30 ± 5%) cultures. These levels did not vary significantly across the 26-day time course, translating into significantly increased levels of ALP staining in $DR3^{wt}$ cultures ($P \leq 0.05$; Figure 2(a)).

To see whether the increase in $DR3^{wt}$ ALP expression was accompanied by an increase in OB activation markers, levels of the $Ca^{2+}$ binding osteoid protein (OPN) and a regulator of bone matrix maturation (pro MMP-9) were measured in culture supernatants [14, 15]. Levels of OPN were significantly elevated in $DR3^{wt}$ cultures compared to $DR3^{ko}$ cultures over the 26-day time course ($P < 0.01$; Figure 2(b)). Levels of OPN peaked at day 17 in the $DR3^{wt}$ (252 ± 55 ng/mL) and at day 23 in the $DR3^{ko}$ cultures (178 ± 70 ng/mL). Levels of pro MMP-9 were initially higher at day 3 in the $DR3^{wt}$ cultures versus $DR3^{ko}$ (3.4 ± 0.5 ng/mL versus 1.5 ± 0.2 ng/mL; $P < 0.01$). $DR3^{wt}$ pro MMP-9 levels peaked at day 7 (4.1 ± 1.7 ng/mL) and decreased over the duration of the time course to 0.7 ± 0.3 ng/mL. In contrast, expression of pro MMP-9 in the $DR3^{ko}$ cultures increased over the culture period and peaked at day 20 (3.8 ± 1.7 ng/mL; Figure 2(c)).

To identify whether the increase in $DR3^{wt}$ OB differentiation and OPN expression correlated with an effect on mineral apposition, levels of mineralization in the cultures were evaluated. At day 17 comparable levels of mineralization were observed between the $DR3^{wt}$ (4±1%) and $DR3^{ko}$ (3±1%) cultures. Mineral apposition was significantly increased in the $DR3^{wt}$ cultures at day 23 (14±2%; $P < 0.001$) and day 26 (14±2%; $P < 0.001$) when compared to $DR3^{ko}$ cultures (5±1% and 5 ± 1%, resp.). This resulted in significantly increased levels of mineral apposition across the time course in the $DR3^{wt}$ cultures ($P < 0.001$; Figure 2(d)). Having demonstrated that DR3 affects OB differentiation and mineral apposition, *in vitro*, we investigated whether it could have a potential effect on OB control of osteoclast formation.

*3.4. Effect of DR3 on OB-Derived RANKL and OPG.* To investigate whether DR3 had a role in regulating OB control of OC formation, levels of OB-derived RANKL and OPG were studied. OPC and OB cell surface RANKL expression were comparable between $DR3^{wt}$ and $DR3^{ko}$ cells (Figure 3(a)). No soluble RANKL was detected in any culture supernatants

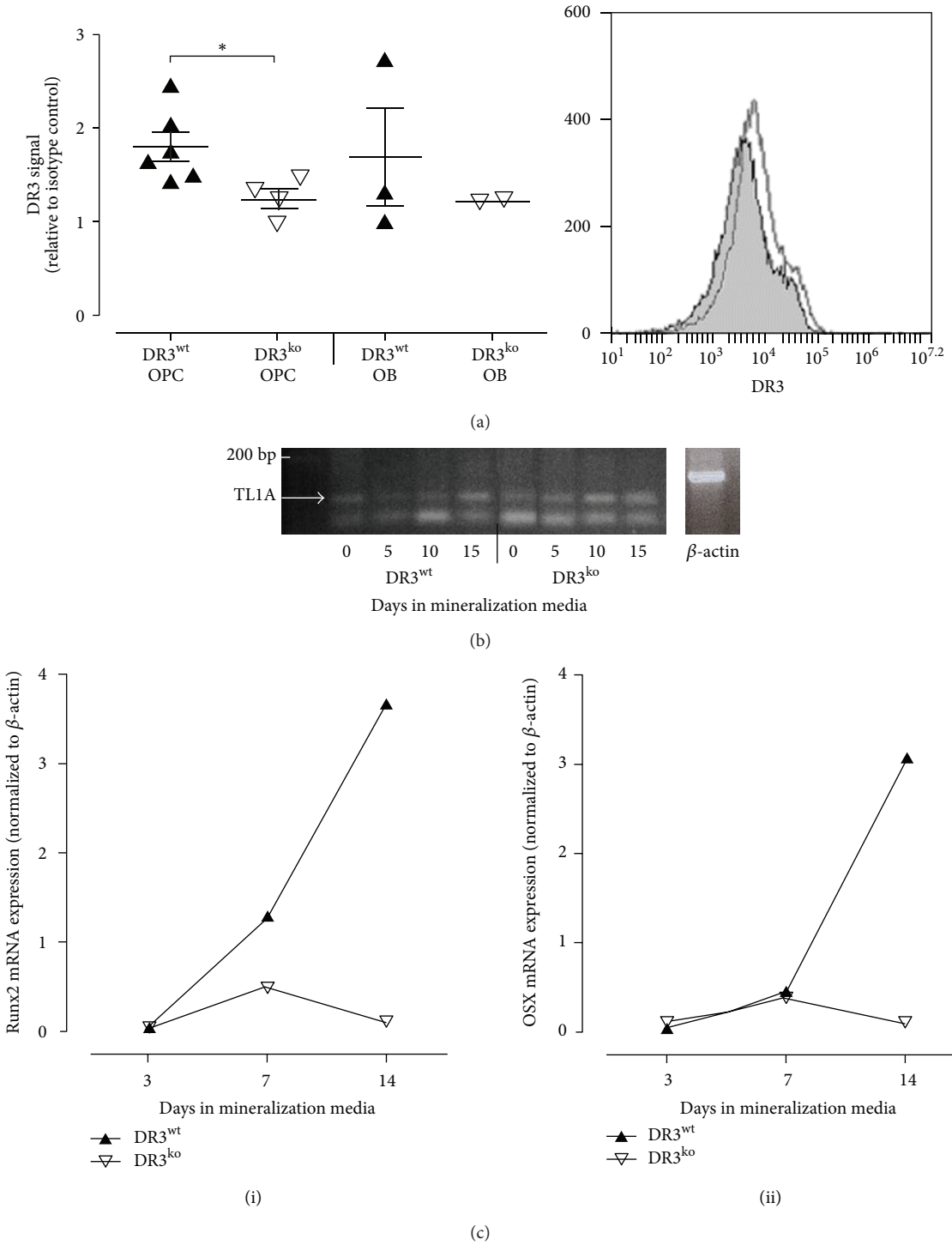


Figure 1: Expression of DR3 by murine OPCs and OBs. (a) DR3$^{wt}$ ($n = 6$) and DR3$^{ko}$ ($n = 4$) OPCs and OB (DR3$^{wt}$ $n = 3$, DR3$^{ko}$ $n = 2$) were analysed for DR3 signal by flow cytometry. DR3 signal was observed on DR3$^{wt}$ OPCs and OBs. A small subpopulation of DR3$^{wt}$ OPCs displayed higher levels of DR3 signal (shaded area = isotype control, black outline = DR3 antibody). (b) RNA was extracted from DR3$^{wt}$ and DR3$^{ko}$ OPCs and OB and tested for *TL1A* mRNA by RT-PCR. A 91 bp band corresponding to TL1A mRNA was detected at all time-points demonstrating constitutive expression of *TL1A* mRNA by DR3$^{wt}$ and DR3$^{ko}$ OPCs and OB. (c) Samples (DR3$^{wt}$ $n = 1$, DR3$^{ko}$ $n = 1$) were tested by qPCR at time points indicated to determine whether DR3 signalling modulates (i) Runx2 or (ii) OSX gene expression. Increased expression of Runx2 was detected at day 7 and day 14 while increased OSX expression was only detected at day 14 in the DR3$^{wt}$ compared to the DR3$^{ko}$.

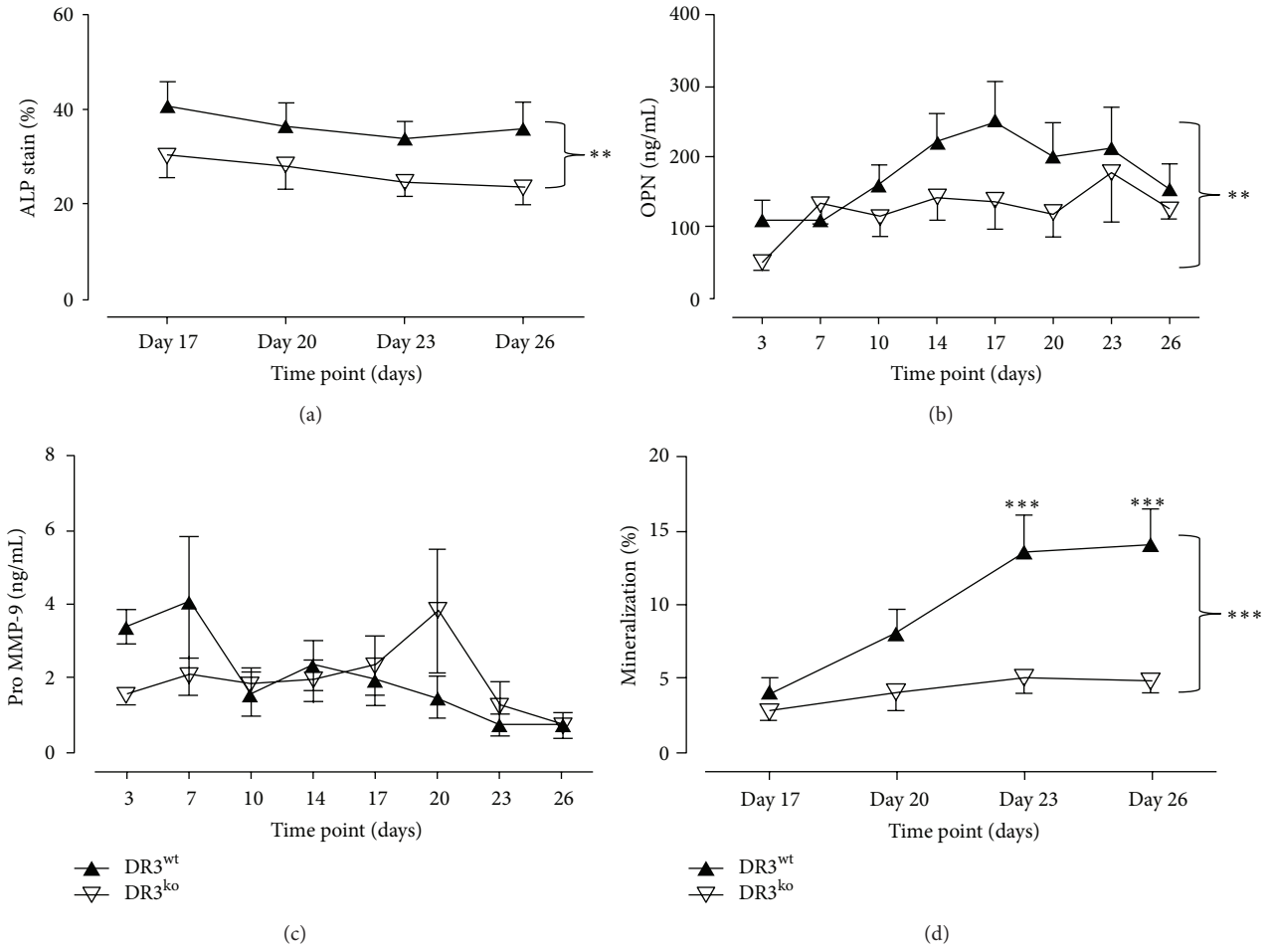


Figure 2: Expression of ALP, OPN, pro MMP-9, and mineral apposition in DR3$^{wt}$ and DR3$^{ko}$ osteoblast cultures. (a) DR3$^{wt}$ ($n = 5$) and DR3$^{ko}$ ($n = 5$) derived OB were cultured and the percentage of well area covered by ALP$^{+}$ cells was calculated. ALP staining was significantly higher in the DR3$^{wt}$ compared to the DR3$^{ko}$ cultures across the 26-day time course ($P \leq 0.01$). Supernatants from OB cultures were tested for soluble levels of (b) OPN and (c) pro MMP-9. Across the time course significantly increased levels of OPN were detected in DR3$^{wt}$ versus DR3$^{ko}$ cultures ($P \leq 0.01$). (d) Levels of mineral apposition in the cultures were tested by alizarin-red staining. Significantly increased levels of mineralization were observed in the DR3$^{wt}$ cultures at days 23 and 26 ($P \leq 0.001$) compared to the DR3$^{ko}$. This translated into a significant increase in the level of mineral apposition across the 26-day time course ($P \leq 0.001$).

(data not shown). In contrast, significantly increased levels of OPG were detected across the 26-day time course in DR3$^{wt}$ compared to DR3$^{ko}$ cultures ($P < 0.01$; Figure 3(b)). Levels of OPG peaked at day 14 ($62 \pm 37$ ng/mL) in DR3$^{wt}$ cultures, while in DR3$^{ko}$ cultures OPG levels reached a peak of $19 \pm 7$ ng/mL on day 17.

*3.5. Assessing the Impact of DR3 on Bone Formation In Vivo.* *In vitro* DR3 has been shown to regulate OB differentiation and mineral apposition while promoting expression of the antiosteoclastogenic cytokine OPG. To identify what effect this would have on *in vivo* bone formation male DBA/1 mice were aged for 10 months as a spontaneous model of ankylosing enthesopathy [12]. Bone formation over a 24-hour period was measured at three sites (knee, tail, and thoracic vertebra) via the incorporation of the fluorescent probe Bone-Tag. Significantly increased fluorescence efficiency values were observed in the DR3$^{wt}$ thoracic spine ($2.94 \pm 0.20 \times 10^{-5}$; $P < 0.05$) compared to the DR3$^{ko}$ thoracic spine ($1.76 \pm 0.26 \times 10^{-5}$; Figure 4(c)), suggesting DR3$^{ko}$ mice exhibit some resistance to the long-term development of spontaneous ankylosing enthesopathy observed in DBA/1 strain wild-type animals.

## 4. Discussion

Spondyloarthritides such as AS and PsA are characterized by the formation of syndesmophytes [3–5]. Mechanisms that control this increased aberrant bone apposition are not clear. This study provides the first direct evidence of DR3's involvement in OB-dependent new bone formation *in vitro* and *in vivo*. We demonstrate (using DR3$^{ko}$ mice) that DR3 has an important role in the differentiation of OB and subsequent mineralization of the organic matrix by OB. We also reveal

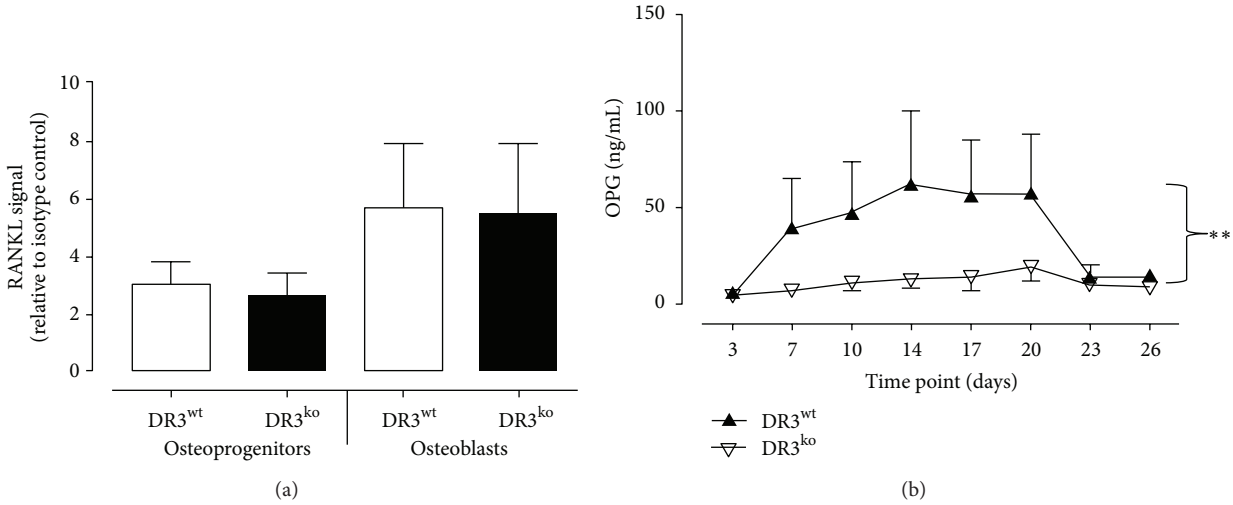


Figure 3: Effect of DR3 on OB-derived RANKL and OPG. (a) Cell surface expression of RANKL was analyzed on DR3$^{wt}$- and DR3$^{ko}$-derived OPCs and OB by flow cytometry. Both DR3$^{wt}$ and DR3$^{ko}$ OPCs displayed a 3-fold increase in RANKL signal over isotype control. RANKL signal increased to 5-fold over isotype in DR3$^{wt}$ and DR3$^{ko}$ OB. (b) Levels of soluble OPG were measured in culture supernatants by ELISA (DR3$^{wt}$ $n = 5$, DR3$^{ko}$ $n = 5$). Expression of OPG was significantly elevated in the DR3$^{wt}$ compared to DR3$^{ko}$ OB cultures over the 26-day time course ($P \leq 0.01$).

a novel pathway whereby DR3 increases OPG production by OB thereby potentially decreasing OC differentiation. *In vivo*, we show that gene deletion of DR3 protects the axial skeleton against excessive bone formation, a bone phenotype previously observed in aging DBA/1 male mice [12].

In the current study we reveal that DR3 is expressed on the cell surface of primary murine OPCs and OBs. DR3 expression on mouse OPC was shown to be comparable to that reported on the human OB MG63 cell line [11]. Cell surface expression of DR3 was reduced on mineralizing OB suggesting that DR3 may be temporally regulated during OB differentiation. In addition to DR3, the current study identified for the first the time expression of TL1A mRNA by primary OPCs and OB. These data suggest a potential autocrine role for the DR3/TL1A pathway in modulating OB differentiation and mineral apposition.

The notion that DR3 signalling has a role in OB differentiation was supported by the mineralization assay results. Levels of ALP expression and mineral apposition were significantly increased in the DR3$^{wt}$ cultures. This could be explained by DR3 signalling increasing either OB differentiation or proliferation, as has been described for effector T cells [16–18]. Total OB cell numbers at experiment end-point were, however, comparable between the DR3$^{wt}$ and DR3$^{ko}$ cultures (data not shown), suggesting that the increase in ALP expressing cells was due to DR3 promoting differentiation. This result is at odds with data described by Borysenko et al. [11], who demonstrated that cross-linking of DR3 with an antibody on MG63 cells induced apoptosis and inhibited differentiation. However, Borysenko and colleagues were unable to reproduce their results with TL1A or in primary human OB, suggesting that activation following receptor cross-linking may differ from actions of the natural ligand. Further support for DR3 having a role in the regulation of OB differentiation is provided by the analysis of Runx2 and OSX gene expression. Runx2 and OSX are critical transcription factors involved in OB differentiation, the absence of which results in the maturational arrest of the OB [19–21]. In the present study both Runx2 and OSX were elevated in the DR3$^{wt}$, but not DR3$^{ko}$, cultures at later time points. These data suggest a possible mechanism through which OB-dependent DR3 signalling potentially modulates OB differentiation.

The DR3-dependent increase in OB differentiation in the DR3$^{wt}$ was associated with increasing mineral apposition in the cultures. This elevation in mineral apposition corresponded with a significant increase in OPN expression in the DR3$^{wt}$ cultures. OPN is suggested to play a role in osteogenesis by attachment of the OB to the organic matrix and to regulate crystal size during mineralization [14, 22]. Interestingly OPN is in part transcriptionally regulated by Runx2 [22, 23] suggesting that in OBs, DR3 modulates OPN expression indirectly via this transcription factor. Before the OB can commence with the formation of newly mineralized bone the organic matrix must first undergo a maturation process. In a study by Filanti et al. [15] members of the matrix metalloproteinase (MMP) family, including MMP-2, -9, and -14, were increased in OB cultures at the time of nodule formation suggesting that they play a significant role in this matrix maturation process. Previous studies have demonstrated that DR3 signalling has a role in MMP-9 production; the THP-1 cell line has been shown to increase MMP-9 expression following treatment with TL1A [24, 25]. In addition, a study by Wang et al. [26] described significantly lower levels of MMP-9 in the joints of DR3$^{ko}$ mice with antigen induced arthritis compared to their DR3$^{wt}$ counterparts during the early stages of disease. In the present study we sought to determine

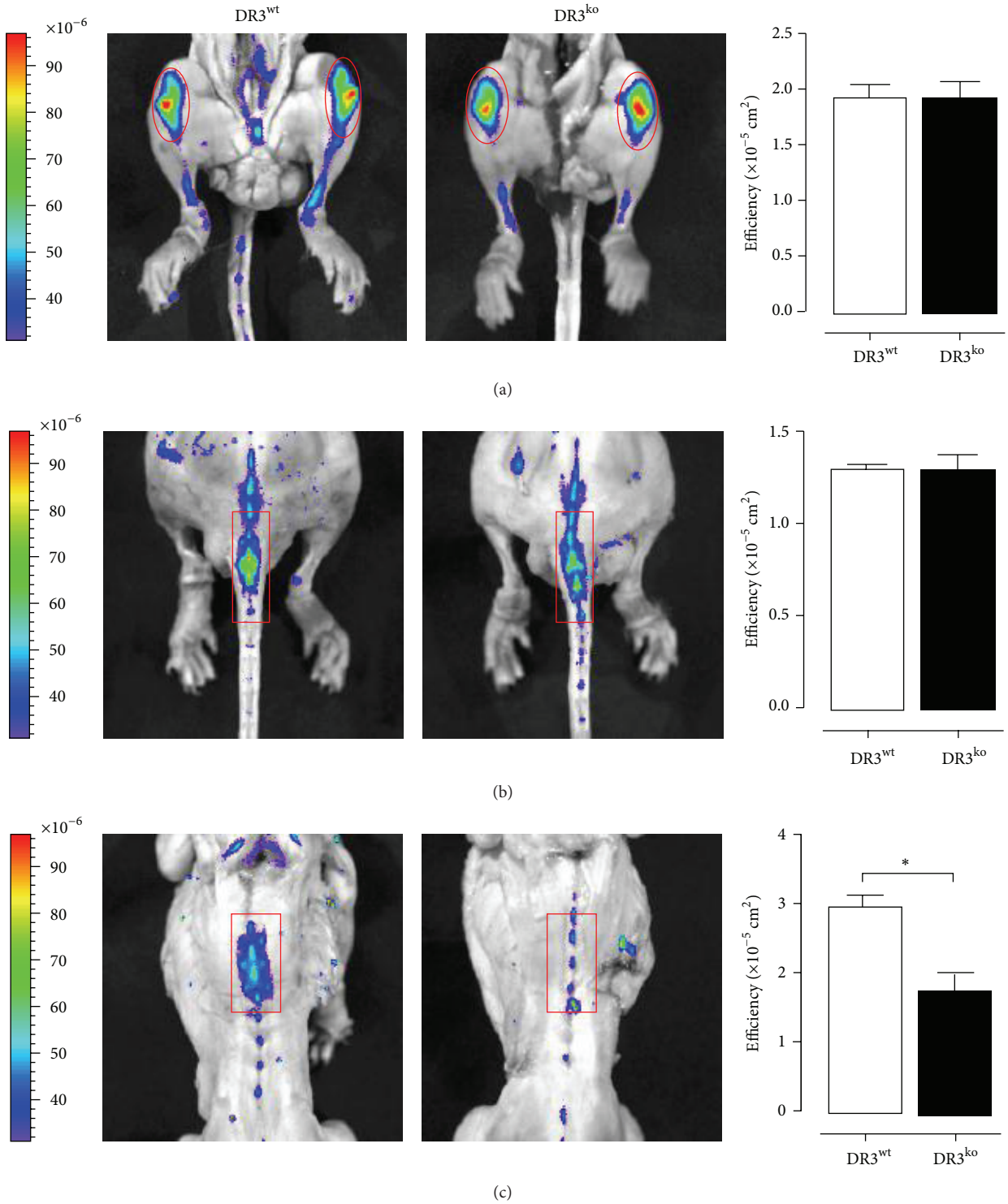


FIGURE 4: Incorporation of BoneTag into the knee, tail, and thoracic spine of 10-month-old DR3$^{wt}$ and DR3$^{ko}$ mice (DR3$^{wt}$ $n = 3$, DR3$^{ko}$ $n = 3$). Mice were injected with the fluorescent probe BoneTag and *in vivo* bone formation was analyzed. No differences in bone formation were observed in the (a) knee or (b) tail. A significant increase in bone formation was observed in the (c) thoracic spine of DR3$^{wt}$ compared to DR3$^{ko}$ mice ($P \leq 0.05$). Area quantified illustrated by red circle/box.

whether OB-dependent DR3 signalling affected osteoblastic MMP-9 expression, which would potentially modulate OB mineral apposition. A difference in the MMP-9 expression pattern was observed between the $DR3^{wt}$ and $DR3^{ko}$ cultures; with higher levels of pro MMP-9 observed at the early time points in the $DR3^{wt}$ cultures. This early expression of MMP-9 shows similarity to the phenotype described by Wang et al. [26] and taken together the results suggest that DR3 signalling has a central role in controlling levels of MMP-9. Unpublished work from our laboratory also suggests that the DR3 pathway may have a key role in the activation of MMP-9 from the pro form.

In addition to an OB specific effect, we have shown *in vitro* that OB DR3-dependent signalling results in significantly elevated expression of the soluble RANKL inhibitor OPG. This data suggests that OB DR3 signalling has the potential to in-directly modulate OC formation; where the increased OB-derived OPG inhibits OC formation and subsequently bone resorption [27]. This is in contrast to the direct contribution of DR3 signalling on osteoclastogenesis during inflammatory arthritis where DR3 has been revealed to enhance OC formation [28]. Interestingly, significantly increased levels of OPG have been detected in the serum from AS patients and are linked to poor physical mobility [29].

To investigate whether DR3 regulation of OB differentiation and mineral apposition could contribute towards the increased bone formation associated with the spondyloarthritides, we examined the effect of DR3 on *in vivo* bone formation in the spontaneous ankylosing enthesopathy model [12]. The results from the 10-month-old mice are consistent with the hypothesis that OB-dependent DR3 signalling has a significant role in regulating the atypical bone formation observed in the axial skeleton of mice with spontaneous ankylosing enthesopathy. The increased bone formation observed in the thoracic spine of DBA/1 $DR3^{wt}$ mice is analogous to the pattern of marginal/nonmarginal syndesmophyte formation observed in the spine of PsA and AS patients [30, 31]; thus this observation could have important implications in the understanding of the pathogenesis of these diseases.

The results from the present study provide the first evidence for a functional role for DR3 signalling in new bone formation. We have shown that cell surface DR3 is temporally expressed by OPCs and OB while TL1A mRNA is constitutively expressed and that an autocrine signalling loop may promote Runx2 and OSX expression, consequently modulating OB differentiation and mineral apposition. OB-dependent DR3 signalling increased levels of the soluble inhibitor OPG and could, therefore, indirectly regulate OC formation and bone resorption. Finally, *in vivo*, the absence of DR3 leads to differences in bone formation compared to wild-type DBA/1 mice, which in 10-month-old animals suggest resistance to the development of spontaneous ankylosing enthesopathy that has been previously described in this strain [12]. These data, taken with the study by Konsta et al. [7], suggest that OB-dependent DR3 signalling could be an important contributory factor in the atypical bone pathology associated with diseases such as PsA and AS.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Eddie C. Y. Wang and Anwen S. Williams contributed equally to this work and are cosenior authors.

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## References

[1] G. Karsenty, H. M. Kronenberg, and C. Settembre, "Genetic control of bone formation," *Annual Review of Cell and Developmental Biology*, vol. 25, pp. 629–648, 2009.

[2] S. L. Teitelbaum, "Osteoclasts: what do they do and how do they do it?" *The American Journal of Pathology*, vol. 170, no. 2, pp. 427–435, 2007, http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1851862/?tool=pmcentrez.

[3] M. A. Khan, "Update on spondyloarthropathies," *Annals of Internal Medicine*, vol. 136, no. 12, pp. 896–907, 2002.

[4] E. Dakwar, J. Reddy, F. L. Vale, and J. S. Uribe, "A review of the pathogenesis of ankylosing spondylitis," *Neurosurgical Focus*, vol. 24, no. 1, p. E2, 2008.

[5] P. Ory, "Radiography in the assessment of musculoskeletal conditions," *Best Practice & Research Clinical Rheumatology*, vol. 17, no. 3, pp. 495–512, 2003.

[6] E. Zinovieva, C. Bourgain, A. Kadi et al., "Comprehensive linkage and association analyses identify haplotype, near to the TNFSF15 gene, significantly associated with spondyloarthritis," *PLoS Genetics*, vol. 5, no. 6, Article ID e1000528, 2009.

[7] M. Konsta, G. Bamias, M. G. Tektonidou, P. Christopoulos, A. Iliopoulos, and P. P. Sfikakis, "Increased levels of soluble TNF-like cytokine 1A in ankylosing spondylitis," *Rheumatology*, vol. 52, no. 3, pp. 448–451, 2013.

[8] M. Schirmer, C. Goldberger, R. Würzner et al., "Circulating cytotoxic $CD8^+$ $CD28^-$ T cells in ankylosing spondylitis," *Arthritis Research*, vol. 4, no. 1, pp. 71–76, 2002.

[9] J. P. Twohig, M. Marsden, and S. M. Cuff, "The death receptor 3/TL1A pathway is essential for efficient development of antiviral $CD4^+$ and $CD8^+$ T-cell immunity," *The FASEB Journal*, vol. 26, no. 8, pp. 3575–3586, 2012, http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3460213&tool=pmcentrez&rendertype=abstract.

[10] R. Bu, C. W. Borysenko, Y. Li, L. Cao, A. Sabokbar, and H. C. Blair, "Expression and function of TNF-family proteins and receptors in human osteoblasts," *Bone*, vol. 33, no. 5, pp. 760–770, 2003.

[11] C. W. Borysenko, V. García-Palacios, R. D. Griswold et al., "Death receptor-3 mediates apoptosis in human osteoblasts under narrowly regulated conditions," *Journal of Cellular Physiology*, vol. 209, no. 3, pp. 1021–1028, 2006.

[12] R. J. U. Lories, P. Matthys, K. De Vlam, I. Derese, and F. P. Luyten, "Ankylosing enthesitis, dactylitis, and onychoperiostitis in male DBA/1 mice: a model of psoriatic arthritis," *Annals of the Rheumatic Diseases*, vol. 63, no. 5, pp. 595–598, 2004.

[13] E. C. Wang, A. Thern, A. Denzel, J. Kitson, S. N. Farrow, and M. J. Owen, "DR3 regulates negative selection during thymocyte development," *Molecular and Cellular Biology*, vol. 21, no. 10, pp. 3451–3461, 2001.

[14] P. Robey and A. Boskey, "Extracellular matrix and biomineralisation of bone," in *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*, pp. 12–19, 2006.

[15] C. Filanti, G. R. Dickson, D. Di Martino et al., "The expression of metalloproteinase-2, -9, and -14 and of tissue inhibitors-1 and-2 is developmentally modulated during osteogenesis in vitro, the mature osteoblastic phenotype expressing metalloproteinase-14," *Journal of Bone and Mineral Research*, vol. 15, no. 11, pp. 2154–2168, 2000.

[16] F. Meylan, T. S. Davidson, E. Kahle, M. Kinder, K. Acharya, and D. Jankovic, "The TNF-family receptor DR3 is essential for diverse T cell-mediated inflammatory diseases," *Immunity*, vol. 29, no. 1, pp. 79–89, 2008.

[17] G. Bamias, M. Mishina, M. Nyce, W. G. Ross, G. Kollias, and J. Rivera-Nieves, "Role of TL1A and its receptor DR3 in two models of chronic murine ileitis," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 22, pp. 8441–8446, 2006.

[18] T. J. Slebioda, T. F. Rowley, J. R. Ferdinand et al., "Triggering of TNFRSF25 promotes $CD8^+$ T-cell responses and anti-tumor immunity," *European Journal of Immunology*, vol. 41, no. 9, pp. 2606–2611, 2011.

[19] T. Komori, H. Yagi, S. Nomura et al., "Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts," *Cell*, vol. 89, no. 5, pp. 755–764, 1997.

[20] P. Ducy, R. Zhang, V. Geoffroy, A. L. Ridall, and G. Karsenty, "Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation," *Cell*, vol. 89, no. 5, pp. 747–754, 1997.

[21] K. Nakashima, X. Zhou, G. Kunkel, Z. Zhang, J. M. Deng, and R. R. Behringer, "The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation," *Cell*, vol. 108, no. 1, pp. 17–29, 2002.

[22] Q. Shen and S. Christakos, "The vitamin D receptor, Runx2, and the Notch signaling pathway cooperate in the transcriptional regulation of osteopontin," *The Journal of Biological Chemistry*, vol. 280, no. 49, pp. 40589–40598, 2005.

[23] C. K. Inman and P. Shore, "The osteoblast transcription factor Runx2 is expressed in mammary epithelial cells and mediates osteopontin expression," *The Journal of Biological Chemistry*, vol. 278, no. 49, pp. 48684–48689, 2003, http://www.ncbi.nlm.nih.gov/pubmed/14506237.

[24] Y.-J. Kang, W.-J. Kim, H.-U. Bae et al., "Involvement of TL1A and DR3 in induction of pro-inflammatory cytokines and matrix metalloproteinase-9 in atherogenesis," *Cytokine*, vol. 29, no. 5, pp. 229–235, 2005.

[25] S. H. Kim, W. H. Lee, B. S. Kwon, G. T. Oh, Y. H. Choi, and J. E. Park, "Tumor necrosis factor receptor superfamily 12 may destabilize atherosclerotic plaques by inducing matrix metalloproteinases," *Japanese Circulation Journal*, vol. 65, no. 2, pp. 136–138, 2001.

[26] E. C. Y. Wang, Z. Newton, O. A. Hayward et al., "Regulation of early cartilage destruction in inflammatory arthritis by death receptor 3," *Arthritis & Rheumatology*, vol. 66, no. 10, pp. 2762–2772, 2014.

[27] S. L. Teitelbaum, "Bone resorption by osteoclasts," *Science*, vol. 289, no. 5484, pp. 1504–1508, 2000.

[28] M. J. Bull, A. S. Williams, Z. Mecklenburgh et al., "The death receptor 3-TNF-like protein 1A pathway drives adverse bone pathology in inflammatory arthritis," *The Journal of Experimental Medicine*, vol. 205, no. 11, pp. 2457–2464, 2008.

[29] C.-H. Chen, H.-A. Chen, H.-T. Liao, C.-H. Liu, C.-Y. Tsai, and C.-T. Chou, "Soluble receptor activator of nuclear factor-kappaB ligand (RANKL) and osteoprotegerin in ankylosing spondylitis: OPG is associated with poor physical mobility and reflects systemic inflammation," *Clinical Rheumatology*, vol. 29, no. 10, pp. 1155–1161, 2010.

[30] F. Cantini, L. Niccoli, C. Nannini, O. Kaloudi, M. Bertoni, and E. Cassarà, "Psoriatic arthritis: a systematic review," *International Journal of the Rheumatic Diseases*, vol. 13, no. 4, pp. 300–317, 2010.

[31] I. van Echteld, A. Cieza, A. Boonen et al., "Identification of the most common problems by patients with ankylosing spondylitis using the International Classification of Functioning, Disability and Health," *Journal of Rheumatology*, vol. 33, no. 12, pp. 2475–2483, 2006.

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*Review Article*

# Mechanisms of Bone Resorption in Periodontitis

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Alveolar bone loss is a hallmark of periodontitis progression and its prevention is a key clinical challenge in periodontal disease treatment. Bone destruction is mediated by the host immune and inflammatory response to the microbial challenge. However, the mechanisms by which the local immune response against periodontopathic bacteria disturbs the homeostatic balance of bone formation and resorption in favour of bone loss remain to be established. The osteoclast, the principal bone resorptive cell, differentiates from monocyte/macrophage precursors under the regulation of the critical cytokines macrophage colony-stimulating factor, RANK ligand, and osteoprotegerin. TNF-$\alpha$, IL-1, and $PGE_2$ also promote osteoclast activity, particularly in states of inflammatory osteolysis such as those found in periodontitis. The pathogenic processes of destructive inflammatory periodontal diseases are instigated by subgingival plaque microflora and factors such as lipopolysaccharides derived from specific pathogens. These are propagated by host inflammatory and immune cell influences, and the activation of T and B cells initiates the adaptive immune response via regulation of the Th1-Th2-Th17 regulatory axis. In summary, Th1-type T lymphocytes, B cell macrophages, and neutrophils promote bone loss through upregulated production of proinflammatory mediators and activation of the RANK-L expression pathways.

## 1. Introduction

Bone resorption is a basic physiologic process that is central to the understanding of many key pathologies, with its most common oral manifestation seen as the alveolar bone destruction in periodontitis [1–4]. This review aims to describe the prevailing understanding of mechanisms of bone resorption as related to periodontal disease, at the molecular and cellular levels. It outlines some of the newer advances in the field of osteoimmunology, and sheds light on recent research contributions and future directions from a clinical perspective [5–8]. Understanding the biological mechanisms that control the immunopathogenesis of the remodelling and resorptive processes will clarify not only the local control of bone cell function but also the pathophysiology of accelerated bone loss, as seen in periodontal disease and other immunoinflammatory diseases of bone such as osteoporosis and rheumatoid arthritis [9–11].

## 2. Bone Homeostasis and Maintenance

Bone is a remarkably dynamic and active tissue, undergoing constant renewal in response to mechanical, nutritional, and hormonal influences. A balance between the coupled processes of bone resorption by osteoclasts and bone formation by osteoblasts is required in a healthy adult [3, 12–14]. Under physiologic conditions, these processes are very carefully regulated by systemic hormones and local factors and orchestrated by osteocytes and bone lining cells which fine-tune interstitial fluid and plasma calcium levels [3]. Thus, bone resorption plays a major role in the homeostasis of skeletal and serum calcium levels, and the regulated coupling of resorption to new bone formation by osteoblasts is required for proper growth, remodelling, and skeletal maintenance [12–14]. The overall quality and quantity of bone will be affected by any factors that influence either of these processes or perturb this balance.

## 3. Bone Cells

Preosteoblasts, osteoblasts, osteocytes, and bone lining cells all arise from the osteogenic line of cells, which, in turn, arise from primitive mesenchymal cells in bone marrow stroma and from pericytes adjacent to connective tissue blood vessels. Their differentiation requires activation of

the Osf2/Cbfa gene, which activates expression of osteocalcin, bone sialoprotein (BSP), osteopontin (OPN), and collagen synthesis, and is followed by stimulation from bone morphogenetic protein- (BMP-) 2 and transforming growth factor beta (TGF-$\beta$) [15–17]. Besides their primary role in bone formation, osteoblasts express chemokines, prostaglandins, and growth factors (e.g., BMPs, TGF-$\beta$, colony-stimulating factor- (CSF-) 1, granulocyte colony-stimulating factor (G-CSF), basic fibroblast growth factor (basic FGF), and insulin-like growth factor (IGF)) with autocrine, self-regulatory, and/or paracrine activity that regulate osteogenic as well as osteoclastic cells [18]. Osteoblastic cells have a major influence on the environmental responsiveness of osteoclasts through localisation, induction, stimulation, and inhibition of resorption [8, 13].

Osteocytes are mature bone cells that have become entrapped in bone matrix and mobilise calcium from matrix for transport and exchange with body fluids in response to systemic demand [19]. They too respond to systemic influences as evidenced by increased levels of cyclic AMP and act as transducers to modulate local bone remodelling activity [19]. They are liberated by osteoclasts during resorption, with the eventual fate of apoptotic cell death. Bone lining cells regulate the ionic composition of bone fluid, protect the bone surface from osteoclasts, and regulate new bone formation or resorption [12, 14].

Osteoclasts are highly specialised motile migratory bone resorptive cells, derived from haematopoietic stem cells [12–14, 20–22]. They are responsible for the degradation of mineralized bone and are, therefore, critical for normal skeletal growth and development, maintenance of bone integrity throughout life, calcium metabolism through remodelling, and homeostasis and repair [12, 14, 20]. As osteoclasts are the prime bone resorptive cells, local stimulation of their activity is an essential requirement for alveolar bone loss [23]. In response to key factors, such as M-CSF/CSF-1, osteoclast differentiation factor (ODF/RANKL), interleukins (IL), tumor necrosis factor (TNF), and contact with mineralized bone particles containing osteocalcin, haematopoietic precursors may undergo differentiation into monocyte and macrophage derived colony-forming cells, circulating peripheral blood monocytes and tissue macrophages, and finally fuse into mature multicellular osteoclasts [17, 20–22, 24–28].

One of the first events in the triggering of preosteoclasts is the contraction of the osteoblast actin and myosin cytoskeleton in response to local and systemic influences, for example, parathyroid hormone (PTH), retinoid acid, and vitamin D3 stimulation [8, 27]. This increases the width of intercellular spaces, exposing more osteoid to interstitial fluid. Osteoblasts also secrete collagenase and plasminogen activator [29]. IL-1, TNF, and epidermal growth factor (EGF) have been shown to deactivate osteoblasts and increase release of CSF-1 and RANKL [19, 27].

## 4. Cellular Mechanisms of Bone Remodelling: Resorption and Formation

The bone remodelling cycle operates continually as osteoclasts are constantly removing mature bone, with new bone simultaneously formed by osteoblasts [14]. This occurs throughout the skeleton in focal units called bone remodelling units (BRU), with each unit of activity lasting three to four months [9]. This multistep process functions in four distinct phases of activation, resorption, reversal, and formation.

"Activation" is the initiating event that converts a resting bone surface into a remodelling surface [12, 20]. It involves the recruitment of mononuclear osteoclast precursors to the bone surface and their differentiation and fusion into functional osteoclasts [8]. Terminal differentiation and mononuclear cell fusion is mediated by cell-to-cell interactions between osteoclast progenitors and osteoblasts/stromal cells and by contact with the mineral phase, particularly with osteocalcin [17, 27, 30]. Both CSF-1 and IL-1 stimulate preosteoclast fusion [26, 28]. E-cadherin is important for cell-to-cell adhesion associated with the fusion of preosteoclasts. Nonmineralised osteoid covering the mineralized bone matrix must be dissolved before the osteoclasts can attach to the mineralised matrix and initiate resorption [8]. Osteoblast proteases are responsible for dissolving this osteoid. Following this, the activated osteoclasts attach to the bone matrix and their cytoskeleton reorganizes; they take on a polarized morphology and form a sealing zone to isolate the resorption site and develop ruffled borders which secrete protease enzymes [9, 14, 20, 31].

During the "resorption" phase, osteoclasts work in concert removing both mineral and organic components of the bone matrix [14]. The hallmark of the resorbing surface is the appearance of scalloped erosion, called Howship's or resorption lacuna [12, 20]. The resorption phase lasts about 8–10 days, presumably the life span of the osteoclast [14].

Once most of the mineral and organic matrix has been resolved, there is a "reversal" phase lasting 7–14 days, marking the transition from destruction to repair. Here, the coupling of resorption to formation takes place [15]. After completion of one resorption lacuna, the osteoclast can move along the bone surface and restart resorption or undergo apoptosis [14].

Numerous paracrine and autocrine chemical signalling factors are involved in all aspects of remodelling, resorption, proliferation, and coupling. Coupling factors are released from their binding proteins during resorption by the acidic environment created by osteoclasts, and they further inhibit resorption via negative feedback, suppressing osteoclast formation and stimulating osteoblastogenesis [4, 19, 32]. Thus, in a series of locally controlled autoregulated cell activation events, a ten-day osteoclastic resorptive phase is usually followed by a repair phase of three months [19]. During repair, a cascade of differentiation events including chemotaxis, cell attachment, mitosis, and differentiation of osteoblast precursors takes place, leading to new bone deposition [19].

## 5. Bone Formation

Formation of new bone, a two-stage process, begins after a short reversal phase, commencing with the deposition of osteoid. The initial organic matrix consisting primarily (90%) of type 1 collagen and various other components is subsequently mineralised over a period of about 20 days [19]. Two theories generally elucidate how calcification proceeds:

the matrix vesicle theory and the nucleation theory; it is speculated that both theories work in parallel depending on the type of skeletal tissue involved [3, 5, 12]. After the mineralisation process is triggered, the mineral content rapidly increases over the first few days to 75% of final mineral content, taking up to a year for the matrix to reach maximum mineral content. The primary constituent of the mature mineral phase is hydroxyapatite [9, 10, 19].

Noncollagenous bone matrix proteins play a key role in matrix mineralisation, cellular adhesion, and regulation of cell activity during coupling of formation and resorption. Osteocalcin, one of the most abundant of these proteins, has a vital role in mineralization, may act as a chemoattractant, and may be essential for osteoclast differentiation. Bone sialoprotein (BSP), a highly specific bone protein, has high calcium-binding potential, thus inhibiting mineral deposition. In addition, it promotes adhesion of osteoclasts to bone matrix molecules through the key RGD (arginine-glycine-aspartic acid) peptide sequence and may regulate osteoclast formation. Osteopontin and osteonectin too are important in osteogenic cell activity [15–17, 19].

## 6. Degradation of the Mineral and Organic Matrix

Osteoclasts resorb bone in resorption lacunae by generating a pH gradient between the cell and bone surface, favouring the mineral-dissolving action of the osteoclast proteinases. Carbonic anhydrase (CA) II is the main cytoplasmic source of protons for the acidification of the lacuna. This hydrates carbon dioxide to carbonic acid, which ionizes into carbonate and hydrogen ions [9, 10, 12, 14, 19, 22]. A vacuolar-type proton pump, V-ATPase, transports the protons generated by CAII into intracellular vesicles. These are then transported and fused to the RB membrane, releasing their proton content to the lacuna. Acidification is subsequently completed by passive potential driven chloride transport. The chloride channel of the ruffled border is identified as ClC-7, and it is transported along with the proton pump to the RB via endosomes [5, 9, 19, 33, 34].

The basolateral membrane exchangers $Na^+$ and $Cl^-$ $HCO_3{}^+$ maintain internal pH at physiologic levels. Calmodulin, a cytoplasmic calcium-binding protein concentrated in the osteoclast cytoplasm adjacent to the RB, regulates the effects of intracellular calcium and the ATP-dependent proton transport across the RB. As resorption proceeds, the increase in cytoplasmic calcium ultimately deactivates the osteoclast, triggering cell detachment from the bone matrix and loss of the RB [9, 19, 33, 34]. Solubilization of hydroxyapatite is followed by digestion of the exposed organic matrix by lysosomal enzymes, bone-derived collagenases, and proteinases [14, 29]. Osteoclasts contain the highest concentration of mitochondria of any cell type, thus generating the ATP required for the carbonic anhydrase-catalysed production of hydrogen ions [9].

Degradation products are removed by transcytosis and finally released into the extracellular space. The specific enzyme TRAP (tartrate-resistant acid phosphatase) is located in cytoplasmic vesicles, which fuse to the transcytotic vesicles to destroy the endocytosed material. When the osteoclast moves away from the resorption lacuna, phagocytes clean up the debri, and osteoblasts move in to begin bone formation anew [19]. The dissolution of the mineral phase in the acidic microenvironment below the RB exposes collagen fibrils to the enzymatic attack of cathepsins B, E, K, S, and L. These cysteine proteases are secreted by osteoclasts to degrade native collagen at an acidic pH of 4.5 [19]. Thereafter, matrix metalloproteinases (MMPs), such as gelatinase A (MMP-2), stromelysin (MMP-3), and collagenase (MMP-1), continue with the matrix degradation process. Thus, calmodulin antagonists and MMP inhibitors can block resorption by inhibiting acidification of the resorptive compartment [19, 29].

## 7. Regulation of Osteoclastic Bone Resorption

The rate of bone resorption can be regulated either at the level of differentiation of osteoclasts from their hematopoietic precursor pool or through the regulation of key functional proteins which control the attachment, migration, and resorptive activities of the mature cell [8, 10, 12, 22]. It is becoming evident that many of the cellular events involved in resorption of bone are modulated by a group of local osteotropic factors which have extremely potent effects on bone cells both *in vitro* and *in vivo*. It must be recognized that many of these cytokines and growth factors exhibit significant redundancy and pleiotropy or overlap in their local effects [18, 19, 35].

As emphasised previously, in states of disease, a disturbance in the homeostatic balance that is essential for functional bone turnover results in destructive osteolytic processes. In inflammatory periodontal disease, both microbial and host-derived factors are implicated in the bone resorption and remodelling processes [2, 4, 8]. These chemical modulators play highly complex roles, and several cell types are often involved. It is difficult to definitively ascertain the precise role of a cytokine or growth factor *in vitro*, and even less so *in vivo*, as multiple local factors often modify the *in vivo* effect [7, 20]. Some factors act directly on osteoclastic cells, whereas others act indirectly through other cell types in the local environment or through secondary production of additional factors [18].

Cytokine regulation is likely to be more important for trabecular bone, which is closer to the cytokine-rich marrow than cortical bone [3, 19]. Many potent osteotropic cytokines, such as IL-1, IL-6, TNF-$\alpha$, and TGF-$\beta$, mediate a multitude of effects in the body in addition to their effects on bone cells [19]. The production of cytokines by osteoblasts is regulated by various hormones and cytokines along with bacteria and lipopolysaccharide [19, 28]. These too can act synergistically with local factors to influence the bone homeostatic balance [20]. Thus, numerous hormones, growth factors, and cytokines modulate osteoclast activity by regulating their differentiation, activation, life span, and function. These include parathyroid hormone (PTH), calcitriol, PTH-related protein, $PGE_2$, thyroxine, and IL-11 [3, 19, 36].

The proinflammatory cytokines (IL-1 and IL-6, TNFs) have been implicated in the stimulation of osteoclastic

resorption in periodontitis. The functions of the immunoregulatory cytokines (IL-2 and IL-4, interferon gamma) are less clear, but low levels of these may contribute to periodontitis. Genetic factors have been shown to account for up to 80% of control of bone mineral density, thus playing a major role in determining variation. However, it is the rate of bone formation rather than the rate of resorption that is influenced by genes [2, 4, 19]. Some individuals demonstrate aggressive bone destruction and high levels of proinflammatory and bone resorptive cytokines that cannot be completely explained by presence of pathogenic bacteria alone. Genetic variation, termed single-nucleotide polymorphisms, of key immune or inflammatory regulatory factors may explain these variances in periodontal disease manifestation, as well as the familial aggregation of aggressive forms of the disease [19].

Systemic influences on bone resorption may be exerted by several mediators, including PTH, IL-1, TNF, TGF, and 1,25-dihydroxyvitamin $D_3$. These factors may affect osteoclast number and activity, directly influence osteogenic cells to cause cytoplasmic contraction and secretion of collagenase, tissue plasminogen activator and RANK-L. Of note, the C-terminal fraction of PTH has been shown to increase osteoclast formation and activity in the presence of osteoblasts and accelerate osteoclast-like cell formation from hematopoietic precursors in the absence of osteoblasts. Calcitonin, interferon gamma (IFN $\gamma$), and TGF $\beta$ are potent inhibitors of osteoclast activity and differentiation [27, 29, 36–38]. The hormones PTH and calcitonin act in concert to maintain blood calcium concentrations at normal physiological levels (0.5–10.5 mg/dL), with actions on intestinal absorption and renal excretion as well as bone cells. There is evidence to support a direct effect of PTH on osteoclasts; however, there is much evidence that supports an indirect mechanism, whereby PTH stimulates osteoblasts to release RANKL, which subsequently activates osteoclasts. PTH also stimulates osteoblastic production of IL-6, which increases osteoclastic differentiation, and causes osteoblasts to contract making the bone surface more susceptible to resorption [6, 19, 25, 34, 36].

The polypeptide calcitonin increases cellular calcium and cAMP and disrupts the clear zone cytoskeleton by decreasing the size of the RB and altering podosome binding ability. It blocks proton extrusion and decreases osteopontin expression; hence osteoclasts are seen to detach from bone surfaces within 15 minutes of its administration. The sex steroids exert an anabolic effect by stimulating osteoblast proliferation and differentiation, as well as decreasing IL-6 transcription. Postmenopausal women experience osteoporosis due to increased osteoclastic resorption and decreased osteoblast proliferation [9, 19, 32, 33].

## 8. Local Mediators of Bone Resorption

Local formation of osteoclasts and their stimulation are required for alveolar bone loss. It has been shown that multiple mediators, such as IL-1, IL-6, IL-11, IL-17, TNF-$\alpha$, TNF-beta, TGF-$\beta$, kinins, and thrombin, can stimulate bone resorption [4, 7, 13, 23]. Bone resorption is also directly regulated locally by ionized calcium generated as a result of osteoclastic resorption, and new evidence indicates that endothelial cells may also play a part via mediators including nitric oxide and endothelin [33]. Lipid mediators, such as bacterial lipopolysaccharide, host-derived platelet-activating factor, and prostaglandins, may also be involved in stimulation of bone resorption. Reactive oxygen intermediates and extracellular nucleotides, both present at sites of inflammation, have also been implicated [8].

The roles of local inflammatory mediators generated by macrophages and T lymphocytes in bone resorption have been extensively studied. Their effector functions on tissue can be direct or indirect as recently reported in relation to the osteoblast stimulated RANKL-production pathway. Alveolar bone resorption in periodontitis can thus be directly or indirectly induced by the cellular inflammatory infiltrate [4, 11, 23, 24].

The gingival crevicular fluid (GCF) has been shown to contain a complex array of protein components that not only irrigate the gingival sulcus but also are released into the oral cavity [39, 40]. GCF is derived from gingival capillary beds (serum components) and from both resident and emigrating inflammatory cells. This fluid contains an array of innate, inflammatory, and adaptive immune molecules and cells whose role is to contribute to the interaction of host and bacteria in this ecological niche [40]. Studies demonstrate that GCF contains mediators that can stimulate bone resorption *in vitro*. The primary factor responsible appears to be IL-1$\alpha$, with IL-1$\beta$ and $PGE_2$ also significant. This exudate, with diagnostic and prognostic potential, is an accessible source of extracellular matrix derived biologic markers of periodontal bone resorption [18, 24, 41–43].

Analysis of GCF has identified cell and humoral responses in both healthy individuals and those with periodontal disease. Although there is no direct evidence of a relationship between GCF cytokine levels and disease, interleukin-1 alpha (IL-1$\alpha$) and IL-1$\beta$ are known to increase the binding of PMNs and monocytes/macrophages to endothelial cells, stimulate the production of $PGE_2$ and the release of lysosomal enzymes, and stimulate bone resorption [42]. Preliminary evidence also indicates the presence of interferon-$\alpha$ in GCF, which may have a protective role in periodontal disease because of its ability to inhibit the bone resorption activity of IL-1$\beta$ [44, 45]. Pyridinoline cross-links, in particular, are specific for bone resorption and thus useful in differentiating gingival inflammation from bone destruction in active lesions [18].

## 9. Roles of Receptor Activator of Nuclear Factor-$\kappa$B Ligand (RANKL) and OPG

The activation and differentiation of osteoclasts are modulated by three members of the TNF ligand and receptor superfamilies: the osteoclastogenesis inducers RANKL, RANK, and OPG. Identification of these three peptides has contributed enormously to our understanding of the molecular mechanisms of osteoclast differentiation and activity [6, 11, 22, 24, 25, 35, 46–48]. RANKL (receptor activator of nF-$\kappa$B

ligand) is a member of the TNF superfamily (also known as osteoclast differentiation factor: ODF, TRANCE, and TNFSF-11). It is expressed as a membrane bound protein (mRANKL) or in soluble form (sRANKL) by osteoblasts or stromal cells. When RANKL binds to its receptor, RANK, on osteoclast and preosteoclast cell surfaces, it promotes osteoclast formation by stimulating proliferation and differentiation [9, 22, 25, 27, 47].

Osteoprotegerin (OPG), its decoy receptor, is a circulating protein, produced by a variety of cell types including osteoblasts and marrow stromal cells, which inhibits osteoclast formation by binding mRANKL, thereby preventing the stimulatory cell-to-cell interaction with preosteoclasts and inhibiting RANKL/RANK interactions. Hence, these three proteins are essential for osteoclast differentiation directed by osteoblasts, and the balance between RANKL and OPG in osteoblasts directs new osteoclast recruitment [7, 25, 32, 46, 47].

Other resident periodontal cells including ligament and gingival fibroblasts also participate in the regulation of bone remodelling and resorption. Infiltrated leukocytes produce inflammatory mediators, for example, IL-1 and $PGE_2$, which affect RANKL and OPG expression by osteoblasts, periodontal ligament fibroblasts, and gingival fibroblasts. RANKL is also expressed in activated T cells [11, 24, 25, 46, 48, 49].

A key finding of recent studies is that sRANKL in combination with CSF-1/M-CSF stimulates osteoclast development from peripheral blood cell precursors by binding to its receptor. It has been established that osteoblasts are responsible for producing CSF-1 and that contact between osteoblasts and osteoclast precursors, mediated by critical survival factor CSF-1 and its receptor, promotes osteoclast development [5, 26–28, 38, 49].

Activation of the RANKL receptor increases the expression of TRAP, $\beta_3$ integrins, cathepsin K, and calcitonin receptors on preosteoclasts. Thus, OPG is a negative regulator and RANKL a positive regulator of osteoclastogenesis through interaction with appropriate receptors on cells of the monocyte and macrophage cell lineage. In addition, many of the local and systemic regulators of osteoclastic resorption have been shown to act via the RANKL/OPG and CSF-1 pathways [24, 27, 46, 47].

## 10. Immunopathogenesis of Periodontal Disease

In chronic periodontal disease, biologically active substances within bacterial plaque induce a local inflammatory response in the gingival soft tissues and periodontium [2]. The resultant influx of inflammatory cells produces a host of cytokines, for example, $PGE_2$, IL-1, and RANK-L, that promote resorption through osteoclasts, the primary bone resorbing cell. Thus, in pathologic inflammatory conditions, stimulatory inflammatory cell products initiate osteoclast activity and disturb the fine balance between protective and destructive processes [3, 6, 14, 22, 25, 36]. This is termed the immunopathogenesis of periodontal disease, and pioneering research in this area over the last decade has spawned a whole new field termed "osteoimmunology" [37, 50].

In periodontal disease, the cellular inflammatory infiltrate of T cells, B cells, macrophages, and neutrophils within gingival connective tissue is increased, with a concurrent increase in the secretion of inflammatory mediators [1, 51]. These inflammatory cells also interact with stromal cells, such as osteoblasts, periodontal ligament, and gingival fibroblasts. RANKL-mediated osteoclastogenesis plays a pivotal role in inflammatory bone resorption, and its expression is increased in periodontitis [22, 24]. While lymphocytes produce RANKL, they might not be involved in bone resorption under physiological conditions. However, in inflammatory pathological resorptive states, activated T lymphocytes may mediate bone resorption through excessive production of sRANKL, and findings suggest that activated T and B lymphocytes are one of the major RANKL-expressing sources in diseased periodontal tissue [11, 22, 25, 27]. Numerous animal models support this association. The majority of RANKL produced by T cells may be soluble, as the expression of mRANKL on T cells is limited. Another key finding is an increase in osteoclast numbers on the alveolar bone crest of animals receiving antigen-specific lymphocytes, which can be suppressed by OPG [11, 47, 48].

Gingival fibroblasts are heterogenic in that they produce OPG in response to LPS and IL-1, suggesting a protective role to suppress osteoclast formation; however, they may also augment chronic inflammatory processes through IL-6 and IFN production. The periodontopathic bacteria *Aggregatibacter actinomycetemcomitans* (*Aa*) and *Porphyromonas gingivalis* (*Pg*) have unique mechanisms to induce RANKL in osteoblasts and gingival fibroblasts. When stimulated with LPS and IL-1, osteoblasts and periodontal ligament fibroblasts express RANKL. RANKL and OPG expression may also be related to the function of amelogenin and regulation of odontoclast formation [11, 25, 28, 30]. Recently, it has been shown that RANKL is upregulated whereas OPG is downregulated in periodontitis compared to periodontal health, resulting in an increased RANKL/OPG ratio. This ratio is further upregulated in smokers and diabetics [52]. It has also been reported that the molecular mechanisms of T cell mediated regulation of osteoclast formation occurs through cross-talk signalling between RANKL and IFN-$\gamma$. Indeed, IFN-$\gamma$ produced by T cells induces rapid degradation of the RANK adapted protein, TNF receptor associated factor 6 (TRAF6), which results in strong inhibition of RANKL induced activation of the transcription factor NF-$\kappa$B and c-Jun N-terminal kinase [9].

## 11. Role of Specific Immune Cells

CD4+ and CD8+ T cells are present in periodontal lesions, as are memory and activated T lymphocytes, and different T cell subsets appear involved in either up- or downregulation of RANKL-mediated periodontal bone resorption. Moreover, Th1- and Th2-type T lymphocytes and their associated cytokines may be present, with a polarization towards a Th1 profile [9, 48, 49, 53]. It has been proposed that Th1-type cells promote bone loss, as RANK-L appears to be predominantly expressed on Th1-type cells, while regulatory

T cells suppress T helper type 1 mediated bone loss. The production of proinflammatory cytokines IL-1 and TNF-$\alpha$ is upregulated by Th1-type T cells. These can induce bone resorption indirectly by stimulation of osteoclast precursors and subsequent activation of osteoclasts through RANK-L production by osteoblasts [49, 53]. Activated T cells can also, through production and expression of OPG, directly promote osteoclast differentiation. These direct and indirect modes of T cell involvement in periodontal bone resorption appear dependent on the extent of Th1-type T cell recruitment in inflamed tissues [53]. It has been well recognized that control of this shift is mediated by a balance between the so-called Th1 and Th2 subsets of T cells, with chronic periodontitis being mediated by Th2 cells [50]. More recently T regulatory (Treg) and Th17 cells have been demonstrated in periodontal tissues, suggesting a role for these mediators in the immunoregulation of the disease [1, 50]. However, Th17 and IL-17 have also been shown to display a protective role as well as a destructive role in periodontal bone resorption [54, 55]. Different models of inflammation report opposite functional roles of IL-17 in terms of its effects on bone destruction. In a recent study it was concluded that IL-17 is protective in the development of periapical lesions depending on its regulation of myeloid cell mediated inflammation. However, the authors noted that the detailed mechanism behind the IL-17 signal-mediated protection in periapical lesions remains unclear [54].

## 12. Bacterial Influence

Similar to other polymicrobial diseases, periodontitis is now characterized as a microbial-shift disease owing to a well-characterized change in the microorganisms that are present (from mostly Gram-positive to mostly Gram-negative species) during the transition from periodontal health to periodontal disease [56, 57].

In a milestone study by Socransky et al. using whole-genome DNA probes, several bacterial complexes associated with either periodontal health or disease were identified. This included three bacterial species that were designated, the "red-complex" periopathogens—*Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*—which grouped together in diseased sites and showed a strong association with disease [58]. Much research has been directed towards understanding the pathogenic mechanisms and virulence determinants of these three bacterial species in the context of a conventional host-pathogen interaction, as exemplified by diseases with single-infective agent aetiology [59]. Support for the alternative hypothesis that periodontal pathogens transform the normally symbiotic microbiota into a dysbiotic state, which leads to a breakdown in the normal homeostatic relationship with the host, comes from evidence that *P. gingivalis* has evolved sophisticated strategies to evade or subvert components of the host immune system (e.g., Toll-like receptors (TLRs) and complement), rather than acting directly as a proinflammatory bacterium [60]. In other words, *P. gingivalis* could be a keystone pathogen of the disease-provoking periodontal microbiota [61–63].

The pathogenic processes of periodontal diseases are primarily due to the host response, which propagates the destruction initiated by microbes. Harmful pathogenic products and enzymes such as hyaluronidases, collagenases, and proteases break down extracellular matrix components in order to produce nutrients for their growth [2, 6, 11]. Arg- and Lys-gingipain cysteine proteinases produced by *P. gingivalis* are key virulence factors that lead to host tissue invasion. Once immunoinflammatory processes begin, various molecules (e.g., proteases, MMPs, cytokines, prostaglandins, and host enzymes) are released from leukocytes and fibroblasts. An imbalance between the level of activated tissue-destroying MMPs and their endogenous inhibitors (TIMPs) has been demonstrated. Thus, the connective tissue attachment and alveolar bone are destroyed, and the junctional epithelium and the inflammatory infiltrate migrate apically [7, 34, 40, 64, 65]. In addition, osteoclasts are activated, initiating bone destruction through direct mechanisms and indirectly through RANKL, RANK, and OPG modulation. In the presence of periodontopathogens, CD4+ T cells show increased RANKL expression [11, 24, 25, 46, 49]. As the destructive pattern continues, subsequent increase in microbial density propagates the periodontal lesion. The flora progressively becomes more anaerobic, and the host response becomes more destructive and chronic. Eventually bone loss and the destructive lesion progress to an extent that can lead to tooth loss [2, 7].

Bacterial virulence factors are capable of potentiating bone resorption themselves. Endotoxin from Gram-negative cell walls activates CD4+ T cells to stimulate resorption via their interaction with macrophages. *Pg* produces a fimbrial protein that is a potent osteoclast stimulator via a tyrosine kinase mechanism [8], antibodies against which prevented bone loss in infected animals. *Aa* produces a 62 kDa heat shock protein associated with the ability to stimulate bone resorption at picomolar concentrations, as well as a peptide that acts as a potent IL-6 inducer in fibroblasts and monocytes [7, 9, 10]. Other virulence factors of *Treponema denticola* and *T. forsythia* include the binding of FH, a negative regulator of complement, to spirochetal surface proteins, that correlates with complement resistance [66–68]. Recently, *T. denticola* has been shown to produce cystalysin, an enzyme that catalyzes the $\alpha,\beta$ elimination of L-cysteine to produce pyruvate, ammonia, and sulfide, which in turn enables the bacterium to produce sulfide at millimolar concentrations in the periodontal pocket. Sulfide is responsible for hemolytic and hemoxidative activities and for the damage to the gingival and periodontal tissues. Moreover, sulfide creates an ecological niche that selectively benefits *T. denticola* [69].

*T. forsythia* expresses a uniquely glycosylated surface envelope, known as the surface- (S-) layer, which plays an immunomodulatory role in influencing the immune response [70]. This S-layer has recently been shown to be important in delaying the cytokine responses of monocyte and macrophage cells *in vitro* [71, 72]. Settem et al. demonstrated in a mouse model of periodontitis that a terminal pseudaminic acid and N-acetylmannosaminuronic acid containing trisaccharide branch on an O-glycan core linked to the *Tannerella* surface proteins plays a role in dampening Th17 differentiation and mitigating neutrophil infiltration into the gingival tissue [73].

## 13. The Innate Immune Response, TLRs, and PAMPs

The host response against periodontopathic bacteria consists of innate and acquired immunity. The innate response meets the challenge of discriminating among large numbers of pathogens through recognition of conserved evolutionary molecular motifs called PAMPs (pathogen associated molecular patterns), which are expressed on pathogens but not by the host. The recently discovered Toll-like receptors (TLRs) are pattern-recognition receptors with key roles in detecting microbes and initiating inflammatory and host defense responses [74]. These signalling receptors are critical in pathogen recognition by the host, through specificity of recognition for several important PAMPs [7, 57]. TLRs are expressed by myelomonocytic cells, endothelial cells, epithelial cells, and other cells, including gingival fibroblasts [7]. Examples of PAMPs that are recognized by TLRs include peptidoglycan bacterial lipoproteins and *Pg* LPS (TLR-2), double stranded ribonucleic acid, LPS and heat shock proteins (TLR-4), and flagellin (TLR-5) [57, 74].

The TLR-PAMP interaction results in the recruitment of specific adapter molecules, which then bind the IL-1R-associated kinase. The signal is transmitted through a chain of signalling molecules common to all TLRs, involving tumor necrosis factor receptor-associated factor-6 (TRAF6) and mitogen activated protein kinases (MAPKs) [30, 74]. Subsequent activation of RANK and activated protein-1 leads to transcription of genes involved in stimulating the innate defenses, for example, expression of intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function associated antigen-1 (LFA-1), causing greater attachment and migration of leukocytes to tissues, as well as increased expression of proinflammatory cytokines involved in significant downstream bone resorption [30, 53]. IL-8 expression attracts neutrophils, and activation of endothelial cells, macrophages, dendritic cells, and neutrophils stimulates matrix metalloproteinase production, with a direct mechanism of tissue damage. Macrophages too when stimulated by pathogenic peptides are directly activated, producing various cytokines and biological mediators, for example, MMP-1 and nitric oxide [7, 11, 75].

Collective data over the last few years provide evidence that gingival fibroblasts and periodontal ligament cells are equipped to respond to LPS stimulation through TLR-PAMP recognition and involvement of the RANKL-mediated responses, producing various inflammatory cytokines, such as IL-1, IL-6, and IL-8, when stimulated by oral bacterial LPS fractions from pathogens such as *Pg*, *Pi* (*Prevotella intermedia*), or *Aa* [7, 11, 74–76].

Osteoblasts, which are highly sensitive to PAMPS, can also be induced to produce mediators and cytokines that are involved in bone resorption, as well as inhibition of the protective factor OPG. *Pi* LPS inhibits differentiation of osteoblasts and mineralisation of bone. Cementoblasts, however, when stimulated by LPS, exhibit decreased levels of RANKL and increased expression of both OPG and osteopontin, suggesting a protective mechanism against bone and root resorption [7, 30, 40].

In addition, LPS from different periodontopathogens, CPg DNA, and *Aa* capsular polysaccharide promote osteoclast differentiation from bone marrow cells. Monocytes stimulated by PAMPS demonstrate increased differentiation into osteoclasts, and induced RANKL expression plays a central role [30, 74]. Costimulatory factors, for example, GM-CSF and M-CSF, are also important as is the secretion of IL-12, which activates T cells to produce IFN gamma, leading to development of the cell mediated Th1 T cell response. In contrast, without costimulatory factors, the Th2 response predominates [28, 49, 50]. Recently, the effects of *P. gingivalis* LPS1435/1449 and LPS1690 on the expression of TLR2 and TLR4 signal transduction and the activation of proinflammatory cytokines IL-6 and IL-8 in human gingival fibroblasts were investigated and it was suggested that these lipid *Aa* structures differentially activate the TLR4-mediated NF-$\kappa$B signaling pathway and significantly modulate the expression of IL-6 and IL-8 [77]. A study aimed at identifying ligands on the surfaces of intact *P. gingivalis* cells that determine their ability to activate TLR2 found that it is due to a lipoprotein contaminant [78]. Further, a number of reports have proposed that the expression of an antagonistic or immunologically inert lipid A by *P. gingivalis* is a mechanism for evasion of TLR4 signaling [79].

Thus, in summary, LPS induced disease leads to the initiation of a local host response in gingival tissues that involves recruitment of inflammatory cells, generation of prostanoids and cytokines, elaboration of lytic enzymes, and activation of osteoclasts. Specifically, LPS increases osteoblastic expression of RANKL, IL-1, $PGE_2$, and TNF-$\alpha$, each of which is known to induce osteoclastic activity, viability, and differentiation [11, 28, 30, 50, 75, 76]. A variety of immune associated cell populations are responsible for the pathogenic processes in periodontal tissues, including specific CD4+ T cells, recruited monocytes, macrophages, and fibroblasts. These produce cytokines (TNF-$\alpha$, IL-1 $\beta$, etc.) within the lesion, which can be monitored and detected in the circulating GCF. In turn, these cytokines are pivotal to the destructive cascade and ultimately trigger the production of MMPs, prostaglandins, and osteoclasts. The end result is irreversible damage to the tooth supporting soft tissues and alveolar bone [23, 40, 43].

## 14. Conclusion

Bone resorption via osteoclasts and bone formation via osteoblasts are coupled, and their dysregulation is associated with numerous diseases of the skeletal system [3, 4, 13]. A wide range of host and microbial factors contribute to alveolar bone loss in periodontitis [2, 4, 23]. Yet, much remains to be understood about the complex mechanisms whereby these factors regulate bone resorption in periodontitis [7, 75]. Recent developments in the area of biological processes and mediators of osteoclast differentiation and activity have expanded our knowledge of resorption processes and set the stage for new diagnostic and therapeutic modalities to treat situations of localized bone loss as seen in periodontal disease [7, 25, 52, 62, 75, 80, 81].

## Conflict of Interests

The authors do not have any conflicts of interest to disclose in relation to this paper.

## References


[1] A. Di Benedetto, I. Gigante, S. Colucci, and M. Grano, "Periodontal disease: linking the primary inflammation to bone loss," *Clinical and Developmental Immunology*, vol. 2013, Article ID 503754, 7 pages, 2013.

[2] A. Nanci and D. D. Bosshardt, "Structure of periodontal tissues in health and disease," *Periodontology 2000*, vol. 40, no. 1, pp. 11–28, 2006.

[3] T. W. Oates and D. L. Cochran, "Bone cell interactions and regulation by inflammatory mediators," *Current Opinion in Periodontology*, vol. 3, pp. 34–44, 1996.

[4] Z. Schwartz, J. Goultschin, D. D. Dean, and B. D. Boyan, "Mechanisms of alveolar bone destruction in periodontitis," *Periodontology 2000*, vol. 14, no. 1, pp. 158–172, 1997.

[5] R. Gruber, *Molecular and Cellular Basis of Bone Resorption*, Wiener Medizinische Wochenschrift, 2014.

[6] L. K. McCauley and R. M. Nohutcu, "Mediators of periodontal osseous destruction and remodeling: principles and implications for diagnosis and therapy," *Journal of Periodontology*, vol. 73, no. 11, pp. 1377–1391, 2002.

[7] M. G. Newman, H. Takei, P. R. Klokkevold, and F. A. Carranza, *Carranza's Clinical Periodontology*, Elsevier Saunders, St. Louis, Mo, USA, 11th edition, 2012.

[8] S. H. Wiebe, M. Hafezi, H. S. Sandhu, S. M. Sims, and S. J. Dixon, "Osteoclast activation in inflammatory periodontal diseases," *Oral Diseases*, vol. 2, no. 2, pp. 167–180, 1996.

[9] R. Baron, "Molecular mechanisms of bone resorption by the osteoclast," *Anatomical Record*, vol. 224, no. 2, pp. 317–324, 1989.

[10] R. Baron, "Molecular mechanisms of bone resorption. An update," *Acta Orthopaedica Scandinavica, Supplement*, vol. 66, no. 266, pp. 66–70, 1995.

[11] M. A. Taubman, P. Valverde, X. Han, and T. Kawai, "Immune response: the key to bone resorption in periodontal disease," *Journal of Periodontology*, vol. 76, no. 11, supplement, pp. 2033–2041, 2005.

[12] A. Bruzzaniti and R. Baron, "Molecular regulation of osteoclast activity," *Reviews in Endocrine and Metabolic Disorders*, vol. 7, no. 1-2, pp. 123–139, 2006.

[13] S. L. Teitelbaum, "Bone resorption by osteoclasts," *Science*, vol. 289, no. 5484, pp. 1504–1508, 2000.

[14] S. L. Teitelbaum, "Osteoclasts: what do they do and how do they do it?" *The American Journal of Pathology*, vol. 170, no. 2, pp. 427–435, 2007.

[15] P. Gehron Robey, "The biochemistry of bone," *Endocrinology and Metabolism Clinics of North America*, vol. 18, no. 4, pp. 858–902, 1989.

[16] C. Raynal, P. D. Delmas, and C. Chenu, "Bone sialoprotein stimulates in vitro bone resorption," *Endocrinology*, vol. 137, no. 6, pp. 2347–2354, 1996.

[17] H. I. Roach, "Why does bone matrix contain non-collagenous proteins? The possible roles of osteocalcin, osteonectin, osteopontin and bone sialoprotein in bone mineralisation and resorption," *Cell Biology International*, vol. 18, no. 6, pp. 617–628, 1994.

[18] W. V. Giannobile, K. F. Al-Shammari, and D. P. Sarment, "Matrix molecules and growth factors as indicators of periodontal disease activity," *Periodontology 2000*, vol. 31, pp. 125–134, 2003.

[19] P. R. Garant, *Oral Cells and Tissues*, Quintessence Publishing Co., 2003.

[20] Z. Bar-Shavit, "The osteoclast: a multinucleated, hematopoietic-origin, bone-resorbing osteoimmune cell," *Journal of Cellular Biochemistry*, vol. 102, no. 5, pp. 1130–1139, 2007.

[21] J. M. W. Quinn, S. Neale, Y. Fujikawa, J. D. McGee, and N. A. Athanasou, "Human osteoclast formation from blood monocytes, peritoneal macrophages, and bone marrow cells," *Calcified Tissue International*, vol. 62, no. 6, pp. 527–531, 1998.

[22] H. Yasuda, N. Shima, N. Nakagawa et al., "Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 95, no. 7, pp. 3597–3602, 1998.

[23] J.-L. Saffar, J.-J. Lasfargues, and M. Cherruau, "Alveolar bone and the alveolar process: the socket that is never stable," *Periodontology 2000*, vol. 14, no. 1, pp. 76–90, 1997.

[24] D. Liu, J. K. Xu, L. Figliomeni et al., "Expression of RANKL and OPG mRNA in periodontal disease: possible involvement in bone destruction," *International Journal of Molecular Medicine*, vol. 11, no. 1, pp. 17–21, 2003.

[25] T. Nagasawa, M. Kiji, R. Yashiro et al., "Roles of receptor activator of nuclear factor-$\kappa$B ligand (RANKL) and osteoprotegerin in periodontal health and disease," *Periodontology 2000*, vol. 43, no. 1, pp. 65–84, 2007.

[26] S. Tanaka, N. Takahashi, N. Udagawa et al., "Macrophage colony-stimulating factor is indispensable for both proliferation and differentiation of osteoclast progenitors," *Journal of Clinical Investigation*, vol. 91, no. 1, pp. 257–263, 1993.

[27] N. Udagawa, N. Takahashi, E. Jimi et al., "Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony-stimulating factor: receptor activator of NF-kappa B ligand," *Bone*, vol. 25, no. 5, pp. 517–523, 1999.

[28] W. Zou and Z. Bar-Shavit, "Dual modulation of osteoclast differentiation by lipopolysaccharide," *Journal of Bone and Mineral Research*, vol. 17, no. 7, pp. 1211–1218, 2002.

[29] S. Bord, A. Horner, R. M. Hembry, J. J. Reynolds, and J. E. Compston, "Production of collagenase by human osteoblasts and osteoclasts in vivo," *Bone*, vol. 19, no. 1, pp. 35–40, 1996.

[30] N. Udagawa, N. Sato, S. Yang et al., "Signal transduction of lipopolysaccharide-induced osteoclast differentiation," *Periodontology 2000*, vol. 43, no. 1, pp. 56–64, 2007.

[31] P. T. Lakkakorpi and H. K. Väänänen, "Cytoskeletal changes in osteoclasts during the resorption cycle," *Microscopy Research and Technique*, vol. 33, no. 2, pp. 171–181, 1996.

[32] A. M. Parfitt, "The coupling of bone formation to bone resorption: a critical analysis of the concept and of its relevance to the pathogenesis of osteoporosis," *Metabolic Bone Disease and Related Research*, vol. 4, no. 1, pp. 1–6, 1982.

[33] M. Zaidi, A. S. Alam, V. S. Shankar et al., "Cellular biology of bone resorption," *Biological Reviews of the Cambridge Philosophical Society*, vol. 68, no. 2, pp. 197–264, 1993.

[34] M. Zaidi, H. C. Blair, B. S. Moonga, E. Abe, and C. L.-H. Huang, "Osteoclastogenesis, bone resorption, and osteoclast-based therapeutics," *Journal of Bone and Mineral Research*, vol. 18, no. 4, pp. 599–609, 2003.

[35] T. J. Chambers, "The pathobiology of the osteoclast," *Journal of Clinical Pathology*, vol. 38, no. 3, pp. 241–252, 1985.

[36] B. R. MacDonald, "Parathyroid hormone, prostaglandins and bone resorption," *World Review of Nutrition and Dietetics*, vol. 47, pp. 163–201, 1986.

[37] R. Gruber, "Cell biology of osteoimmunology," *Wiener Medizinische Wochenschrift*, vol. 160, no. 17-18, pp. 438–445, 2010.

[38] H. Kaji, T. Sugimoto, M. Kanatani et al., "Carboxyl-terminal parathyroid hormone fragments stimulate osteoclast-like cell formation and osteoclastic activity," *Endocrinology*, vol. 134, no. 4, pp. 1897–1904, 1994.

[39] J. L. Ebersole, "Humoral immune responses in gingival crevice fluid: local and systemic implications," *Periodontology 2000*, vol. 31, pp. 135–166, 2003.

[40] J. L. Ebersole, D. R. Dawson, L. A. Morford, R. Peyyala, C. S. Miller, and O. A. Gonzaléz, "Periodontal disease immunology: "double indemnity" in protecting the host," *Periodontology 2000*, vol. 62, no. 1, pp. 163–202, 2013.

[41] W. V. Giannobile, "Crevicular fluid biomarkers of oral bone loss," *Current Opinion in Periodontology*, vol. 4, pp. 11–20, 1997.

[42] G. Gupta, "Gingival crevicular fluid as a periodontal diagnostic indicator-II: inflammatory mediators, host-response modifiers and chair side diagnostic aids," *Journal of Medicine and Life*, vol. 6, no. 1, pp. 7–13, 2013.

[43] A. Holmlund, L. Hänström, and U. H. Lerner, "Bone resorbing activity and cytokine levels in gingival crevicular fluid before and after treatment of periodontal disease," *Journal of Clinical Periodontology*, vol. 31, no. 6, pp. 475–482, 2004.

[44] M. Gowen and G. R. Mundy, "Actions of recombinant interleukin 1, interleukin 2, and interferon-$\gamma$ on bone resorption in vitro," *Journal of Immunology*, vol. 136, no. 7, pp. 2478–2482, 1986.

[45] I. B. Lamster and M. J. Novak, "Host mediators in gingival crevicular fluid: implications for the pathogenesis of periodontal disease," *Critical Reviews in Oral Biology & Medicine*, vol. 3, no. 1-2, pp. 31–60, 1992.

[46] L. C. Hofbauer, S. Khosla, C. R. Dunstan, D. L. Lacey, W. J. Boyle, and B. L. Riggs, "The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption," *Journal of Bone and Mineral Research*, vol. 15, no. 1, pp. 2–12, 2000.

[47] Q. Jin, J. A. Cirelli, C. H. Park et al., "RANKL inhibition through osteoprotegerin blocks bone loss in experimental periodontitis," *Journal of Periodontology*, vol. 78, no. 7, pp. 1300–1308, 2007.

[48] T. Kawai, T. Matsuyama, Y. Hosokawa et al., "B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease," *The American Journal of Pathology*, vol. 169, no. 3, pp. 987–998, 2006.

[49] X. Han, T. Kawai, and M. A. Taubman, "Interference with immune-cell-mediated bone resorption in periodontal disease," *Periodontology 2000*, vol. 45, no. 1, pp. 76–94, 2007.

[50] E. J. Ohlrich, M. P. Cullinan, and G. J. Seymour, "The immunopathogenesis of periodontal disease," *Australian Dental Journal*, vol. 54, supplement 1, pp. S2–S10, 2009.

[51] T. Yucel-Lindberg and T. Bage, "Inflammatory mediators in the pathogenesis of periodontitis," *Expert Reviews in Molecular Medicine*, vol. 15, p. e7, 2013.

[52] G. N. Belibasakis and N. Bostanci, "The RANKL-OPG system in clinical periodontology," *Journal of Clinical Periodontology*, vol. 39, no. 3, pp. 239–248, 2012.

[53] M. A. Taubman and T. Kawai, "Involvement of T-lymphocytes in periodontal disease and in direct and indirect induction of bone resorption," *Critical Reviews in Oral Biology and Medicine*, vol. 12, no. 2, pp. 125–135, 2001.

[54] E. AlShwaimi, E. Berggreen, H. Furusho et al., "IL-17 receptor a signaling is protective in infection-stimulated periapical bone destruction," *Journal of Immunology*, vol. 191, no. 4, pp. 1785–1791, 2013.

[55] S. L. Gaffen and G. Hajishengallis, "A new inflammatory cytokine on the block: re-thinking periodontal disease and the Th1/Th2 paradigm in the context of Th17 cells and IL-17," *Journal of Dental Research*, vol. 87, no. 9, pp. 817–828, 2008.

[56] P. D. Marsh, "Microbial ecology of dental plaque and its significance in health and disease," *Advances in Dental Research*, vol. 8, no. 2, pp. 263–271, 1994.

[57] R. P. Darveau, "Periodontitis: a polymicrobial disruption of host homeostasis," *Nature Reviews Microbiology*, vol. 8, no. 7, pp. 481–490, 2010.

[58] S. S. Socransky, A. D. Haffajee, M. A. Cugini, C. Smith, and R. L. Kent Jr., "Microbial complexes in subgingival plaque," *Journal of Clinical Periodontology*, vol. 25, no. 2, pp. 134–144, 1998.

[59] S. C. Holt and J. L. Ebersole, "*Porphyromonas gingivalis, Treponema denticola*, and *Tannerella forsythia*: the "red complex", a prototype polybacterial pathogenic consortium in periodontitis," *Periodontology 2000*, vol. 38, no. 1, pp. 72–122, 2005.

[60] G. Hajishengallis, S. Liang, M. A. Payne et al., "Low-abundance biofilm species orchestrates inflammatory periodontal disease through the commensal microbiota and complement," *Cell Host and Microbe*, vol. 10, no. 5, pp. 497–506, 2011.

[61] G. Hajishengallis, "Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response," *Trends in Immunology*, vol. 35, no. 1, pp. 3–11, 2014.

[62] G. Hajishengallis, R. P. Darveau, and M. A. Curtis, "The keystone-pathogen hypothesis," *Nature Reviews Microbiology*, vol. 10, no. 10, pp. 717–725, 2012.

[63] G. Hajishengallis and S. E. Sahingur, "Novel inflammatory pathways in periodontitis," *Advances in Dental Research*, vol. 26, no. 1, pp. 23–29, 2014.

[64] R. Claesson, A. Johansson, G. Belibasakis, L. Hänström, and S. Kalfas, "Release and activation of matrix metalloproteinase 8 from human neutrophils triggered by the leukotoxin of *Actinobacillus actinomycetemcomitans*," *Journal of Periodontal Research*, vol. 37, no. 5, pp. 353–359, 2002.

[65] J. S. Kinney, T. Morelli, M. Oh et al., "Crevicular fluid biomarkers and periodontal disease progression," *Journal of Clinical Periodontology*, vol. 41, no. 2, pp. 113–120, 2014.

[66] S. Grosskinsky, M. Schott, C. Brenner, S. J. Cutler, M. M. Simon, and R. Wallich, "Human complement regulators C4b-binding protein and C1 esterase inhibitor interact with a novel outer surface protein of *Borrelia recurrentis*," *PLoS Neglected Tropical Diseases*, vol. 4, no. 6, article no. e698, 2010.

[67] T. Meri, H. Amdahl, M. J. Lehtinen et al., "Microbes bind complement inhibitor factor H *via* a common site," *PLoS Pathogens*, vol. 9, no. 4, Article ID e1003308, 2013.

[68] D. P. Miller, J. V. Mcdowell, J. K. Bell, M. P. Goetting-Minesky, J. C. Fenno, and R. T. Marconi, "Analysis of the complement sensitivity of oral treponemes and the potential influence of FH binding, FH cleavage and dentilisin activity on the pathogenesis of periodontal disease," *Molecular Oral Microbiology*, vol. 29, no. 5, pp. 194–207, 2014.

[69] F. Spyrakis, B. Cellini, S. Bruno et al., "Targeting Cystalysin, a virulence factor of treponema denticola-supported periodontitis," *ChemMedChem*, vol. 9, no. 7, pp. 1501–1511, 2014.

[70] R. P. Settem, K. Honma, and A. Sharma, "Neutrophil mobilization by surface-glycan altered Th17-skewing bacteria mitigates periodontal pathogen persistence and associated alveolar bone loss," *PLoS ONE*, vol. 9, no. 9, Article ID e108030, 2014.

[71] G. Posch, G. Sekot, V. Friedrich et al., "Glycobiology aspects of the periodontal pathogen *Tannerella forsythia*," *Biomolecules*, vol. 2, no. 4, pp. 467–482, 2012.

[72] G. Sekot, G. Posch, P. Messner et al., "Potential of the *Tannerella forsythia* S-layer to delay the immune response," *Journal of Dental Research*, vol. 90, no. 1, pp. 109–114, 2011.

[73] R. P. Settem, K. Honma, T. Nakajima et al., "A bacterial glycan core linked to surface (S)-layer proteins modulates host immunity through Th17 suppression," *Mucosal Immunology*, vol. 6, no. 2, pp. 415–426, 2013.

[74] D. M. Underhill and A. Ozinsky, "Toll-like receptors: key mediators of microbe detection," *Current Opinion in Immunology*, vol. 14, no. 1, pp. 103–110, 2002.

[75] P. Mark Bartold and T. E. van Dyke, "Periodontitis: a host-mediated disruption of microbial homeostasis. Unlearning learned concepts," *Periodontology 2000*, vol. 62, no. 1, pp. 203–217, 2013.

[76] A. L. Dumitrescu, S. A. El-Aleem, B. Morales-Aza, and L. F. Donaldson, "A model of periodontitis in the rat: effect of lipopolysaccharide on bone resorption, osteoclast activity, and local peptidergic innervation," *Journal of Clinical Periodontology*, vol. 31, no. 8, pp. 596–603, 2004.

[77] T. D. K. Herath, R. P. Darveau, C. J. Seneviratne, C.-Y. Wang, Y. Wang, and L. Jin, "Tetra- and penta-acylated lipid A structures of *Porphyromonas gingivalis* LPS differentially activate TLR4-mediated NF-kappaB signal transduction cascade and immuno-inflammatory response in human gingival fibroblasts," *PLoS ONE*, vol. 8, no. 3, Article ID e58496, 2013.

[78] S. Jain, S. R. Coats, A. M. Chang, and R. P. Darveau, "A novel class of lipoprotein lipase-sensitive molecules mediates toll-like receptor 2 activation by *Porphyromonas gingivalis*," *Infection and Immunity*, vol. 81, no. 4, pp. 1277–1286, 2013.

[79] C. Slocum, S. R. Coats, N. Hua et al., "Distinct lipid a moieties contribute to pathogen-induced site-specific vascular inflammation," *PLoS Pathogens*, vol. 10, no. 7, Article ID e1004215, 2014.

[80] G. Hajishengallis and J. D. Lambris, "Complement-targeted therapeutics in periodontitis," *Advances in Experimental Medicine and Biology*, vol. 735, pp. 197–206, 2013.

[81] G. Hajishengallis and R. J. Lamont, "Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology," *Molecular Oral Microbiology*, vol. 27, no. 6, pp. 409–419, 2012.

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*Review Article*

# Innate and Adaptive Immunity in Calcific Aortic Valve Disease

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Calcific aortic valve disease (CAVD) is the most common heart valve disorder. CAVD is a chronic process characterized by a pathologic mineralization of valve leaflets. Ectopic mineralization of the aortic valve involves complex relationships with immunity. Studies have highlighted that both innate and adaptive immunity play a role in the development of CAVD. In this regard, accumulating evidence indicates that fibrocalcific remodelling of the aortic valve is associated with activation of the NF-$\kappa$B pathway. The expression of TNF-$\alpha$ and IL-6 is increased in human mineralized aortic valves and promotes an osteogenic program as well as the mineralization of valve interstitial cells (VICs), the main cellular component of the aortic valve. Different factors, including oxidized lipid species, activate the innate immune response through the Toll-like receptors. Moreover, VICs express 5-lipoxygenase and therefore produce leukotrienes, which may amplify the inflammatory response in the aortic valve. More recently, studies have emphasized that an adaptive immune response is triggered during CAVD. Herein, we are reviewing the link between the immune response and the development of CAVD and we have tried, whenever possible, to keep a translational approach.

## 1. Introduction

Ectopic cardiovascular mineralization occurs in different disorders and is associated with a substantial morbidity. Long seen as a passive process ectopic pathologic mineralization is now considered as a highly regulated process at the molecular and cellular levels [1]. Calcific aortic valve disease (CAVD) is a chronic disorder that involves a mineralization of the valvular tissue. Fibrosis often accompanies pathologic mineralization and both processes contribute to the fibrocalcific remodelling of the aortic valve. CAVD is the most common heart valve disease and encompasses a wide variety of clinical presentations. As such, there is a continuum from aortic valve sclerosis to severe aortic stenosis with a common underlying process: ectopic mineralization of the aortic valve [2]. One key central question, still unresolved, is what drives the fibrocalcific remodelling of the aortic valve. Is the mineralization of the aortic valve triggered and enhanced by factors that normally promote the mineralization of bone? Studies performed in the last decade have unravelled important key underlying mechanisms involved in the ectopic mineralization of the aortic valve. These works have highlighted that though there are, to some extent, similarities with the ossification process, the mineralization of the aortic valve relies on specific mechanisms that differ from bone ossification. In fact, the response to different stimuli may diverge considerably in VICs when compared to osteoblasts. These findings may thus explain the association between osteoporosis and ectopic vascular/valvular mineralization [3]. The tissue architecture and specific mechanical strain imposed on the aortic valve drive specific molecular events that may promote pathologic mineralization [4]. Apart from mineralization, key features of CAVD include the abnormal accumulation of different lipid species and inflammatory cells in explanted human pathologic samples [5].

Immunity, both innate and adaptive, has been shown to play an important role in different chronic disorders including atherosclerosis. In CAVD, accumulating evidence clearly indicates that inflammation is involved in the development and possibly in the progression of the disease process [6]. Innate response to different factors has been shown to promote the mineralization of valve interstitial cells (VICs)

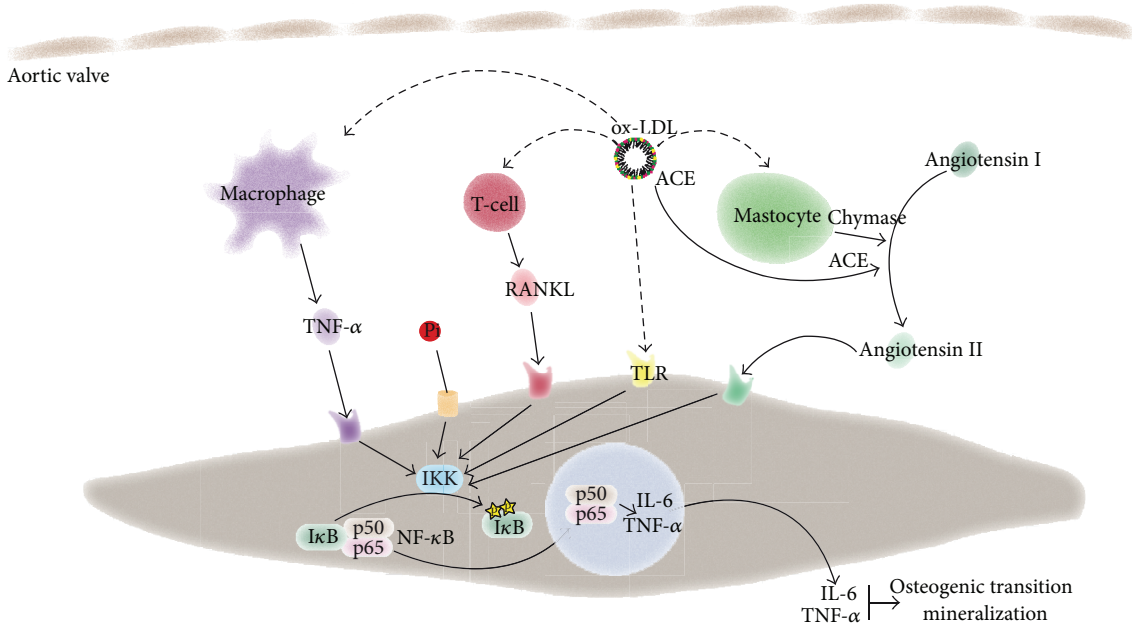



Figure 1: Scheme showing the interaction between inflammatory cells and valve interstitial cells (VICs) and its role during CAVD.

(Figure 1), the main cellular component of the aortic valve. On the other hand, emerging evidence suggests that specific adaptive immunity could play a substantial role in orchestrating an immune response during CAVD (Figure 1). In this review, we describe, at the molecular and cellular levels, the role of immunity in CAVD.

## 2. Pathobiology of CAVD: An Overview

The aortic valve is normally a thin structure that accomplishes the important task of promoting the unidirectional blood flow from the left ventricle to the aorta. The histological structure of the aortic valve is classically divided into three different layers: the fibrosa, the spongiosa, and the ventricularis. The fibrosa and the ventricularis are the outermost external layers that, respectively, face the aorta and the left ventricle in diastole. The fibrosa mostly contains collagen fibers with dispersed VICs, whereas the ventricularis has a high content of elastic fibers and is populated by both VICs and smooth muscle cells (SMCs). The central layer, the spongiosa, has a high content in glycosaminoglycans (GAGs) and is thought to play an important role in the biomechanical properties of the aortic valve in absorbing a part of the mechanical load during the cardiac cycle [7, 8]. The aortic valve is covered with an endothelium and studies have highlighted that the aortic and ventricular endothelium have different biological properties, which may impact on the development of CAVD [9].

The microscopic analyses of human mineralized aortic valves, obtained from surgeries, have revealed some important key features of CAVD. First, lipids infiltrate the valve tissue often in the vicinity of mineralized areas [10]. Second, dense inflammatory infiltrates are present in some valves where oxidized lipids and ectopic mineralization are observed [6, 11]. Three, in about 15% of mineralized aortic valves osteogenic metaplasia is observed [12]. Coté et al. showed in 285 mineralized valves that the presence of dense chronic inflammatory infiltrates, present in 28% of valves, was associated with osseous metaplasia, neovascularization, and a higher level of tissue remodeling [6]. Furthermore, the density of leukocytes in mineralized aortic valves was associated with a faster progression rate of aortic stenosis. Hence, these data suggest that CAVD has possibly important relationships with inflammation.

## 3. NF-Kappa B Pathway and CAVD

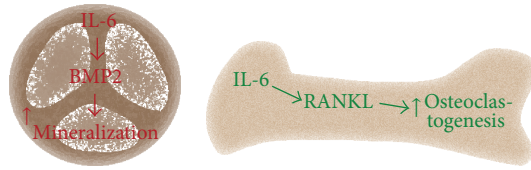
*3.1. Regulation of the NF-κB Pathway.* The nuclear factor-κ B (NF-κB) is at the center stage of inflammation. The NF-κB pathway is classically divided into the canonical and noncanonical cascades. The canonical pathway, which is activated by tumor necrosis factor alpha (TNF-$\alpha$) and IL-1$\beta$ among others, relies on activation of the heterodimer formed by p65 (RelA) and p50, which control the expression of several genes involved in the inflammatory response [13]. Upstream, the inhibitor of IκB kinase (IKK), controls the activation of the cascade. IKK is a multisubunit complex, which consists of IKK$\alpha$, IKK$\beta$, and IKK$\gamma$ (NEMO) [14]. IKK$\beta$ catalytic activity is promoted by IKK$\gamma$, a regulatory protein that promotes activity of the IKK complex. In turn, the IKK complex phosphorylates IκB$\alpha$ on Ser residues 32 and 36. As a result, IκB$\alpha$ is targeted for degradation and the p65/p50 heterodimer is liberated from the cytosol and migrates to the nucleus where it controls the expression of target genes [15]. It is worth underlining that posttranscriptional modification of p65 subunit is also important to control the

nuclear translocation and transcriptional activities. Twelve phosphosites for p65 have been described so far and have been shown to either enhance or decrease transcriptional activity in a gene-specific and possibly cell-specific manner [16]. For instance, in U937 macrophages stimulated with LPS, Ser 536 phosphorylated p65 is specifically recruited on IL-8 promoter and not on IL-6 and TNF-$\alpha$ promoters [17]. Phosphorylation of Ser 276 on p65 by the catalytic unit of protein kinas A (PKAc) has been shown to promote the acetylation of p65 and to allow a stable association with its cofactors CREB-binding protein/p300 and chromatin targets [18]. Reactive oxygen species (ROS) have been shown to act as cofactor in PKAc-mediated phosphorylation on Ser 276 of p65 [19]. Of note, ROS are generated in mineralized aortic valves and promote the expression of osteogenic genes [20]. On the other hand, the noncanonical pathway relies on the phosphorylation of IKK$\alpha$ by its upstream kinase NF-$\kappa$B-inducing kinase (NIK/MEKK14) and is independent from IKK$\gamma$ [21]. Activation of this pathway by different signals, such as lymphotoxin B, CD40L, or B cell activating factor, promotes the nuclear translocation of RelB/p52 [22].

*3.2. Phosphate and Activation of the NF-κB Cascade.* By producing a high level of cytokines VICs play a crucial role in the regulation of inflammation. In explanted human mineralized aortic valves the levels of phosphorylated I$\kappa$B (Ser 32) are increased significantly [23]. One fact, which is often underappreciated, is that phosphate (Pi) is an important promoter of inflammation in isolated VICs [23]. Phosphates are produced in fairly good amounts by VICs during mineralization owing to a high expression of nucleotide metabolizing enzymes, the ectonucleotidases [24]. During the mineralization of the aortic valve, alkaline phosphatase (ALP) and ectonucleotide phosphodiesterase/pyrophosphatase-1 (ENPP1) are overexpressed. The ectonucleotidases generate Pi at the cell membrane [25–27]. In turn, Pi is internalized by VICs through the sodium-dependent phosphate cotransporter Pit1 (SLC20A1), which is overexpressed in stenotic aortic valves [28]. Though the molecular details are lacking, the intracellular channelling of Pi is associated with a lower level of Akt, a kinase involved in cell survival. We recently identified that Akt prevents the activation of the NF-$\kappa$B pathway in VICs [23]. Also, the overexpression of ectonucleotidases contributes to the depletion of the extracellular levels of nucleotides, which, in turn, decrease the signalling through the P2Y2 receptor (P2Y2R) [24]. In VICs, secretion of ATP gives a survival signal through the P2Y2R and Akt. Hence, a lower signalling through Akt promotes a sustained activation of the NF-$\kappa$B pathway. The mechanism whereby Akt decreases NF-$\kappa$B signalling in VICs remains to be fully investigated, but overexpression of Akt in VICs prevents the nuclear translocation of NF-$\kappa$B-p65 in response to phosphate and inhibits the mineralization of cell cultures [23].

*3.3. IL-6 Regulates the Mineralization of the Aortic Valve.* One important target of NF-$\kappa$B-p65 is interleukin-6 (IL-6), a pleiotropic cytokine often used as a blood plasma marker of inflammation and cardiovascular risk [29]. However, little is known on the role of IL-6 in CAVD. By using a whole-genome transcriptomic approach El Husseini et al. identified that, among the family of cytokines that signal through gp130, IL-6 is highly expressed in mineralized aortic valves [23]. Evidence indicates that the NF-$\kappa$B pathway is activated in human explanted aortic valves and thus directly contributes to the secretion of IL-6. IL-6 is produced and secreted in large amounts by VICs. As highlighted above several factors may contribute to promote the production of IL-6 by VICs. In this regard, a high activity of ectonucleotidases contributes to decreasing the signalling through Akt, which in VICs normally represses the NF-$\kappa$B pathway [23]. Moreover, although not studied in VICs, it is possible that other factors including cytokines such as TNF-$\alpha$ and oxidized lipid species may also contribute to trigger the secretion of IL-6. In turn, IL-6 exerts an important control over the osteogenic transition of VICs through auto/paracrine effects. In this regard, silencing of IL-6 in VICs prevented phosphate-induced mineralization of cell cultures [23]. Moreover, the silencing of IL-6 in VICs abrogated the rise of osteogenic transcripts following a treatment with the phosphate-containing medium [23]. Conversely, treatment of VICs with exogenous IL-6 increased the expression of osteogenic genes severalfold. BMP2 is the gene which is the most highly upregulated following a treatment of VIC cultures with IL-6. Inhibition of BMP2 with Noggin, an antagonist of BMP2 and BMP4, prevented IL-6-induced mineralization of VICs [23]. Taken together, a high production of IL-6 by VICs is conducive to an osteogenic transition and mineralization of the aortic valve through a BMP2 pathway [23]. Recently, it has been identified that IL-6 promotes in the aortic valve the endothelial-mesenchymal transition (EndoMT) [30]. Hence, in the aortic valve it is possible that IL-6 increases the amount of mesenchymal cells with osteogenic properties through the EndoMT process. Of interest, in bone, IL-6 stimulates osteoclast activity. Of note, this effect is indirect as it needs the presence of osteoblasts. In this regard, IL-6 induces the expression of receptor activator of nuclear factor-kappa B ligand (RANKL) by osteoblasts whereby osteoclastogenesis is promoted [31]. Chronic and systemic elevation of IL-6 is one important feature of several clinical conditions, including obesity, which are linked with osteoporosis and a higher incidence of CAVD [32]. Hence, though it remains to be investigated it is possible that IL-6 explains, at least in part, the so-called calcification paradox, where bone demineralization occurs at the same time that valvular/vascular mineralization develops (Figure 2).

*3.4. TNF Family of Cytokines.* TNF-$\alpha$ strongly activates the canonical NF-$\kappa$B pathway and promotes the mineralization of VICs [33, 34]. TNF-$\alpha$ is first synthesized as a type II transmembrane protein, which upon cleavage by TNF-$\alpha$ converting enzyme (TACE) is released in the extracellular space [35]. It is produced by different cells including monocytes/macrophages, smooth muscle cells, and adipocytes among others. When stimulated, the ubiquitous TNF receptor 1 (TNFR1) activates and recruits TNFR-associated death domain (TRADD), which, in turn, recruits the Fas-associated protein with death domain (FADD) [36, 37]. As a result,




(a) CAVD (b) Bone

Figure 2: The calcification paradox could be explained by interleukin-6 (IL-6), which mediates the mineralization of the aortic valve and in bone activates osteoclastogenesis.

FADD activates caspase-3 and caspase-8. Therefore, though not fully investigated, it is possible that TNF-$\alpha$-induced mineralization of VIC culture is dependent on an apoptotic process. To this effect, apoptosis is one important process involved in the mineralization of the aortic valve [24]. Apoptotic bodies derived from plasma membrane and rich in ectonucleotidases promote the nucleation of hydroxyapatite and the formation of spheroid mineralized microparticles (2–5 $\mu$m), which incidentally are the basic unit of mineralized material formed in CAVD [4, 38]. On the other hand, in response to TNF-$\alpha$, TNFR1 may also recruit a receptor associated complex formed by TNF receptor associated factors (TRAF) and TRADD proteins, which activates transforming growth factor $\beta$ activated kinase-1 (TAK1) [39]. TAK1 phosphorylates IKK$\beta$ and triggers activation of NF-$\kappa$B. In vascular smooth muscle cells (VSMCs), TNF-$\alpha$ induces the expression of Msx2, a homeobox transcription factor involved in osteogenic transition [40]. Msx2 is increased in mineralized aortic valve and promotes activation of the Wnt pathway, which is by the way involved in the development of CAVD [41]. TNF-$\alpha$ has also crosstalk with interleukin-1$\beta$ (IL-1$\beta$). In this regard, in mice deficient for the IL-1 receptor antagonist, IL-1RA, the circulating levels of TNF-$\alpha$ are increased and the aortic valve is thickened [42]. The double knockout mice IL-1RA$^{-/-}$ TNF-$\alpha^{-/-}$, however, do not develop fibrosis of the aortic valve. On the other hand, in isolated VICs, IL-1$\beta$ induces the expression of MMP-1, suggesting that it may participate in the remodelling process of the aortic valve [43]. Hence, it is likely that IL-1$\beta$ and TNF-$\alpha$ have reciprocal crosstalk, which promotes CAVD in mice. Recently, the expression of TNF-related apoptosis-inducing ligand (TRAIL) has been shown to be elevated in mineralized aortic valves [44]. TRAIL, a member of the TNF superfamily, has been shown to promote apoptosis-mediated mineralization of VICs through the death receptor 4, which is overexpressed by VICs during mineralization. Hence, overexpression of the TNF family of cytokines in CAVD plays an important role in promoting fibrosis/mineralization of the aortic valve through both apoptosis and an osteogenic program.

RANKL is expressed by activated CD4+, CD8+ cells, osteoblast, and bone marrow stromal cells. It is a type 2 transmembrane protein, which can be cleaved by metalloproteinase [45]. RANKL binds to its receptor RANK whereby TNFR-associated factors 2 and 6 (TRAFF2-6) are recruited [46]. This leads to a sequence of events that mediates the activation of AP-1, c-Src, and c-Cbl [47]. Osteoprotegerin (OPG) is a soluble decoy receptor for RANKL and is thus a negative regulator of RANKL signaling [48]. In bone, stimulation of RANK activates osteoclast activity. The OPG$^{-/-}$ mice develop osteoporosis and vascular calcification [49]. In the aortic valve, a study based on immunodetection has revealed that RANKL is overexpressed in mineralized aortic valves, whereas the expression of OPG is decreased in the same valves [50]. In isolated VIC cultures, the stimulation of cells with RANKL activates mineralization and the expression of ALP [50]. In LDLR$^{-/-}$ mice under high fat diet, the administration of OPG reduced mineralization of the aortic valve and decreased the expression of osteogenic genes, such as osterix and osteocalcin [51]. Taken together, these observations suggest that RANK signaling may play a permissive role in the development of CAVD.

*3.5. Angiotensin II.* The renin angiotensin system (RAS) is activated in patients with visceral obesity, a condition associated with the development of CAVD [32]. Studies have underscored that enzymes, which generate angiotensin II, are present in stenotic mineralized aortic valves [52]. Angiotensin converting enzyme (ACE) is present in human explanted stenotic aortic valves and colocalizes with low-density lipoprotein (LDL). Experiments with isolated LDL fraction showed that ACE was present in this fraction, suggesting that the angiotensin II-generating enzyme is possibly transported in the aortic valve. Moreover, mastocytes producing chymase, an angiotensin II-generating enzyme, are present in mineralized aortic valves and their density correlates with several indices of disease severity [53, 54]. As such, both ACE and chymase contribute to produce angiotensin II in the aortic valve. Moreover, Côté et al. identified in prehypertensive men with CAVD that the circulating levels of angiotensin II correlate with the valvular mRNA levels of TNF-$\alpha$ and IL-6 [55]. Moreover, immunohistological analyses revealed in stenotic aortic valves that angiotensin II was present in the vicinity of mineralized nodules and colocalized with TNF-$\alpha$ and IL-6 [55]. Patients under a therapy with angiotensin receptor blockers (ARBs) have a lower level of transcript encoding for IL-6 in their aortic valves [56]. Moreover, a retrospective study showed that ARBs are associated with a slower progression rate of aortic stenosis [57]. In mice, the administration of angiotensin II promoted the fibrotic remodelling of the aortic valve [58]. Also, the administration of olmesartan, an ARB, prevented the fibrotic remodelling of the aortic valve in the hypercholesterolemic

rabbit [59]. Taken together, these data suggest that both systemic and valvular production of angiotensin II may contribute to CAVD. It is worth pointing out that angiotensin II is a potent activator of the NF-$\kappa$B pathway via the type 1 angiotensin receptor (AT1AR). The activation of NF-$\kappa$B by angiotensin II is relatively complex and differs from the signalling induced by TNF-$\alpha$. Stimulation of AT1AR, a Gq protein coupled receptor, activates phospholipase C $\beta$ (PLC$\beta$), which generates inositol triphosphate and diacylglycerol (DAG). In turn, DAG activates a typical protein kinase C (PKC). PKC phosphorylates an adaptor protein of the membrane guanylate kinase family, CARMA3 [60]. Next, activated CARMA3 forms a complex with Bcl10 and mucosa-associated lymphoid tissue lymphoma translocation 1 (MALT1), which deubiquitylates several members of the activating pathway of NF-$\kappa$B including IKK$\gamma$. As a result, the accumulation of IKK$\gamma$ promotes the nuclear translocation of p65. Moreover, in VSMCs angiotensin II activates RhoA, which leads to the phosphorylation of p65 on Ser 536. It is suspected that NIK may mediate the phosphorylation of p65 on Ser 536 [61]. This sequence of events has possibly important repercussion as p65 Ser 536 is not inhibited by I$\kappa$B [62]. This creates a nuclear pool of p65 Ser 536, which is recycled with the chromatin-bound promoter [63]. Hence, this chain of events promotes a sustained activation of target genes of p65. However, whether a similar pathway of activation is present in VICs remains to be explored.

## 4. Innate Immune Response to Oxidized Lipid Species and Ectopic Mineralization of the Aortic Valve

*4.1. Interaction between Lipid Retention and Toll-Like Receptors.* The Toll-like receptors (TLRs), which are expressed by VICs, play a key role in driving the inflammatory reaction in response to several stimuli. In this regard, several lipid species can activate TLRs. Early investigations of CAVD have pointed out that apoB, oxidized-low-density lipoproteins (ox-LDL), and Lp(a) infiltrate mineralized aortic valves [64]. So far, several factors have been identified to play a role in lipid infiltration/retention of the aortic valve. To this effect, a high proportion of circulating small, dense, LDL has been associated with a higher accumulation of ox-LDL in the aortic valve and with an elevated level of TNF-$\alpha$ [10]. Small, dense LDLs have a higher oxidation rate and have a greater ability to infiltrate tissues. Of note, the small, dense LDL phenotype is one key feature of diabetes and the metabolic syndrome (MetS), two important risk factors for the development and progression of CAVD [65]. Also, during CAVD a higher production of proteoglycans (PGs) may help to promote the retention of lipids. The expression of decorin is increased in aortic valves and histological studies showed a colocalization of decorin with lipoprotein lipase (LPL), which is secreted by macrophages [66]. Studies have highlighted that interaction between LPL and decorin promotes the retention of LDL. The expression of biglycan is increased in human pathological samples of CAVD and participates in the retention of lipids within the aortic valve [67]. Moreover, emerging evidence indicates that biglycan is a potent agonist of TLRs. In this regard, in isolated VICs biglycan stimulates TLR-2, which leads to the secretion of phospholipid transfer protein (PLTP). In turn, PLTP may associate with apoA1 of high-density lipoproteins (HDLs), which may impede their function, namely, the reverse cholesterol transport (RCT) [67]. More recently, biglycan has been shown to promote the osteogenic transition of VICs through TLR-2, the extracellular signal-regulated protein kinase 1/2 (ERK1/2), and the NF-$\kappa$B pathways [68]. In isolated VICs, biglycan also induced the secretion of monocyte chemoattractant protein-1 (MCP-1) [69]. Also, it is worth underlining that ox-LDL has been shown to stimulate TLR-4, whereby the mineralization of VICs is promoted [70]. Zeng et al. reported in VICs that Notch1 promotes the activation of the NF-$\kappa$B pathway following stimulation of TLR-4 with bacterial lipopolysaccharide (LPS) [71]. The authors documented that the Notch intracellular domain (NICD) interacted with I$\kappa$B kinase $\alpha$ (IKK$\alpha$) and in doing so promoted the nuclear translocation of p65 subunit of NF-$\kappa$B. However, the molecular mechanism whereby the NICD may impact on IKK and its phosphorylation status was not examined. The same group also identified that ox-LDL promoted the secretion of Jagged 1 by VICs, which ultimately led to increased levels of NICD and activation of an osteogenic response [72]. However, it is worth highlighting that frameshift mutations of the *NOTCH1* gene have been associated with bicuspid aortic valves (BAV) and mineralization of the aortic valve [73]. Investigations have shown that the NICD, which is the intracellular portion of the Notch1 receptor cleaved by the $\gamma$-secretase following stimulation of the Notch receptor, activates the expression of the hairy family of repressors that inhibit the expression of BMP2 and Runx-2, respectively, a bone morphogen and master transcription factor involved in osteogenesis [74]. Hence, Notch1 delivers signals that prevent the osteogenic transition of VICs. The apparent discrepancy between studies that have investigated the role of Notch in CAVD may result from different cell culture conditions and may be context dependent. Further work is needed to explore the interactions between Notch, inflammation, and the mineralization of the aortic valve.

The retention of lipids in the extracellular matrix of the aortic valve is also promoted by the elongation of GAG chains, which is enhanced by transforming growth factor $\beta$1 (TGF-$\beta$1) [75]. The retention of lipids in the aortic valve promotes, in turn, the oxidation of lipid species, which are potent agonists of the TLRs. During CAVD, the uncoupling of nitric oxide synthase (NOS) contributes to the increase of the oxidative stress [76]. Though the mechanisms of NOS uncoupling in the aortic valve have not been investigated, it is possible that a decreased bioavailability of NOS substrate, L-arginine, or cofactors, such as tetrahydrobiopterin, contribute to the increase of the production of ROS [77]. As a result, the increased production of ROS in mineralized aortic valves promotes the formation of lipid peroxidation products with proinflammatory activities. Recently, pathways leading to the production of highly reactive lipid species, derived from ox-LDL, have been identified and may play an important role in the immune response and the mineralization of the aortic valve.

*4.2. Lp(a), Lipoprotein-Associated Phospholipase A2, and Inflammation-Mediated Mineralization of the Aortic Valve.* In a genome-wide association study (GWAS), Thanassoulis et al. have recently identified that single nucleotide polymorphisms (SNPs) of the *LPA* gene encoding for Lp(a) were associated with CAVD [78]. Using a Mendelian randomization study design two other independent studies have since then corroborated the positive association between Lp(a) and CAVD [79, 80]. These studies thus suggest a causal role for Lp(a) in the development of CAVD. Though the physiological role of Lp(a) is largely unknown, it is well accepted that it is a major carrier of oxidized-phospholipids (ox-PLs) in the blood plasma [81]. Ox-PLs are potent stimulators of inflammation through their metabolism by phospholipase enzymes [82]. Mahmut et al. have recently discovered that lipoprotein-associated phospholipase A2 (Lp-PLA2) is overexpressed in mineralized aortic valve [83]. Immunohistological analyses suggest that Lp-PLA2 is transported in the aortic valve by lipoproteins and also secreted by infiltrating macrophages. The transcript level of Lp-PLA2 in stenotic aortic valves correlated with several indices of tissue remodelling and mineralization of the aortic valve. Of note, ox-PLs are the natural substrates for Lp-PLA2 and are hydrolyzed into lysophosphatidylcholine (LPC), which are highly reactive and proinflammatory. In isolated VICs, LPC promoted the expression of ALP, ENPP1, and the sodium-phosphate cotransporter Pit1 [83]. Of note, the expressions of ALP, ENPP1, and Pit1 are increased in mineralized aortic valves and contribute actively to the mineralization process [28]. Hence, it is likely that ox-PLs transported by Lp(a) are transformed into LPC by Lp-PLA2 in the aortic valve, whereby inflammation and mineralization are triggered.

*4.3. Lipoxygenase and Leukotrienes: Role in CAVD.* Arachidonate is a polyunsaturated fatty acid (20 : 4), which is produced from phospholipids and the action of PLA2 and lipoprotein lipase (LPL), which are incidentally overexpressed in the aortic valve during CAVD [66, 84]. Arachidonate is a powerful metabolite that exerts an important control over inflammation. In mineralized aortic valve the expressions of 5-lipoxygenase (5-LO) and LTC4S are increased and correlate with echocardiographic indices of aortic stenosis severity [85]. Moreover, the expression of 5-LO was documented in both macrophages and VICs. The combined action of 5-LO and LTC4S generates leukotriene C(4) (LTC4), which has potent proinflammatory activities [86]. *In vitro*, LTC4 induced oxidative stress in VICs with the loss of mitochondrial membrane potential and the concomitant expression of BMP2 and BMP6 [85]. Hence, the expression of 5-LO by VICs promotes the production of leukotrienes, which constitute short acting messengers that drives inflammation of tissues. Whether the 5-LO pathway plays a crucial role in the mineralization of the aortic valve remains to be investigated.

*4.4. Remodelling, Neoangiogenesis, and MMPs.* The activation of innate immunity leads to the expression of several factors involved in tissue remodelling. TGF-$\beta$1, which is overexpressed in CAVD, activates VICs and promotes their transformation into secretory myofibroblast-like cells [87]. Of interest, the inhibition of serotoninergic receptor 5-$HT_{2B}$ prevents TGF-$\beta$1-induced transformation of VICs into myofibroblast [88]. This latter finding may have pathobiological significance, since mast cells are present in mineralized aortic valves and may thus affect TGF-$\beta$ signalling through the production of serotonin. One simple observation of explanted human aortic valves is that neoangiogenesis is associated with the development of CAVD. Of note, the density of neovessels correlates with the presence of chronic inflammatory infiltrates [6]. The expression of heat shock protein 60 (HSP60), a marker of inflammation, correlates with the development of neoangiogenesis in stenotic aortic valves [89]. In mineralized aortic valves, endothelial progenitor cells CD34+ are present in valvular tissue [90]. In mice defective for chondromodulin-1, a protein with antiangiogenic properties, there is a neovascularization of the aortic valve and mineralization of leaflets [91]. Though the role of angiogenesis in CAVD is not elucidated yet, one hypothesis is that it contributes to the recruitment of inflammatory cells in a positive feedback loop. Osteonectin (SPARC), a matricellular protein highly expressed in stenotic aortic valves, is cleaved by metalloproteinases into a proangiogenic peptide [90]. The expression of several MMPs, including MMP2-3 and MMP9, is elevated in mineralized aortic valves [92]. In addition, a recent study has identified that MMP12, which is a potent elastase, is overexpressed in stenotic aortic valves [93]. Also, in human mineralized aortic valves the expression of cathepsins K, V, and S is increased [94]. In $apoE^{-/-}$ mice with 5/6 nephrectomy, cathepsin S induced elastolysis and promoted the mineralization of the aortic valve [95]. These studies thus suggest that during the remodelling process elastic fibers fragments with osteogenic properties are generated and may participate in the mineralization of the aortic valve.

## 5. Adaptive Immunity

From the above discussion it is clear that there is an activation of innate immunity in CAVD. Though it has been observed more than a decade ago that CD4+ and CD8+ T cells infiltrate the aortic valve during CAVD, it is only recently that investigations have shown a clonal expansion of T cells in mineralized aortic valves. In a seminal work, Winchester et al. showed that the proportion of circulating CD3+ T cells expressing HLA-DR was increased in subjects with CAVD [96]. Also, the proportion of circulating CD8+ CD57+ T cell subset expressing HLA-DR was elevated during CAVD. These findings thus suggest that a subset of memory T cells is activated in patients with CAVD. In mineralized aortic valves, both bicuspid and tricuspid, the clonal expansions of the TCR repertoire were documented. Immunohistological analyses of explanted mineralized aortic valves showed that CD8+ CD28 null cells were present at the proximity of mineralized nodules [96, 97]. These data thus suggest that a systemic adaptive immunity, coupled to lymphocytic infiltration of the aortic valve, is activated during CAVD. Though the antigen(s) responsible for this response in CAVD remains

to be determined, it is possible that oxidatively modified epitopes may play a role [98]. Several questions though remain to be studied with regard to the role of adaptive immunity in the development of CAVD. The identification of epitopes that induce an adaptive immune response, as well as the role of this response in fibrocalcific remodelling of the aortic valve, remains to be explored.

## 6. An Integrative View of Inflammation in CAVD and Potential Therapeutic Opportunities

Several factors may promote inflammation in CAVD, but an important culprit is possibly the oxidized lipids [99]. In this regard, ox-LDL is a potent trigger of inflammation through the Toll-like receptors. Also, ox-LDL generates epitopes, which may activate adaptive immunity [100]. In addition, the overexpression of Lp-PLA2, LPL, and 5-LO in CAVD contributes to generate bioactive lipid-derived species, which amplify inflammation. These factors along with angiotensin II contribute to the activation of the NF-$\kappa$B cascade. The activation of NF-$\kappa$B in CAVD is substantiated by an elevated level of phosphorylated I$\kappa$B$\alpha$ (Ser32) along with the overexpression of target genes such as IL-6 [23]. In this scheme of things, it is possible that NF-$\kappa$B represents a hub of signalling, which may drive, at least in part, the fibrocalcific remodelling process of the aortic valve. Different therapeutic alternatives could be examined in order to prevent the progression of CAVD. In this regard, investigations to decrease activation of the RAS or blocking enzyme pathways that promote the production of highly reactive lipid species are needed. The recent discovery that IL-6 may represent an effector cytokine in promoting the osteogenic transition of VICs warrants further investigation. The use of monoclonal antibodies directed against TNF-$\alpha$ or IL-6 could be examined in preclinical animal models. The cardiovascular inflammation reduction trial (CIRT) will examine if the administration of methotrexate can reduce cardiovascular events [101]. This study will randomize 7000 patients to a placebo or methotrexate. Though this study is not designed and powered to study CAVD, it will examine as a secondary outcome the rate of CAVD in both arms. Though the mechanism by which methotrexate reduces inflammation remains, to some extent, obscure, in the hypercholesterolemic rabbit, it decreased the size of atherosclerotic plaques and *in vitro* it reduced the expression of TNF-$\alpha$, IL1$\beta$, and CXCL2 in human umbilical vein endothelial cells treated with TNF-$\alpha$ [102]. The repositioning of drugs with an anti-inflammatory effect in CAVD is a potential therapeutic avenue, which needs further exploration [103]. However, basic and translational works are clearly needed in this field in order to tease out the key underpinning processes that link immunity with CAVD.

## 7. Conclusion

Research in the last several years has clearly identified that CAVD is an active disorder, which has an immune component. Both innate and adaptive immunity are activated during CAVD. Several upstream factors converge on the NF-$\kappa$B. Oxidized lipid species and angiotensin II promote activation of the NF-$\kappa$B cascade, which increases the expression of different cytokines. Both apoptosis-mediated mineralization and osteogenic transition of VICs are activated by NF-$\kappa$B and promote the ectopic mineralization of the aortic valves. Recent discoveries linking the immune response with CAVD should spur more translational work in order to develop novel therapeutic alternatives for this chronic process affecting our aging societies.

## Disclosure

Patrick Mathieu has patent application for the use of ectonucleotidases and Lp-PLA2 inhibitors in the treatment of CAVD.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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## References

[1] P. Mathieu, M. C. Boulanger, and R. Bouchareb, "Molecular biology of calcific aortic valve disease: towards new pharmacological therapies," *Expert Review of Cardiovascular Therapy*, vol. 12, no. 7, pp. 851–862, 2014.

[2] N. M. Rajamannan, F. J. Evans, E. Aikawa et al., "Calcific aortic valve disease: not simply a degenerative process: a review and agenda for research from the national heart and lung and blood institute aortic stenosis working group. Executive summary: calcific aortic valve disease—2011 update," *Circulation*, vol. 124, no. 16, pp. 1783–1791, 2011.

[3] V. Persy and P. D'Haese, "Vascular calcification and bone disease: the calcification paradox," *Trends in Molecular Medicine*, vol. 15, no. 9, pp. 405–416, 2009.

[4] R. Bouchareb, M.-C. Boulanger, D. Fournier, P. Pibarot, Y. Messaddeq, and P. Mathieu, "Mechanical strain induces the production of spheroid mineralized microparticles in the aortic valve through a RhoA/ROCK-dependent mechanism," *Journal of Molecular and Cellular Cardiology*, vol. 67, pp. 49–59, 2014.

[5] K. D. O'Brien, D. D. Reichenbach, S. M. Marcovina, J. Kuusisto, C. E. Alpers, and C. M. Otto, "Apolipoproteins B, (a), and E accumulate in the morphologically early lesion of 'degenerative' valvular aortic stenosis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 16, no. 4, pp. 523–532, 1996.

[6] N. Coté, A. Mahmut, Y. Bosse et al., "Inflammation is associated with the remodeling of calcific aortic valve disease," *Inflammation*, vol. 36, no. 3, pp. 573–581, 2013.

[7] J.-H. Chen and C. A. Simmons, "Cell-matrix interactions in the pathobiology of calcific aortic valve disease: critical

roles for matricellular, matricrine, and matrix mechanics cues," *Circulation Research*, vol. 108, no. 12, pp. 1510–1524, 2011.

[8] J. T. Butcher, C. A. Simmons, and J. N. Warnock, "Mechanobiology of the aortic heart valve," *Journal of Heart Valve Disease*, vol. 17, no. 1, pp. 62–73, 2008.

[9] P. F. Davies, A. G. Passerini, and C. A. Simmons, "Aortic valve: turning over a new leaf(let) in endothelial phenotypic heterogeneity," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 24, no. 8, pp. 1331–1333, 2004.

[10] D. Mohty, P. Pibarot, J.-P. Després et al., "Association between plasma LDL particle size, valvular accumulation of oxidized LDL, and inflammation in patients with aortic stenosis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 28, no. 1, pp. 187–193, 2008.

[11] C. M. Otto, J. Kuusisto, D. D. Reichenbach, A. M. Gown, and K. D. O'Brien, "Characterization of the early lesion of 'degenerative' valvular aortic stenosis: histological and immunohistochemical studies," *Circulation*, vol. 90, no. 2, pp. 844–853, 1994.

[12] I. Steiner, P. Kašparová, A. Kohout, and J. Dominik, "Bone formation in cardiac valves: a histopathological study of 128 cases," *Virchows Archiv*, vol. 450, no. 6, pp. 653–657, 2007.

[13] A. R. Brasier, "The nuclear factor-B-interleukin-6 signalling pathway mediating vascular inflammation," *Cardiovascular Research*, vol. 86, no. 2, pp. 211–218, 2010.

[14] F. Liu, Y. Xia, A. S. Parker, and I. M. Verma, "IKK biology," *Immunological Reviews*, vol. 246, no. 1, pp. 239–253, 2012.

[15] A. A. Beg, T. S. Finco, P. V. Nantermet, and A. S. Baldwin Jr., "Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I$\kappa$B$\alpha$: a mechanism for NF-$\kappa$B activation," *Molecular and Cellular Biology*, vol. 13, no. 6, pp. 3301–3310, 1993.

[16] K. Hochrainer, G. Racchumi, and J. Anrather, "Site-specific phosphorylation of the p65 protein subunit mediates selective gene expression by differential NF-$\kappa$B and RNA polymerase II promoter recruitment," *The Journal of Biological Chemistry*, vol. 288, no. 1, pp. 285–293, 2013.

[17] C. C. Ghosh, S. Ramaswami, A. Juvekar et al., "Gene-specific repression of proinflammatory cytokines in stimulated human macrophages by nuclear I$\kappa$B$\alpha$," *Journal of Immunology*, vol. 185, no. 6, pp. 3685–3693, 2010.

[18] H. Zhong, R. E. Voll, and S. Ghosh, "Phosphorylation of NF-$\kappa$B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300," *Molecular Cell*, vol. 1, no. 5, pp. 661–671, 1998.

[19] M. Jamaluddin, S. Wang, I. Boldogh, B. Tian, and A. R. Brasier, "TNF-$\alpha$-induced NF-$\kappa$B/RelA Ser276 phosphorylation and enhanceosome formation is mediated by an ROS-dependent PKAc pathway," *Cellular Signalling*, vol. 19, no. 7, pp. 1419–1433, 2007.

[20] E. Branchetti, R. Sainger, P. Poggio et al., "Antioxidant enzymes reduce DNA damage and early activation of valvular interstitial cells in aortic valve sclerosis," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 33, no. 2, pp. e66–e74, 2013.

[21] G. Xiao, A. Fong, and S.-C. Sun, "Induction of p100 processing by NF-$\kappa$B-inducing kinase involves docking I$\kappa$B kinase $\alpha$ (IKK$\alpha$) to p100 and IKK$\alpha$-mediated phosphorylation," *Journal of Biological Chemistry*, vol. 279, no. 29, pp. 30099–30105, 2004.

[22] M. D. Morrison, W. Reiley, M. Zhang, and S.-C. Sun, "An atypical Tumor Necrosis Factor (TNF) receptor-associated factor-binding motif of B cell-activating factor belonging to the TNF Family (BAFF) receptor mediates induction of the noncanonical NF-$\kappa$B signaling pathway," *Journal of Biological Chemistry*, vol. 280, no. 11, pp. 10018–10024, 2005.

[23] D. El Husseini, M.-C. Boulanger, A. Mahmut et al., "P2Y2 receptor represses IL-6 expression by valve interstitial cells through Akt: implication for calcific aortic valve disease," *Journal of Molecular and Cellular Cardiology*, vol. 72, pp. 146–156, 2014.

[24] N. Côté, D. El Husseini, A. Pépin et al., "ATP acts as a survival signal and prevents the mineralization of aortic valve," *Journal of Molecular and Cellular Cardiology*, vol. 52, no. 5, pp. 1191–1202, May 2012.

[25] P. Mathieu, P. Voisine, A. Pépin, R. Shetty, N. Savard, and F. Dagenais, "Calcification of human valve interstitial cells is dependent on alkaline phosphatase activity," *Journal of Heart Valve Disease*, vol. 14, no. 3, pp. 353–357, 2005.

[26] N. Côté, D. El Husseini, A. Pépin et al., "Inhibition of ectonucleotidase with ARL67156 prevents the development of calcific aortic valve disease in warfarin-treated rats," *European Journal of Pharmacology*, vol. 689, no. 1–3, pp. 139–146, 2012.

[27] P. Mathieu, "Pharmacology of ectonucleotidases: relevance for the treatment of cardiovascular disorders," *European Journal of Pharmacology*, vol. 696, no. 1–3, pp. 1–4, 2012.

[28] D. El Husseini, M.-C. Boulanger, D. Fournier et al., "High expression of the Pi-transporter SLC20A1/Pit1 in calcific aortic valve disease promotes mineralization through regulation of Akt-1," *PLoS ONE*, vol. 8, no. 1, Article ID e53393, 2013.

[29] P. Mathieu, I. Lemieux, and J.-P. Després, "Obesity, inflammation, and cardiovascular risk," *Clinical Pharmacology & Therapeutics*, vol. 87, no. 4, pp. 407–416, 2010.

[30] G. J. Mahler, E. J. Farrar, and J. T. Butcher, "Inflammatory cytokines promote mesenchymal transformation in embryonic and adult valve endothelial cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 33, no. 1, pp. 121–130, 2013.

[31] T. Wada, T. Nakashima, N. Hiroshi, and J. M. Penninger, "RANKL-RANK signaling in osteoclastogenesis and bone disease," *Trends in Molecular Medicine*, vol. 12, no. 1, pp. 17–25, 2006.

[32] P. Mathieu, P. Poirier, P. Pibarot, I. Lemieux, and J.-P. Després, "Visceral obesity the link among inflammation, hypertension, and cardiovascular disease," *Hypertension*, vol. 53, no. 4, pp. 577–584, 2009.

[33] Z. Yu, K. Seya, K. Daitoku, S. Motomura, I. Fukuda, and K.-I. Furukawa, "Tumor necrosis factor-$\alpha$ accelerates the calcification of human aortic valve interstitial cells obtained from patients with calcific aortic valve stenosis via the BMP2-Dlx5 pathway," *Journal of Pharmacology and Experimental Therapeutics*, vol. 337, no. 1, pp. 16–23, 2011.

[34] J. J. Kaden, R. Kiliç, A. Sarikoç et al., "Tumor necrosis factor alpha promotes an osteoblast-like phenotype in human aortic valve myofibroblasts: a potential regulatory mechanism of valvular calcification," *International Journal of Molecular Medicine*, vol. 16, no. 5, pp. 869–872, 2005.

[35] R. A. Black, C. T. Rauch, C. J. Kozlosky et al., "A metalloproteinase disintegrin that releases tumour-necrosis factor-$\alpha$ from cells," *Nature*, vol. 385, no. 6618, pp. 729–733, 1997.

[36] H. Hsu, J. Xiong, and D. V. Goeddel, "The TNF receptor 1-associated protein TRADD signals cell death and NF-$\kappa$B activation," *Cell*, vol. 81, no. 4, pp. 495–504, 1995.

[37] H. Hsu, H.-B. Shu, M.-G. Pan, and D. V. Goeddel, "TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways," *Cell*, vol. 84, no. 2, pp. 299–308, 1996.

[38] S. Bertazzo, E. Gentleman, K. L. Cloyd, A. H. Chester, M. H. Yacoub, and M. M. Stevens, "Nano-analytical electron

microscopy reveals fundamental insights into human cardiovascular tissue calcification," *Nature Materials*, vol. 12, no. 6, pp. 576–583, 2013.

[39] A. Adhikari, M. Xu, and Z. J. Chen, "Ubiquitin-mediated activation of TAK1 and IKK," *Oncogene*, vol. 26, no. 22, pp. 3214–3226, 2007.

[40] H.-L. Lee, K. M. Woo, H.-M. Ryoo, and J.-H. Baek, "Tumor necrosis factor-$\alpha$ increases alkaline phosphatase expression in vascular smooth muscle cells via MSX2 induction," *Biochemical and Biophysical Research Communications*, vol. 391, no. 1, pp. 1087–1092, 2010.

[41] Z. Al-Aly, J.-S. Shao, C.-F. Lai et al., "Aortic Msx2-Wnt calcification cascade is regulated by TNF-$\alpha$-dependent signals in diabetic Ldlr$^{-/-}$ mice," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 12, pp. 2589–2596, 2007.

[42] K. Isoda, T. Matsuki, H. Kondo, Y. Iwakura, and F. Ohsuzu, "Deficiency of interleukin-1 receptor antagonist induces aortic valve disease in BALB/c Mice," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 30, no. 4, pp. 708–715, 2010.

[43] J. J. Kaden, C.-E. Dempfle, R. Grobholz et al., "Interleukin-1 beta promotes matrix metalloproteinase expression and cell proliferation in calcific aortic valve stenosis," *Atherosclerosis*, vol. 170, no. 2, pp. 205–211, 2003.

[44] A. Galeone, G. Brunetti, A. Oranger et al., "Aortic valvular interstitial cells apoptosis and calcification are mediated by TNF-related apoptosis-inducing ligand," *International Journal of Cardiology*, vol. 169, no. 4, pp. 296–304, 2013.

[45] L. Lum, B. R. Wong, R. Josien et al., "Evidence for a role of a tumor necrosis factor-$\alpha$ (TNF-$\alpha$)-converting enzyme-like protease in shedding of TRANCE, a TNF family member involved in osteoclastogenesis and dendritic cell survival," *Journal of Biological Chemistry*, vol. 274, no. 19, pp. 13613–13618, 1999.

[46] B. R. Wong, R. Josien, S. Y. Lee, M. Vologodskaia, R. M. Steinman, and Y. Choi, "The TRAF family of signal transducers mediates NF-$\kappa$B activation by the TRANCE receptor," *The Journal of Biological Chemistry*, vol. 273, no. 43, pp. 28355–28359, 1998.

[47] B. R. Wong, D. Besser, N. Kim et al., "TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src," *Molecular Cell*, vol. 4, no. 6, pp. 1041–1049, 1999.

[48] W. S. Simonet, D. L. Lacey, C. R. Dunstan et al., "Osteoprotegerin: a novel secreted protein involved in the regulation of bone density," *Cell*, vol. 89, no. 2, pp. 309–319, 1997.

[49] N. Bucay, I. Sarosi, C. R. Dunstan et al., "Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification," *Genes and Development*, vol. 12, no. 9, pp. 1260–1268, 1998.

[50] J. J. Kaden, S. Bickelhaupt, R. Grobholz et al., "Receptor activator of nuclear factor $\kappa$B ligand and osteoprotegerin regulate aortic valve calcification," *Journal of Molecular and Cellular Cardiology*, vol. 36, no. 1, pp. 57–66, 2004.

[51] R. M. Weiss, D. D. Lund, Y. Chu et al., "Osteoprotegerin inhibits aortic valve calcification and preserves valve function in hypercholesterolemic mice," *PLoS ONE*, vol. 8, no. 6, Article ID e65201, 2013.

[52] K. D. O'Brien, D. M. Shavelle, M. T. Caulfield et al., "Association of angiotensin-converting enzyme with low-density lipoprotein in aortic valvular lesions and in human plasma," *Circulation*, vol. 106, no. 17, pp. 2224–2230, 2002.

[53] S. Helske, K. A. Lindstedt, M. Laine et al., "Induction of local angiotensin II-producing systems in stenotic aortic valves," *Journal of the American College of Cardiology*, vol. 44, no. 9, pp. 1859–1866, 2004.

[54] E. Wypasek, J. Natorska, G. Grudzień, G. Filip, J. Sadowski, and A. Undas, "Mast cells in human stenotic aortic valves are associated with the severity of stenosis," *Inflammation*, vol. 36, no. 2, pp. 449–456, 2013.

[55] N. Côté, P. Pibarot, A. Pépin et al., "Oxidized low-density lipoprotein, angiotensin II and increased waist cirumference are associated with valve inflammation in prehypertensive patients with aortic stenosis," *International Journal of Cardiology*, vol. 145, no. 3, pp. 444–449, 2010.

[56] N. Côté, A. Mahmut, D. Fournier et al., "Angiotensin receptor blockers are associated with reduced fibrosis and interleukin-6 expression in calcific aortic valve disease," *Pathobiology*, vol. 81, no. 1, pp. 15–24, 2013.

[57] R. Capoulade, M.-A. Clavel, P. Mathieu et al., "Impact of hypertension and renin-angiotensin system inhibitors in aortic stenosis," *European Journal of Clinical Investigation*, vol. 43, no. 12, pp. 1262–1272, 2013.

[58] T. Fujisaka, M. Hoshiga, J. Hotchi et al., "Angiotensin II promotes aortic valve thickening independent of elevated blood pressure in apolipoprotein-E deficient mice," *Atherosclerosis*, vol. 226, no. 1, pp. 82–87, 2013.

[59] K. Arishiro, M. Hoshiga, N. Negoro et al., "Angiotensin receptor-1 blocker inhibits atherosclerotic changes and endothelial disruption of the aortic valve in hypercholesterolemic rabbits," *Journal of the American College of Cardiology*, vol. 49, no. 13, pp. 1482–1489, 2007.

[60] L. M. McAllister-Lucas, J. Ruland, K. Siu et al., "CARMA3/Bcl10/MALT1-dependent NF-$\kappa$B activation mediates angiotensin II-responsive inflammatory signaling in nonimmune cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 1, pp. 139–144, 2007.

[61] S. Choudhary, M. Lu, R. Cui, and A. R. Brasier, "Involvement of a novel Rac/RhoA guanosine triphosphatase-nuclear factor-$\kappa$B inducing kinase signaling pathway mediating angiotensin II-induced RelA transactivation," *Molecular Endocrinology*, vol. 21, no. 9, pp. 2203–2217, 2007.

[62] C. Y. Sasaki, T. J. Barberi, P. Ghosh, and D. L. Longo, "Phosphorylation of Re1A/p65 on serine 536 defines an I$\kappa$B$\alpha$-independent NF-$\kappa$B pathway," *Journal of Biological Chemistry*, vol. 280, no. 41, pp. 34538–34547, 2005.

[63] D. Bosisio, I. Marazzi, A. Agresti, N. Shimizu, M. E. Bianchi, and G. Natoli, "A hyper-dynamic equilibrium between promoter-bound and nucleoplasmic dimers controls NF-$\kappa$B-dependent gene activity," *The EMBO Journal*, vol. 25, no. 4, pp. 798–810, 2006.

[64] M. Olsson, J. Thyberg, and J. Nilsson, "Presence of oxidized low density lipoprotein in nonrheumatic stenotic aortic valves," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 19, no. 5, pp. 1218–1222, 1999.

[65] M. Briand, I. Lemieux, J. G. Dumesnil et al., "Metabolic syndrome negatively influences disease progression and prognosis in aortic stenosis," *Journal of the American College of Cardiology*, vol. 47, no. 11, pp. 2229–2236, 2006.

[66] A. Mahmut, M.-C. Boulanger, D. Fournier et al., "Lipoprotein lipase in aortic valve stenosis is associated with lipid retention and remodelling," *European Journal of Clinical Investigation*, vol. 43, no. 6, pp. 570–578, 2013.

[67] H. Derbali, Y. Bosse, N. Cote et al., "Increased biglycan in aortic valve stenosis leads to the overexpression of phospholipid transfer protein via toll-like receptor 2," *The American Journal of Pathology*, vol. 176, no. 6, pp. 2638–2645, 2010.

[68] R. Song, Q. Zeng, L. Ao et al., "Biglycan induces the expression of osteogenic factors in human aortic valve interstitial cells via toll-like receptor-2," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 32, no. 11, pp. 2711–2720, 2012.

[69] R. Song, L. Ao, K.-S. Zhao et al., "Soluble biglycan induces the production of ICAM-1 and MCP-1 in human aortic valve interstitial cells through TLR2/4 and the ERK1/2 pathway," *Inflammation Research*, vol. 63, no. 9, pp. 703–710, 2014.

[70] N. A. Nadlonek, J. H. Lee, M. J. Weyant, X. Meng, and D. A. Fullerton, "Ox-LDL induces PiT-1 expression in human aortic valve interstitial cells," *Journal of Surgical Research*, vol. 184, no. 1, pp. 6–9, 2013.

[71] Q. Zeng, R. Song, L. Ao et al., "Notch1 promotes the pro-osteogenic response of human aortic valve interstitial cells via modulation of erk1/2 and nuclear factor-κb activation," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 33, no. 7, pp. 1580–1590, 2013.

[72] Q. Zeng, C. Jin, L. Ao et al., "Cross-talk between the toll-like receptor 4 and notch1 pathways augments the inflammatory response in the interstitial cells of stenotic human aortic valves," *Circulation*, vol. 126, no. 11, supplement 1, pp. S222–S230, 2012.

[73] V. Garg, A. N. Muth, J. F. Ransom et al., "Mutations in NOTCH1 cause aortic valve disease," *Nature*, vol. 437, no. 7056, pp. 270–274, 2005.

[74] V. Nigam and D. Srivastava, "Notch1 represses osteogenic pathways in aortic valve cells," *Journal of Molecular and Cellular Cardiology*, vol. 47, no. 6, pp. 828–834, 2009.

[75] N. Osman, K. J. Grande-Allen, M. L. Ballinger et al., "Smad2-dependent glycosaminoglycan elongation in aortic valve interstitial cells enhances binding of LDL to proteoglycans," *Cardiovascular Pathology*, vol. 22, no. 2, pp. 146–155, 2013.

[76] J. D. Miller, Y. Chu, R. M. Brooks, W. E. Richenbacher, R. Peña-Silva, and D. D. Heistad, "Dysregulation of antioxidant mechanisms contributes to increased oxidative stress in calcific aortic valvular stenosis in humans," *Journal of the American College of Cardiology*, vol. 52, no. 10, pp. 843–850, 2008.

[77] H. Li and U. Forstermann, "Uncoupling of endothelial NO synthase in atherosclerosis and vascular disease," *Current Opinion in Pharmacology*, vol. 13, no. 2, pp. 161–167, 2013.

[78] G. Thanassoulis, C. Y. Campbell, D. S. Owens et al., "Genetic associations with valvular calcification and aortic stenosis," *The New England Journal of Medicine*, vol. 368, no. 6, pp. 503–512, 2013.

[79] P. R. Kamstrup, A. Tybjærg-Hansen, and B. G. Nordestgaard, "Elevated lipoprotein(a) and risk of aortic valve stenosis in the general population," *Journal of the American College of Cardiology*, vol. 63, no. 5, pp. 470–477, 2014.

[80] B. J. Arsenault, S. M. Boekholdt, M. P. Dube et al., "Lipoprotein(a) levels, genotype, and incident aortic valve stenosis: a prospective mendelian randomization study and replication in a case-control cohort," *Circulation: Cardiovascular Genetics*, vol. 7, no. 3, pp. 304–310, 2014.

[81] M.-Y. Hung, J. L. Witztum, and S. Tsimikas, "New therapeutic targets for calcific aortic valve stenosis: the lipoprotein(a)-lipoprotein-associated phospholipase $A_2$-oxidized phospholipid axis," *Journal of the American College of Cardiology*, vol. 63, no. 5, pp. 478–480, 2014.

[82] Y. I. Miller, S.-H. Choi, P. Wiesner et al., "Oxidation-specific epitopes are danger-associated molecular patterns recognized by pattern recognition receptors of innate immunity," *Circulation Research*, vol. 108, no. 2, pp. 235–248, 2011.

[83] A. Mahmut, M.-C. Boulanger, D. El Husseini et al., "Elevated expression of lipoprotein-associated phospholipase A2 in calcific aortic valve disease: implications for valve mineralization," *Journal of the American College of Cardiology*, vol. 63, no. 5, pp. 460–469, 2014.

[84] A. Mahmut, H. Mahjoub, M.-C. Boulanger et al., "Lp-PLA2 is associated with structural valve degeneration of bioprostheses," *European Journal of Clinical Investigation*, vol. 44, no. 2, pp. 136–145, 2014.

[85] E. Nagy, D. C. Andersson, K. Caidahl et al., "Upregulation of the 5-lipoxygenase pathway in human aortic valves correlates with severity of stenosis and leads to leukotriene-induced effects on valvular myofibroblasts," *Circulation*, vol. 123, no. 12, pp. 1316–1325, 2011.

[86] D. Poeckel and C. D. Funk, "The 5-lipoxygenase/leukotriene pathway in preclinical models of cardiovascular disease," *Cardiovascular Research*, vol. 86, no. 2, pp. 243–253, 2010.

[87] J. H. Chen, W. L. K. Chen, K. L. Sider, C. Y. Yip, and C. A. Simmons, "$\beta$-catenin mediates mechanically regulated, transforming growth factor-$\beta$1-induced myofibroblast differentiation of aortic valve interstitial cells," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 3, pp. 590–597, 2011.

[88] J. D. Hutcheson, L. M. Ryzhova, V. Setola, and W. D. Merryman, "5-$HT_{2B}$ antagonism arrests non-canonical TGF-$\beta$1-induced valvular myofibroblast differentiation," *Journal of Molecular and Cellular Cardiology*, vol. 53, no. 5, pp. 707–714, 2012.

[89] A. Mazzone, M. C. Epistolato, R. De Caterina et al., "Neoangiogenesis, T-lymphocyte infiltration, and heat shock protein-60 are biological hallmarks of an immunomediated inflammatory process in end-stage calcified aortic valve stenosis," *Journal of the American College of Cardiology*, vol. 43, no. 9, pp. 1670–1676, 2004.

[90] A. Charest, A. Pépin, R. Shetty et al., "Distribution of SPARC during neovascularisation of degenerative aortic stenosis," *Heart*, vol. 92, no. 12, pp. 1844–1849, 2006.

[91] M. Yoshioka, S. Yuasa, K. Matsumura et al., "Chondromodulin-I maintains cardiac valvular function by preventing angiogenesis," *Nature Medicine*, vol. 12, no. 10, pp. 1151–1159, 2006.

[92] O. Fondard, D. Detaint, B. Iung et al., "Extracellular matrix remodelling in human aortic valve disease: the role of matrix metalloproteinases and their tissue inhibitors," *European Heart Journal*, vol. 26, no. 13, pp. 1333–1341, 2005.

[93] I. Perrotta, E. Russo, C. Camastra et al., "New evidence for a critical role of elastin in calcification of native heart valves: Immunohistochemical and ultrastructural study with literature review," *Histopathology*, vol. 59, no. 3, pp. 504–513, 2011.

[94] S. Helske, S. Syväranta, K. A. Lindstedt et al., "Increased expression of elastolytic cathepsins S, K, and V and their inhibitor cystatin C in stenotic aortic valves," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 26, no. 8, pp. 1791–1798, 2006.

[95] E. Aikawa, M. Aikawa, G. Rusanescu et al., "Arterial and aortic valve calcification abolished by elastolytic cathepsin S deficiency in chronic renal disease," *Circulation*, vol. 119, no. 13, pp. 1785–1794, 2009.

[96] R. Winchester, M. Wiesendanger, W. O'Brien et al., "Circulating activated and effector memory T cells are associated with

calcification and clonal expansions in bicuspid and tricuspid valves of calcific aortic stenosis," *Journal of Immunology*, vol. 187, no. 2, pp. 1006–1014, 2011.

[97] H. D. Wu, M. S. Maurer, R. A. Friedman et al., "The lymphocytic infiltration in calcific aortic stenosis predominantly consists of clonally expanded T cells," *The Journal of Immunology*, vol. 178, no. 8, pp. 5329–5339, 2007.

[98] R. Wu, R. Giscombe, G. Holm, and A. K. Lefvert, "Induction of human cytotoxic T lymphocytes by oxidized low density lipoproteins," *Scandinavian Journal of Immunology*, vol. 43, no. 4, pp. 381–384, 1996.

[99] C. Cote, P. Pibarot, J. P. Despres et al., "Association between circulating oxidised low-density lipoprotein and fibrocalcific remodelling of the aortic valve in aortic stenosis," *Heart*, vol. 94, no. 9, pp. 1175–1180, 2008.

[100] S. Stemme, B. Faber, J. Holm, O. Wiklund, J. L. Witztum, and G. K. Hansson, "T lymphocytes from human atherosclerotic plaques recognize oxidized low density lipoprotein," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 9, pp. 3893–3897, 1995.

[101] P. M. Ridker, "Testing the inflammatory hypothesis of atherothrombosis: scientific rationale for the cardiovascular inflammation reduction trial (CIRT)," *Journal of Thrombosis and Haemostasis*, vol. 7, supplement 1, pp. 332–339, 2009.

[102] A. Bulgarelli, A. A. Martins Dias, B. Caramelli, and R. C. Maranhão, "Treatment with methotrexate inhibits atherogenesis in cholesterol-fed rabbits," *Journal of Cardiovascular Pharmacology*, vol. 59, no. 4, pp. 308–314, 2012.

[103] M. R. Hurle, L. Yang, Q. Xie, D. K. Rajpal, P. Sanseau, and P. Agarwal, "Computational drug repositioning: from data to therapeutics," *Clinical Pharmacology & Therapeutics*, vol. 93, no. 4, pp. 335–341, 2013.

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*Review Article*

# Recognition of Immune Response for the Early Diagnosis and Treatment of Osteoarthritis

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Osteoarthritis is a common and debilitating joint disease that affects up to 30 million Americans, leading to significant disability, reduction in quality of life, and costing the United States tens of billions of dollars annually. Classically, osteoarthritis has been characterized as a degenerative, wear-and-tear disease, but recent research has identified it as an immunopathological disease on a spectrum between healthy condition and rheumatoid arthritis. A systematic literature review demonstrates that the disease pathogenesis is driven by an early innate immune response which progressively catalyzes degenerative changes that ultimately lead to an altered joint microenvironment. It is feasible to detect this infiltration of cells in the early, and presumably asymptomatic, phase of the disease through noninvasive imaging techniques. This screening can serve to aid clinicians in potentially identifying high-risk patients, hopefully leading to early effective management, vast improvements in quality of life, and significant reductions in disability, morbidity, and cost related to osteoarthritis. Although the diagnosis and treatment of osteoarthritis routinely utilize both invasive and non-invasive strategies, imaging techniques specific to inflammatory cells are not commonly employed for these purposes. This review discusses this paradigm and aims to shift the focus of future osteoarthritis-related research towards early diagnosis of the disease process.

## 1. Introduction

Osteoarthritis (OA) is a painful and debilitative joint disease that commonly affects the hand, hip, and knee joints of aging adults. Disease progression is a leading cause of hospitalization and ultimately requires joint replacement surgery which costs the US healthcare industry over $42 billion in 2009 for the hip and knee joints alone [1]. Clinical OA affects up to 30 million Americans including one-third of seniors aged 65 or older and 13.9% of all adults at least 25 years of age [2]. While disease-modifying antirheumatic drugs (DMARDs) have been identified for rheumatoid arthritis (RA), an inflammatory joint disease often studied and characterized in comparison with OA, similar therapy for OA has yet to be identified [3, 4]. The classical definition of OA as a wear-and-tear, noninflammatory disease has recently transitioned to an inflammatory disease lying on a spectrum between normal control and RA. Despite the fact that the immune system plays a significant role in both diseases, DMARDs effective in the treatment of RA, including tumor necrosis factor $\alpha$ (TNF$\alpha$) and interleukin-1 (IL-1) inhibitors, have so far proven unsuccessful in slowing disease progression and clinical deterioration of OA patients. This paper will characterize the key players in OA pathogenesis and identify disease-modifying therapeutic strategies which could be reasonably accommodated in the setting of a prevalent, high-morbidity, and costly disease in the United States of America.

Recent research has established that multiple cells, cytokines, chemokines, complement, and other aspects of the immune system are involved in the pathogenesis of OA, with the roles of integral cells and proteins summarized in Tables 1 and 2, respectively. There exists a continuum of inflammation along the spectrum of normal, OA, and RA, with progressive increases in cytokines and other mediators of inflammation along with leukocyte infiltration [5]. OA pathogenesis is multifactorial and complex with evidence pointing towards unique phenotypes and seemingly discrete stages: early, intermediate, and late. Numerous pathways exist

Table 1: Role of the essential cells implicated in OA pathogenesis.

| Cell type | Role | Comments |
|---|---|---|
| Macrophages | (i) Line intimal layer [5, 14]<br>(ii) Required for production of MMPs and cartilage damage [5, 14]<br>(iii) TNF$\alpha$, IL-1, MMP, TGF$\beta$, IL-10, IL-12, and chemokine production [29]<br>(iv) Mediators of TGF$\beta$ induced osteophyte formation [76]<br>(v) Possess TLRs [14] | |
| T cell (TCR = T-cell receptor) | (i) Line subintimal layer [5]<br>(ii) Present in different stages of activation: early ($CD69^+$), intermediate ($CD25,38^+$), and late ($CD45RO^+$) [34]<br>(iii) Increased $CD4^+/CD8^+$ ratio in OA knees versus control [35]<br>(iv) Th1 > Th2 subset, as well as increased Th1 cytokine product IFN$\gamma$ [34, 36]<br>(v) Increased $CD3\varepsilon^+$ T cells and $CD3\varepsilon^+/CD3\zeta^+$ T cell ratio in OA synovium and decreased ratio of $CD3\zeta^+$ T cells [65]<br>(vi) Produce chemokines attractant to macrophages [65]<br>(vii) CDR3 similarity in TCR [77]<br>(viii) Several autoantigens including those on chondrocyte membrane have been identified [31, 78, 79]<br>(ix) Cartilage linking protein and G1 domain of aggrecan have shown promise as potential autoantigens to follow [31, 78, 79] | (i) Suggestive of chronic inflammation<br>(ii) Suggestive of oligoclonal expansion and antigen-driven response |
| Mast cell (MC) | (i) Numbers are at least as high as those in RA synovium [5]<br>(ii) Mostly in subintimal layer and around blood vessels [22]<br>(iii) Levels positively correlated with total cellular infiltrate, however no correlation with ESR [22]<br>(iv) Degranulated MCs found most commonly in intimal layer [23]<br>(v) Higher ratio of tryptase to tryptase/chymase phenotype in OA than controls [24]<br>(vi) Selective expansion of tryptase MC phenotype [24] | (i) MCs lie around blood vessels and mediate vascular permeability hinting at crucial role of MCs, however not related to ESR and degranulated phenotype seen in intimal layer<br>(ii) Tryptase phenotype suggestive of degranulation |
| B cells | (i) Not always present or may be present in small numbers [24]<br>(ii) Mostly in subintimal layer [5]<br>(iii) Undergo oligoclonal expansion with similar CDR3 regions [80, 81]<br>(iv) Evidence of somatic hypermutation [80, 81]<br>(v) In patients with moderate to strong infiltration, there were presence of germinal centers and increased T-cell populations [80, 81]<br>(vi) Potential role as antigen presenting cell [80, 81]<br>(vii) Several autoantigens have been identified including those on the chondrocyte membrane but multiple antigens have been discovered and patterns have yet to be characterized [31]<br>(viii) Elevated antibody titer to cartilage membrane in OA patients versus control [82]<br>(ix) Secrete IL-6 [83] | (i) Suggestive of antigen-driven response |
| Fibroblast | (i) Activated by both IL-1$\beta$ and TNF$\alpha$ and must neutralize both to decrease activation [29]<br>(ii) Produces MMPs, IL-6, IL-8, ADAMTS-4,5, and MCP-1 [29] | |
| NK cell | (i) May have role in early pathogenesis of OA: found to have $CD16^+CD56^+$ phenotype [19] positive for granzymes A and B [19, 21] and $CD16^-CD56^+$ phenotype negative for granzymes A and B [20]<br>(ii) Poor *in vitro* IFN$\gamma$ production upon stimulation in late OA patients [20]<br>(iii) Stimulated by IL-15 [66] | (i) Suggestive of activation/exhaustion phenotypes |
| Neutrophil (PMN) | (i) Generally not found in OA synovial tissue, but sometimes present [5]<br>(ii) HNP1-3 (mainly produced by PMNs) found in synovia of OA patients, with levels inhibited by TNF$\alpha$ stimulation [27]<br>(iii) NGAL (mainly produced by PMNs) found in OA synovia complexed with MMP-9, decreasing its degradation and increasing glycosaminoglycan levels released from cartilage explants [27] | (i) PMNs may play a role in the earliest stages of OA and therefore might not be expected to be identified in most studies of established OA samples |

Table 2: Role of dominant effectors involved in OA pathogenesis.

| Protein | Role | Comments |
|---|---|---|
| IL-1$\beta$ | (i) Produced by macrophages [29]<br>(ii) Receptors upregulated in OA chondrocytes and fibroblasts [84]<br>(iii) Stimulates production of MMPs [85], ADAMTS-4 [86], and chemokines [87]<br>(iv) Inhibits proteoglycan and type II collagen via repressing GlcAT-1 [88]<br>(v) Induces apoptosis in chondrocytes via upregulation of Bcl-2 family of proteins, mitochondrial depolarization [89], and perhaps ROS [90] and NO production [91] | (i) GlcAT-1 is an important enzyme for production of glycosaminoglycan |
| TNF$\alpha$ | (i) Produced by macrophages [29]<br>(ii) Promotes resorption and inhibits production of proteoglycan in cartilage [30]<br>(iii) Stimulates MMP and chemokine production [32]<br>(iv) Decreases collagen production [32]<br>(v) May form a negative feedback loop with HNP1-3 [25] | |
| IL-6 | (i) Produced by fibroblasts [14], chondrocytes [92, 93], and B cells [83]<br>(ii) Production by chondrocytes induced by $PGE_2$ [92], TNF$\alpha$, and IL-1$\beta$ [93]<br>(iii) Found to be present in intimal layer and produced mostly by plasma cells when detected in high levels (>600 pg/mL) in synovial fluid [83]<br>(iv) Activates JAK/STAT to inhibit aggrecan core and link protein and type II collagen gene expression; blocking STAT phosphorylation inhibits this downregulation [94]<br>(v) After binding to its receptor, it binds and inactivates transcription factor for COL2A1 gene, which encodes procollagen chain of triple helix of type II collagen [95]<br>(vi) Upregulates expression of MMPs in conjunction with IL-1 [96] | |
| Complement | (i) Expression and activation abnormally high in OA synovium, significantly in early OA [17]<br>(ii) MAC present around chondrocytes and in synovium in late OA [17]<br>(iii) MAC stimulates MMP, ADAMTS, and chemokine production in chondrocytes [17]<br>(iv) Cartilage ECM, fibromodulin, and aggrecan induced formation of C5b-9 [17]<br>(v) $C5^-$ knockout mice showed no significant synovitis or cartilage loss versus control $C5^+$ mice 8–12 weeks s/p medial meniscectomy [17]<br>(vi) $C6^-$ mice developed roughly half the degeneration from synovitis as $C6^+$ mice s/p medial meniscectomy [17]<br>(vii) $CD59^-$ mice developed more severe OA [17]<br>(viii) C1s cleaves IGFBP-5 which is chondroprotective [18]<br>(ix) C1s inhibition shown to promote better joint architecture in dogs [18] | |
| TLR | (i) Activated by DAMPs released from ECM in joint damage [14]<br>(ii) Induce proinflammatory cytokine production (IL-1$\beta$, TNF$\alpha$, MMP, etc.) by macrophages [14]<br>(iii) Induce catabolic pathways in chondrocytes [15]<br>(iv) Upregulated on chondrocytes in advanced OA [15]<br>(v) TLR4 on OA chondrocytes more sensitive to S100 than control [16] | (i) S100 is a DAMP |
| $PGE_2$ | (i) Upregulated in OA joints [92]<br>(ii) Inhibits proteoglycan synthesis by suppressing aggrecan gene transcription [92]<br>(iii) EP2,4 receptors upregulated in joint cartilage as OA progresses [92]<br>(iv) Decreases collagen type II/type I ratio [92]<br>(v) When coupled with IL-1 stimulation, it greatly increases expression of IL-6 and iNOs [92] | |

Table 2: Continued.

| Protein | Role | Comments |
|---|---|---|
| ADAMTS | (i) ADAMTS-4 can be downregulated by inhibiting TNF$\alpha$ and/or IL-1$\beta$ while ADAMTS-5 is constitutive in human [29] | (i) Uncertainty over which of the two is more significant in OA pathogenesis |
| TGF$\beta$ | (i) Osteophyte formation [76] | |
| VEGF | (i) Promotes angiogenesis and MMP production [14] | |
| IL-4,7,8,10,13,15,17,18, adipokines, and leukemia inhibitory factor | (i) Detected in synovium [5]<br>(ii) IL-17 works synergistically with TNF$\alpha$ and IL-1 and is released by mast cells [97]<br>(iii) Increased levels of IL-15 in early versus late OA [66] | |

and not all may be implicated in specific joints or individuals, but all eventually lead to the endpoint of joint degeneration.

## 2. Immune Response

*2.1. Early Innate Response.* Both the innate and adaptive immune systems have been implicated in OA pathogenesis, but of particular interest is the role of the innate immune system in early OA. Pathogenesis begins with trauma to the joint, which may constitute repetitive microtrauma accumulated throughout a lifetime or a major traumatic event such as articular fracture. Trauma to the joint is absorbed by subchondral bone [6] and joint-associated fat pads [7], respectively. Subchondral bone releases cytokines while the fat pads release adipokines such as leptin, resistin, adiponectin, visfatin, and chemerin [7]. Although the role of adipokines in OA remains to be conclusively elucidated, many studies have implied that they may act as chemokines and increase matrix-degrading enzymes matrix metalloproteinase (MMP) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) [7–9], nitric oxide synthase (NOS) [10], Toll-like receptor (TLR) [7], and other cytokine production [7, 11]. Additionally, joint-associated fat pads are innervated by C-fiber neurons which release substance P, thereby increasing pain sensitivity, proinflammatory cytokine production, and vascular permeability [12, 13]. This series of events leads to the release of damage-associated molecular patterns (DAMPs), or alarmins, from the extracellular matrix (ECM) by both direct trauma and the action of MMPs and ADAMTS, as well as from neutrophils and monocytes. DAMPs stimulate TLRs on macrophages and chondrocytes, inducing a strong upregulation of catabolic markers (MMPs 1, 3, 9, and 13, IL-6, IL-8, and monocyte chemotactic protein 1) and cytokines TNF$\alpha$ and IL-1$\beta$ by way of NF$\kappa$B activation, which is the master regulator in immune response [14–16]. This chronic activation of TLRs leads to their upregulation in chondrocytes [15] and increased sensitivity [16].

The actions of complement are further demonstrating the significant role played by the innate immune system in early OA. Wang et al. reported that complement expression and activation were abnormally high in OA synovium, especially in early OA, seen in Figure 1. Additionally, the membrane attack complex (MAC, C5b-9) was present surrounding chondrocytes in late OA [17]. MAC directly damages the cell membrane but also stimulates MMP, ADAMTS, and chemokine production in chondrocytes, leading to increased chondrocyte destruction, catabolism of cartilage, and leukocyte infiltration. MMPs release components of the extracellular matrix, such as fibromodulin and aggrecan, which further induce MAC formation. To further assess the role of complement, Wang et al. knocked out C5 in mice and observed that, compared to $C5^+$ controls, the $C5^-$ mice showed no significant synovitis or cartilage loss 8–12 weeks status post (s/p) medial meniscectomy, a surgery that can induce OA. Furthermore, $C6^-$ mice developed about half the synovial degeneration as $C6^+$ mice s/p medial meniscectomy. CD59, a MAC inhibitor, was also knocked out in another mouse model, and these mice developed more severe OA compared to controls [17]. In another study, Busby Jr. et al. found that

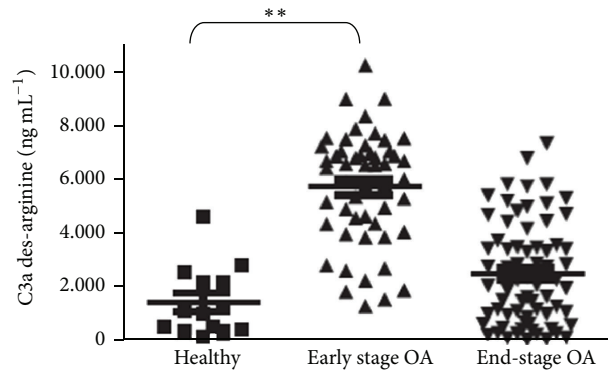


Figure 1: Complement synovial infiltration in the early pathogenesis of OA. ELISA quantification of C3a des-arginine in synovial fluid of healthy ($n = 14$), early-stage OA ($n = 52$), and end-stage OA ($n = 69$) patients. C3a des-arginine is a carboxypeptidase-cleaved, stable form of C3a that is generated from C3 during activation of the complement cascade. $^{**}P \leq 0.01$ by one-way analysis of variance (ANOVA) and Dunnett's post hoc test (reproduction of image with permission and modified caption from Wang et al. [17]).

inhibiting C1s, a serine protease involved in the initiation of the classic activation pathway, promoted favorable joint architecture in dogs. One mechanism by which C1s exerts its effects is by cleaving chondroprotective IGFBP-5 [18].

Other innate immune cells have also been found to play a role in pathogenesis. NK cells have been found in the synovium of OA patients, in one study exhibiting a $CD16^+CD56^+$ phenotype both with and without granzymes A and B [19]. Granzyme A and B expression correlates with cytolytic potency *in vitro* [19]. In another study, NK cells were identified within OA synovia with a $CD16^-CD56^+$ phenotype without granzyme expression. Additionally, these cells demonstrated poor production of interferon $\gamma$ (IFN$\gamma$), a cytokine central to osteoclastogenesis, upon stimulation *in vitro* [20]. In yet another study, granzymes A and B could be identified in the synovia from OA, RA, and reactive arthritis patients [21]. These findings imply that, in OA joints, NK cells can be of an active, cytolytic phenotype, or of an exhaustive, postactivation versus immunoregulatory phenotype. Granzymes A and B, exclusively produced by cytolytic lymphocytes, were identified both intracellularly in NK cells and in the synovia of OA patients [19, 21]. While granzyme presence in the synovium could be explained by T cells, the exclusiveness of this is unlikely. The production and release of granzymes [19, 21] support the notion of an activation/postactivation phenotype theory of NK cell involvement [20]. Of note, Huss et al., who identified mostly $CD16^-CD56^+$ NK cells negative for granzymes and suggested that NK cells are of the immunoregulatory phenotype [20], performed their analysis on patients undergoing primary or revision joint replacement, indicative of late OA patients. Concordantly, IFN$\gamma$ production and degranulation of NK cells were significantly lower after *in vitro* stimulation of synovial tissue taken from revision versus primary joint replacement patients (degranulation of 2% and 7%, resp., $P < 0.05$) [20]. The decreased sensitivity of synovial NK cells

to stimulation in revision versus primary joint replacement patients demonstrates evidence for an exhaustive NK cell phenotype in late OA. Most likely there is a combination of both activating and immunoregulatory roles played by NK cells in OA pathogenesis.

Mast cells have been identified in the synovium of OA patients [22–24], and in one study their counts were found to have a positive correlation with total cellular infiltrate ($r_s$ = 0.82, $P$ = 0.0141) [19]. Interestingly, no correlation between ESR and mast cell count or total cellular infiltrate was found, suggesting only local effects in the joint microenvironment inconsistent with markers of systemic inflammation or disease process [22]. This point is a major barrier to diagnosing and monitoring OA and is expounded upon in later sections. Mast cells are a potent regulator of vascular permeability, and they may play a crucial role in leukocyte recruitment to OA joints. Degranulated mast cells have been found in OA synovium [23], and Buckley et al. discovered a selective expansion and higher ratio of mast cell tryptase phenotype in OA synovium, a phenotype consistent with degranulation [24].

While the significance of neutrophils in synovial disease is well characterized in RA, the role of neutrophils in OA is relatively unknown. Neutrophils are found in varying levels in the synovium of OA patients but generally are found only in small numbers if present at all [5]. However, human neutrophil peptides 1–3 (HNP1–3), $\alpha$-defensins, were found in the synovial tissue of both OA and RA patients in one study [25]. Interestingly, stimulation with TNF$\alpha$ led to the inhibition of HNP1–3 levels in the synovium of OA patients but not RA patients. The authors concluded that this was most likely due to desensitization of TNF receptors in RA synovia. Paired with the finding that HNP1–3 stimulates macrophages to release TNF$\alpha$ [26], the authors concluded that TNF forms a negative feedback loop with HNP1–3 [25]. If HNP1–3 release does precede the actions of TNF$\alpha$, this would suggest that neutrophils play a role in early OA pathogenesis, as TNF$\alpha$ is a central mediator of the disease process. In another study, neutrophil gelatinase-associated lipocalin (NGAL) was found in complex with MMP-9 in OA synovia. NGAL served to decrease degradation of MMP-9 [27], found to be the predominant gelatinase in actively resorbing cartilage [28]. In the presence of NGAL-MMP-9, increased levels of glycosaminoglycan were released from cartilage explants *in vitro* [27]. The role of the innate immune response in early OA pathogenesis is summarized in a stepwise fashion below.

(I) Trauma to the joint is absorbed by subchondral bone and fat pads.

(II) Cytokines, MMPs, and ADAMTS are released.

(III) Direct trauma and MMP/ADAMTS activity release DAMPs which stimulate TLRs.

(IV) TLR activation stimulates NF$\kappa$B, the release of cytokines (mainly TNF$\alpha$ and IL-1$\beta$), macrophages, complement, catabolic pathways in chondrocytes, other innate immune cells, and ultimately the adaptive immune response.

(V) Chronic cascading increases TLR expression and receptor sensitivity, further increasing inflammation.

*2.2. Adaptive Response.* Actions of the innate immune system inevitably lead to activation of the adaptive immune system, increasing inflammation and damage to the joints. TNF$\alpha$ and IL-1$\beta$ are the dominant and most abundant cytokines implicated in OA [5]. They act independently of each other and additively to shift synovial tissue homeostasis towards catabolism [29, 30]. Mechanisms of this shift include increased resorption and inhibition of proteoglycans in cartilage, production of MMPs and chemokines, endothelium activation, and induction of apoptosis in chondrocytes [31, 32]. This leads to increased macrophage and $CD4^+$ T cell infiltration, blood vessel formation by increased vascular endothelial growth factor (VEGF), and increased cyclooxygenase-2 level [33]. Macrophages and T cells, specifically of the $CD4^+$ Th1 subtype [34, 35], are the most abundant cell types found in the synovium of OA patients [5, 36]. Their activation initiates a repetitive cascade of events, activating both the innate and adaptive immune systems, and this propagating inflammation destroys increasing amounts of cartilage, decreasing function and increasing morbidity. T cells are responsible for enhanced stimulation of macrophages and the activation of B cells. Autoreactive B cells further damage cellular integrity and increase inflammation by producing autoantibodies specific for cartilage cell surface proteins such as osteopontin and collagen. Elevated titers of these autoantibodies were found in the sera from OA patients compared to controls [31]. The adaptive immune response is summarized in stepwise fashion below.

(I) Cytokine release and increased vascular permeability lead to T-cell infiltration.

(II) T cells release chemokines and cytokines including IFN$\gamma$, further stimulating macrophages.

(III) Antigen presentation activates B cells.

(IV) B cells release IL-6, increasing acute phase reactants, and produce autoantibodies causing direct damage to cartilage.

(V) Lymphocyte and macrophage activation in the joint microenvironment lead to a chronic, relapsing course of inflammation.

## 3. Early Diagnosis and Treatment

*3.1. Imaging Techniques.* Anatomic imaging techniques, such as radiography and magnetic resonance imaging (MRI), are currently used for epidemiological studies and clinical trials [37, 38]. Plain radiography is the traditional approach to monitoring progression of disease by clinicians; however, the drawbacks of this approach are apparent: insensitivity to change, nonspecificity, susceptibility to measurement error due to change in positioning, and inability to detect early stages of disease [39, 40]. MRI is regarded as sensitive, valid, and reproducible in that it can assess abnormalities of the whole-joint structure including cartilage degeneration [41], subchondral bone marrow lesions [42, 43], meniscal defects

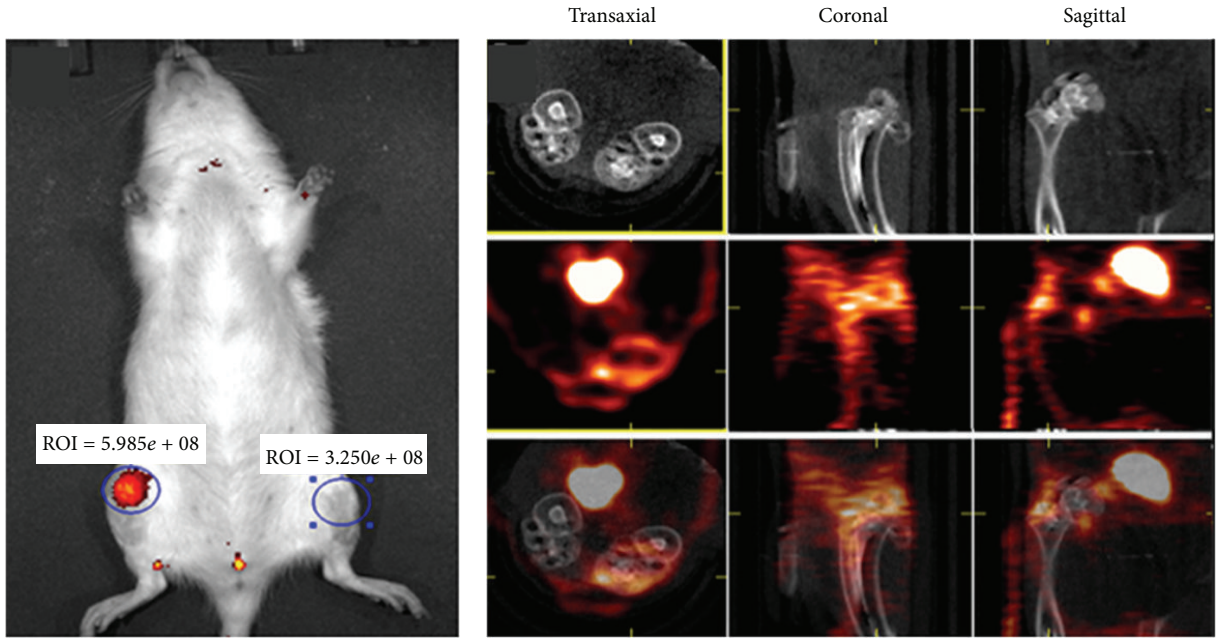


Figure 2: *In vivo* imaging of inflammation with two cFLFLF-derived probes in the rat knee joints treated with (right knee) or without (left knee) monoiodoacetate (MIA). (a) CFLFLF-PEG-Cy 7 probe with animal back down, at day 5 after MIA injection; (b) cFLFLF-PEG-DOTA-$^{64}$Cu with animal back up, at day 5 after MIA injection (upper column: micro-CT; middle column: micro-PET; lower column: fused).

[44], and joint effusion and synovitis [45]. However, even MRI is not sensitive enough to detect the early immune cell infiltration of joints in OA, as inflammation far precedes cartilage destruction marked by radiographic change [46].

There is a substantial need to develop imaging techniques that can visualize the activity of the disease process itself, rather than measure structural changes that are a result of the disease process [47]. In this regard, a few reports have been published on the use of functional nuclear imaging techniques, such as positron emission tomography (PET) and planar or single-photon emission computed tomography (SPECT), for monitoring the inflammatory process of OA [48]. $^{18}$F-2-Fluoro-2-deoxy-D-glucose and $^{111}$In-diethylene triamine pentaacetic acid-folates have been explored as imaging tracers for OA because of respective increased metabolism of glucoses and elevated expression of folate receptors in activated immune cells [49]. Although these tracers have demonstrated some promise in clinical trials as well as in experimental OA models, they are likely not in use due to the lack of an inflammation-specific window of opportunity for imaging.

Alternatively, formyl peptide receptor (FPR) is primarily expressed on activated leukocytes as a defense mechanism to detect and trigger an immune cell response to inflammation caused by infections in a time and concentration dependent manner [50]. In the past years, based on a FPR-specific binding peptide, cFLFLF, we have successfully utilized the cFLFLF-PEG modules to build PET (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid-$^{64}$Cu, also known as DOTA-$^{64}$Cu), SPECT ($^{99m}$Tc), and optical (cyanine-5 and cyanine-7) imaging probes and exhibited excellent imaging in a variety of animal inflammation models [51–55]. The cFLFLFK-Cy-7 probe is now commercially available (Kerafast, Inc.) and cFLFLF-based probes have been developed enthusiastically for animal imaging by the broader research community [56–58].

We are currently exploring if a cFLFLF-based SPECT imaging approach is feasible to monitor aseptic inflammation with a particular interest in OA. To this end, an acute model was created by intra-articular injection of monoiodoacetate for near-infrared fluorescence (NIRF) or PET imaging of inflammatory cells during OA development in rat knee joints. As shown in Figure 2, the inflamed joints were well imaged by either a NIRF probe cFLFLF-PEG-Cy 7 (Figure 2(a)) or a PET probe cFLFLF-PEG-DOTA-$^{64}$Cu (Figure 2(b)). If available in the clinic, use of this SPECT technique can facilitate early detection and monitoring of the recruitment of innate leukocytes during OA development, allow correct characterization and diagnosis to direct early appropriate intervention, and improve long-term outcomes in OA patients [59].

As a caveat, FPR expression in fibroblasts and mesenchymal stem cells (MSCs) has been demonstrated [60, 61]. However, these MSCs and fibroblasts likely serve to repair tissue, initiate tissue remodeling, and mediate leukocyte infiltration in response to the acute chemotactic stimuli of formyl peptide, thereby still reflecting early changes on imaging. The actions of fibroblasts are noted in Tables 1 and 2, respectively. MSCs decrease inflammation, and overexpression of FPR in these cells is currently being studied as potential therapy in chronic disease such as cystic fibrosis [61]. Additionally, FPR ligands have been shown to decrease inflammation in joints and have even been suggested as potential therapies for RA

Table 3: Probes for mediators of inflammation in modern imaging techniques.

| Cell type or protein | Probe |
|---|---|
| Macrophage | (i) $^{18}$F-FDG (PET) [98, 99] |
| CD4$^+$ T cell | (i) $^{64}$Cu-PTSM (PET) [100]<br>(ii) $^{18}$F-FB-IL-2 (PET) [99] |
| B cell | (i) $^{124}$I-rituximab (PET) [101] |
| Neutrophil | (i) cFLFLF-PEG-Cy 7 (NIRF)<br>(ii) cFLFLF-PEG-DOTA-$^{64}$Cu (PET) |
| Mast cell | (i) Ligand 1 (*in vitro*) [102] |
| TNF$\alpha$ | (i) $^{64}$Cu-DOTA-etanercept (PET) [103] |
| Complement | (i) USPIO-conjugated anti-C3mab (T2-MRI) [104] |
| MMP | (i) $^{124}$I-HO-MPI (CGS 27023A) (PET) [105] |

[62]. Regarding the utilization of FPR as an imaging target in early OA, the actions of MSCs, fibroblasts, and FPR ligands, while noteworthy, should have little to no effect or have not yet been discovered. Probes for many cell types and mediators of inflammation mentioned in this paper are displayed in Table 3 and Figure 3.

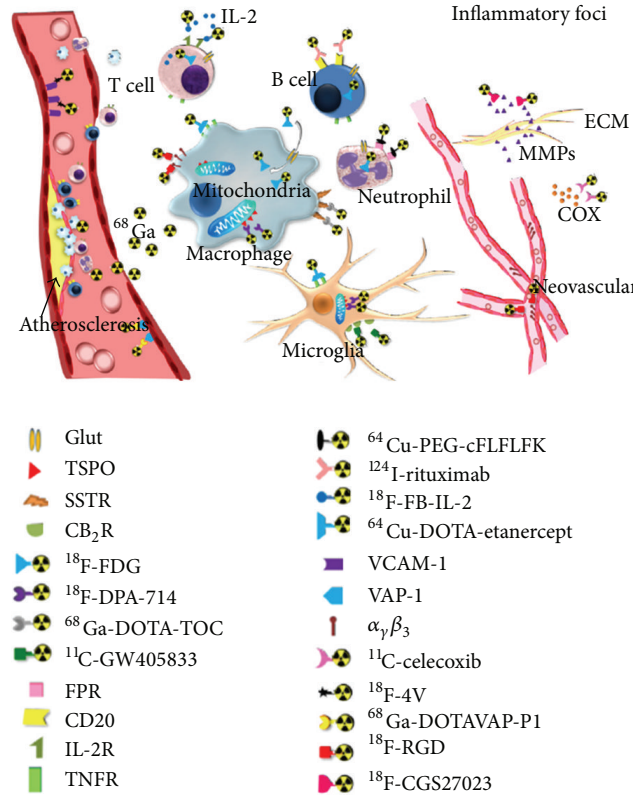


Figure 3: Inflammatory biomarkers in PET imaging (reproduced with permission from Wu et al. [99]).

*3.2. Biomarkers.* To date, many barriers exist in identifying biomarkers reflective of OA severity; histochemical findings have yet to be linked to clinical traits such as pain and function. Foremost, as evidenced in the next section, inflammation in OA is not only local but also systemic, making standard systemic measurements from individual to individual difficult. The confounding factors in systemic inflammation are immeasurable: age, genetics, diet, activity, kidney function, liver function, weight, and other comorbidities to name a few [63]. Numerous biomarkers have been thought to show promise in recent studies, such as serum cartilage oligomeric matrix protein and urine C-terminal cross-linked telopeptide type II collagen levels, but these are nonspecific to cartilage [63, 64]. Complicating the lack in specificity of inflammatory biomarkers is that measurements in OA patients are drawn once disease is already established. The ability is compromised to determine baseline patient values, cut-off values distinguishing normal from abnormal, and markers that are pathological rather than released naturally or concurrently. Another major barrier limiting the identification of both biomarkers and effective treatment is the unfortunate discrepancy between *in vitro* and *in vivo* studies. Decreasing specific mediators of inflammation has thus far not led to improved pain score or prognosis *in vivo*.

For these reasons testing biomarker levels in synovial fluid seems appropriate. However, the natural microenvironment between individual joints varies, making standard measurements difficult to implement [63]. This is evidenced by the unique infrapatellar fat pad of the knee, which greatly contributes to OA pathogenesis by way of adipokine release. Additionally, extracting synovial fluid is restricted to the larger joints and carries risks as compared to drawing blood. Finally, different phenotypes of disease presumably involve diverse biomarkers, pathways, and sequelae [63]. As evidenced by Table 1, T cells [34, 65] and NK cells [19, 20, 66] have been shown to possess exhaustive and chronic phenotypes, respectively, in late versus early disease, demonstrating that early disease is the primary mechanism responsible for changes in the joint microenvironment, underlining the importance of identifying these changes.

*3.3. Hurdles to Treatment.* The significance of the innate immune system in early OA becomes evident, as it leads to direct chondrocyte and cartilage destruction as well as NF$\kappa$B activation with pronounced redundancy and perpetuation. As stated previously, NF$\kappa$B is the master regulator of the immune response. It is involved in the activation of complement, defensins, adhesions, and caspase-1, as well as the production of cytokines, reactive oxygen species (ROS), and NO. Despite the attractiveness of targeting NF$\kappa$B in disease-modifying therapy, it is an unreliable target in large part due to its universal role in normal cellular signaling. Its modulation has a significant side effect profile; however natural health products such as those found in grapes and green tea have shown promise but need further study [67].

The difficulty in treating OA is that once local and systemic inflammation is established, debilitative changes in affected joints are difficult to control. OA is both affected by and contributing to a baseline proinflammatory state, such as that seen in senescence, metabolic syndrome, and

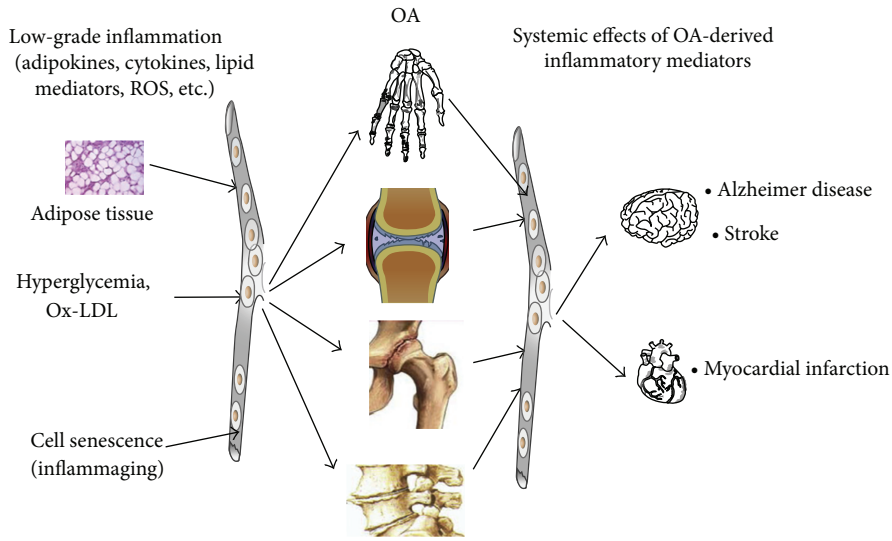


Figure 4: Model for role of systemic proinflammatory state and OA. Inflammatory mediators released into blood enter the joint exacerbating OA, which releases its own mediators of inflammation leading to increased systemic inflammation (reproduced with permission from Berenbaum [6]).

Alzheimer's disease amongst others (Figure 4) [4]. For example, Berenbaum et al. found that a high fat diet increased inflammation in the acute phase of OA [7]. In another study, Kyrkanides et al. found that inducing OA in mice genetically susceptible to Alzheimer's disease exacerbated and accelerated neuroinflammation, increasing the number and size of amyloid plaques [68]. Many therapies, including anti-TNF$\alpha$ and anti-IL-1$\beta$ therapy, have been shown to decrease inflammation but fail to significantly improve function or prognosis in established OA patients [3, 4]. Pain levels have been shown to have a statistically significant correlation with level of change in synovitis ($r = 0.21$, $P = 0.0003$), but not cartilage destruction or baseline level of synovitis [69]. This correlation is only modest and does nothing but supporting the notion that a relative increase in inflammation will increase perception of pain. There is a disconnection between biomarkers of disease, radiographic change, and symptomology, complicating treatment. Degenerative change in OA can occur under two months following trauma [70], and epigenetics has been shown to play a role in mediating the acute inflammatory changes driven by the altered joint microenvironment [71]. It is for these reasons that we hypothesize that addressing early inflammatory change in the synovium consistent with OA is crucial in modulating disease progression and therefore patient disability. Therapies that have failed to show benefit to date may be effective when implemented at an appropriate stage of disease. Future research should be targeted toward identifying at-risk patients and early intervention.

## 4. Perspectives

Pharmacological treatment to date has had varying effects on symptomology, but disease modulation has yet to be attained. Common modalities include NSAIDS, corticosteroids, chondroitin sulfate, and glucosamine [72]. These treatments are variably effective on an individual basis and often only provide temporary relief and are needed to be repeated chronically. Trials of anti-TNF$\alpha$ and anti-IL-1$\beta$ therapy for disease modulation have been unsuccessful despite the dominance of TNF$\alpha$ and IL-1$\beta$ in pathogenesis [4]. Chevalier et al. concluded that IL-1$\beta$ antagonism may benefit patients with baseline high levels of pain if administered in low, frequent intra-articular (IA) injections to avoid neutropenia and serious infection [73]. One *in vivo* study revealed that IA injection of lubricin up to two weeks after injury reduced severity of OA in mice, while local antioxidants such as N-acetylcysteine after injury showed promise *in vitro* [74, 75]. The proposed benefit of these treatments administered soon after injury in injury-induced OA underlines the significance of early intervention in OA pathogenesis. Treatment with fibroblast growth factor 18, which is specific for the anabolic FGFR-3 versus the catabolic FGFR-1, is currently on trial [71].

We believe that regular screening is needed and is justified as OA is ubiquitous in seniors aged over 65, is clinically present in 13.9% of US adults aged 25 or above, and is a leading cause of disability and hospitalization in the USA [1, 2, 72]. However, further studies are needed in order to establish guidelines for screening. We recommend that regular screening for OA be implemented on an outpatient basis. Special attention should be given to patients of 65 years or above and patients with metabolic syndrome, Alzheimer's disease, or other systemic proinflammatory states. Candidate markers for screening should continue to be researched with particular attention paid to local articular levels. IL-6, complement, and ratio of FGFR-3/FGFR-1 should be considered.

Additionally, the role of physical, imaging, or combination diagnostic paradigms must be considered. Contrast-enhanced MRI and power Doppler ultrasound are the leading imaging modalities for synovitis [46]. Identifying early and specific changes in OA may best be visualized

using PET, NIRF, or SPECT imaging. Many probes for cells and proteins involved in OA pathogenesis are listed in Table 3 and Figure 3. We are currently developing a Tc99m-cFLFLF/SPECT technique to visualize early leukocyte recruitment in OA joints based on a preliminary *in vivo* study (Figure 2). While our probe is not 100% specific for leukocytes, we are currently in the process of identifying more specific receptors.

As changes in the joint consistent with OA can occur rapidly following injury and are associated with inflammation, intervention should be aimed at the early reactive phase of OA pathogenesis [70]. Importantly, past therapeutic trials may have failed due to attempted intervention at irreversible stages of disease. Wang et al. showed that knocking out components of the complement cascade greatly reduced incidence of OA in mice [17] and this strategy for treatment management should be further researched. A study assessing whether there is an increased relative risk of OA diagnosis and severity in patients with paroxysmal nocturnal hemoglobinuria could be beneficial in this regard. Anticomplement therapy should initially be attempted locally to narrow the focus of treatment and lower the incidence of potential severe infection.

While disease modification in OA still eludes the medical community, recent advances in pathogenesis and understanding of the disease process beseech hope to solving the riddle of a ubiquitous, costly disease that significantly diminishes quality of life in millions of patients. With guided further research and international collaboration, we believe that early detection and intervention in OA are possible. Due to the lack of success and discrepancy of disease modulation between *in vivo* and *in vitro* studies, the significance of identifying patients in the early phase of disease becomes paramount in experimenting with detection and treatment of the disease process. Screening must be implemented in high-risk patients, and early, aggressive treatment is necessary and mandated to avoid substantial morbidity.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## References


[1] L. Murphy and C. G. Helmick, "The impact of osteoarthritis in the United States: a population-health perspective: a population-based review of the fourth most common cause of hospitalization in U.S. adults," *American Journal of Nursing*, vol. 112, no. 3, pp. S13–S19, 2012.

[2] R. C. Lawrence, D. T. Felson, C. G. Helmick et al., "Estimates of the prevalence of arthritis and other rheumatic conditions in the United States. Part II," *Arthritis and Rheumatism*, vol. 58, no. 1, pp. 26–35, 2008.

[3] C. J. Malemud, "Anticytokine therapy for osteoarthritis: evidence to date," *Drugs and Aging*, vol. 27, no. 2, pp. 95–115, 2010.

[4] A. L. G. Calich, D. S. Domiciano, and R. Fuller, "Osteoarthritis: can anti-cytokine therapy play a role in treatment?" *Clinical Rheumatology*, vol. 29, no. 5, pp. 451–455, 2010.

[5] B. J. E. de Lange-Brokaar, A. Ioan-Facsinay, G. J. V. M. van Osch et al., "Synovial inflammation, immune cells and their cytokines in osteoarthritis: a review," *Osteoarthritis and Cartilage*, vol. 20, no. 12, pp. 1484–1499, 2012.

[6] F. Berenbaum, "Osteoarthritis as an inflammatory disease (osteoarthritis is not osteoarthrosis!)," *Osteoarthritis and Cartilage*, vol. 21, no. 1, pp. 16–21, 2013.

[7] F. Berenbaum, F. Eymard, and X. Houard, "Osteoarthritis, inflammation and obesity," *Current Opinion in Rheumatology*, vol. 25, no. 1, pp. 114–118, 2013.

[8] K. O. Yaykasli, O. F. Hatipoglu, E. Yaykasli et al., "Leptin induces ADAMTS-4, ADAMTS-5, and ADAMTS-9 genes expression by mitogen-activated protein kinases and NF-$\kappa$B signaling pathways in human chondrocytes," *Cell Biology International*, 2014.

[9] W. Hui, G. J. Litherland, M. S. Elias et al., "Leptin produced by joint white adipose tissue induces cartilage degradation via upregulation and activation of matrix metalloproteinases," *Annals of the Rheumatic Diseases*, vol. 71, no. 3, pp. 455–462, 2012.

[10] M. Otero, R. Lago, R. Gómez, F. Lago, J. J. Gomez-Reino, and O. Gualillo, "Phosphatidylinositol 3-kinase, MEK-1 and p38 mediate leptin/interferon-gamma synergistic NOS type II induction in chondrocytes," *Life Sciences*, vol. 81, no. 19-20, pp. 1452–1460, 2007.

[11] P.-J. Francin, A. Abot, C. Guillaume et al., "Association between adiponectin and cartilage degradation in human osteoarthritis," *Osteoarthritis and Cartilage*, vol. 22, no. 3, pp. 519–526, 2014.

[12] S. Clockaerts, Y. M. Bastiaansen-Jenniskens, J. Runhaar et al., "The infrapatellar fat pad should be considered as an active osteoarthritic joint tissue: a narrative review," *Osteoarthritis and Cartilage*, vol. 18, no. 7, pp. 876–882, 2010.

[13] M. Bohnsack, F. Meier, G. F. Walter et al., "Distribution of substance-P nerves inside the infrapatellar fat pad and the adjacent synovial tissue: a neurohistological approach to anterior knee pain syndrome," *Archives of Orthopaedic and Trauma Surgery*, vol. 125, no. 9, pp. 592–597, 2005.

[14] D. H. Sohn, J. Sokolove, O. Sharpe et al., "Plasma proteins present in osteoarthritic synovial fluid can stimulate cytokine production via Toll-like receptor 4," *Arthritis Research and Therapy*, vol. 14, article R7, 2012.

[15] S.-L. Su, C.-D. Tsai, C.-H. Lee, D. M. Salter, and H.-S. Lee, "Expression and regulation of Toll-like receptor 2 by IL-1$\beta$ and fibronectin fragments in human articular chondrocytes," *Osteoarthritis and Cartilage*, vol. 13, no. 10, pp. 879–886, 2005.

[16] R. F. P. Schelbergen, A. B. Blom, M. H. J. van den Bosch et al., "Alarmins S100A8 and S100A9 elicit a catabolic effect in human osteoarthritic chondrocytes that is dependent on toll-like receptor 4," *Arthritis & Rheumatism*, vol. 64, no. 5, pp. 1477–1487, 2012.

[17] Q. Wang, A. L. Rozelle, C. M. Lepus et al., "Identification of a central role for complement in osteoarthritis," *Nature Medicine*, vol. 17, no. 12, pp. 1674–1679, 2011.

[18] W. H. Busby Jr., S. A. Yocum, M. Rowland et al., "Complement 1s is the serine protease that cleaves IGFBP-5 in human osteoarthritic joint fluid," *Osteoarthritis and Cartilage*, vol. 17, no. 4, pp. 547–555, 2009.

[19] J. A. Kummer, P. P. Tak, B. M. N. Brinkman et al., "Expression of granzymes A and B in synovial tissue from patients with rheumatoid arthritis and osteoarthritis," *Clinical Immunology and Immunopathology*, vol. 73, no. 1, pp. 88–95, 1994.

[20] R. S. Huss, J. I. Huddleston, S. B. Goodman, E. C. Butcher, and B. A. Zabel, "Synovial tissue-infiltrating natural killer cells in osteoarthritis and periprosthetic inflammation," *Arthritis & Rheumatism*, vol. 62, no. 12, pp. 3799–3805, 2010.

[21] P. P. Tak, L. Spaeny-Dekking, M. C. Kraan, F. C. Breedveld, C. J. Froelich, and C. E. Hack, "The levels of soluble granzyme A and B are elevated in plasma and synovial fluid of patients with rheumatoid arthritis (RA)," *Clinical and Experimental Immunology*, vol. 116, no. 2, pp. 366–370, 1999.

[22] T. E. Damsgaard, F. B. Sørensen, T. Herlin, and P. O. Schiøtz, "Stereological quantification of mast cells in human synovium," *APMIS*, vol. 107, no. 3, pp. 311–317, 1999.

[23] G. Dean, J. A. Hoyland, J. Denton, R. P. Donn, and A. J. Freemont, "Mast cells in the synovium and synovial fluid in osteoarthritis," *British Journal of Rheumatology*, vol. 32, no. 8, pp. 671–675, 1993.

[24] M. G. Buckley, P. J. Gallagher, and A. F. Walls, "Mast cell subpopulations in the synovial tissue of patients with osteoarthritis: selective increase in numbers of tryptase-positive, chymase-negative mast cells," *The Journal of Pathology*, vol. 186, no. 1, pp. 67–74, 1998.

[25] B. Riepl, S. Grässel, R. Wiest, M. Fleck, and R. H. Straub, "Tumor necrosis factor and norepinephrine lower the levels of human neutrophil peptides 1–3 secretion by mixed synovial tissue cultures in osteoarthritis and rheumatoid arthritis," *Arthritis Research and Therapy*, vol. 12, article R110, 2010.

[26] O. Soehnlein, Y. Kai-Larsen, R. Frithiof et al., "Neutrophil primary granule proteins HBP and HNP1-3 boost bacterial phagocytosis by human and murine macrophages," *The Journal of Clinical Investigation*, vol. 118, no. 10, pp. 3491–3502, 2008.

[27] K. Gupta, M. Shukla, J. B. Cowland, C. J. Malemud, and T. M. Haqqi, "Neutrophil gelatinase-associated lipocalin is expressed in osteoarthritis and forms a complex with matrix metalloproteinase 9," *Arthritis & Rheumatism*, vol. 56, no. 10, pp. 3326–3335, 2007.

[28] J. M. Milner, A. D. Rowan, T. E. Cawston, and D. A. Young, "Metalloproteinase and inhibitor expression profiling of resorbing cartilage reveals pro-collagenase activation as a critical step for collagenolysis," *Arthritis Research & Therapy*, vol. 8, no. 5, article R142, 2006.

[29] J. Bondeson, A. B. Blom, S. Wainwright, C. Hughes, B. Caterson, and W. B. van den Berg, "The role of synovial macrophages and macrophage-produced mediators in driving inflammatory and destructive responses in osteoarthritis," *Arthritis and Rheumatism*, vol. 62, no. 3, pp. 647–657, 2010.

[30] J. Saklatvala, "Tumour necrosis factor $\alpha$ stimulates resorption and inhibits synthesis of proteoglycan in cartilage," *Nature*, vol. 322, no. 6079, pp. 547–549, 1986.

[31] A. Haseeb and T. M. Haqqi, "Immunopathogenesis of osteoarthritis," *Clinical Immunology*, vol. 146, no. 3, pp. 185–196, 2013.

[32] V. Lefebvre, C. Peeters-Joris, and G. Vaes, "Modulation by interleukin 1 and tumor necrosis factor $\alpha$ of production of collagenase, tissue inhibitor of metalloproteinases and collagen types in differentiated and dedifferentiated articular chondrocytes," *Biochimica et Biophysica Acta*, vol. 1052, no. 3, pp. 366–378, 1990.

[33] M. J. Benito, D. J. Veale, O. FitzGerald, W. B. van den Berg, and B. Bresnihan, "Synovial tissue inflammation in early and late osteoarthritis," *Annals of the Rheumatic Diseases*, vol. 64, no. 9, pp. 1263–1267, 2005.

[34] L. I. Sakkas, C. Scanzello, N. Johanson et al., "T cells and T-cell cytokine transcripts in the synovial membrane in patients with osteoarthritis," *Clinical and Diagnostic Laboratory Immunology*, vol. 5, no. 4, pp. 430–437, 1998.

[35] I. Saito, T. Koshino, K. Nakashima, M. Uesugi, and T. Saito, "Increased cellular infiltrate in inflammatory synovia of osteoarthritic knees," *Osteoarthritis and Cartilage*, vol. 10, no. 2, pp. 156–162, 2002.

[36] F. Pessler, L. X. Chen, L. Dai et al., "A histomorphometric analysis of synovial biopsies from individuals with Gulf War Veterans' Illness and joint pain compared to normal and osteoarthritis synovium," *Clinical Rheumatology*, vol. 27, no. 9, pp. 1127–1134, 2008.

[37] F. Eckstein, W. Wirth, and M. C. Nevitt, "Recent advances in osteoarthritis imaging—the Osteoarthritis Initiative," *Nature Reviews Rheumatology*, vol. 8, no. 10, pp. 622–630, 2012.

[38] C. Ding, Y. Zhang, and D. Hunter, "Use of imaging techniques to predict progression in osteoarthritis," *Current Opinion in Rheumatology*, vol. 25, no. 1, pp. 127–135, 2013.

[39] A. Guermazi, F. W. Roemer, D. Burstein, and D. Hayashi, "Why radiography should no longer be considered a surrogate outcome measure for longitudinal assessment of cartilage in knee osteoarthritis," *Arthritis Research and Therapy*, vol. 13, article 247, 2011.

[40] V. B. Kraus, "Waiting for action on the osteoarthritis front," *Current Drug Targets*, vol. 11, no. 5, pp. 518–520, 2010.

[41] F. W. Roemer, C. K. Kwoh, M. J. Hannon et al., "Risk factors for magnetic resonance imaging-detected patellofemoral and tibiofemoral cartilage loss during a six-month period: the Joints on Glucosamine study," *Arthritis and Rheumatism*, vol. 64, no. 6, pp. 1888–1898, 2012.

[42] S. K. Tanamas, A. E. Wluka, J.-P. Pelletier et al., "Bone marrow lesions in people with knee osteoarthritis predict progression of disease and joint replacement: a longitudinal study," *Rheumatology*, vol. 49, no. 12, pp. 2413–2419, 2010.

[43] C. K. Kwoh, "Osteoarthritis: clinical relevance of bone marrow lesions in OA," *Nature Reviews Rheumatology*, vol. 9, no. 1, pp. 7–8, 2013.

[44] L. Sharma, J. S. Chmiel, O. Almagor et al., "Significance of preradiographic magnetic resonance imaging lesions in persons at increased risk of knee osteoarthritis," *Arthritis & Rheumatology*, vol. 66, no. 7, pp. 1811–1819, 2014.

[45] F. W. Roemer, A. Guermazi, D. T. Felson et al., "Presence of MRI-detected joint effusion and synovitis increases the risk of cartilage loss in knees without osteoarthritis at 30-month follow-up: the MOST study," *Annals of the Rheumatic Diseases*, vol. 70, no. 10, pp. 1804–1809, 2011.

[46] J. Sokolove and C. M. Lepus, "Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations," *Therapeutic Advances in Musculoskeletal Disease*, vol. 5, no. 2, pp. 77–94, 2013.

[47] H.-J. Park, S. S. Kim, S.-Y. Lee et al., "A practical MRI grading system for osteoarthritis of the knee: association with Kellgren-Lawrence radiographic scores," *European Journal of Radiology*, vol. 82, no. 1, pp. 112–117, 2013.

[48] H. J. Braun and G. E. Gold, "Diagnosis of osteoarthritis: imaging," *Bone*, vol. 51, no. 2, pp. 278–288, 2012.

[49] H. Nakamura, K. Masuko, K. Yudoh et al., "Positron emission tomography with $^{18}$F-FDG in osteoarthritic knee," *Osteoarthritis and Cartilage*, vol. 15, no. 6, pp. 673–681, 2007.

[50] C. Y. J. Wenham and P. G. Conaghan, "New horizons in osteoarthritis," *Age and Ageing*, vol. 42, no. 3, pp. 272–278, 2013.

[51] Y. Zhang, B. Kundu, K. D. Fairchild et al., "Synthesis of novel neutrophil-specific imaging agents for Positron Emission Tomography (PET) imaging," *Bioorganic and Medicinal Chemistry Letters*, vol. 17, no. 24, pp. 6876–6878, 2007.

[52] L. W. Locke, M. D. Chordia, Y. Zhang et al., "A novel neutrophil-specific PET imaging agent: cFLFLFK-PEG-$^{64}$Cu," *The Journal of Nuclear Medicine*, vol. 50, no. 5, pp. 790–797, 2009.

[53] L. Xiao, Y. Zhang, Z. Liu, M. Yang, L. Pu, and D. Pan, "Synthesis of the Cyanine 7 labeled neutrophil-specific agents for noninvasive near infrared fluorescence imaging," *Bioorganic and Medicinal Chemistry Letters*, vol. 20, no. 12, pp. 3515–3517, 2010.

[54] Y. Zhang, L. Xiao, M. D. Chordia et al., "Neutrophil targeting heterobivalent SPECT imaging probe: CFLFLF-PEG-TKPPR-$^{99m}$Tc," *Bioconjugate Chemistry*, vol. 21, no. 10, pp. 1788–1793, 2010.

[55] L. Xiao, Y. Zhang, S. S. Berr et al., "A novel near-infrared fluorescence imaging probe for in vivo neutrophil tracking," *Molecular Imaging*, vol. 11, no. 5, pp. 372–382, 2012.

[56] S. Massey, K. Johnston, T. M. Mott et al., "*In vivo* bio luminescence imaging of *Burkholderia mallei* respiratory infection and treatment in the mouse model," *Frontiers in Microbiology*, vol. 2, article 174, 2011.

[57] G. J. Stasiuk, H. Smith, M. Wylezinska-Arridge et al., "$Gd^{3+}$ cFLFLFK conjugate for MRI: a targeted contrast agent for FPR1 in inflammation," *Chemical Communications*, vol. 49, no. 6, pp. 564–566, 2013.

[58] J. Zhou, Y.-T. Tsai, H. Weng et al., "Real-time detection of implant-associated neutrophil responses using a formyl peptide receptor-targeting NIR nanoprobe," *International Journal of Nanomedicine*, vol. 7, pp. 2057–2068, 2012.

[59] D. A. Dorward, C. D. Lucas, A. G. Rossi, C. Haslett, and K. Dhaliwal, "Imaging inflammation: molecular strategies to visualize key components of the inflammatory cascade, from initiation to resolution," *Pharmacology and Therapeutics*, vol. 135, no. 2, pp. 182–199, 2012.

[60] S. E. VanCompernolle, K. L. Clark, K. A. Rummel, and S. C. Todd, "Expression and function of formyl peptide receptors on human fibroblast cells," *The Journal of Immunology*, vol. 171, no. 4, pp. 2050–2056, 2003.

[61] A. Viswanathan, R. G. Painter, N. A. Lanson Jr., and G. Wang, "Functional expression of *N*-formyl peptide receptors in human bone marrow-derived mesenchymal stem cells," *Stem Cells*, vol. 25, no. 5, pp. 1263–1269, 2007.

[62] W. Kao, R. Gu, Y. Jia et al., "A formyl peptide receptor agonist suppresses inflammation and bone damage in arthritis," *British Journal of Pharmacology*, vol. 171, no. 17, pp. 4087–4096, 2014.

[63] F. P. J. G. Lafeber and W. E. van Spil, "Osteoarthritis year 2013 in review: biomarkers; reflecting before moving forward, one step at a time," *Osteoarthritis and Cartilage*, vol. 21, no. 10, pp. 1452–1464, 2013.

[64] M. Attur, S. Krasnokutsky-Samuels, J. Samuels, and S. B. Abramson, "Prognostic biomarkers in osteoarthritis," *Current Opinion in Rheumatology*, vol. 25, no. 1, pp. 136–144, 2013.

[65] L. I. Sakkas, G. Koussidis, E. Avgerinos, J. Gaughan, and C. D. Platsoucas, "Decreased expression of the CD3$\zeta$ chain in T cells infiltrating the synovial membrane of patients with osteoarthritis," *Clinical and Diagnostic Laboratory Immunology*, vol. 11, no. 1, pp. 195–202, 2004.

[66] C. R. Scanzello, E. Umoh, F. Pessler et al., "Local cytokine profiles in knee osteoarthritis: elevated synovial fluid interleukin-15 differentiates early from end-stage disease," *Osteoarthritis and Cartilage*, vol. 17, no. 8, pp. 1040–1048, 2009.

[67] S. Khalifé and M. Zafarullah, "Molecular targets of natural health products in arthritis," *Arthritis Research & Therapy*, vol. 13, article 102, 2011.

[68] S. Kyrkanides, R. H. Tallents, J.-N. H. Miller et al., "Osteoarthritis accelerates and exacerbates Alzheimer's disease pathology in mice," *Journal of Neuroinflammation*, vol. 8, article 112, 2011.

[69] C. L. Hill, D. J. Hunter, J. Niu et al., "Synovitis detected on magnetic resonance imaging and its relation to pain and cartilage loss in knee osteoarthritis," *Annals of the Rheumatic Diseases*, vol. 66, no. 12, pp. 1599–1603, 2007.

[70] M. L. Schenker, R. L. Mauck, and S. Mehta, "Pathogenesis and prevention of posttraumatic osteoarthritis after intra-articular fracture," *Journal of the American Academy of Orthopaedic Surgeons*, vol. 22, no. 1, pp. 20–28, 2014.

[71] R. F. Loeser, "Osteoarthritis year in review 2013: biology," *Osteoarthritis and Cartilage*, vol. 21, no. 10, pp. 1436–1442, 2013.

[72] E. F. Goljan, *Rapid Review Pathology*, Elsevier/Saunders, Philadelphia, Pa, USA, 4th edition, 2014.

[73] X. Chevalier, T. Conrozier, and P. Richette, "Desperately looking for the right target in osteoarthritis: the anti-IL-1 strategy," *Arthritis Research and Therapy*, vol. 13, article 124, 2011.

[74] M. Z. C. Ruan, A. Erez, K. Guse et al., "Proteoglycan 4 expression protects against the development of osteoarthritis," *Science Translational Medicine*, vol. 5, no. 176, Article ID 176ra34, 2013.

[75] J. A. Martin, D. McCabe, M. Walter, J. A. Buckwalter, and T. O. McKinley, "*N*-acetylcysteine inhibits post-impact chondrocyte death in osteochondral explants," *The Journal of Bone and Joint Surgery A*, vol. 91, no. 8, pp. 1890–1897, 2009.

[76] P. L. E. M. Van Lent, A. B. Blom, P. Van Der Kraan et al., "Crucial role of synovial lining macrophages in the promotion of transforming growth factor $\beta$-mediated osteophyte formation," *Arthritis and Rheumatism*, vol. 50, no. 1, pp. 103–111, 2004.

[77] H. Nakamura, S. Yoshino, T. Kato, J. Tsuruha, and K. Nishioka, "T-cell mediated inflammatory pathway in osteoarthritis," *Osteoarthritis and Cartilage*, vol. 7, no. 4, pp. 401–402, 1999.

[78] A. Guerassimov, Y. Zhang, A. Cartman et al., "Immune responses to cartilage link protein and the G1 domain of proteoglycan aggrecan in patients with osteoarthritis," *Arthritis & Rheumatism*, vol. 42, no. 3, pp. 527–533, 1999.

[79] H. de Jong, S. E. Berlo, P. Hombrink et al., "Cartilage proteoglycan aggrecan epitopes induce proinflammatory autoreactive T-cell responses in rheumatoid arthritis and osteoarthritis," *Annals of the Rheumatic Diseases*, vol. 69, no. 1, pp. 255–262, 2010.

[80] R.-R. Da, Y. Qin, D. Baeten, and Y. Zhang, "B cell clonal expansion and somatic hypermutation of Ig variable heavy chain genes in the synovial membrane of patients with osteoarthritis," *The Journal of Immunology*, vol. 178, no. 1, pp. 557–565, 2007.

[81] S. Shiokawa, N. Matsumoto, and J. Nishimura, "Clonal analysis of B cells in the osteoarthritis synovium," *Annals of the Rheumatic Diseases*, vol. 60, no. 8, pp. 802–805, 2001.

[82] J. Mollenhauer, K. von der Mark, G. Burmester, K. Gluckert, E. Lutjen-Drecoll, and K. Brune, "Serum antibodies against chondrocyte cell surface proteins in osteoarthritis and rheumatoid arthritis," *The Journal of Rheumatology*, vol. 15, no. 12, pp. 1811–1817, 1988.

[83] F. Doss, J. Menard, M. Hauschild et al., "Elevated IL-6 levels in the synovial fluid of osteoarthritis patients stem from plasma cells," *Scandinavian Journal of Rheumatology*, vol. 36, no. 2, pp. 136–139, 2007.

[84] M. D. Smith, S. Triantafillou, A. Parker, P. P. Youssef, and M. Coleman, "Synovial membrane inflammation and cytokine production in patients with early osteoarthritis," *The Journal of Rheumatology*, vol. 24, no. 2, pp. 365–371, 1997.

[85] M. Kobayashi, G. R. Squires, A. Mousa et al., "Role of interleukin-1 and tumor necrosis factor $\alpha$ in matrix degradation of human osteoarthritic cartilage," *Arthritis and Rheumatism*, vol. 52, no. 1, pp. 128–135, 2005.

[86] Z. Fan, B. Bau, H. Yang, S. Soeder, and T. Aigner, "Freshly isolated osteoarthritic chondrocytes are catabolically more active than normal chondrocytes, but less responsive to catabolic stimulation with interleukin-1$\beta$," *Arthritis & Rheumatism*, vol. 52, no. 1, pp. 136–143, 2005.

[87] N. Akhtar and T. M. Haqqi, "Epigallocatechin-3-gallate suppresses the global interleukin-1beta-induced inflammatory response in human chondrocytes," *Arthritis Research and Therapy*, vol. 13, no. 3, article R93, 2011.

[88] J. N. Gouze, K. Bordji, S. Gulberti et al., "Interleukin-1beta down-regulates the expression of glucuronosyltransferase I, a key enzyme priming glycosaminoglycan biosynthesis: influence of glucosamine on interleukin-1beta-mediated effects in rat chondrocytes," *Arthritis & Rheumatism*, vol. 44, no. 2, pp. 351–360, 2001.

[89] F. Heraud, A. Heraud, and M.-F. Harmand, "Apoptosis in normal and osteoarthritic human articular cartilage," *Annals of the Rheumatic Diseases*, vol. 59, no. 12, pp. 959–965, 2000.

[90] M. Mathy-Hartert, L. Hogge, C. Sanchez, G. Deby-Dupont, J. M. Crielaard, and Y. Henrotin, "Interleukin-1$\beta$ and interleukin-6 disturb the antioxidant enzyme system in bovine chondrocytes: a possible explanation for oxidative stress generation," *Osteoarthritis and Cartilage*, vol. 16, no. 7, pp. 756–763, 2008.

[91] J.-P. Pelletier, F. Mineau, P. Ranger, G. Tardif, and J. Martel-Pelletier, "The increased synthesis of inducible nitric oxide inhibits IL-1ra synthesis by human articular chondrocytes: possible role in osteoarthritic cartilage degradation," *Osteoarthritis and Cartilage*, vol. 4, no. 1, pp. 77–84, 1996.

[92] X. Li, M. Ellman, P. Muddasani et al., "Prostaglandin $E_2$ and its cognate EP receptors control human adult articular cartilage homeostasis and are linked to the pathophysiology of osteoarthritis," *Arthritis & Rheumatism*, vol. 60, no. 2, pp. 513–523, 2009.

[93] P.-A. Guerne, B. L. Zuraw, J. H. Vaughan, D. A. Carson, and M. Lotz, "Synovium as a source of interleukin 6 in vitro. Contribution to local and systemic manifestations of arthritis," *The Journal of Clinical Investigation*, vol. 83, no. 2, pp. 585–592, 1989.

[94] F. Legendre, J. Dudhia, J.-P. Pujol, and P. Bogdanowicz, "JAK/STAT but not ERK1/ERK2 pathway mediates interleukin (IL)-6/soluble IL-6R down-regulation of type II collagen, aggrecan core, and link protein transcription in articular chondrocytes. Association with a down-regulation of Sox9 expression," *The Journal of Biological Chemistry*, vol. 278, no. 5, pp. 2903–2912, 2003.

[95] B. Porée, M. Kypriotou, C. Chadjichristos et al., "Interleukin-6 (IL-6) and/or soluble IL-6 receptor down-regulation of human type II collagen gene expression in articular chondrocytes requires a decrease of Sp1.Sp3 ratio and of the binding activity of both factors to the COL2A1 promoter," *The Journal of Biological Chemistry*, vol. 283, no. 8, pp. 4850–4865, 2008.

[96] T. E. Cawston, V. A. Curry, C. A. Summers et al., "The role of oncostatin M in animal and human connective tissue collagen turnover and its localization within the rheumatoid joint," *Arthritis & Rheumatology*, vol. 41, pp. 1760–1771, 1998.

[97] J. Suurmond, A. L. Dorjee, M. R. Boon et al., "Mast cells are the main interleukin-17-positive cells in anti-citrullinated protein antibody-positive and -negative rheumatoid arthritis and osteoarthritis synovium," *Arthritis Research & Therapy*, vol. 13, no. 5, article R150, 2011.

[98] T. L. Y. Brown, H. J. Spencer, K. E. Beenken et al., "Evaluation of dynamic [$^{18}$F]-FDG-PET imaging for the detection of acute post-surgical bone infection," *PLoS ONE*, vol. 7, no. 7, Article ID e41863, 2012.

[99] C. Wu, F. Li, G. Niu, and X. Chen, "PET imaging of inflammation biomarkers," *Theranostics*, vol. 3, no. 7, pp. 448–466, 2013.

[100] C. M. Griessinger, R. Kehlbach, D. Bukala et al., "In vivo tracking of th1 cells by PET reveals quantitative and temporal distribution and specific homing in lymphatic tissue," *The Journal of Nuclear Medicine*, vol. 55, no. 2, pp. 301–307, 2014.

[101] L. Tran, A. D. R. Huitema, M. H. Van Rijswijk et al., "CD20 antigen imaging with $^{124}$I-rituximab PET/CT in patients with rheumatoid arthritis," *Human Antibodies*, vol. 20, no. 1-2, pp. 29–35, 2011.

[102] Q. Wang, X. Shi, X. Zhu, M. Ehlers, J. Wu, and C. Schmuck, "A fluorescent light-up probe as an inhibitor of intracellular $\beta$-tryptase," *Chemical Communications*, vol. 50, no. 46, pp. 6120–6122, 2014.

[103] Q. Cao, W. Cai, Z.-B. Li et al., "PET imaging of acute and chronic inflammation in living mice," *European Journal of Nuclear Medicine and Molecular Imaging*, vol. 34, no. 11, pp. 1832–1842, 2007.

[104] G. Girardi, J. Fraser, R. Lennen, R. Vontell, M. Jansen, and G. Hutchison, "Imaging of activated complement using ultrasmall superparamagnetic iron oxide particles (USPIO)—conjugated vectors: an *in vivo in utero* non-invasive method to predict placental insufficiency and abnormal fetal brain development," *Molecular Psychiatry*, 2014.

[105] D. Hartung, M. Schäfers, S. Fujimoto et al., "Targeting of matrix metalloproteinase activation for noninvasive detection of vulnerable atherosclerotic lesions," *European Journal of Nuclear Medicine and Molecular Imaging*, vol. 34, supplement 1, pp. S1–S8, 2007.

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*Research Article*

# Osteoprotegerin Polymorphisms in a Mexican Population with Rheumatoid Arthritis and Generalized Osteoporosis: A Preliminary Report

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Bone disease in rheumatoid arthritis (RA) is a complex phenomenon where genetic risk factors have been partially evaluated. The system formed by receptor activator for nuclear factor-$\kappa$B (RANK), receptor activator for nuclear factor-$\kappa$B ligand (RANKL), and osteoprotegerin (OPG): RANK/RANKL/OPG is a crucial molecular pathway for coupling between osteoblasts and osteoclasts, since OPG is able to inhibit osteoclast differentiation and activation. We aim to evaluate the association between SNPs C950T (rs2073617), C209T (rs3134069), T245G (rs3134070) in the *TNFRSF11B* (OPG) gene, and osteoporosis in RA. We included 81 women with RA and 52 healthy subjects in a cross-sectional study, genotyped them, and measured bone mineral density (BMD) at the lumbar spine and the femoral neck. Mean age in RA was 50 ± 12 with disease duration of 12 ± 8 years. According to BMD results, 23 (33.3%) were normal and 46 (66.7%) had osteopenia/osteoporosis. We found a higher prevalence of C allele for C950T SNP in RA.

Polymorphisms C209T and T245G did not reach statistical significance in allele distribution. Further studies including patients from other regions of Latin America with a multicenter design to increase the sample size are required to confirm our findings and elucidate if C950T SNP could be associated with osteoporosis in RA.

## 1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects synovial joints [1]. Its prevalence in Mexico is considerably elevated ranging from 0.3 to 1% [2]. Currently, the diagnosis of osteoporosis is made on the basis of a measurement by dual-energy x-ray absorptiometry (DXA) at the spine and hip showing a decrement in bone mineral density (BMD) lower than 2.5 standard deviations in comparison with healthy young adults [3]. However, bone involvement in RA is complex; an increased proportion of patients may suffer from osteoporosis; nevertheless causal effects are considered to be multifactorial. Previous studies performed in our center showed a prevalence of axial osteoporosis in Mexican women with RA of 31% [4].

Genetic risk factors constitute a variable that contributes to influencing the development of osteoporosis in RA, in this context; some genetic polymorphisms may influence the rate of presentation of osteoporosis. Nevertheless such genetic changes do not explain entirely osteoporosis presence or fracture related events. Strong evidence supports the fact that osteoclasts have an important role in osteoporosis and bone erosion formation [5, 6].

The system conformed by nuclear factor-$\kappa$B (RANK)/receptor activator for nuclear factor-$\kappa$B ligand (RANKL) and osteoprotegerin (OPG) are crucial, for the bone remodeling process. Furthermore, it appeared that not only osteoblasts but also activated *T* lymphocytes play a crucial role in the pathogenesis of rheumatoid arthritis (RA) and osteoporosis, since they can produce RANKL, which stimulates the differentiation and activation of osteoclasts [7]. These three proteins belong to the superfamily of the TNF-$\alpha$ and have different patterns of expression; RANKL is expressed in osteoblasts and its function is to participate in osteoclasts differentiation [7, 8]. Osteoprotegerin (OPG) lacks a transmembrane domain, and hence it is a receptor that can be secreted. OPG recognizes and is able to bind RANKL, blocking its interaction with RANK inhibiting osteoclastic differentiation and activation [9–11]. Since OPG is homologous to RANK it acts as a false receptor for RANKL [12–14]. OPG is secreted mainly by stromal cells but can also be secreted by other types of cells [15]. Different studies have been able to identify single nucleotide polymorphisms (SNPs) in *TNFSF11* (RANKL) and *TNFRSF11B* (OPG) genes in association with osteoporosis or low bone density [12, 16]. It has been documented that OPG overexpression induces an increase in bone density and protects from the development to osteoporosis. In contrast mice that are OPG-deficient develop osteoporosis very rapidly [14, 15, 17]. OPG overexpression blocks osteoclasts maturation; contrariwise, an underexpression of this molecule results in an excessive increase of bone resorption and osteoporosis [18]. Mutations in the promoter region of *TNFRSF11B* could have influence on the transcription and translation rate of OPG. A study in European population (Eslovenia) showed that two SNPs named rs3134069 and rs3134070 in the *TNFRSF11B* could form a haplotype with susceptibility to osteoporosis [16, 19]. To date, there is a lack of information evaluating the relationship between polymorphisms of OPG gene and bone mineral density.

Therefore, the aim of the present study was to assess if there is an association between SNPs C950T (rs2073617), C2097 (rs3134069), and T245G (rs3134070) in the *TNFRSF11B* (OPG) gene and osteoporosis in rheumatoid arthritis patients.

## 2. Patients and Methods

*2.1. Study Subjects.* We carried out a cross-sectional study that included 81 consecutive unrelated women who were diagnosed with RA from March 2007 to March 2009. These patients were referred from an outpatient rheumatology clinic in a secondary care center in Guadalajara, Mexico (Hospital General Regional 110, IMSS). Inclusion criteria were as follows: age $\geq$ 18 years at entry; self-identified race Mexican Mestizo (defined as individuals who were born in Mexico and were descendants of the original autochthonous inhabitants of the region and of individuals mainly Spaniards); fulfillment of the 1987 American College of Rheumatology (ACR) classification criteria [20]; and no previous BMD measurement. Patients were excluded if pregnancy was present and if they had an overlap syndrome, were receiving bisphosphonates or parathyroid hormone therapy, or had a comorbidity associated with low BMD, such as diabetes mellitus, thyroid disease, or chronic renal failure; patients were not related to each other.

A healthy control group matched by age and sex was obtained from blood donors who attended to "Centro Médico Nacional de Occidente" blood bank; all blood donors were of Mexican Mestizo origin and denied at the time of the study having any disease (acute or chronic).

*2.2. Clinical Assessment.* Each patient was interviewed using a structured questionnaire to record demographic information, general risk factors for osteoporosis (i.e., age, weight, and height), clinical characteristics, and RA treatment. At the time of the evaluation two trained researchers assessed RA disease activity by systematical evaluation of the DAS-28 index [21]; to determine patient disability the Spanish modified version of the Health Assessment Questionnaire-Disability Index (HAQ-DI) [22] was used. Global functional status was evaluated according to the Steinbrocker classification [23]. Rheumatoid factor and C-reactive protein (CRP) were measured by nephelometry using commercially available kits. Erythrocyte sedimentation rate (ESR, mm/h) was measured using the Wintrobe method.

Table 1: Genotyping strategies for *TNFRSF11B* polymorphism variants detection.

| SNP | Primers | Band size (bp) | Restriction enzyme | Recognized sequence | Band size after digestion (bp) |
|---|---|---|---|---|---|
| C950T rs2073617 | 5′-TGCGTCCGGATCTTGGCTGGATCGG-3′<br>5′-ACTTACCACGAGCGCGCAGCACAGCAA-3′ | 548 | *Hinc II* | GTY RAC | **C** 248 and 287<br>**T** 548 |
| C209T rs3134070 | 5′-CGAACCCTAGAGCAAAGTGC-3′<br>5′-TGTCTGATTGGCCCTAAAGC-3′ | 271 | *Taq I* | T CGA | **C** 212, 31 and 28<br>**T** 212 and 59 |
| T245G rs3134069 | 5′-CGAACCCTAGAGCAAAGTGC-3′<br>5′-TGTCTGATTGGCCCTAAAGC-3′ | 271 | *Hinf I* | G ANTC | **A** 195, and 176<br>**C** 127, 76 and 69 |

SNP: single nucleotide polymorphism and bp: base pair.

*2.3. BMD Measurement.* BMD was measured (g/cm$^2$) by DEXA using a Lunar Prodigy densitometer (GE Medical Systems Lunar, Madison, WI, software V 8.8; GE Medical Systems); the anatomical regions assessed were lumbar spine in the posterior-anterior projection (L1-L4) and femoral neck. The coefficient of variation during the measurement of a standard phantom in our laboratory is 0.7%. The coefficient of variation was 2.4% at the lumbar spine and 1.6% at the femoral neck. All scans were performed by the same experienced technician, who was blinded to the characteristics of patients. Each patient was classified into one of the following categories proposed by WHO: normal BMD, defined as having a measurement of BMD within 1 SD of the BMD of normal young adult women (equivalent to $T$ score $> -1$); osteopenia, defined as having a $T$ score in BMD between $-1$ and $-2.4$ SD; and osteoporosis, defined as having a $T$ score in BMD $\leq -2.5$ SD. Patients with a $T$ score $< -1.0$ were considered to have low BMD in consequence; for analysis purposes in our study we decided to include osteopenia and osteoporosis in one single group denominated as "low BMD".

*2.4. Genotyping.* DNA from 81 patients with rheumatoid arthritis was extracted from blood samples using conventional methods [24] and stored frozen at −80°C.

We chose three SNPs that are present in the promoter region (5′UTR) of the *TNFRSF11B* (OPG) gene; such polymorphisms were previously reported in association with osteoporosis in postmenopausal women or related conditions [19, 25–28]. We performed 3 polymerase chain reaction-restriction fragment length polymorphisms (PCR_RFLPs) protocols as previously described [26, 29, 30]. Table 1 shows primer sequence, products obtained after PCR, restriction enzymes used, their recognition site, and end products after restriction.

Each PCR reaction was carried out in 10 $\mu$L final volume containing (final concentrations) 1X buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl, and 4 mM $MgCl_2$); 5 pmol/mL each of the pair primers according to polymorphism (Table 1); 10 mM each of the four deoxyribonucleoside triphosphates; 1 U of taq DNA polymerase (Invitrogen, Carlsbad, CA, USA); and 200 to 300 ng DNA template. The PCR products were visualized by electrophoresis in 10% polyacrylamide gels at 150 V for 90 min, followed by silver staining.

*2.5. Statistical Analysis.* Allelic and genotypic frequencies for each SNP were determined by gene count. Hardy-Weinberg equilibrium was tested, using $\chi^2$ test. Genotypic differences between RA patients with and without osteoporosis were evaluated by Mantel-Haenszel test using the EPI INFO (version 6.04d) statistical program. For allelic differences, we used Fisher's exact test. Odds ratios (OR) and 95% confidence interval (95% CI) were computed to evaluate the risk for osteoporosis conferred by presence of the risk alleles. All analyses used two-sided tails with a $P$ value of ≤0.05 used as significance criterion.

## 3. Results

*3.1. Demographics and Clinical.* We included 81 patients, classified as RA according to ACR classification criteria [20], and 52 healthy subjects. For the RA group, mean age was 50 ± 12 years, 100% of the studied subjects were women, mean duration disease was 12 ± 8 years, and all of them were receiving treatment, some of them with monotherapy and others combined therapy; the number and percentages of patients taking a specific drug are listed in Table 2.

RA patients were evaluated for BMD according to the classification criteria of the WHO for osteoporosis. According to the results of bone densitometry 23 patients (33.3%) had normal BMD, 31 (44.9%) had osteopenia (low bone density), and 15 (21.7%) had osteoporosis. For study purposes we grouped osteopenia with osteoporosis in one group constituted by 46 patients (66.7%) (see Table 2). From these, 23 patients (32.9%) had osteopenia and 13 (18.6%) had osteoporosis in the lumbar spine, whereas 35 patients (50.7%) had osteopenia and 7 (10.1%) had osteoporosis of the femoral neck. Clinical risk factors and factors related to RA severity for low BMD were registered and are listed in Table 2.

*3.2. Molecular Analysis.* We performed at least three PCR-RFLP tests for every subject included in the present study but were able to obtain complete genotyping results in 77 DNA samples from RA patients and 52 healthy subjects. Healthy subjects group was in Hardy-Weinberg equilibrium (data not shown).

In Table 3 genotype and allele frequencies of three SNPs in the OPG gene in healthy subjects and rheumatoid arthritis

Table 2: Clinical characteristics of study patients.

| Characteristic | $n = 81$ |
|---|---|
| Age (years), median (range) | 49.9 (21–79) |
| Menopausal, *n* (%) | 43 (53.1) |
| Weight (kg), median (range) | 65.8 (43–94) |
| BMI, median (range) | 26.87 (18–38) |
| Oral contraceptives use, *n* (%) | 41 (50.6) |
| Current smoker, *n* (%) | 17 (21) |
| Family history of fractures *n* (%) | 6 (7.4) |
| Alcohol consumption 3 or more units/day, *n* (%) | 3 (3.37) |
| Duration of RA (years), median (range) | 11.8 (1.5–35) |
| DAS-28 score, median (range) | 3.8 (0–6.7) |
| Global functional status III-IV, *n* (%) | 7 (7.8) |
| HAQ-DI score, median (range) | 0.7 (0–2.8) |
| Medications | *n* (%) |
| Glucocorticoid | 72 (88.9) |
| Prednisone dosage (mg/day), median (range) | 4.4 (0–10) |
| Methotrexate | 56 (70.9) |
| Sulfasalazine | 39 (49.4) |
| Chloroquine | 27 (34.2) |
| Azathioprine | 25 (31.6) |
| D-Penicillamine | 10 (12.7) |
| Biologic agents | 8 (10.1) |
| BMD (WHO classification) | *n* (%) |
| Normal | 23 (33.3) |
| Osteopenia/osteoporosis | 46 (66.7) |

BMI: body mass index, RA: rheumatoid arthritis, DAS-28: disease activity score, HAQ-DI: health assessment questionnaire-disability index, BMD: bone mineral density, and WHO: World Health Organization.

patients are shown; we found significant difference in the distribution of the C950T (rs2073617) alleles, the C allele being more prevalent in the RA group. Polymorphisms C209T and T245G did not reach statistical significance.

We further analyzed genetic variants in RA patients and divided this group with respect to bone mass index status according to the WHO classification in normal or osteopenia/osteoporosis; there was no significant difference in allele distribution between RA patients with and without osteoporosis; Table 4 shows genotype and allele frequencies in these subgroups.

## 4. Discussion

Osteoporosis represents a major health problem throughout the world and the most serious consequence of osteoporosis is hip fracture, which has a high associated morbidity and mortality [31]. Systemic effects of RA in bone remodeling include loss of axial and appendicular bone mass, associated with an increase in fracture risk (9.6 per 1,000 person-years) [32]. Clinical patterns of bone mass loss include generalized osteoporosis (axial and peripheral), juxta-articular osteoporosis which is adjacent to synovial membrane, and marginal and subchondral erosions which are directly associated with inflamed synovial tissue [33]. A high proportion of patients will suffer from hip and spine fractures causing limitations in physical abilities and social capabilities. Osteoporosis presence in RA patients involves multiple factors, such as disease activity, the use of some prescribed drugs particularly DMARDs and corticosteroids, female sex, older age, and menopause [34].

The development of erosive lesions as well as generalized osteoporosis in RA results from an imbalance between bone mass formation and bone resorption process. In RA the unsteadiness between osteoblastic bone formation (mesenchymal origin) and osteoclastic bone resorption (derived from monocytes/macrophages) promotes bone resorption by osteoclasts and as a consequence loss in bone strength, which may lead to the development of osteoporosis and fractures.

Multiple interactions between bone and the immune system have been described; bone is a major target of chronic inflammation, since it increases bone resorption and results in suppressed local bone formation, causing a wide spectrum of bone involvement in RA [35]. Lymphocyte or macrophage-derived cytokines are among the most potent mediators of inflammation, involved in upregulation of osteoclasts formation, activation, and signaling pathways [36].

Osteoclasts progenitors are recruited under physiological conditions from the haematopoyetic system and can get stimulated by some cytokines and hormones to form precursors that will eventually express cell surface markers of a well-differentiated osteoclast; furthermore, some inflammatory synovial cells, present in RA, particularly mononuclear cells and giant multinucleated cells can be differentiated into osteoclasts [5], under proinflammatory cytokines stimuli, including tumor necrosis factor alpha (TNF-$\alpha$), interleukin 1 (IL-1), and interleukin 6 (IL-6) [6].

Recently Pathak et al. described that sera from RA active patients contribute to bone loss by two mechanisms: (1) directly inhibiting osteoblasts proliferation and differentiation and (2) enhancing osteoblast-mediated osteoclastogenesis via RANKL and IL-6 [37].

Another key component that has influence over the osteoclastic differentiation is RANK, a receptor for TNF, attached to the membrane of osteoclast precursor cells that can bind to RNAKL through interaction with osteoblastic stromal cells [9, 38, 39]. RANK is essential for signal transduction that will lead to ostoclastic differentiation. Osteoprotegerin (OPG) also known as inhibition factor for osteoclastogenesis is a member of the TNFRSF, located in chromosome 8q24 [17] with a length of 29 Kb [13]; it is synthesized originally as a propeptide of 400 aa and is secreted as a soluble protein after the cleavage of 21 aa that corresponds to the transmembrane and cytoplasmic domains [17]. OPG mRNA is expressed in different human tissues (lung, heart, kidney, liver, intestines, stomach, cerebrum, thyroid gland, and bone marrow) apart from the bone, where its primary function appears to be inhibition of osteoclasts maturation and activation. In this study, we evaluated the association of three *TNFRSF11B* promoter

Table 3: Genotype and allele frequencies of polymorphisms in the *TNFRSF11B* (OPG) gene in healthy subjects and rheumatoid arthritis (RA) patients.

| SNP | Genotypes | HS | RA | Allele | HS | RA | *P* | OR (95% CI) |
|---|---|---|---|---|---|---|---|---|
| C950T rs2073617 | CC | 9 (17.6) | 28 (43.1) | C | 47 (46.1) | 82 (63.1) | 0.01 | 0.50 (0.30–0.85) |
| | CT | 29 (56.9) | 26 (40) | T | 55 (53.9) | 48 (36.9) | | |
| | TT | 13 (25.5) | 11 (16.9) | | | | | |
| C209T rs3134070 | CC | 39 (75.0) | 52 (72.2) | C | 89 (85.6) | 122 (84.7) | 1.0 | 1.1 (0.53–2.2) |
| | CT | 11 (21.2) | 18 (25.0) | T | 15 (14.4) | 22 (15.3) | | |
| | TT | 2 (3.8) | 2 (2.8) | | | | | |
| T245G rs3134069 | AA | 41 (78.8) | 66 (85.7) | A | 93 (89.4) | 142 (92.2) | 0.51 | 0.71 (0.30–1.69) |
| | AC | 11 (21.2) | 10 (13) | C | 11 (10.6) | 12 (7.8) | | |
| | CC | 0 (0) | 1 (1.3) | | | | | |

OPG: osteoprotegerin, SNP: single nucleotide polymorphism, RA: rheumatoid arthritis, HS: healthy subjects, OR: odds ratios, and 95% CI: 95% confidence intervals. In order to compute OR (95% CI) the following alleles were used as reference: in correspondence with the allele of risk: rs2073617 T, rs3134070 T, and rs3134069 C.

Table 4: Genotype and allele frequencies of polymorphisms in the *TNFRSF11B* (OPG) gene in rheumatoid arthritis (RA) patients with normal and low bone mineral density (BMD).

| SNP | Genotypes | RA with normal BMD | RA with low BMD | Allele | RA with normal BMD | RA with low BMD | *P* | OR (95% CI) |
|---|---|---|---|---|---|---|---|---|
| C950T rs2073617 | CC | 9 (50.0) | 16 (44.4) | C | 25 (69.4) | 46 (63.9) | 0.67 | 1.3 (0.55–3.03) |
| | CT | 7 (38.9) | 14 (38.9) | T | 11 (30.6) | 26 (36.1) | | |
| | TT | 2 (11.1) | 6 (16.7) | | | | | |
| C209T rs3134070 | CC | 17 (81.0) | 29 (69.0) | C | 37 (88.1) | 70 (83.3) | 0.60 | 1.5 (0.49–4.43) |
| | CT | 3 (14.3) | 12 (28.6) | T | 5 (11.9) | 14 (16.7) | | |
| | TT | 1 (4.8) | 1 (2.4) | | | | | |
| T245G rs3134069 | AA | 20 (90.9) | 39 (88.6) | A | 42 (95.5) | 82 (93.2) | 0.72 | 1.5 (0.29–7.95) |
| | AC | 2 (9.1) | 4 (9.1) | C | 2 (4.5) | 6 (6.8) | | |
| | CC | 0 (0) | 1 (2.3) | | | | | |

OPG: osteoprotegerin, SNP: single nucleotide polymorphism, RA: rheumatoid arthritis, BMD: bone mineral density, OR: odds ratios, and 95% CI: 95% confidence intervals. In order to compute OR (95% CI) the following alleles were used as reference: in correspondence with the allele of risk: rs2073617 T, rs3134070 T, and rs3134069 C.

polymorphisms with presence of osteoporosis in RA. Our study demonstrated that the C allele of the C950T was more frequent in RA compared with HS; thereafter we analyzed the RA group to seek for differences in allele distribution between patients with and without osteoporosis; nevertheless we did not observe a significant difference. The other two polymorphisms did not reach statistical significance.

The observed association in the present study could be caused by direct functional effect of the SNP or to linkage disequilibrium (LD) with another functional variant. According to the HapMap data, there is a certain amount of LD between these SNPs, indicating that although it is possible that different causative variants exist in *TNFRSF11B*, it is also possible that only one of them turns out to have functional consequences. The C950T SNP is located in the promoter region, near the TGF-$\beta$ response area and 129 bp upstream the TATA box [40]. A functional study of *TNFRSF11B* polymorphisms through luciferase reporter assays revealed that gene expression was affected by the C950T polymorphism, since it was significantly higher in the presence of the C allele [27].

Previous association studies that include polymorphisms in the *TNFRSF11B* gene were performed in osteoporotic patients. In 2002 Ohmori et al. showed a significant association between C950T SNP and lower BMD [25]; in contrast with such results in 2004 Brändström et al. showed that C950T was not associated with bone mineral density in elderly Swedish women [41], and later Arko et al. in 2005 tested the three polymorphisms included in the present study and others searching for an association with bone mineral density in postmenopausal women; the linkage disequilibrium was confirmed but no associations were found [26]. For SNP T245G, similar frequencies as obtained in this study have been described previously by Zajíčková et al. in postmenopausal women [42]. Only a few studies have looked for an association between these SNPs and RA; in 2010 Assmann et al. performed a case-control study and found an association between SNPs in the RANK and RANKL gene but

not in the OPG gene; nevertheless none of the SNPs included in our study were genotyped by them [43]. In a recent study the SNP A163G in the osteoprotegerin gene was associated with osteoporosis in RA [44]. This polymorphism was not included in the present study but a lack of association with this particular polymorphism and osteoporosis in RA was previously confirmed by our study group [45].

Because this work constitutes a preliminary report, a limitation in our study is a small sample size that may limit our statistical power to detect small differences between groups although we consider that our sample would be sufficient to identify polymorphisms that may confer a high risk for low BMD in RA and did not show any trend to suspect that increasing the sample size a statistical significance would be achieved. We recognize that future multicenter studies should be performed, increasing the sample size, with our results being useful as a referent for Mexican patients with RA to identify that these polymorphisms probably do not confer a high risk for low BMD in our population with RA. Further studies in other populations are required to confirm our findings.

In conclusion, we confirmed the association of C allele of the C950T SNP in the *TNFRSF11B* gene with RA patients, but we were not able to support our main hypothesis that this polymorphism in the OPG gene is associated with osteoporosis in RA. Further studies including other regions of Mexico and Latin America with an increased sample size are required to confirm our findings and further elucidate if C950T SNP in the OPG gene could be associated with osteoporosis and not only with RA presence.

## Ethical Approval

This research was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all participants, the study protocol was approved by the Local Committees for Ethical and Health Research (CLIEIS 2007-1301-1), and all data was managed anonymously.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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## References

[1] M. Akil and R. S. Amos, "Rheumatoid arthritis, I: clinical features and diagnosis," *British Medical Journal*, vol. 310, no. 6979, pp. 587–590, 1995.

[2] M. H. Cardiel and J. Rojas-Serrano, "Community based study to estimate prevalence, burden of illness and help seeking behavior in rheumatic diseases in Mexico City. A COPCORD study," *Clinical and Experimental Rheumatology*, vol. 20, no. 5, pp. 617–624, 2002.

[3] J. A. Kanis, L. J. Melton III, C. Christiansen, C. C. Johnston, and N. Khaltaev, "The diagnosis of osteoporosis," *Journal of Bone and Mineral Research*, vol. 9, no. 8, pp. 1137–1141, 1994.

[4] F. Alcaraz-Lopez Miriam, G.-L. L. Aguilar-Chavez Erika, A. Lopez-Olivo Maria, C. Loaiza-Cardenas, and I. Gamez-Nava Jorge, "Performance of albrand index for identifying low bone density in rheumatoid arthritis," *The Journal of Rheumatology*, vol. 33, no. 2, p. 408, 2006.

[5] E. M. Gravallese, "Bone destruction in arthritis," *Annals of the Rheumatic Diseases*, vol. 61, supplement 2, pp. ii84–ii86, 2002.

[6] S. Roux and P. Orcel, "Bone loss: factors that regulate osteoclast differentiation: an update," *Arthritis Research*, vol. 2, no. 6, pp. 451–456, 2000.

[7] D. L. Lacey, E. Timms, H.-L. Tan et al., "Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation," *Cell*, vol. 93, no. 2, pp. 165–176, 1998.

[8] N. Udagawa, N. Takahashi, H. Yasuda et al., "Osteoprotegerin produced by osteoblasts is an important regulator in osteoclast development and function," *Endocrinology*, vol. 141, no. 9, pp. 3478–3484, 2000.

[9] H. Hsu, D. L. Lacey, C. R. Dunstan et al., "Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 7, pp. 3540–3545, 1999.

[10] M. C. Bezerra, J. F. Carvalho, A. S. Prokopowitsch, and R. M. R. Pereira, "RANK, RANKL and osteoprotegerin in arthritic bone loss," *Brazilian Journal of Medical and Biological Research*, vol. 38, no. 2, pp. 161–170, 2005.

[11] D. H. Jones, Y.-Y. Kong, and J. M. Penninger, "Role of RANKL and RANK in bone loss and arthritis," *Annals of the Rheumatic Diseases*, vol. 61, supplement 2, pp. ii32–ii39, 2002.

[12] S.-S. Dong, X.-G. Liu, Y. Chen et al., "Association analyses of RANKL/RANK/OPG gene polymorphisms with femoral neck compression strength index variation in caucasians," *Calcified Tissue International*, vol. 85, no. 2, pp. 104–112, 2009.

[13] Y.-H. Hsu, T. Niu, H. A. Terwedow et al., "Variation in genes involved in the RANKL/RANK/OPG bone remodeling pathway are associated with bone mineral density at different skeletal sites in men," *Human Genetics*, vol. 118, no. 5, pp. 568–577, 2006.

[14] C. Liu, T. S. Walter, P. Huang et al., "Structural and functional insights of RANKL-RANK interaction and signaling," *Journal of Immunology*, vol. 184, no. 12, pp. 6910–6919, 2010.

[15] A.-P. Trouvin and V. Goëb, "Receptor activator of nuclear factor-$\kappa$B ligand and osteoprotegerin: maintaining the balance to prevent bone loss," *Clinical Interventions in Aging*, vol. 5, pp. 345–354, 2010.

[16] J. B. Richards, F. K. Kavvoura, F. Rivadeneira et al., "Collaborative meta-analysis: associations of 150 candidate genes with osteoporosis and osteoporotic fracture," *Annals of Internal Medicine*, vol. 151, no. 8, pp. 528–537, 2009.

[17] P. Narducci, R. Bareggi, and V. Nicolin, "Receptor Activator for Nuclear Factor kappa B Ligand (RANKL) as an osteoimmune key regulator in bone physiology and pathology," *Acta Histochemica*, vol. 113, no. 2, pp. 73–81, 2011.

[18] W. J. Boyle, W. S. Simonet, and D. L. Lacey, "Osteoclast differentiation and activation," *Nature*, vol. 423, no. 6937, pp. 337–342, 2003.

[19] B. Arko, J. Preelj, R. Komel, A. Kocijani, P. Hudler, and J. Marc, "Sequence variations in the osteoprotegerin gene promoter in patients with postmenopausal osteoporosis," *Journal of Clinical*

*Endocrinology and Metabolism*, vol. 87, no. 9, pp. 4080–4084, 2002.

[20] F. C. Arnett, S. M. Edworthy, D. A. Bloch et al., "The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 31, no. 3, pp. 315–324, 1988.

[21] M. L. L. Prevoo, M. A. van'T Hof, H. H. Kuper, M. A. van Leeuwen, L. B. A. van de Putte, and P. L. C. M. van Riel, "Modified disease activity scores that include twenty-eight-joint counts development and validation in a prospective longitudinal study of patients with rheumatoid arthritis," *Arthritis & Rheumatism*, vol. 38, no. 1, pp. 44–48, 1995.

[22] M. H. Cardiel, M. Abello-Banfi, R. Ruiz-Mercado, and D. Alarcon-Segovia, "How to measure health status in rheumatoid arthritis in non-English speaking patients: validation of a Spanish version of the Health Assessment Questionnaire Disability Index (Spanish HAQ-DI)," *Clinical and Experimental Rheumatology*, vol. 11, no. 2, pp. 117–121, 1993.

[23] M. C. Hochberg, R. W. Chang, I. Dwosh, S. Lindsey, T. Pincus, and F. Wolfe, "The American College of Rheumatology 1991 revised criteria for the classification of global functional status in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 35, no. 5, pp. 498–502, 1992.

[24] S. Gustincich, G. Manfioletti, G. del Sal, C. Schneider, and P. Carninci, "A fast method for high-quality genomic DNA extraction from whole human blood," *BioTechniques*, vol. 11, no. 3, pp. 298–302, 1991.

[25] H. Ohmori, Y. Makita, M. Funamizu et al., "Linkage and association analyses of the osteoprotegerin gene locus with human osteoporosis," *Journal of Human Genetics*, vol. 47, no. 8, pp. 400–406, 2002.

[26] B. Arko, J. Preželj, A. Kocijančič, R. Komel, and J. Marc, "Association of the osteoprotegerin gene polymorphisms with bone mineral density in postmenopausal women," *Maturitas*, vol. 51, no. 3, pp. 270–279, 2005.

[27] C. Vidal, R. Formosa, and A. Xuereb-Anastasi, "Functional polymorphisms within the TNFRSF11B (osteoprotegerin) gene increase the risk for low bone mineral density," *Journal of Molecular Endocrinology*, vol. 47, no. 3, pp. 327–333, 2011.

[28] G. Beyens, A. Daroszewska, F. de Freitas et al., "Identification of sex-specific associations between polymorphisms of the Osteoprotegerin gene, TNFRSF11B, and Paget's disease of bone," *Journal of Bone and Mineral Research*, vol. 22, no. 7, pp. 1062–1071, 2007.

[29] M. Soufi, M. Schoppet, A. M. Sattler et al., "Osteoprotegerin gene polymorphisms in men with coronary artery disease," *Journal of Clinical Endocrinology and Metabolism*, vol. 89, no. 8, pp. 3764–3768, 2004.

[30] D. Pitocco, G. Zelano, G. Gioffrè et al., "Association between osteoprotegerin G1181C and T245G polymorphisms and diabetic charcot neuroarthropathy: a case-control study," *Diabetes Care*, vol. 32, no. 9, pp. 1694–1697, 2009.

[31] C. Cooper, G. Campion, and L. J. Melton III, "Hip fractures in the elderly: a world-wide projection," *Osteoporosis International*, vol. 2, no. 6, pp. 285–289, 1992.

[32] S. Y. Kim, S. Schneeweiss, J. Liu et al., "Risk of osteoporotic fracture in a large population-based cohort of patients with rheumatoid arthritis," *Arthritis Research and Therapy*, vol. 12, no. 4, article R154, 2010.

[33] S. R. Goldring, "The final pathogenetic steps in focal bone erosions in rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 59, no. 1, pp. i72–i74, 2000.

[34] R. M. R. Pereira, J. F. de Carvalho, and E. Canalis, "Glucocorticoid-induced osteoporosis in rheumatic diseases," *Clinics*, vol. 65, no. 11, pp. 1197–1205, 2010.

[35] P. Geusens and W. F. Lems, "Osteoimmunology and osteoporosis," *Arthritis Research and Therapy*, vol. 13, no. 5, article 242, 2011.

[36] G. Mori, P. D'Amelio, R. Faccio, and G. Brunetti, "The interplay between the bone and the immune system," *Clinical and Developmental Immunology*, vol. 2013, Article ID 720504, 16 pages, 2013.

[37] J. L. Pathak, N. Bravenboer, P. Verschueren et al., "Inflammatory factors in the circulation of patients with active rheumatoid arthritis stimulate osteoclastogenesis via endogenous cytokine production by osteoblasts," *Osteoporosis International*, vol. 25, no. 10, pp. 2453–2463, 2014.

[38] J. Li, I. Sarosi, X.-Q. Yan et al., "RANK is the intrinsic hematopoietic cell surface receptor that controls osteoclastogenesis and regulation of bone mass and calcium metabolism," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 4, pp. 1566–1571, 2000.

[39] T. L. Burgess, Y.-X. Qian, S. Kaufman et al., "The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts," *Journal of Cell Biology*, vol. 145, no. 3, pp. 527–538, 1999.

[40] K. Thirunavukkarasu, R. R. Miles, D. L. Halladay et al., "Stimulation of osteoprotegerin (OPG) gene expression by transforming growth factor-$\beta$ (TGF-$\beta$). Mapping of the OPG promoter region that mediates TGF-$\beta$ effects," *The Journal of Biological Chemistry*, vol. 276, no. 39, pp. 36241–36250, 2001.

[41] H. Brändström, P. Gerdhem, F. Stiger et al., "Single nucleotide polymorphisms in the human gene for osteoprotegerin are not related to bone mineral density or fracture in elderly women," *Calcified Tissue International*, vol. 74, no. 1, pp. 18–24, 2004.

[42] K. Zajíčková, A. Zemanová, M. Hill, and I. Žofková, "Is A163G polymorphism in the osteoprotegerin gene associated with heel velocity of sound in postmenopausal women?" *Physiological Research*, vol. 57, no. 1, pp. S153–S157, 2008.

[43] G. Assmann, J. Koenig, M. Pfreundschuh et al., "Genetic variations in genes encoding RANK, RANKL, and OPG in rheumatoid arthritis: a case-control study," *Journal of Rheumatology*, vol. 37, no. 5, pp. 900–904, 2010.

[44] Y. M. Hussien, A. Shehata, R. A. Karam, S. S. Alzahrani, H. Magdy, and A. M. El-Shafey, "Polymorphism in vitamin D receptor and osteoprotegerin genes in Egyptian rheumatoid arthritis patients with and without osteoporosis," *Molecular Biology Reports*, vol. 40, no. 5, pp. 3675–3680, 2013.

[45] A. J. L. Brambila-Tapia, J. Durán-González, L. Sandoval-Ramírez et al., "MTHFR C677T, MTHFR A1298C, and OPG A163G polymorphisms in Mexican patients with rheumatoid arthritis and osteoporosis," *Disease Markers*, vol. 32, no. 2, pp. 109–114, 2012.

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*Review Article*

# The Impact of Immune System in Regulating Bone Metastasis Formation by Osteotropic Tumors

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Bone metastases are frequent and debilitating consequence for many tumors, such as breast, lung, prostate, and kidney cancer. Many studies report the importance of the immune system in the pathogenesis of bone metastasis. Indeed, bone and immune system are strictly linked to each other because bone regulates the hematopoietic stem cells from which all cells of the immune system derive, and many immunoregulatory cytokines influence the fate of bone cells. Furthermore, both cytokines and factors produced by immune and bone cells promote the growth of tumor cells in bone, contributing to supporting the vicious cycle of bone metastasis. This review summarizes the current knowledge on the interactions among bone, immune, and tumor cells aiming to provide an overview of the osteoimmunology field in bone metastasis from solid tumors.

## 1. Introduction

The skeleton is the most common site of metastasis and bone in turn is the main responsible of death since the presence of bone metastases makes the primary disease no longer curable [1]. Symptoms like bone pain, hypercalcemia, fracture, and spinal cord compression appear in this type of metastasis, causing a decline in the quality of life [2]. Some types of tumors are characterized by a selective bone tropism, out of which are prostate, breast, lung, and kidney cancers. Bone metastases can give osteolytic, osteosclerotic, or mixed lesions. Osteolytic metastases are due to an enhanced activity of bone-resorbing cells, the osteoclasts (OCs), which cause bone destruction [3, 4]. Typically, breast, lung, and kidney cancers metastasize to bone with osteolytic lesions, whereas prostate cancer metastasizes with osteosclerotic ones. Lung and kidney tumors metastasize in an early phase of the disease, while breast cancer metastasizes with a slower and less aggressive growth. At least 65–75% of breast and prostate cancer patients develop bone metastases during the course of their disease, and breast cancer patients show a relatively long median survival time after diagnosis of bone metastases [5–7]. Bone metastases from prostate cancer are typically osteosclerotic and are caused by an increased activity of bone-forming cells, the osteoblasts (OBs), leading to enhanced bone formation [3, 4].

Approximately 30–40% of NSCLC patients develop bone metastasis during their disease, with a median survival time measured in months [1]. About 20–35% of kidney cancer patients develop bone metastases, which are particularly destructive, with a rate of skeletal complications higher than other tumors [8, 9]. Accumulating evidences suggest the importance of the immune cell response to factors in the tumor microenvironment as main regulator of cancer progression and metastases. The bone marrow is a reservoir for immune cells such as macrophages, dendritic cells (DCs), myeloid derived suppressor cells (MDSCs), and different T cell subsets that can directly impair the so called "tumor/bone vicious cycle" [10]. This review focuses on the current knowledge of the role of the immune cells in controlling tumor spreading to bone.

## 2. Bone Marrow Is an Attractive Soil for Cancer Cells

Bone marrow (BM) microenvironment is a fertile soil for homing, survival, and proliferation of circulating cancer cells. It provides both endosteal and vascular niches, which support

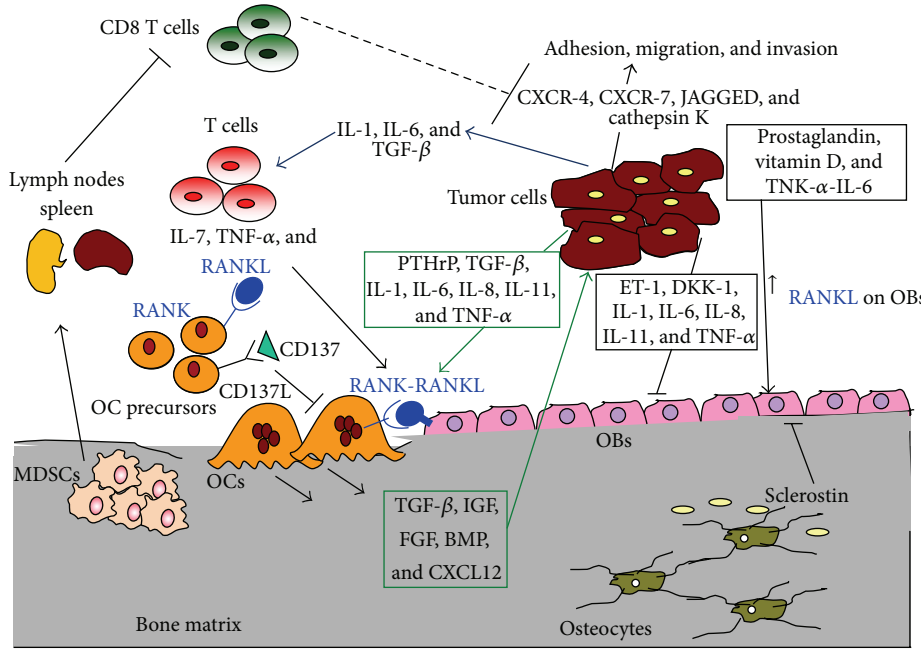


Figure 1: Interactions among bone, immune, and tumor cells sustain the vicious cycle of bone metastasis. Tumor cells release cytokines that activate T cells to produce proosteoclastogenic factors, such as RANKL, which activate OCs. In turn, the release of bone matrix growth factors during bone resorption enhances the tumor growth. MDSCs originate from BM and migrate to secondary lymphoid organs where they inhibit the antitumor immune response mediated by CD8 T cells. Consequently, the increased tumor growth induces the production of osteolytic factors which activates the OCs, the cells responsible for bone destruction.

hematopoietic and nonhematopoietic stem cells such as mesenchymal stem cells [11]. OCs degrade endosteal components and promote mobilization of hematopoietic progenitor cells [12], whereas OBs on the endosteal surface of bone are critical supporting cells for hematopoietic stem cells (HSCs) in BM [13, 14]. Indeed, stimulation of the PTH (parathyroid hormone) receptor on OBs increased the number of HSCs in BM [13] and also the size of HSC niche, which promotes skeletal localization of prostate cancer cells [15]. Shiozawa et al. demonstrated that, after injection in a mouse model of bone metastasis, human prostate cancer cells occupy mouse HSC niche, displacing HSCs. Thus, the HSC niche is a direct target of prostate cancer cells during dissemination and plays a pivotal role in bone metastases [16]. BM tissue is constituted by red and yellow marrows. Red marrow contains HSCs and yellow marrow mainly consists of fat cells [17]. Red marrow is particularly vascularised; thus, it is a common site of metastasis. Recently, an important role of yellow marrow in the pathogenesis of bone metastasis has also been recognized because bone marrow adipocytes promote the growth of metastatic tumor cells in bone [18].

In physiological conditions, bone undergoes a constant remodelling through OC-mediated bone resorption and OB-mediated bone regeneration in a coupled manner to maintain homeostasis. However, during tumor growth in the bone, dysregulation of this process leads to osteolytic or osteosclerotic phenotypes [19]. Indeed, cancer cells express adhesion molecules which bind their ligand on BM stromal cells, releasing angiogenic and bone-resorbing factors, which disrupt the normal homeostasis of BM microenvironment causing bone metastasis (Figure 1) [20, 21]. For instance, vascular endothelial molecule-1 (VCAM-1) is expressed in breast cancer and binds $\alpha 4\beta 7$ and $\alpha 4\beta 1$ (VLA-4) integrins on OC precursors with high affinity, causing osteoclastogenesis. $\alpha 4$ or VCAM-1 blocking antibodies effectively inhibit bone metastasis [22]. $\alpha v\beta 3$ is another integrin expressed by breast cancer cells [23], which is particularly important for OC adhesion to bone [23]. CD44 is a molecule highly expressed by breast cancer cells, which promotes invasion and adhesion to BM [24]. Moreover, CD44 is also expressed by breast cancer stem cells which can lie in a dormant state in the BM [25] and then directly induce bone metastasis [26].

CXCL12, expressed by OBs and endothelial cells in BM, and its receptors CXCR-4 regulate cell migration and bone metastasis from prostate cancer [27, 28]. When cancer cells reach the bone microenvironment, they induce the release of different factors enmeshed in the bone matrix, such as bone morphogenetic proteins (BMPs), transforming growth factor-$\beta$ (TGF-$\beta$), insulin-like growth factor (IGF), and fibroblast growth factor (FGF) as well as others that stimulate the growth of metastatic tumor cells (Figure 1) [29]. The last, in turn, secretes prostaglandins, PTH, PTH-related peptide, activated vitamin D, interleukin-6 (IL-6), and tumor necrosis factor (TNF), leading to an increase in receptor activator of nuclear factor NF-kB ligand (RANKL) expression on OBs and BM stromal cells [4], which stimulates the OC number and survival and activity (Figure 1). Interestingly, prostate and breast cancer cells respond to these factors activating different OB transcription factors [30, 31]; thus cancer cells can differentiate into an osteoblastic bone-forming phenotype.

This phenomenon is called osteomimicry and it has been observed in bone metastatic prostate and breast cancer cell lines [32–34].

Progenitors and mature cells in the BM frequently expressed the receptor Notch [35], whereas the Notch ligand Jagged is overexpressed by bone metastatic tumor cells [36]. Thus, cancer cells directly activate osteolysis through the Notch-Jagged interactions in the BM. In particular, Jagged1, which is a downstream mediator of the prometastatic TGF-$\beta$, directly activates OC differentiation and promotes tumor growth stimulating IL-6 production by OBs [37].

## 3. Bone and Immune System Cross Talk

Bone and immune and hematopoietic systems are tightly linked since bone cells and hematopoietic cells are in deep physical contact, are reciprocally regulated, are interconnected in their function, and share several common pathways [38]. Indeed, bone cells express surface molecules regulating the expansion of HSCs from which all cells of the mammalian immune system derive, whereas many immunoregulatory cytokines directly act on bone cells [39, 40]. OBs and OCs both affect the maintenance and the mobilization of HSCs [13, 14]. OBs control the proliferation of hematopoietic progenitors [41] and support commitment and differentiation of all stages of B cell development. Indeed *in vitro* production of B cell precursors from progenitors required contact with OBs and expression of CXCL12 and interleukin-7 (IL-7), which was induced by PTH [13, 42]. Moreover, OC precursors, T, B, and NK cells originate from the same stem cell; thus, some of the receptors and ligands that mediate the immune process also regulate the maturation of OC precursors and the ability of OCs to degrade bone. Circulating OC precursors are a reservoir of the pre-OC pool in the BM, but they are also an abundant source of pre-OCs that can be recruited into bone or joint tissue in response to reparative or pathological signals.

RANKL, its receptor RANK, and the natural decoy receptor osteoprotegerin (OPG) [43] form a crucial molecular link between the immune system and bone [44]. The membrane RANKL is expressed by OBs/stromal cells; the soluble RANKL was originally cloned from T cells [45, 46], but it is also expressed by B cells [47], whereas the receptor RANK is expressed by DCs, monocytes, macrophages, and tumor cells [44, 48]. Activated T cells produce RANKL, which directly regulates osteoclastogenesis and bone remodelling, explaining why different pathological conditions, such as cancer, result in systemic and local bone loss. The RANKL to OPG ratio in serum is a determinant factor for OC activation at bone level: a higher serum RANKL to OPG ratio is an index for upregulation of osteoclastogenesis [49].

Many immune factors, including costimulatory receptors and cytokines such as interferon-$\gamma$ (IFN-$\gamma$) and TNF regulate bone cell development, bone turnover, and pathogenesis of bone diseases [50]. The role of IFN-$\gamma$ in osteoclastogenesis is controversial; indeed, in studies conducted *in vitro* [51] and *in vivo*, in animal model, it shows an antiosteoclastogenic effect [52], whereas, in humans, IFN-$\gamma$ increases in presence of bone loss due to oestrogen deficiency and rheumatoid arthritis [53, 54]. IFN-$\gamma$ influences osteoclastogenesis directly by blocking OC formation through inhibition of OC maturation [55] and indirectly by stimulating T cell activation with a consequent increase of proosteoclastogenic factors [56].

*3.1. The Interactions between T Cells and Osteoclast Precursors Regulate Bone Resorption in Bone Metastasis.* A direct regulation of bone resorption by T cell has been widely described for bone metastasis by both solid tumors and multiple myeloma (MM) [57, 58]. Indeed, studies on peripheral blood mononuclear cells (PBMCs), isolated from patients affected by breast, prostate, and lung cancer with bone metastases, demonstrated an increase of circulating OC precursors in these patients compared to patients without bone metastases and healthy controls [58, 59]. In bone metastatic patients, OC precursors differentiate into mature OCs *in vitro* in presence of T cells without adding M-CSF and RANKL, but T cell depletion results in the absence of OC formation without exogenous stimulation [58].

Another important mediator of the interactions between T and bone cells is IL-7, a cytokine produced by stromal cells and by cells at inflammatory site, with different effects on hematopoietic and immunologic systems [60]. The main function of IL-7 is the control of B and T lymphopoiesis [61], but it is also important for the tumor process [62] and the correct bone homeostasis [63, 64]. According to the model considered, IL-7 displays either inhibitory or activator effects on OCs [63, 65]. Some studies demonstrated that IL-7, produced by T cells, promotes osteoclastogenesis by upregulating T cell-derived cytokines, such as RANKL and TNF$\alpha$ [66–68], and that its production is increased by oestrogen deficiency [69]. Furthermore, in bone metastatic patients, IL-7 serum levels were significantly higher than those in nonbone metastatic patients and in healthy controls [59, 68, 70]. This increase of serum IL-7 is at least in part dependent on IL-7 production by tumor cells as demonstrated in a human-in-mice model of bone metastasis from lung cancer [71]. All these data confirm the T cell modulatory activity on OCs. Nevertheless, also OCs affect T cell activity, because they present antigenic peptides to T cells and induce FoxP3 expression in CD8 T cells, which regulate an inappropriate activation of the immune response [72]. The cellular responses in cell-to-cell interactions between T cells and OCs are regulated through reciprocal CD137/CD137L and RANK/RANKL interactions [73]. CD137 is a costimulatory member of the TNF receptor induced by T cell receptor activation. Its ligand CD137L is expressed on OC precursors: *in vitro* CD137L ligation suppresses osteoclastogenesis through the inhibition of OCs precursor fusion. On the other hand, RANKL expressed on T cells binds to RANK on OCs, producing a reverse signal in T cells able to enhance apoptosis [73].

*3.2. T Cells Regulate Tumor Growth in Bone.* Many data suggest that T cells can regulate tumor growth in bone also independently from their interaction with bone cells. Indeed, memory T cells have been found in the BM of breast cancer patients suggesting their role in cancer immune surveillance

[74]. Moreover, the RANKL-RANK interaction between CD4 T cells and breast cancer cells promotes invasion, dissemination, and metastasis formation in an animal model [75]. Some antibone metastatic therapies show immunomodulatory effects; for instance, the blockade of TGF-$\beta$ at metastatic sites may locally activate an antitumor T cell response, because, normally, TGF-$\beta$, released in BM by OC activity, inhibits T cell proliferation [76].

Zoledronic acid, an antiresorptive agent, can activate cytotoxic $\gamma/\delta$-T cells and inhibit populations of myeloid derived cells with T cell suppressor capabilities [77]. Modulation of antitumor T cell responses alters tumor growth in bone. Indeed, by using mice models $Lyn^{-/-}$, which have more OCs and a hyperactive myeloid population with an increased T cell responses, Zhang et al. reported a reduced tumor growth in bone despite enhanced osteolysis [78]. Lyn is a member of the Src family tyrosine kinases, which inhibits OC differentiation by downregulating PLC$\gamma$2 activation, which regulates the OC formation and function [79]. PLC$\gamma 2^{-/-}$ mice have an increased bone tumor burden despite protection from bone loss, because they have dysfunctional OCs and impaired T cell activation mediated by DCs. Importantly, injection of antigen-specific wild-type cytotoxic CD8 T cells in both these mice models reduces the growth of tumor cells in the bone, regardless of OC functionality. According to these data, a condition of immune deficiency can interfere with the antitumor effects of OC blockade [78]. In particular, cytotoxic CD8 T cells seem to be critical regulators of tumor growth in bone, since their activation diminishes and their depletion enhances bone metastases, even with zoledronic acid.

*3.3. Myeloid Derived Suppressor Cells Regulate Cancer Progression.* Myeloid derived suppressor cells are a heterogeneous population of immature myeloid cells identified by the coexpression of Gr-1 and CD11b in mice and CD11b and CD33 in humans [80–83]. MDSCs are significantly overproduced in tumor-bearing mice and cancer patients and they represent a prognostic indicator in various osteotropic tumors including breast, lung and MM [84, 85]. Emerging evidences suggest the importance of the MDSCs in driving the progression of cancer disease by suppressing both the innate and the adaptive immune response. Thus, MDSCs exert their proneoplastic effects through the impairment of T cell/antigen recognition, the release of small soluble oxidizers, and depletion of essential amino acids from the local extracellular environment [86–88]. Besides suppressing CD4 and CD8 T cell populations, MDSCs promote the activation and expansion of regulatory T cells (Treg) and thus mediate immunosuppression.

Finally, all these mechanisms contribute to tumor progression and metastasis spreading to many organs, especially to bone (Figure 1).

Bone metastases are associated with an increase in OC activation and since MDSCs are progenitors of the OC precursors, it is not surprising that they are found to be largely increased in bone metastatic patients. Strikingly, Sawant et al. confirmed that MDSCs isolated from tumor-bone microenvironment can differentiate into mature and functional OCs *in vitro* and *in vivo* in a mouse model of breast cancer bone metastases [89]. MDSCs from mice bearing bone metastases also induce osteolysis in syngenic animals, indicating that these cells are primed as OC progenitors and the bone microenvironment triggers their activation in functional OCs. It has been also suggested that cancer cells release different soluble factors in the bone, which promotes MDSCs to differentiate into OCs. Thus, breast cancer cells can secrete CCL2, CCL5, or osteopontin which promotes the expression of cathepsin K and matrix metalloproteinase 9 (MMP9), thus enhancing OC functions [90]. On the other hand, MDSC expresses several proosteoclastogenic factors as CCR2, the receptor of CCL2, showing the responsiveness of these cells to the chemokine. Similarly, in the MM model, Zhuang et al. discovered that tumor induced MDSCs were responsible to induce osteolytic lesions by acting as OC precursors [91]. Additionally, only MDSCs isolated from bone are capable of becoming active OCs, suggesting the importance of the bone microenvironment in driving OC maturation.

Despite the critical role for OCs in the establishment of bone metastatic vicious cycle, the PLC$\gamma 2^{-/-}$ mouse model, bearing severe OC defects, suggests that MDSCs can enhance tumor growth in bone independently of their ability to differentiate into OCs [78]. Interestingly, the increased PLC$\gamma 2^{-/-}$ tumor growth was the result of a higher MDSCs accumulation in secondary lymphoid organs, leading to a strong inhibition of the antitumor T cell response (Figure 1). Despite the importance of MDSCs expansion as a crucial event in the pathogenesis of tumor progression, little is known about the mechanisms leading to this process. Capietto et al. have recently shown $\beta$-catenin as a crucial modulator of MDSC accumulation in response to tumor [92]. The downregulation of $\beta$-catenin signaling in MDSC promotes their expansion and consequently increases tumor growth in both mice and humans. On the contrary, expression of a constitutively activate form of $\beta$-catenin in mice decreased the number of MDSCs and tumor growth. Importantly, the downregulation of $\beta$-catenin can also occur in MDSCs from WT mice during tumor dissemination to bone, indicating that $\beta$-catenin pathway modulates MDSC expansion in both primary and metastatic solid tumors.

## 4. Conclusions

The rapidly developing field of osteoimmunology shows the importance of the deep interconnection between skeletal and immune system. This relationship results in the generation of several cellular pathways, which provides the discovery of new potential targets for the prevention and treatment of bone metastasis.

The bone marrow represents an active and hospitable microenvironment, allowing multiple cell interactions which are critical in the pathogenesis of tumor progression. Thus, additional studies to elucidate new mechanisms promoting the accumulation of bone marrow derived cells such as MDSCs are mandatory to address the critical steps of tumor

progression in bone. The design of new drugs must consider the potential effects on both immune system and bone; thus further investigations to understand the osteoimmune system are even more important.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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## References

[1] R. E. Coleman, "Clinical features of metastatic bone disease and risk of skeletal morbidity," *Clinical Cancer Research*, vol. 12, part 2, pp. 6243s–6249s, 2006.

[2] N. Sethi and Y. Kang, "Dysregulation of developmental pathways in bone metastasis," *Bone*, vol. 48, no. 1, pp. 16–22, 2011.

[3] G. R. Mundy, "Metastasis to bone: causes, consequences and therapeutic opportunities," *Nature Reviews Cancer*, vol. 2, no. 8, pp. 584–593, 2002.

[4] G. D. Roodman, "Mechanisms of bone metastasis," *The New England Journal of Medicine*, vol. 350, no. 16, pp. 1655–1664, 2004.

[5] R. E. Coleman, "Metastatic bone disease: clinical features, pathophysiology and treatment strategies," *Cancer Treatment Reviews*, vol. 27, no. 3, pp. 165–176, 2001.

[6] A. Lipton, "Bisphosphonates and breast carcinoma: present and future," *Cancer*, vol. 88, no. 12, pp. 3033–3037, 2000.

[7] R. E. Coleman, "Bisphosphonates: clinical experience," *Oncologist*, vol. 9, supplement 4, pp. 14–27, 2004.

[8] S. L. Wood and J. E. Brown, "Skeletal metastasis in renal cell carcinoma: current and future management options," *Cancer Treatment Reviews*, vol. 38, no. 4, pp. 284–291, 2012.

[9] M. R. Smith, "Zoledronic acid to prevent skeletal complications in cancer: corroborating the evidence," *Cancer Treatment Reviews*, vol. 31, supplement 3, pp. 19–25, 2005.

[10] L. M. Cook, G. Shay, A. Aruajo, and C. C. Lynch, "Integrating new discoveries into the "vicious cycle" paradigm of prostate to bone metastases," *Cancer and Metastasis Reviews*, vol. 33, no. 2-3, pp. 511–525, 2014.

[11] S. Azizidoost, S. Babashah, F. Rahim, M. Shahjahani, and N. Saki, "Bone marrow neoplastic niche in leukemia," *Hematology*, vol. 19, no. 4, pp. 232–238, 2014.

[12] O. Kollet, A. Dar, S. Shivtiel et al., "Osteoclasts degrade endosteal components and promote mobilization of hematopoietic progenitor cells," *Nature Medicine*, vol. 12, no. 6, pp. 657–664, 2006.

[13] L. M. Calvi, G. B. Adams, K. W. Weibrecht et al., "Osteoblastic cells regulate the haematopoietic stem cell niche," *Nature*, vol. 425, no. 6960, pp. 841–846, 2003.

[14] J. Zhang, C. Niu, L. Ye et al., "Identification of the haematopoietic stem cell niche and control of the niche size," *Nature*, vol. 425, no. 6960, pp. 836–841, 2003.

[15] S. I. Park, C. Lee, W. David Sadler et al., "Parathyroid hormone-related protein drives a $CD11b^{+}Gr1^{+}$ cell-mediated positive feedback loop to support prostate cancer growth," *Cancer Research*, vol. 73, no. 22, pp. 6574–6583, 2013.

[16] Y. Shiozawa, E. A. Pedersen, A. M. Havens et al., "Human prostate cancer metastases target the hematopoietic stem cell niche to establish footholds in mouse bone marrow," *The Journal of Clinical Investigation*, vol. 121, no. 4, pp. 1298–1312, 2011.

[17] J. E. Compston, "Bone marrow and bone: a functional unit," *Journal of Endocrinology*, vol. 173, no. 3, pp. 387–394, 2002.

[18] M. K. Herroon, E. Rajagurubandara, A. L. Hardaway et al., "Bone marrow adipocytes promote tumor growth in bone via FABP4-dependent mechanisms," *Oncotarget*, vol. 4, no. 11, pp. 2108–2123, 2013.

[19] B. F. Boyce, E. Rosenberg, A. E. de Papp, and L. T. Duong, "The osteoclast, bone remodelling and treatment of metabolic bone disease," *European Journal of Clinical Investigation*, vol. 42, no. 12, pp. 1332–1341, 2012.

[20] L. J. Suva, C. Washam, R. W. Nicholas, and R. J. Griffin, "Bone metastasis: mechanisms and therapeutic opportunities," *Nature Reviews Endocrinology*, vol. 7, no. 4, pp. 208–218, 2011.

[21] A. C. Chiang and J. Massagué, "Molecular basis of metastasis," *The New England Journal of Medicine*, vol. 359, no. 26, pp. 2814–2823, 2008.

[22] X. Lu, E. Mu, Y. Wei et al., "VCAM-1 promotes osteolytic expansion of indolent bone micrometastasis of breast cancer by engaging alpha4beta1-positive osteoclast progenitors," *Cancer Cell*, vol. 20, no. 6, pp. 701–714, 2011.

[23] I. Pécheur, O. Peyruchaud, C.-M. Serre et al., "Integrin alpha(v)beta3 expression confers on tumor cells a greater propensity to metastasize to bone," *The FASEB Journal*, vol. 16, no. 10, pp. 1266–1268, 2002.

[24] J. M. Reuben, B.-N. Lee, H. Gao et al., "Primary breast cancer patients with high risk clinicopathologic features have high percentages of bone marrow epithelial cells with ALDH activity and $CD44^{+}CD24^{lo}$ cancer stem cell phenotype," *European Journal of Cancer*, vol. 47, no. 10, pp. 1527–1536, 2011.

[25] S. A. Patel, S. H. Ramkissoon, M. Bryan et al., "Delineation of breast cancer cell hierarchy identifies the subset responsible for dormancy," *Scientific Reports*, vol. 2, article 906, 2012.

[26] L. D'Amico, S. Patanè, C. Grange et al., "Primary breast cancer stem-like cells metastasise to bone, switch phenotype and acquire a bone tropism signature," *British Journal of Cancer*, vol. 108, no. 12, pp. 2525–2536, 2013.

[27] M. K. Conley-LaComb, A. Saliganan, P. Kandagatla, Y. Q. Chen, M. L. Cher, and S. R. Chinni, "PTEN loss mediated Akt activation promotes prostate tumor growth and metastasis via CXCL12/CXCR4 signaling," *Molecular Cancer*, vol. 12, no. 1, article 85, 2013.

[28] R. S. Taichman, C. Cooper, E. T. Keller, K. J. Pienta, N. S. Taichman, and L. K. McCauley, "Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone," *Cancer Research*, vol. 62, no. 6, pp. 1832–1837, 2002.

[29] P. V. Hauschka, A. E. Mavrakos, M. D. Iafrati, S. E. Doleman, and M. Klagsbrun, "Growth factors in bone matrix. Isolation of multiple types by affinity chromatography on heparin-sepharose," *The Journal of Biological Chemistry*, vol. 261, no. 27, pp. 12665–12674, 1986.

[30] A. Bellahcène, M.-P. Merville, and V. Castronovo, "Expression of bone sialoprotein, a bone matrix protein, in human breast cancer," *Cancer Research*, vol. 54, no. 11, pp. 2823–2826, 1994.

[31] A. Bellahcene and V. Castronovo, "Increased expression of osteonectin and osteopontin, two bone matrix proteins, in human breast cancer," *The American Journal of Pathology*, vol. 146, no. 1, pp. 95–100, 1995.

[32] K. S. Koeneman, F. Yeung, and L. W. Chung, "Osteomimetic properties of prostate cancer cells: a hypothesis supporting the predilection of prostate cancer metastasis and growth in the bone environment," *Prostate*, vol. 39, no. 4, pp. 246–261, 1999.

[33] D.-L. Lin, C. P. Tarnowski, J. Zhang et al., "Bone metastatic LNCaP-derivative C4-2B prostate cancer cell line mineralizes in vitro," *The Prostate*, vol. 47, no. 3, pp. 212–221, 2001.

[34] R. F. Cox, A. Jenkinson, K. Pohl, F. J. O'Brien, and M. P. Morgan, "Osteomimicry of mammary adenocarcinoma cells *in vitro*; increased expression of bone matrix proteins and proliferation within a 3D collagen environment," *PLoS ONE*, vol. 7, no. 7, Article ID e41679, 2012.

[35] S. Chiba, "Notch signaling in stem cell systems," *Stem Cells*, vol. 24, no. 11, pp. 2437–2447, 2006.

[36] S. Santagata, F. Demichelis, A. Riva et al., "JAGGED1 expression is associated with prostate cancer metastasis and recurrence," *Cancer Research*, vol. 64, no. 19, pp. 6854–6857, 2004.

[37] N. Sethi, X. Dai, C. G. Winter, and Y. Kang, "Tumor-derived JAGGED1 promotes osteolytic bone metastasis of breast cancer by engaging notch signaling in bone cells," *Cancer Cell*, vol. 19, no. 2, pp. 192–205, 2011.

[38] H. L. Aguila and D. W. Rowe, "Skeletal development, bone remodeling, and hematopoiesis," *Immunological Reviews*, vol. 208, pp. 7–18, 2005.

[39] H. Takayanagi, "New developments in osteoimmunology," *Nature Reviews Rheumatology*, vol. 8, no. 11, pp. 684–689, 2012.

[40] S. Kasagi and W. Chen, "TGF-beta1 on osteoimmunology and the bone component cells," *Cell and Bioscience*, vol. 3, article 4, 2013.

[41] F. Arai, A. Hirao, M. Ohmura et al., "Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche," *Cell*, vol. 118, no. 2, pp. 149–161, 2004.

[42] J. Zhu, R. Garrett, Y. Jung et al., "Osteoblasts support B-lymphocyte commitment and differentiation from hematopoietic stem cells," *Blood*, vol. 109, no. 9, pp. 3706–3712, 2007.

[43] P. J. Kostenuik and V. Shalhoub, "Osteoprotegerin: a physiological and pharmacological inhibitor of bone resorption," *Current Pharmaceutical Design*, vol. 7, no. 8, pp. 613–635, 2001.

[44] L. C. Hofbauer, C. A. Kühne, and V. Viereck, "The OPG/RANKL/RANK system in metabolic bone diseases," *Journal of Musculoskeletal Neuronal Interactions*, vol. 4, no. 3, pp. 268–275, 2004.

[45] B. R. Wong, R. Josien, S. Y. Lee et al., "TRANCE (Tumor necrosis factor [TNF]-related Activation-induced Cytokine), a new TNF family member predominantly expressed in T cells, is a dendritic cell-specific survival factor," *The Journal of Experimental Medicine*, vol. 186, no. 12, pp. 2075–2080, 1997.

[46] D. M. Anderson, E. Maraskovsky, W. L. Billingsley et al., "A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function," *Nature*, vol. 390, no. 6656, pp. 175–179, 1997.

[47] Y.-Y. Kong, H. Yoshida, I. Sarosi et al., "OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis," *Nature*, vol. 397, no. 6717, pp. 315–323, 1999.

[48] D. Santini, G. Perrone, I. Roato et al., "Expression pattern of receptor activator of NF$\kappa$B (RANK) in a series of primary solid tumors and related bone metastases," *Journal of Cellular Physiology*, vol. 226, no. 3, pp. 780–784, 2011.

[49] L. C. Hofbauer, S. Khosla, C. R. Dunstan, D. L. Lacey, W. J. Boyle, and B. L. Riggs, "The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption," *Journal of Bone and Mineral Research*, vol. 15, no. 1, pp. 2–12, 2000.

[50] R. Pacifici, "The immune system and bone," *Archives of Biochemistry and Biophysics*, vol. 503, no. 1, pp. 41–53, 2010.

[51] H. Takayanagi, K. Ogasawara, S. Hida et al., "T-cell-mediated regulation of osteoclastogenesis by signalling cross-talk between RANKL and IFN-$\gamma$," *Nature*, vol. 408, no. 6812, pp. 600–605, 2000.

[52] K. Sato, T. Satoh, K. Shizume et al., "Prolonged decrease of serum calcium concentration by murine $\gamma$-interferon in hypercalcemic, human tumor (EC-GI)-bearing nude mice," *Cancer Research*, vol. 52, no. 2, pp. 444–449, 1992.

[53] Y. Gao, F. Grassi, M. R. Ryan et al., "IFN-$\gamma$ stimulates osteoclast formation and bone loss in vivo via antigen-driven T cell activation," *The Journal of Clinical Investigation*, vol. 117, no. 1, pp. 122–132, 2007.

[54] S. Cenci, G. Toraldo, M. N. Weitzmann et al., "Estrogen deficiency induces bone loss by increasing T cell proliferation and lifespan through IFN-$\gamma$-induced class II transactivator," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 18, pp. 10405–10410, 2003.

[55] S. Abbas and Y. Abu-Amer, "Dominant-negative I$\kappa$B facilitates apoptosis of osteoclasts by tumor necrosis factor-$\alpha$," *Journal of Biological Chemistry*, vol. 278, no. 22, pp. 20077–20082, 2003.

[56] R. Pacifici, "Estrogen deficiency, T cells and bone loss," *Cellular Immunology*, vol. 252, no. 1-2, pp. 68–80, 2008.

[57] S. Colucci, G. Brunetti, R. Rizzi et al., "T cells support osteoclastogenesis in an in vitro model derived from human multiple myeloma bone disease: the role of the OPG/TRAIL interaction," *Blood*, vol. 104, no. 12, pp. 3722–3730, 2004.

[58] I. Roato, M. Grano, G. Brunetti et al., "Mechanisms of spontaneous osteoclastogenesis in cancer with bone involvement," *The FASEB Journal*, vol. 19, no. 2, pp. 228–230, 2005.

[59] I. Roato, E. Gorassini, L. Buffoni et al., "Spontaneous osteoclastogenesis is a predictive factor for bone metastases from non-small cell lung cancer," *Lung Cancer*, vol. 61, no. 1, pp. 109–116, 2008.

[60] P. M. Appasamy, "Biological and clinical implications of interleukin-7 and lymphopolesis," *Cytokines, Cellular and Molecular Therapy*, vol. 5, no. 1, pp. 25–39, 1999.

[61] A. E. Namen, S. Lupton, K. Hjerrild et al., "Stimulation of B-cell progenitors by cloned murine interleukin-7," *Nature*, vol. 333, no. 6173, pp. 571–573, 1988.

[62] M. A. A. Al-Rawi, K. Rmali, G. Watkins, R. E. Mansel, and W. G. Jiang, "Aberrant expression of interleukin-7 (IL-7) and its signalling complex in human breast cancer," *European Journal of Cancer*, vol. 40, no. 4, pp. 494–502, 2004.

[63] C. Miyaura, Y. Onoe, M. Inada et al., "Increased B-lymphopoiesis by interleukin 7 induces bone loss in mice with intact ovarian function: similarity to estrogen deficiency," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 94, no. 17, pp. 9360–9365, 1997.

[64] M. N. Weitzmann, C. Roggia, G. Toraldo, L. Weitzmann, and R. Pacifici, "Increased production of IL-7 uncouples bone formation from bone resorption during estrogen deficiency," *The Journal of Clinical Investigation*, vol. 110, no. 11, pp. 1643–1650, 2002.

[65] S.-K. Lee, J. F. Kalinowski, S. L. Jastrzebski, L. Puddington, and J. A. Lorenzo, "Interleukin-7 is a direct inhibitor of in vitro osteoclastogenesis," *Endocrinology*, vol. 144, no. 8, pp. 3524–3531, 2003.

[66] G. Toraldo, C. Roggia, W.-P. Qian, R. Pacific, and M. N. Weitzmann, "IL-7 induces bone loss in vivo by induction of receptor activator of nuclear factor $\kappa$B ligand and tumor necrosis factor $\alpha$ from T cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 1, pp. 125–130, 2003.

[67] N. Giuliani, S. Colla, R. Sala et al., "Human myeloma cells stimulate the receptor activator of nuclear factor-$\alpha$B ligand (RANKL) in T lymphocytes: a potential role in multiple myeloma bone disease," *Blood*, vol. 100, no. 13, pp. 4615–4621, 2002.

[68] I. Roato, G. Brunetti, E. Gorassini et al., "IL-7 up-regulates TNF-$\alpha$-dependent osteoclastogenesis in patients affected by solid tumor," *PLoS ONE*, vol. 1, article e124, 2006.

[69] P. D'Amelio, A. Grimaldi, S. Di Bella et al., "Estrogen deficiency increases osteoclastogenesis up-regulating T cells activity: a key mechanism in osteoporosis," *Bone*, vol. 43, no. 1, pp. 92–100, 2008.

[70] I. Roato, E. Gorassini, G. Brunetti et al., "IL-7 modulates osteoclastogenesis in patients affected by solid tumors," *Annals of the New York Academy of Sciences*, vol. 1117, pp. 377–384, 2007.

[71] I. Roato, D. Caldo, L. Godio et al., "Bone invading NSCLC cells produce IL-7: mice model and human histologic data," *BMC Cancer*, vol. 10, article 12, 2010.

[72] J. R. Kiesel, Z. S. Buchwald, and R. Aurora, "Cross-presentation by osteoclasts induces FoxP3 in $CD8^+$ T cells," *The Journal of Immunology*, vol. 182, no. 9, pp. 5477–5487, 2009.

[73] R. Senthilkumar and H.-W. Lee, "CD137L- and RANKL-mediated reverse signals inhibit osteoclastogenesis and T lymphocyte proliferation," *Immunobiology*, vol. 214, no. 2, pp. 153–161, 2009.

[74] M. Feuerer, M. Rocha, L. Bai et al., "Enrichment of memory T cells and other profound immunological changes in the bone marrow from untreated breast cancer patients," *International Journal of Cancer*, vol. 92, no. 1, pp. 96–105, 2001.

[75] W. Tan, W. Zhang, A. Strasner et al., "Tumour-infiltrating regulatory T cells stimulate mammary cancermetastasis through RANKL-RANK signalling," *Nature*, vol. 470, no. 7335, pp. 548–553, 2011.

[76] S. H. Wrzesinski, Y. Y. Wan, and R. A. Flavell, "Transforming growth factor-$\beta$ and the immune response: implications for anticancer therapy," *Clinical Cancer Research*, vol. 13, no. 18, pp. 5262–5270, 2007.

[77] K. Schilbach, A. Geiselhart, and R. Handgretinger, "Induction of proliferation and augmented cytotoxicity of $\gamma\delta$ T lymphocytes by bisphosphonate clodronate," *Blood*, vol. 97, no. 9, pp. 2917–2918, 2001.

[78] K. Zhang, S. Kim, V. Cremasco et al., "$CD8^+$ T cells regulate bone tumor burden independent of osteoclast resorption," *Cancer Research*, vol. 71, no. 14, pp. 4799–4808, 2011.

[79] S.-H. Yoon, Y. Lee, H.-J. Kim et al., "Lyn inhibits osteoclast differentiation by interfering with PLC$\gamma$1-mediated $Ca^{2+}$ signaling," *FEBS Letters*, vol. 583, no. 7, pp. 1164–1170, 2009.

[80] V. Bronte, E. Apolloni, A. Cabrelle et al., "Identification of a $CD11b^+$/Gr-$1^+$/$CD31^+$ myeloid progenitor capable of activating or suppressing $CD8^+$ T cells," *Blood*, vol. 96, no. 12, pp. 3838–3846, 2000.

[81] D. I. Gabrilovich, M. P. Velders, E. M. Sotomayor, and W. M. Kast, "Mechanism of immune dysfunction in cancer mediated by immature Gr-$1^+$ myeloid cells," *Journal of Immunology*, vol. 166, no. 9, pp. 5398–5406, 2001.

[82] G. Gallina, L. Dolcetti, P. Serafini et al., "Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on $CD8^+$ T cells," *The Journal of Clinical Investigation*, vol. 116, no. 10, pp. 2777–2790, 2006.

[83] S. Kusmartsev, S. Nagaraj, and D. I. Gabrilovich, "Tumor-associated $CD8^+$ T cell tolerance induced by bone marrow-derived immature myeloid cells," *The Journal of Immunology*, vol. 175, no. 7, pp. 4583–4592, 2005.

[84] B. Almand, J. I. Clark, E. Nikitina et al., "Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer," *The Journal of Immunology*, vol. 166, no. 1, pp. 678–689, 2001.

[85] W. Li, K. Wu, E. Zhao et al., "HMGB1 recruits myeloid derived suppressor cells to promote peritoneal dissemination of colon cancer after resection," *Biochemical and Biophysical Research Communications*, vol. 436, no. 2, pp. 156–161, 2013.

[86] S. Kusmartsev, Y. Nefedova, D. Yoder, and D. I. Gabrilovich, "Antigen-specific inhibition of $CD8^+$ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species," *The Journal of Immunology*, vol. 172, no. 2, pp. 989–999, 2004.

[87] Y. Liu, J. A. Van Ginderachter, L. Brys, P. De Baetselier, G. Raes, and A. B. Geldhof, "Nitric oxide-independent CTL suppression during tumor progression: association with arginase-producing (M2) myeloid cells," *The Journal of Immunology*, vol. 170, no. 10, pp. 5064–5074, 2003.

[88] A. Mazzoni, V. Bronte, A. Visintin et al., "Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism," *The Journal of Immunology*, vol. 168, no. 2, pp. 689–695, 2002.

[89] A. Sawant, J. Deshane, J. Jules et al., "Myeloid-derived suppressor cells function as novel osteoclast progenitors enhancing bone loss in breast cancer," *Cancer Research*, vol. 73, no. 2, pp. 672–682, 2013.

[90] S. Das, R. S. Samant, and L. A. Shevde, "Hedgehog signaling induced by breast cancer cells promotes osteoclastogenesis and osteolysis," *The Journal of Biological Chemistry*, vol. 286, no. 11, pp. 9612–9622, 2011.

[91] J. Zhuang, J. Zhang, S. T. Lwin et al., "Osteoclasts in multiple myeloma are derived from Gr-1+CD11b+myeloid-derived suppressor cells," *PLoS ONE*, vol. 7, no. 11, Article ID e48871, 2012.

[92] A.-H. Capietto, S. Kim, D. E. Sanford et al., "Down-regulation of PLC$\gamma$2-$\beta$-catenin pathway promotes activation and expansion of myeloid-derived suppressor cells in cancer," *Journal of Experimental Medicine*, vol. 210, no. 11, pp. 2257–2271, 2013.

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*Review Article*

# Osteoimmunology: Major and Costimulatory Pathway Expression Associated with Chronic Inflammatory Induced Bone Loss

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The field of osteoimmunology has emerged in response to the range of evidences demonstrating the close interrelationship between the immune system and bone metabolism. This is pertinent to immune-mediated diseases, such as rheumatoid arthritis and periodontal disease, where there are chronic inflammation and local bone erosion. Periprosthetic osteolysis is another example of chronic inflammation with associated osteolysis. This may also involve immune mediation when occurring in a patient with rheumatoid arthritis (RA). Similarities in the regulation and mechanisms of bone loss are likely to be related to the inflammatory cytokines expressed in these diseases. This review highlights the role of immune-related factors influencing bone loss particularly in diseases of chronic inflammation where there is associated localized bone loss. The importance of the balance of the RANKL-RANK-OPG axis is discussed as well as the more recently appreciated role that receptors and adaptor proteins involved in the immunoreceptor tyrosine-based activation motif (ITAM) signaling pathway play. Although animal models are briefly discussed, the focus of this review is on the expression of ITAM associated molecules in relation to inflammation induced localized bone loss in RA, chronic periodontitis, and periprosthetic osteolysis, with an emphasis on the soluble and membrane bound factor osteoclast-associated receptor (OSCAR).

## 1. Introduction

The term osteoimmunology and the study of osteoimmunology have developed due to the close interrelationship between the immune system and bone metabolism [1]. This is evident in immune-mediated diseases, such as rheumatoid arthritis and periodontal disease (periodontitis), where there are local bone erosion and inflammation as reviewed in detail in multiple publications [2–4]. Similarities in the mechanisms of bone loss in disease are likely related to the inflammatory cytokines expressed in a number of bone loss diseases. These cytokines are known to upregulate osteoclast activity via increased expression levels of receptor activator NF kappa B ligand (RANKL) relative to osteoprotegerin (OPG) (as explored below) and increase localized bone loss in diseases such as RA, periodontal disease, and periprosthetic osteolysis [5–10].

This review highlights the role of immune-related cells and factors in modulating bone loss, particularly in these diseases. While the importance of the RANKL-RANK-OPG axis has been appreciated for nearly two decades [11–13], more recent studies have highlighted the importance of factors associated with immunoreceptor tyrosine-based activation motif (ITAM) signalling. This review will briefly discuss the RANKL-RANK-OPG axis but its major focus will be on the role of ITAM-associated factors, the more recently investigated pathway, and how it relates to inflammatory bone loss diseases, in particular osteoclast-associated receptor (OSCAR) [14].

## 2. Chronic Inflammation-Mediated Bone Loss

*2.1. Rheumatoid Arthritis.* Rheumatoid arthritis (RA) affects 1-2% of the population and involves an autoimmune reaction with an autoantibody response to citrullinated proteins (and others such as rheumatoid factor and collagen type II) [15]. RA is characterized by synovitis involving angiogenesis, synovial proliferation, increased infiltration, survival, and decreased apoptosis of inflammatory cells [16]. Further to this there is an increase in osteoclast number and activity leading to focal bone erosions, juxta-articular osteopenia, and joint destruction [17–20]. Animal models suggest that there may also be suppression of localised osteoblast formation of bone [21].

*2.2. Periodontitis and Similarities to RA.* Periodontitis is a chronic inflammatory disease of the gingival tissues, with an associated loss of the supporting structures including the periodontal ligament and alveolar bone. The aetiology involves an inflammatory response to bacterial infection such as *P. gingivalis* and possibly an autoimmune reaction, as reviewed [22]. Periodontitis is the most common and widespread bone loss pathology in humans with 64% of the US population aged 65 years and older reported as having moderate or severe periodontitis [23]. Despite the prevalence of this disease the most common treatment is either mechanical subgingival plaque removal or surgical debridement. Inevitably, in the absence of effective treatment, support structures (periodontium) are compromised and the affected teeth will loosen and fall out.

RA and periodontitis have a similar pathophysiology, characterized by destructive inflammation that culminates in localized bone loss. The citrullination of proteins by *P. gingivalis* and the subsequent generation of autoantigens that drive autoimmunity in RA have been proposed as a possible mechanism linking these two diseases [24]. Similarities in RA and periodontitis may relate to citrullinated enolase as the specific antigen involved as well as cross-reaction between the antibodies directed towards the immunodominant epitope of human citrullinated alpha-enolase and a conserved sequence on citrullinated *P. gingivalis* enolase [25].

New evidence suggests a relationship between the extent and severity of chronic periodontitis and RA [24, 25]. Individuals with advanced RA are more likely to experience more significant periodontal problems compared to their non-RA counterparts and vice versa. This is supported by findings in a study using a combined animal model of RA and periodontitis [26], which demonstrated more severe development of arthritis in mice with periodontitis. Further to this, mice in which periodontitis alone was induced had evidence of radiocarpal bone loss in the absence of arthritic disease [26]. Additionally, mice in which inflammatory arthritis was induced also had evidence of periodontitis [26]. This suggests presence of either RA or periodontitis places the individual at risk of developing the other disease. Both conditions involve an imbalance between proinflammatory and anti-inflammatory cytokines and increased bone-resorbing activity. Cantley et al. (2011) thus proposed that these two diseases are related through a common underlying dysfunction of fundamental inflammatory mechanisms [26]. New treatment strategies are needed for both diseases that target the inhibition of proinflammatory cytokines, destructive enzymes, and bone-resorbing activity. The clinical implications of the current research strongly suggest that patients with RA should be carefully screened for their periodontal status, as reviewed [27].

*2.3. Peri-Prosthetic Osteolysis.* Joint replacement surgery is used as a last resort in osteoarthritis (OA) and RA patients and is a relatively successful operation; however, a large proportion of implants fail within 10–20 years as a result of bone loss and implant loosening [4, 10, 28]. The pathogenesis behind prosthetic implant failure involves wear of prosthetic alloys, such as polyethylene (PE), cobalt chrome, and titanium liberated from the implant surface [29–31]. These particles stimulate a chronic inflammatory response [29], which increases bone-resorbing activity of the osteoclasts [32] and suppresses bone formation by the osteoblast [33, 34] resulting in bone loss [25]. Periprosthetic tissues contain granulomatous lesions dominated by inflammatory cells, particularly macrophages, and foreign-body giant cells [29–31, 35, 36]. It is believed that an inflammatory reaction is initiated within the tissues in an attempt at particle clearance. This then becomes a chronic reaction resulting in a granulomatous lesion. This granulomatous lesion in periprosthetic osteolysis often leads to the formation of pseudosynovium-like structure, in which cells are organized into lining layer, in the membranous tissues adjacent to the failed implant surface [35]. Juxtaposed to this pseudosynovium are fibrous and collagenous regions, possibly scar tissues, which could be indicative of late stage periprosthetic osteolysis. The plethora of factors release in this inflammatory reaction within the tissues contributes towards the promotion of osteoclast formation [9, 37].

Higher numbers of T lymphocytes have been observed in the periprosthetic tissues of human and mouse models containing polyethylene and metal particles compared with normal tissues [38–40] and osteoarthritic tissues [41]. Sandhu et al. (1998) proposed that T cells are indirectly affected by the inflammatory cascade induced by wear particles [40]. It is however important to note that T cells make up less than 10% of total cell population [42] in periprosthetic tissues. Given the low levels of T lymphocytes, the general belief held is that lymphocyte infiltrates are not normally associated with wear-particle induced periprosthetic osteolysis, in particular, in the granulomatous region [29, 32, 42, 43]. The role of T cells may however be more pertinent in RA patients with implants. The prevalence of foreign-body giant cells in response to implant-derived wear debris in RA patients and non-RA patients does not differ but appears to be linked to the amount of polyethylene wear debris [36]. As would be expected, this study reported a high prevalence of plasma cells in lymphocytic infiltrates in untreated RA patients compared with non-RA patients with a different distribution [36]. Whether implant wear is inducing a different reaction in RA versus non-RA patients may potentially have implications for combination and immune targeted treatment of inflammation and osteolysis in these patients.

## 3. RANKL-RANK-OPG Axis

Receptor activator NF kappa B ligand- (RANKL-) RANK signalling has several important roles in the immune system and bone [13, 44–46]. Physiologically, RANKL is required for normal development of lymph nodes [13], as evident in knockout mice. In bone, RANKL interaction with its receptor, RANK, expressed by the osteoclast, induces the transcription factor nuclear factor of activated T cells-1 (NFATc1) [47–49]. NFATc1 is an essential factor for differentiation, multinucleation and activation [47–49]. NFATc1 binds directly to and regulates osteoclast differentiation genes such as tartrate resistant acid phosphatase (TRAP) [50], cathepsin K (Cath K) [51], osteoclast-associated receptor (OSCAR) [52], $\beta$3-integrin [53, 54], and calcitonin receptor (CTR) [50]. NFATc1 is also involved in autoregulation of itself, further enhancing gene expression and osteoclast differentiation [55].

RANK-RANKL interaction is inhibited by the decoy receptor OPG [12] and thus the ratio of RANKL to OPG has a crucial influence on bone resorption [56]. Interactions between RANK expressing cells of the lamina propria and T cells expressing RANKL also play a role in intestinal inflammation [57]. In the vasculature, RANKL interacts with RANK to promote survival of endothelial cells [58]. Additionally, RANKL is upregulated in the keratinocytes of inflamed skin [59]. Further to this, in an inflammatory arthritis model reminiscent of RA, activated T cells exacerbate joint destruction via RANKL upregulation [46].

*3.1. RANKL-RANK-OPG in Bone Pathologies.* The RANK-RANKL-OPG axis is known to regulate not only normal bone physiology but also alterations in RANK-RANKL-OPG interactions that play a role in bone disease. Uncoupling in the balance between the level and activity of these molecules culminates in osteoporotic or osteopetrotic phenotypes due to an increase or decrease in osteoclast formation and activity. This is particularly evident in focal bone loss associated with chronic inflammatory diseases such as rheumatoid arthritis, periodontal disease, and periprosthetic bone loss. In active RA, periodontal disease, and prosthetic loosening, elevated levels of RANKL relative to OPG are observed in the synovial-like soft tissue, gingival tissue, and soft tissues adjacent to sites of osteolysis [5–10]. Further to this the elevated ratio of RANKL : OPG expression is associated with increased differentiation and activity of the bone-resorbing osteoclasts [5, 7–9, 32, 60], suggesting RANKL : OPG ratio as an important indicator for bone erosion.

As RANKL and OPG are key molecules regulating bone loss in diseases, therapeutic interventions targeting these molecules and their signaling cascades are being investigated to treat a wide range of diseases.

*3.2. RANKL-RANK-OPG in RA.* Osteoclasts are the prominent cell eroding bone in inflammatory arthritis [20]. A seminal paper in the field of osteoimmunology used a RANKL knockout background to demonstrate that animals developed an osteopetrotic phenotype and a reduction in bone erosion, characterized by the absence of osteoclasts, whilst inflammation did not differ between wild-type and RANKL knockout mice [61]. In contrast, cartilage erosion was present in both control littermate and RANKL knockout mice [61], suggesting that the RANK-RANKL-OPG axis does not directly affect cartilage metabolism.

In human studies, the levels of soluble RANKL have been found to be higher than OPG in synovial fluids from patients with RA compared with osteoarthritis (OA) patients [62] suggesting a role in increased bone resorption. In support of this, a more recent large center study has reported the ratio of RANKL : OPG and markers of bone and cartilage degradation (such as collagen terminal 1 (CTX-1)) to be predictive of progression of radiological bone damage in RA [60].

In synovial tissues from patients with active RA, RANKL expression is predominantly located in sublining regions [62, 63] concentrated at focal sites of osteoclastic bone erosion in the pannus- bone interface [64]. In contrast, OPG has been described as being in regions of synovium some distance from the sites of bone erosion in RA [64]. We reported OPG is associated with endothelial cells and macrophages in the synovial lining layer of OA and normal tissues whilst absent in patients with active RA [63]. RANKL expressing cells have been detected in a subset of fibroblast-like synoviocytes and infiltrating mononuclear cells [62]. Further to this, activated synoviocytes from RA tissue express RANKL and have decreased OPG and are capable of supporting osteoclastogenesis *in vitro* [65]. RANKL expressing cells are also seen within areas of lymphocyte infiltration and dual immunostaining by ourselves, and others have shown that many of the RANKL-positive cells are a subset of CD3+ and CD4+ cells [6, 62, 63]. Activated T cells from RA patients have increased RANKL are able to induce osteoclast formation *in vitro* [62]. This study also reported a higher ratio of soluble RANKL relative to OPG suggesting T cells as a source of soluble RANKL in RA [62].

NFATc1 is a transcription factor crucial to RANKL-RANK signaling in the osteoclasts [47] and is initially identified as being expressed by T cells and involved in regulation of cytokine transcription [66]. We observed NFATc1 positive cells in lymphocyte aggregates in RA tissues [67]. Many of the NFATc1-immunostained mononuclear cells observed were single nucleated and thus could be either precursor cells of osteoclasts such as macrophages, or lymphocytes. Those with lymphocyte morphology are most likely activated T cells as most of them demonstrated NFATc1 positive staining localized mainly in nucleus [67]. These cells may promote osteoclastogenesis through the RANK/RANKL pathway [39] because as already mentioned, as activated T-cells demonstrate elevated expression of membrane-bound RANKL with the ability to support osteoclastogenesis *in vitro* [62].

*3.3. RANKL-RANK-OPG in Periodontal Disease.* The relative ratio of RANKL to OPG is also a significant indicator in bone loss associated with periodontal disease [5, 69]. Soluble RANKL is significantly higher in gingival crevicular fluid (GCF) of periodontitis patients than in healthy GCF, while OPG is not [69]. Similar to RA, B and T lymphocytes express

RANKL in gingival tissues associated with periodontitis [5, 69] with expression of more than 50 and 90% of T cells and B cells, respectively. Consistent with a role in osteoclast regulation, lymphocytes isolated from gingival tissues of patients induced differentiation of mature osteoclast cells in a RANKL-dependent manner *in vitro* [69]. These results suggest that activated T and B cells can be the cellular source of RANKL and an inducer of bone resorption in periodontal disease.

In a crude mRNA analysis of tissue from dental patients, those with periodontitis exhibited significantly higher NFATc1 gene expression, compared with healthy subjects. Interestingly, NFATc1 and RANKL expression levels strongly correlated with each other and with clinical periodontal parameters [70].

*3.4. RANK-RANKL-OPG in Peri-Prosthetic Osteolysis.* Aseptic bone loss adjacent to orthopedic joint implants is a common cause of joint implant failure in humans. RANK, RANKL, and tumour necrosis factor (TNF-$\alpha$) are key modulators of bone turnover and their expression has been reported by ourselves and others in the tissues near periprosthetic osteolysis in patients undergoing revision of total hip prostheses [9, 71, 72]. These factors were strongly expressed by large multinucleated cells containing polyethylene wear debris in revision tissues [9]. More interestingly a strong statistical correlation was found between volume of bone loss, polyethylene wear debris, and RANK, RANKL, and TNF-$\alpha$ expression [9]. This was consistent with the earlier findings of Stea et al. (2000) [72] where immunohistochemical detection of TNF-$\alpha$ positively correlated with radiographic scores of osteolysis.

In periprosthetic osteolysis, elevated levels of RANKL, relative to its competitor OPG, are associated with increased differentiation and activity of the bone-resorbing osteoclasts [8, 32, 73]. An earlier study had shown that cells isolated from periprosthetic tissues containing wear particles expressed mRNA encoding for the proosteoclastogenic molecules, RANKL, its receptor RANK, monocyte colony-stimulating factor (M-CSF), interleukin- (IL-) 1 beta, TNF-$\alpha$, IL-6, and soluble IL-6 receptor, as well as OPG [8]. Other studies showed that osteoclasts formed from cells isolated from periprosthetic tissues in the presence and absence of human osteoblastic cells *in vitro* [8, 74]. When osteoclasts formed in the absence of osteoblastic cells, markedly higher levels of RANKL mRNA relative to OPG mRNA were expressed. Particles of prosthetic materials also stimulated human monocytes to express both osteoclast-associated genes and osteoclast mediating factors *in vitro* [8]. These findings suggest that ingestion of prosthetic wear particles by macrophages results in expression of osteoclast-differentiating molecules and stimulation of macrophage differentiation into osteoclasts [8]. Subsequent immunohistochemical studies demonstrate significantly higher levels of RANKL in the periprosthetic tissues of patients with implant failure than in similar tissues from osteoarthritic and healthy subjects [32]. In contrast, OPG protein levels were similar in all tissues with the net result of higher RANKL : OPG ratio [32]. Of interest, RANKL protein and mRNA were predominantly associated with macrophage cells containing wear particles in the periprosthetic tissues [32]. These findings support the contention that high levels of RANKL in periprosthetic tissues of patients with prosthetic loosening may significantly contribute to aseptic implant loosening [32].

We observed both mRNA and protein expressions of NFATc1 to be higher in periprosthetic osteolysis than in OA tissues although levels did not reach significance [75]. This is consistent with low T lymphocyte numbers observed in these tissues. This may provide an explanation why lower than expected NFATc1 protein and mRNA levels are found in periprosthetic osteolytic tissues.

## 4. The ITAM Pathway in Osteoimmunology

The ITAM pathway regulates proliferation, survival, and differentiation of effector immune cells and provides osteoclasts with costimulatory signals [14, 76–80]. In preosteoclasts and osteoclasts, innate immune receptors, TREM2 and OSCAR, associate with the ITAM adaptor proteins DAP12 and Fc receptor gamma-chain (FcR$\gamma$), respectively [80, 81]. DAP12 and TREM2 are required for differentiation into multinucleated, bone-resorbing osteoclasts *in vitro* via phosphorylation of the Syk tyrosine kinase [79]. OSCAR on the cell surface mediates signal transduction via FcR$\gamma$ [82, 83] (Figure 1). The induction of intracellular calcium via this pathway is required in conjunction with RANKL-RANK interaction for NFATc1 induction [84].

*4.1. Mutations of ITAM-Associated Molecules and Bone Phenotypes In Vivo.* In the context of human pathology the roles of TREM2 or DAP12 have only begun to be recognized. Studies in diseased tissues, particularly in Nasu-Hakola disease [85–87] and very recently in Alzheimer's disease [88], have shown that these molecules may be involved. Mutations in TREM2 or DAP12 have been associated with bone pathologies such as bone cysts and increased fractures (in addition to presenile dementia) in Nasu-Hakola disease [87, 89]. These studies support a role for DAP12 and a relationship between the skeletal and psychotic characteristics observed in Nasu-Hakola disease and for schizophrenia and presenile dementia [90]. In TREM2 deficient individuals the osteoclast precursors failed to differentiate into effective bone-resorbing cells [91]. Consistent with this, Paloneva et al. [87] demonstrated that function mutations in DAP12 and TREM2 result in an inefficient and delayed differentiation of osteoclasts *in vitro*. In postmenopausal osteoporosis a rare allele (G allele) of OSCAR-2322A> G (SNP in the $5'$ flanking region) has been associated with lower bone mineral density [92].

Although animal models are not the focus of this review it is interesting to note the phenotypes of ITAM related molecules in single and combination knockouts. TREM$^{-/-}$ mice have an osteopenic phenotype similar to Nasu-Hakola disease. *In vitro*, lack of TREM2 impairs proliferation osteoclast precursors and affects the rate of osteoclastogenesis by accelerating differentiation into mature osteoclasts [93] suggesting different effects of knocking out TREM2 *in vivo* and

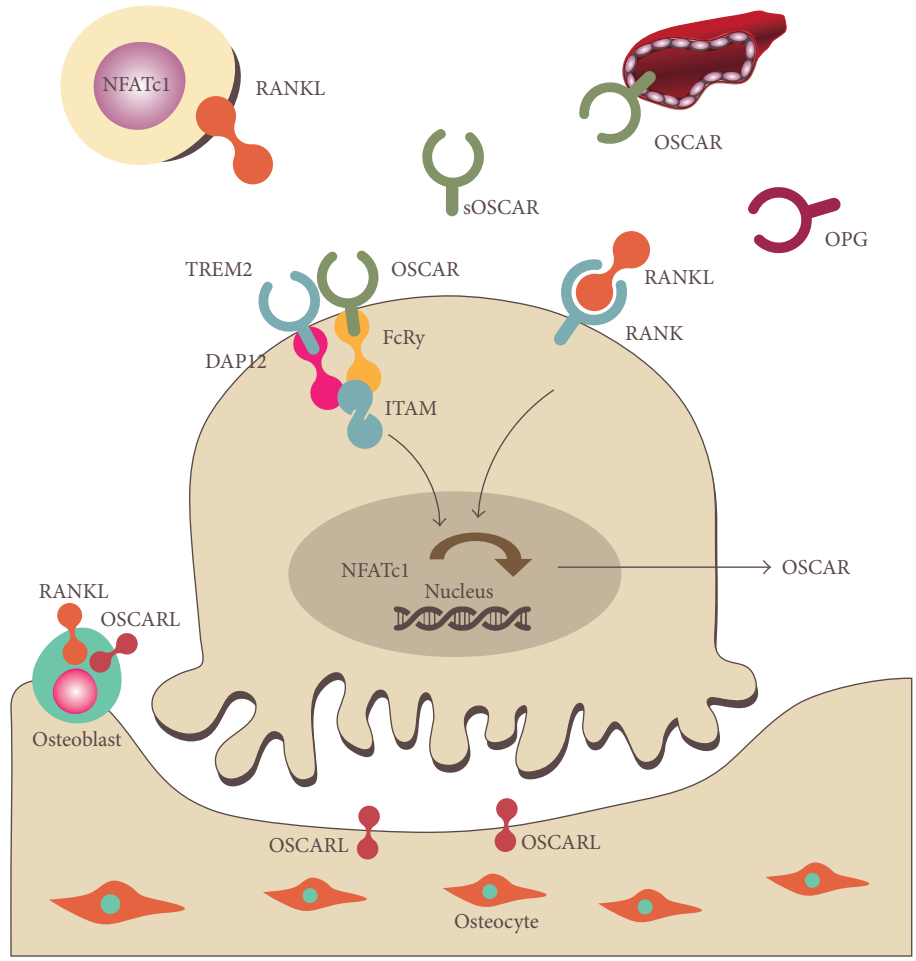



Figure 1: RANGL-RANK-OPG axis and ITAM, the costimulatory pathway, in inflammation induced localised bone loss pathologies.

*in vitro*. In DAP12 deficient mice (−/−) there are an increased bone mass (osteopetrosis) and impeded development of osteoclasts. Mice that are double knockout for the adaptors DAP12$^{-/-}$ and FcR$\gamma^{-/-}$ are severely osteopetrotic and bone marrow derived osteoclast precursors from these mice are unable to differentiate into mature osteoclasts in a RANKL- and M-CSF-mediated culture system [79, 82]. Although there is some redundancy, findings from these studies suggest that DAP12 is the predominant factor responsible for optimal osteoclast differentiation.

*4.2. OSCAR Signalling and Function in Bone Regulation.* OSCAR is an IgG-like receptor expressed by monocytes, macrophages monocyte-derived dendritic cells in humans, and is involved in antigen presentation as well as survival, maturation, and activation of dendritic cells [14, 76, 77, 83, 94, 95]. Ligation of human OSCAR on monocytes and neutrophils results in the induction of a proinflammatory cascade and the initiation of downstream immune responses [95]. Importantly, cell bound OSCAR on osteoclast precursors is an essential costimulatory factor in osteoclast formation but does not bypass the requirement of RANKL. RANKL-RANK induction of NFATc1 expression precedes that of OSCAR [96] and is crucial for induction of OSCAR gene expression [52]. In addition, ligand-activated OSCAR interacts with FcR$\gamma$ to produce an increase in intracellular calcium [95] that augments NFATc1 expression [96]. This establishes a positive feedback loop that results in marked elevation of both OSCAR and NFATc1 expressions in terminal stages of osteoclast formation [52, 96]. These findings demonstrate a significant role for OSCAR in immune modulation as well as osteoclastogenesis.

*In vitro* studies demonstrate that addition of OSCAR-Fc to osteoblast-osteoclast cocultures results in the inhibition of osteoclast differentiation with Kim et al. (2002) suggesting that this was due to OSCAR-Fc blocking an osteoblast derived OSCAR ligand binding to OSCAR [14]. This may be in addition to the recent identification of the motifs within fibrillar collagens in the extracellular matrix (ECM) as OSCAR ligands [97]. The importance of OSCAR is further highlighted by the fact that soluble OSCAR (s)OSCAR, in the form of OSCAR-Fc, has also been shown to inhibit osteoclast differentiation from PBMCs in the presence of RANKL, M-CSF, and TGF-$\beta$ [94].

Costimulatory immune pathways may further increase osteoclast differentiation and activity [81, 82], particularly in

chronic inflammatory diseases with an immune component such as in RA, periprosthetic osteolysis, and periodontal disease. In fact, our studies [67, 75] and those of others suggest that deregulation of ITAM-associated molecules contributes to the pathogenesis and severity of rheumatoid arthritis, periodontal disease, periprosthetic osteolysis, and osteoporosis [10, 67, 75, 92, 94, 98, 99].

*4.3. Expression of ITAM-Associated Molecules in Chronic Inflammation Induced Localised Osteolysis.* We, and others, have demonstrated increased levels of ITAM-related factors, including TREM2, DAP12, OSCAR, and FcR$\gamma$ in human periprosthetic tissues adjacent to sites of osteolysis [75] and in RA synovial tissues [67, 94]. Additionally, we have observed ITAM-related factors expressed in periodontitis tissue adjacent to bone loss (unpublished observations). Of these factors, soluble and membrane-bound OSCAR have been more extensively assessed in the context of RA and vascular disease (expanded on below).

*4.4. Expression of ITAM-Associated Molecules in Rheumatoid Arthritis.* We observed markedly higher levels of TREM2, DAP12, OSCAR, and FcR$\gamma$ in active RA patients compared to synovial tissues from inactive RA, OA, or control healthy joint. Multiple cell types expressed TREM2 including mononuclear cells in lymphoid aggregates and fibroblasts [67]. In OA tissues, TREM2 immunostaining was noticed in monocyte/macrophage-like cells mainly around perivascular areas and on blood vessels (unpublished observations). The positive TREM2 immunostaining on the vasculature was consistent with the finding on expression of TREM2 in endothelial cells that has been documented earlier [100]. TREM2 immunostaining was also occasionally spotted on lymphocyte-like cells in some OA tissues; however, to date there has been no study indicating the expression of TREM2 in lymphocytes but further investigation is needed for confirmation.

Interestingly, DAP12 appeared predominantly associated with macrophage-like cells in the sublining of the synovial tissue, particularly in the macrophage-like cells in the lining of the OA group [67]. More recently, a study by Chen et al. (2014) [101] reported that mRNA expression levels of DAP12 in the peripheral blood mononuclear cells of active RA patients were significantly higher in active RA patients than in inactive RA or OA patients. This is consistent with our observations [67] of higher levels of DAP12 protein expressed in the synovium in active RA patients than in inactive RA or OA patients. They also noted that the levels significantly decreased after effective therapy [101].

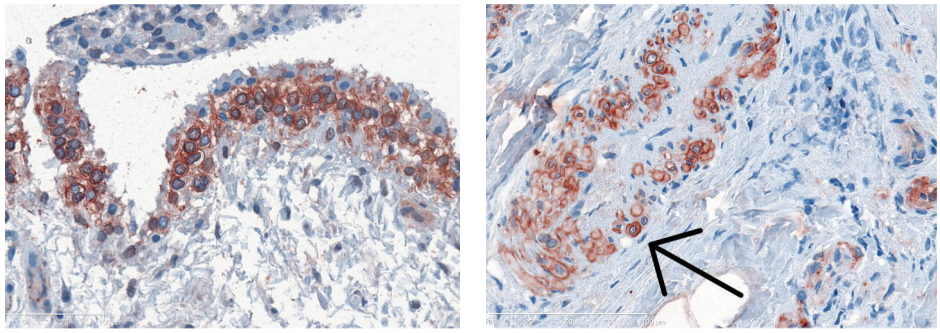
FcR$\gamma$ protein associates with fibroblasts and monocytes of the synovial sublining whilst lymphoid aggregates and the vasculature do not express FcR$\gamma$ [67]. Of note, similar to DAP12, FcR$\gamma$ was associated with macrophage-like synoviocytes in the synovial lining with some scattered monocytes in the sublining of the OA tissue. This increased DAP12 and FcR$\gamma$ expression might indicate a role in the pathogenesis of OA but this is yet to be determined [67].

*4.5. Soluble and Synovial Tissue Levels of OSCAR in RA.* Analysis of human synovial tissue, serum, plasma, and synovial fluid suggests that OSCAR expression is associated with disease activity in RA [67, 94, 98, 99]. Recent studies show that OSCAR protein expression is increased in monocytes from RA patients compared with healthy individuals, correlating with inflammatory disease activity (DAS28) [94]. OSCAR has also been noted to be expressed by mononuclear cells adjacent to synovial microvessels in RA tissues [94]. Consistent with these findings, our immunohistochemical studies show that high levels of OSCAR are associated with mono- and multinuclear cells in active RA tissues compared to tissues from OA and normal patients [67] (Figure 2). Furthermore, semiquantitative analysis confirmed that there is a significant elevation of OSCAR ($P < 0.05$) in active RA synovial tissues compared to osteoarthritis synovial tissues. This increased expression of OSCAR in the synovial tissue of active RA suggests OSCAR regulation by inflammatory cytokines and supports a role for OSCAR in the pathogenesis of RA.

A study investigating the clinical, radiological, and synovial immunopathological responses following anti-rheumatic treatment in RA proposed that high synovial tissue vascularity predicted favorable clinical and radiological responses to treatment [102]. Similar to this, we previously reported the increased OPG staining associated with the vasculature in synovial tissues retrieved from the patient in remission, OA and normal compared with active RA [63]. Furthermore, we have reported increased levels of OPG associated with vasculature following treatment with DMARDS [7].

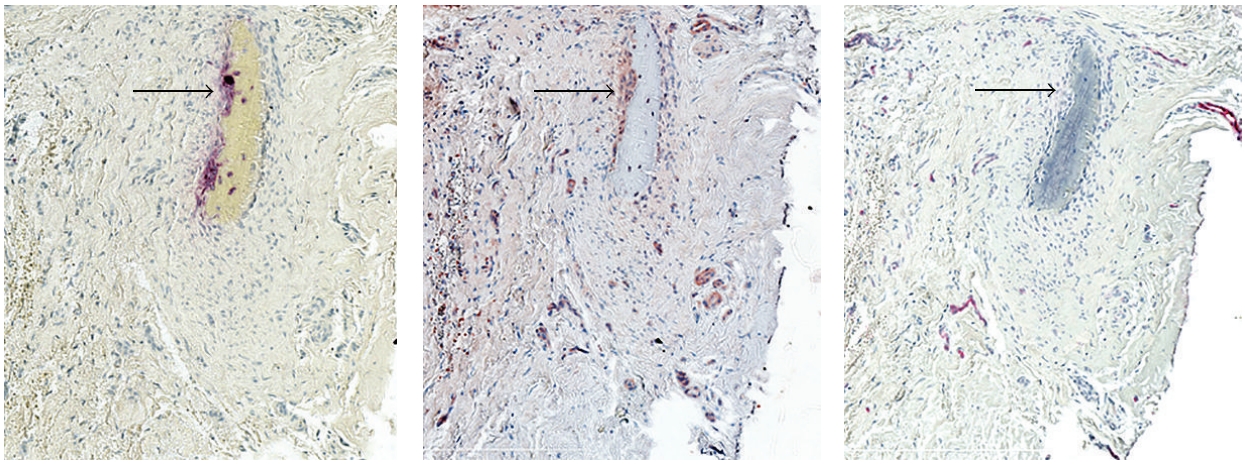
More recently we have detected increased expression of OSCAR protein associated with the microvasculature of synovial tissue from all inactive and active RA patient tissues (9/9) compared to none in the normal synovial tissue group (0/9) [67]. Importantly, in diseased tissues OSCAR was expressed mostly on the luminal side of the microvasculature, consistent with OSCAR expression by endothelial cells [67, 103]. Our findings suggest that OSCAR is associated with the endothelial cells of the microvasculature and is either produced by endothelial cells or secreted by other cells and bound by the endothelial cells in inflammatory states. The marked reduction in the OSCAR associated with endothelial cells observed in OA and healthy synovial tissues compared with active RA indicates an immune modulatory mechanism [67], which may also signal back to the osteoclasts and regulate bone resorption.

Following observations of OSCAR association with blood vessels the expression of OSCAR was investigated in endothelial cell lines *in vitro*. Our analysis on bone marrow-derived endothelial cells (BMECs) challenged with IL-1$\beta$ and TNF-$\alpha$ *in vitro* demonstrated that the inflammatory cytokines increased OSCAR expressed as both mRNA and secreted and membrane-bound proteins [67]. Together with *in vivo* observations on synovial tissues and serum levels these studies suggest that inflammatory cytokines in RA regulate cleavage or secretion of sOSCAR from preosteoclasts or the microvasculature.



(a) (b)

Figure 2: OSCAR positive cells (red) in synovial tissue. (a) OSCAR immunostaining in the lining cells of OA tissues. (b) Mononuclear OSCAR positive cells as indicated by arrow. The magnification was 400x.



(a) (b) (c)

Figure 3: Expression of osteoclast and vascular-associated molecules in mildly inflamed gingival tissues. (a) TRAP (osteoclast marker), (b) OSCAR C. Von Willebrand factor to identify the microvasculature. The magnification was 200x.

The regulation by inflammatory cytokines, such as TNF-$\alpha$, of OSCAR messenger RNA expression has also been observed in monocytes [94]. Interestingly, levels of OSCAR were found to increase in serum from RA patients following anti-TNF treatment [94]. Of note, these studies did not investigate gene or the release of protein OSCAR by human peripheral blood derived osteoclasts in response to TNF-$\alpha$ in conjunction with RANKL.

*4.6. Expression of ITAM-Associated Molecules in Periodontal Disease.* To our knowledge, very limited descriptive or functional studies have investigated ITAM factors in periodontitis and normal gingival tissues. An early study however reported that isolated polymorphonuclear neutrophils from GCF of adult periodontitis patients exhibited higher Fc alpha RI and Fc gamma RI levels and lower Fc gamma RIIa and Fc gamma RIIIb levels than peripheral blood polymorphonuclear neutrophils. They found that GCF derived polymorphonuclear neutrophils had a reduced ability to phagocytose and kill IgG1-opsonized *P. gingivalis* compared to peripheral blood polymorphonuclear neutrophils [104].

Our recent unpublished observations have identified OSCAR colocalizing with TRAP in cells in serial sections of mildly inflamed gingival tissue (Figure 3). Of note, these osteoclast markers are highly expressed in the multinucleated cells on the bone. Similar to the previous observations of expression of ITAM-associated molecules in active and inactive RA patients OSCAR expression was also noted in the microvasculature. Given the similarities in pathogenesis of RA and periodontitis [27] it is worth investigating expression of ITAM factors in periodontitis, gingivitis, and normal gingival tissues.

*4.7. Expression of ITAM-Associated Molecules in Peri-Prosthetic Osteolysis.* We have reported a marked increase in the levels of TREM2 and DAP12, OSCAR, and FcR$\gamma$ in tissues containing PE particles, compared with OA synovial control tissue when assessed by a semiquantitative scoring system [75]. Furthermore, the observed increased levels of these proteins in peri-prosthetic tissues were consistent with the finding that the corresponding mRNA levels were also increased [75]. Of interest, PE-containing osteoclast-like

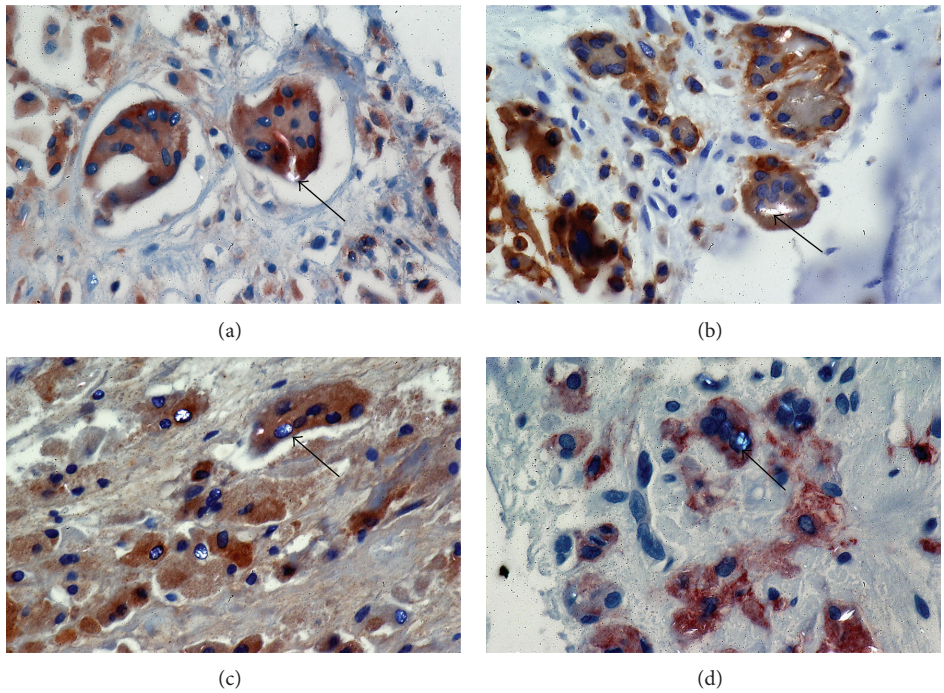


Figure 4: Expression of ITAM-associated molecules in PE-containing tissues from sites of aseptic loosening due to osteolysis. (a) TREM2, (b) DAP12, (c) OSCAR, and (d) FcR$\gamma$ immunostaining. The magnification was 400x.

cells in these tissues were associated with high levels of TREM2 and OSCAR protein and their respective adaptor molecules DAP12 and FcR$\gamma$ [75] (Figure 4). Consistent with these *in vivo* observations, PE particles added to human peripheral blood derived osteoclast cells *in vitro* upregulated ITAM expression [75].

It is important to also understand the role synovial fluid may play in modulating regulators of cartilage and bone destruction in the joint. Andersson et al. (2007) found that synovial fluid from patients with OA stimulated the mRNA expression of OSCAR and NFATc1 in mouse calvarial implants *in vitro*, while mRNA expressions of DAP12 and FcR$\gamma$ were not affected by synovial fluid from either revision or OA patient groups. The authors suggested that perhaps OSCAR and NFATc1 mRNA might be regulated by soluble factors that are present in OA synovial fluid. However, expression of DAP12 and FcR$\gamma$ was not regulated in the same way [105]. This is an area that could further be explored.

Considering that inflammation recruits osteoclast precursors and can induce the differentiation and activation of osteoclasts the enhanced expression of ITAM-related molecules in revision tissues could exacerbate bone loss in this disease.

*4.8. Potential Role for OSCAR in the Clinic.* Previous studies have demonstrated that soluble fusion (OSCAR-fc) protein, comprising the extracellular domain of OSCAR, could inhibit osteoclastogenesis in murine preosteoclast/osteoblast cocultures [14] and PBMCs cultured in the presence of RANKL, M-CSF, and TGF-$\beta$ [94]. In this situation sOSCAR could compete with OSCAR ligand and reduce OSCAR signalling. The ability of soluble sOSCAR *in vitro* to impede osteoclast formation may prove useful in inhibiting osteoclast differentiation and may thus prevent bone damage in diseases such as RA.

There is conflicting data as to whether sOSCAR increases in healthy individuals or it increases as a result of erosive activity in RA. Soluble OSCAR has been detected in serum and reported to be higher in healthy compared to RA patients [94, 99]. Serum levels of sOSCAR were shown to inversely correlate with erosion and disease activity [99]. A recent study, however, reported higher levels of sOSCAR in the plasma of RA patients rather than healthy individuals [98]. We have also detected sOSCAR in the synovial fluid of OA and active RA patients with no significant difference between these diseases [67]. We believe that sOSCAR has the potential to act as decoy receptor for OSCAR ligand within the joint and affect osteoclast development in RA. It is possible that successful treatment results in increased cleavage of cell associated OSCAR resulting in increased sOSCAR levels in the joint. In this way sOSCAR regulates osteoclastic bone resorption and is an early marker that predicts joint damage. The biological effect of serum and synovial fluid-derived OSCAR on osteoclastogenesis is yet to be investigated.

Synovitis and erosion are not always linked with some patients having progressive erosive disease despite being in remission and it is unclear what factors drive this [106]. The discordance between clinical inflammatory disease activity and radiological outcomes emphasizes the need for a validated marker of bone damage in conjunction with current clinical parameters that are routinely assessed [107, 108]. The ability to monitor bone erosion will allow the clinician to

make important decisions on therapy earlier [109] and reduce structural joint damage [110]. Currently unaddressed RA-induced joint damage affects mobility of patients later in life and predisposes to secondary osteoarthritis [110]. In addition, while we have numerous markers that reflect inflammation and related disease activity there are none that monitor joint erosion, other than X-rays which only indicate damage after it has occurred [111]. Therefore, it is essential that an accurate early marker of joint erosion is identified in order to guide effective treatment modalities in order to protect the joint of arthritic patients.

## 5. Conclusion

While the significance of ITAM-associated molecules has been largely established in the context of bone biology and an immunological point of view, limited studies have been carried out on osteoclast ITAM-related molecules in human bone pathologies. The increased levels of ITAM factors in inflamed tissues adjacent to sites of localized bone loss in RA, periodontal disease, and periprosthetic osteolysis may prove indicative of the disease progression. Further to this, levels of the soluble factor, OSCAR, in serum or local fluid, may provide us with a potential bone destructive marker and potential target for modulation of bone erosion.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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## References

[1] J. Rho, M. Takami, and Y. Choi, "Osteoimmunology: interactions of the immune and skeletal systems," *Molecules and Cells*, vol. 17, no. 1, pp. 1–9, 2004.

[2] S. R. Goldring, P. E. Purdue, T. N. Crotti et al., "Bone remodelling in inflammatory arthritis," *Annals of the Rheumatic Diseases*, vol. 72, no. 2, pp. ii52–ii55, 2013.

[3] K. Sato and H. Takayanagi, "Osteoclasts, rheumatoid arthritis, and osteoimmunology," *Current Opinion in Rheumatology*, vol. 18, no. 4, pp. 419–426, 2006.

[4] R. A. Kayal, "The role of osteoimmunology in periodontal disease," *BioMed Research International*, vol. 2013, Article ID 639368, 12 pages, 2013.

[5] T. Crotti, M. D. Smith, R. Hirsch et al., "Receptor activator NF $\kappa$B ligand (RANKL) and osteoprotegerin (OPG) protein expression in periodontitis," *Journal of Periodontal Research*, vol. 38, no. 4, pp. 380–387, 2003.

[6] T. N. Crotti, M. D. Smith, H. Weedon et al., "Receptor activator NF-$\kappa$B ligand (RANKL) expression in synovial tissue from patients with rheumatoid arthritis, spondyloarthropathy, osteoarthritis, and from normal patients: semiquantitative and quantitative analysis," *Annals of the Rheumatic Diseases*, vol. 61, no. 12, pp. 1047–1054, 2002.

[7] D. Haynes, T. Crotti, H. Weedon et al., "Modulation of RANKL and osteoprotegerin expression in synovial tissue from patients with rheumatoid arthritis in response to disease-modifying antirheumatic drug treatment and correlation with radiologic outcome," *Arthritis Care and Research*, vol. 59, no. 7, pp. 911–920, 2008.

[8] D. R. Haynes, T. N. Crotti, A. E. Potter et al., "The osteoclastogenic molecules RANKL and RANK are associated with periprosthetic osteolysis," *Journal of Bone and Joint Surgery*, vol. 83, no. 6, pp. 902–911, 2001.

[9] C. A. Holding, D. M. Findlay, R. Stamenkov et al., "The correlation of RANK, RANKL and TNF$\alpha$ expression with bone loss volume and polyethylene wear debris around hip implants," *Biomaterials*, vol. 27, no. 30, pp. 5212–5219, 2006.

[10] C. Jiang, Z. Li, H. Quan et al., "Osteoimmunology in orthodontic tooth movement," *Oral Diseases*, 2014.

[11] W. S. Simonet, D. L. Lacey, C. R. Dunstan et al., "Osteoprotegerin: a novel secreted protein involved in the regulation of bone density," *Cell*, vol. 89, no. 2, pp. 309–319, 1997.

[12] D. L. Lacey, E. Timms, H.-L. Tan et al., "Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation," *Cell*, vol. 93, no. 2, pp. 165–176, 1998.

[13] Y.-Y. Kong, H. Yoshida, I. Sarosi et al., "OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis," *Nature*, vol. 397, no. 6717, pp. 315–323, 1999.

[14] N. Kim, M. Takami, J. Rho, R. Josien, and Y. Choi, "A novel member of the leukocyte receptor complex regulates osteoclast differentiation," *The Journal of Experimental Medicine*, vol. 195, no. 2, pp. 201–209, 2002.

[15] D. Aletaha, T. Neogi, A. J. Silman et al., "2010 rheumatoid arthritis classification criteria: an American college of rheumatology/European League against rheumatism collaborative initiative," *Arthritis & Rheumatism*, vol. 62, no. 9, pp. 2569–2581, 2010.

[16] M. G. H. van de Sande, M. J. H. de Hair, C. van der Leij et al., "Different stages of rheumatoid arthritis: features of the synovium in the preclinical phase," *Annals of the Rheumatic Diseases*, vol. 70, no. 5, pp. 772–777, 2011.

[17] M.-M. Tămaş, E. Filippucci, A. Becciolini et al., "Bone erosions in rheumatoid arthritis: ultrasound findings in the early stage of the disease," *Rheumatology*, vol. 53, no. 6, Article ID ket484, pp. 1100–1107, 2014.

[18] R. J. Black, L. Spargo, C. Schultz et al., "Decline in hand bone mineral density indicates increased risk of erosive change in early rheumatoid arthritis," *Arthritis Care and Research*, vol. 66, no. 4, pp. 515–522, 2014.

[19] M. Bromley and D. E. Woolley, "Chondroclasts and osteoclasts at subchondrial sites of erosion in the rheumatoid joint," *Arthritis and Rheumatism*, vol. 27, no. 9, pp. 968–975, 1984.

[20] E. M. Gravallese, Y. Harada, J.-T. Wang, A. H. Gorn, T. S. Thornhill, and S. R. Goldring, "Identification of cell types

responsible for bone resorption in rheumatoid arthritis and juvenile rheumatoid arthritis," *The American Journal of Pathology*, vol. 152, no. 4, pp. 943–951, 1998.

[21] N. C. Walsh, S. Reinwald, C. A. Manning et al., "Osteoblast function is compromised at sites of focal bone erosion in inflammatory arthritis," *Journal of Bone and Mineral Research*, vol. 24, no. 9, pp. 1572–1585, 2009.

[22] K. S. Kornman, R. C. Page, and M. S. Tonetti, "The host response to the microbial challenge in periodontitis: assembling the players," *Periodontology 2000*, vol. 14, no. 1, pp. 33–53, 1997.

[23] P. I. Eke, B. A. Dye, L. Wei, G. O. Thornton-Evans, and R. J. Genco, "Prevalence of periodontitis in adults in the united states: 2009 and 2010," *Journal of Dental Research*, vol. 91, no. 10, pp. 914–920, 2012.

[24] G. P. Harvey, T. R. Fitzsimmons, A. A. S. S. K. Dhamarpatni, C. Marchant, D. R. Haynes, and P. M. Bartold, "Expression of peptidylarginine deiminase-2 and -4, citrullinated proteins and anti-citrullinated protein antibodies in human gingiva," *Journal of Periodontal Research*, vol. 48, no. 2, pp. 252–261, 2013.

[25] K. Lundberg, N. Wegner, T. Yucel-Lindberg, and P. J. Venables, "Periodontitis in RA-the citrullinated enolase connection," *Nature Reviews Rheumatology*, vol. 6, no. 12, pp. 727–730, 2010.

[26] M. D. Cantley, D. R. Haynes, V. Marino, and P. M. Bartold, "Pre-existing periodontitis exacerbates experimental arthritis in a mouse model," *Journal of Clinical Periodontology*, vol. 38, no. 6, pp. 532–541, 2011.

[27] P. M. Bartold, R. I. Marshall, and D. R. Haynes, "Periodontitis and rheumatoid arthritis: a review," *Journal of Periodontology*, vol. 76, no. 11, supplement, pp. 2066–2074, 2005.

[28] D. T. Graves, T. Oates, and G. P. Garlet, "Review of osteoimmunology and the host response in endodontic and periodontal lesions," *Journal of Oral Microbiology*, vol. 3, article 5304, 2011.

[29] D. W. Howie, "Tissue response in relation to type of wear particles around failed hip arthroplasties," *The Journal of Arthroplasty*, vol. 5, no. 4, pp. 337–348, 1990.

[30] H.-G. Willert, H. Bertram, and G. Hans Buchhorn, "Osteolysis in alloarthroplasty of the hip: the role of bone cement fragmentation," *Clinical Orthopaedics and Related Research*, no. 258, pp. 108–121, 1990.

[31] H.-G. Willert and G. H. Buchhorn, "The significance of wear and material fatigue in loosening of hip prostheses," *Orthopade*, vol. 18, no. 5, pp. 350–369, 1989.

[32] T. N. Crotti, M. D. Smith, D. M. Findlay et al., "Factors regulating osteoclast formation in human tissues adjacent to peri-implant bone loss: Expression of receptor activator NFκB, RANK ligand and osteoprotegerin," *Biomaterials*, vol. 25, no. 4, pp. 565–573, 2004.

[33] G. J. Atkins, K. J. Welldon, C. A. Holding, D. R. Haynes, D. W. Howie, and D. M. Findlay, "The induction of a catabolic phenotype in human primary osteoblasts and osteocytes by polyethylene particles," *Biomaterials*, vol. 30, no. 22, pp. 3672–3681, 2009.

[34] C. Vermes, K. A. Roebuck, R. Chandrasekaran, J. G. Dobai, J. J. Jacobs, and T. T. Glant, "Particulate wear debris activates protein tyrosine kinases and nuclear factor κB, which down-regulates type I collagen synthesis in human osteoblasts," *Journal of Bone and Mineral Research*, vol. 15, no. 9, pp. 1756–1765, 2000.

[35] S. R. Goldring, A. L. Schiller, M. Roelke, C. M. Rourke, D. A. O'Neil, and W. H. Harris, "The synovial-like membrane at the bone-cement interference in loose total hip replacements and its proposed role in bone lysis," *Journal of Bone and Joint Surgery—Series A*, vol. 65, no. 5, pp. 575–584, 1983.

[36] A. Vasudevan, E. F. DiCarlo, T. Wright et al., "Cellular response to prosthetic wear debris differs in patients with and without rheumatoid arthritis," *Arthritis and rheumatism*, vol. 64, no. 4, pp. 1005–1014, 2012.

[37] E. M. Greenfield, Y. Bi, A. A. Ragab, V. M. Goldberg, and R. R. van De Motter, "The role of osteoclast differentiation in aseptic loosening," *Journal of Orthopaedic Research*, vol. 20, no. 1, pp. 1–8, 2002.

[38] B. Hercus and P. A. Revell, "Phenotypic characteristics of T lymphocytes in the interfacial tissue of aseptically loosened prosthetic joints," *Journal of Materials Science: Materials in Medicine*, vol. 12, no. 10–12, pp. 1063–1067, 2001.

[39] I. Roato, D. Caldo, L. D'Amico et al., "Osteoclastogenesis in peripheral blood mononuclear cell cultures of periprosthetic osteolysis patients and the phenotype of T cells localized in periprosthetic tissues," *Biomaterials*, vol. 31, no. 29, pp. 7519–7525, 2010.

[40] J. Sandhu, J. E. Waddell, M. Henry, and E. L. Boynton, "The role of T cells in polyethylene particulate induce inflammation," *The Journal of Rheumatology*, vol. 25, no. 9, pp. 1794–1799, 1998.

[41] S. Landgraeber, M. Toetsch, C. Wedemeyer et al., "Over-expression of p53/BAK in aseptic loosening after total hip replacement," *Biomaterials*, vol. 27, no. 15, pp. 3010–3020, 2006.

[42] W. A. Jiranek, M. Machado, M. Jasty et al., "Production of cytokines around loosened cemented acetabular components: analysis with immunohistochemical techniques and in situ hybridization," *Journal of Bone and Joint Surgery—Series A*, vol. 75, no. 6, pp. 863–879, 1993.

[43] F. F. Buechel, D. Drucker, M. Jasty, W. Jiranek, and W. H. Harris, "Osteolysis around uncemented acetabular components of cobalt-chrome surface replacement hip arthroplasty," *Clinical Orthopaedics and Related Research*, no. 298, pp. 202–211, 1994.

[44] T. Nakashima and H. Takayanagi, "Osteoimmunology: crosstalk between the immune and bone systems," *Journal of Clinical Immunology*, vol. 29, no. 5, pp. 555–567, 2009.

[45] W. C. Dougall, M. Glaccum, K. Charrier et al., "RANK is essential for osteoclast and lymph node development," *Genes & Development*, vol. 13, no. 18, pp. 2412–2424, 1999.

[46] Y.-Y. Kung, U. Felge, I. Sarosi et al., "Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand," *Nature*, vol. 402, no. 6759, pp. 304–309, 1999.

[47] H. Takayanagi, S. Kim, T. Koga et al., "Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts," *Developmental Cell*, vol. 3, no. 6, pp. 889–901, 2002.

[48] H. Hirotani, N. A. Tuohy, J.-T. Woo, P. H. Stern, and N. A. Clipstone, " The calcineurin/nuclear factor of activated T cells signaling pathway regulates osteoclastogenesis in RAW264.7 cells," *The Journal of Biological Chemistry*, vol. 279, no. 14, pp. 13984–13992, 2004.

[49] N. Ishida, K. Hayashi, M. Hoshijima et al., "Large scale gene expression analysis of osteoclastogenesis *in vitro* and elucidation of NFAT2 as a key regulator," *The Journal of Biological Chemistry*, vol. 277, no. 43, pp. 41147–41156, 2002.

[50] K. Matsuo, D. L. Galson, C. Zhao et al., "Nuclear factor of activated T-cells (NFAT) rescues osteoclastogenesis in precursors lacking c-Fos," *Journal of Biological Chemistry*, vol. 279, no. 25, pp. 26475–26480, 2004.

[51] M. Matsumoto, M. Kogawa, S. Wada et al., "Essential role of p38 mitogen-activated protein kinase in cathepsin K gene expression during osteoclastogenesis through association of NFATc1 and PU.1," *The Journal of Biological Chemistry*, vol. 279, no. 44, pp. 45969–45979, 2004.

[52] Y. Kim, K. Sato, M. Asagiri, I. Morita, K. Soma, and H. Takayanagi, "Contribution of nuclear factor of activated T cells c1 to the transcriptional control of immunoreceptor osteoclast-associated receptor but not triggering receptor expressed by myeloid cells-2 during osteoclastogenesis," *The Journal of Biological Chemistry*, vol. 280, no. 38, pp. 32905–32913, 2005.

[53] T. N. Crotti, M. Flannery, N. C. Walsh, J. D. Fleming, S. R. Goldring, and K. P. McHugh, "NFATc1 regulation of the human $\beta$3 integrin promoter in osteoclast differentiation," *Gene*, vol. 372, no. 1-2, pp. 92–102, 2006.

[54] T. N. Crotti, S. M. Sharma, J. D. Fleming et al., "PU.1 and NFATc1 mediate osteoclastic induction of the mouse $\beta_3$ integrin promoter," *Journal of Cellular Physiology*, vol. 215, no. 3, pp. 636–644, 2008.

[55] M. Asagiri, K. Sato, T. Usami et al., "Autoamplification of NFATc1 expression determines its essential role in bone homeostasis," *The Journal of Experimental Medicine*, vol. 202, no. 9, pp. 1261–1269, 2005.

[56] T.-Y. Ho, K. Santora, J. C. Chen, A.-L. Frankshun, and C. A. Bagnell, "Effects of relaxin and estrogens on bone remodeling markers, receptor activator of NF-kB ligand (RANKL) and osteoprotegerin (OPG), in rat adjuvant-induced arthritis," *Bone*, vol. 48, no. 6, pp. 1346–1353, 2011.

[57] T. Totsuka, T. Kanai, Y. Nemoto et al., "Rank-rankl signaling pathway is critically involved in the function of $CD4^+CD25^+$ regulatory T cells in chronic colitis," *The Journal of Immunology*, vol. 182, no. 10, pp. 6079–6087, 2009.

[58] H.-H. Kim, H. S. Shin, H. J. Kwak et al., "RANKL regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway," *The FASEB Journal*, vol. 17, no. 14, pp. 2163–2165, 2003.

[59] K. Loser, A. Mehling, S. Loeser et al., "Epidermal RANKL controls regulatory T-cell numbers via activation of dendritic cells," *Nature Medicine*, vol. 12, no. 12, pp. 1372–1379, 2006.

[60] L. H. D. van Tuyl, A. E. Voskuyl, M. Boers et al., "Baseline RANKL:OPG ratio and markers of bone and cartilage degradation predict annual radiological progression over 11 years in rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 69, no. 9, pp. 1623–1628, 2010.

[61] A. R. Pettit, H. Ji, D. Von Stechow et al., "TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis," *The American Journal of Pathology*, vol. 159, no. 5, pp. 1689–1699, 2001.

[62] S. Kotake, N. Udagawa, M. Hakoda et al., "Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients," *Arthritis & Rheumatism*, vol. 44, no. 5, pp. 1003–1012, 2001.

[63] D. R. Haynes, E. Barg, T. N. Crotti et al., "Osteoprotegerin expression in synovial tissue from patients with rheumatoid arthritis, spondyloarthropathies and osteoarthritis and normal controls," *Rheumatology*, vol. 42, no. 1, pp. 123–134, 2003.

[64] A. R. Pettit, N. C. Walsh, C. Manning, S. R. Goldring, and E. M. Gravallese, "RANKL protein is expressed at the pannus-bone interface at sites of articular bone erosion in rheumatoid arthritis," *Rheumatology*, vol. 45, no. 9, pp. 1068–1076, 2006.

[65] H. Takayanagi, H. Iizuka, T. Juji et al., "Involvement of receptor activator of nuclear factor kappaB ligand/osteoclast differentiation factor in osteoclastogenesis from synoviocytes in rheumatoid arthritis," *Arthritis & Rheumatism*, vol. 43, no. 2, pp. 259–269, 2000.

[66] S. Monticelli and A. Rao, "NFAT1 and NFAT2 are positive regulators of IL-4 gene transcription," *European Journal of Immunology*, vol. 32, no. 10, pp. 2971–2978, 2002.

[67] T. N. Crotti, A. A. S. S. K. Dharmapatni, E. Alias, A. C. W. Zannettino, M. D. Smith, and D. R. Haynes, "The immunoreceptor tyrosine-based activation motif (ITAM)-related factors are increased in synovial tissue and vasculature of rheumatoid arthritic joints," *Arthritis Research & Therapy*, vol. 14, no. 6, article R245, 2012.

[68] N. J. Horwood, V. Kartsogiannis, J. M. W. Quinn, E. Romas, T. J. Martin, and M. T. Gillespie, "Activated T lymphocytes support osteoclast formation *in vitro*," *Biochemical and Biophysical Research Communications*, vol. 265, no. 1, pp. 144–150, 1999.

[69] T. Kawai, T. Matsuyama, Y. Hosokawa et al., "B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease," *The American Journal of Pathology*, vol. 169, no. 3, pp. 987–998, 2006.

[70] G. N. Belibasakis, G. Emingil, B. Saygan, O. Turkoglu, G. Atilla, and N. Bostanci, "Gene expression of transcription factor NFATc1 in periodontal diseases," *APMIS*, vol. 119, no. 3, pp. 167–172, 2011.

[71] J. Chiba, H. E. Rubash, and Y. Iwaki, "The characterization of cytokines in the interface tissue obtained from failed cementless total hip arthroplasty with and without femoral osteolysis," *Clinical Orthopaedics and Related Research*, no. 300, pp. 304–312, 1994.

[72] S. Stea, M. Visentin, D. Granchi et al., "Cytokines and osteolysis around total hip prostheses," *Cytokine*, vol. 12, no. 10, pp. 1575–1579, 2000.

[73] D. R. Haynes, T. N. Crotti, and H. Zreiqat, "Regulation of osteoclast activity in peri-implant tissues," *Biomaterials*, vol. 25, no. 20, pp. 4877–4885, 2004.

[74] A. Sabokbar, O. Kudo, and N. A. Athanasou, "Two distinct cellular mechanisms of osteoclast formation and bone resorption in periprosthetic osteolysis," *Journal of Orthopaedic Research*, vol. 21, no. 1, pp. 73–80, 2003.

[75] E. Alias, A. S. S. K. Dharmapatni, A. C. Holding et al., "Polyethylene particles stimulate expression of ITAM-related molecules in peri-implant tissues and when stimulating osteoclastogenesis in vitro," *Acta Biomaterialia*, vol. 8, no. 8, pp. 3104–3112, 2012.

[76] E. Merck, B. De Saint-Vis, M. Scuiller et al., "Fc receptor $\gamma$-chain activation via hOSCAR induces survival and maturation of dendritic cells and modulates Toll-like receptor responses," *Blood*, vol. 105, no. 9, pp. 3623–3632, 2005.

[77] E. Merck, C. Gaillard, D. M. Gorman et al., "OSCAR is an FcR$\gamma$-associated receptor that is expressed by myeloid cells and is involved in antigen presentation and activation of human dendritic cells," *Blood*, vol. 104, no. 5, pp. 1386–1395, 2004.

[78] K. Nemeth, M. Schoppet, N. Al-Fakhri et al., "The role of osteoclast-associated receptor in osteoimmunology," *The Journal of Immunology*, vol. 186, no. 1, pp. 13–18, 2011.

[79] A. Mócsai, M. B. Humphrey, J. A. G. van Ziffle et al., "The immunomodulatory adapter proteins DAP12 and Fc receptor $\gamma$-chain (FcR$\gamma$) regulate development of functional osteoclasts through the Syk tyrosine kinase," *Proceedings of the National*

*Academy of Sciences of the United States of America*, vol. 101, no. 16, pp. 6158–6163, 2004.

[80] M. B. Humphrey, L. L. Lanier, and M. C. Nakamura, "Role of ITAM-containing adapter proteins and their receptors in the immune system and bone," *Immunological Reviews*, vol. 208, no. 1, pp. 50–65, 2005.

[81] H. Takayanagi, "Mechanistic insight into osteoclast differentiation in osteoimmunology," *Journal of Molecular Medicine*, vol. 83, no. 3, pp. 170–179, 2005.

[82] T. Koga, M. Inui, K. Inoue et al., "Costimulatory signals mediated by the ITAM motif cooperate with RANKL for bone homeostasis," *Nature*, vol. 428, no. 6984, pp. 758–763, 2004.

[83] S. Ishikawa, N. Arase, T. Suenaga et al., "Involvement of FcR$\gamma$ in signal transduction of osteoclast-associated receptor (OSCAR)," *International Immunology*, vol. 16, no. 7, pp. 1019–1025, 2004.

[84] K. Kim, J. H. Kim, J. Lee et al., "Nuclear factor of activated T cells c1 induces osteoclast-associated receptor gene expression during tumor necrosis factor-related activation-induced cytokine-mediated osteoclastogenesis," *The Journal of Biological Chemistry*, vol. 280, no. 42, pp. 35209–35216, 2005.

[85] Y. Numasawa, C. Yamaura, S. Ishihara et al., "Nasu-Hakola disease with a splicing mutation of TREM2 in a Japanese family," *European Journal of Neurology*, vol. 18, no. 9, pp. 1179–1183, 2011.

[86] J. Paloneva, T. Manninen, G. Christman et al., "Mutations in two genes encoding different subunits of a receptor signaling complex result in an identical disease phenotype," *American Journal of Human Genetics*, vol. 71, no. 3, pp. 656–662, 2002.

[87] J. Paloneva, J. Mandelin, A. Kiialainen et al., "DAP12/TREM2 deficiency results in impaired osteoclast differentiation and osteoporotic features," *The Journal of Experimental Medicine*, vol. 198, no. 4, pp. 669–675, 2003.

[88] B. Melchior, A. E. Garcia, B.-K. Hsiung et al., "Dual induction of TREM2 and tolerance-related transcript, Tmem176b, in amyloid transgenic mice: implications for vaccine-based therapies for Alzheimer's disease," *ASN Neuro*, vol. 2, no. 3, Article ID e00037, 2010.

[89] J. Paloneva, M. Kestilä, J. Wu et al., "Loss-of-function mutations in TYROBP (DAP12) result in a presenile dementia with bone cysts," *Nature Genetics*, vol. 25, no. 3, pp. 357–361, 2000.

[90] T. Kaifu, J. Nakahara, M. Inui et al., "Osteopetrosis and thalamic hypomyelinosis with synaptic degeneration in DAP12-deficient mice," *The Journal of Clinical Investigation*, vol. 111, no. 3, pp. 323–332, 2003.

[91] M. Cella, C. Buonsanti, C. Strader, T. Kondo, A. Salmaggi, and M. Colonna, "Impaired differentiation of osteoclasts in TREM-2-deficient individuals," *The Journal of Experimental Medicine*, vol. 198, no. 4, pp. 645–651, 2003.

[92] G. S. Kim, J.-M. Koh, J. S. Chang et al., "Association of the OSCAR promoter polymorphism with BMD in postmenopausal women," *Journal of Bone and Mineral Research*, vol. 20, no. 8, pp. 1342–1348, 2005.

[93] K. Otero, M. Shinohara, H. Zhao et al., "TREM2 and beta-catenin regulate bone homeostasis by controlling the rate of osteoclastogenesis," *Journal of Immunology*, vol. 188, no. 6, pp. 2612–2621, 2012.

[94] S. Herman, R. B. Müller, G. Krönke et al., "Induction of osteoclast-associated receptor, a key osteoclast costimulation molecule, in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 58, no. 10, pp. 3041–3050, 2008.

[95] E. Merck, C. Gaillard, M. Scuiller et al., "Ligation of the FcR$\gamma$ chain-associated human osteoclast-associated receptor enhances the proinflammatory responses of human monocytes and neutrophils," *Journal of Immunology*, vol. 176, no. 5, pp. 3149–3156, 2006.

[96] K. Kim, J. H. Kim, J. Lee et al., "Nuclear factor of activated T cells c1 induces osteoclast-associated receptor gene expression during tumor necrosis factor-related activation-induced cytokine-mediated osteoclastogenesis," *The Journal of Biological Chemistry*, vol. 280, no. 42, pp. 35209–35216, 2005.

[97] A. D. Barrow, N. Raynal, T. L. Andersen et al., "OSCAR is a collagen receptor that costimulates osteoclastogenesis in DAP12-deficient humans and mice," *Journal of Clinical Investigation*, vol. 121, no. 9, pp. 3505–3516, 2011.

[98] N. Ndongo-Thiam, G. de Sallmard, J. Kastrup, and P. Miossec, "Levels of soluble osteoclast-associated receptor (sOSCAR) in rheumatoid arthritis: Link to disease severity and cardiovascular risk," *Annals of the Rheumatic Diseases*, vol. 73, no. 6, pp. 1276–1277, 2014.

[99] S. Zhao, Y.-Y. Guo, N. Ding, L.-L. Yang, and N. Zhang, "Changes in serum levels of soluble osteoclast-associated receptor in human rheumatoid arthritis," *Chinese Medical Journal*, vol. 124, no. 19, pp. 3058–3060, 2011.

[100] L. C. Chen, J. D. Laskin, M. K. Gordon, and D. L. Laskin, "Regulation of TREM expression in hepatic macrophages and endothelial cells during acute endotoxemia," *Experimental and Molecular Pathology*, vol. 84, no. 2, pp. 145–155, 2008.

[101] D.-Y. Chen, L. Yao, Y.-M. Chen et al., "A potential role of myeloid DAP12-associating lectin (MDL)-1 in the regulation of inflammation in rheumatoid arthritis patients," *PLoS ONE*, vol. 9, no. 1, Article ID e86105, 2014.

[102] A. R. Pettit, H. Weedon, M. Ahern et al., "Association of clinical, radiological and synovial immunopathological responses to anti-rheumatic treatment in rheumatoid arthritis," *Rheumatology*, vol. 40, no. 11, pp. 1243–1255, 2001.

[103] C. Goettsch, M. Rauner, K. Sinningen et al., "The Osteoclast-Associated Receptor (OSCAR) is a novel receptor regulated by oxidized low-density lipoprotein in human endothelial cells," *Endocrinology*, vol. 152, no. 12, pp. 4915–4926, 2011.

[104] T. Kobayashi, K. Yamamoto, N. Sugita et al., "Effective in vitro clearance of *Porphyromonas gingivalis* by Fc$\alpha$ receptor I (CD89) on gingival crevicular neutrophils," *Infection and Immunity*, vol. 69, no. 5, pp. 2935–2942, 2001.

[105] M. K. Andersson, P. Lundberg, A. Ohlin et al., "Effects on osteoclast and osteoblast activities in cultured mouse calvarial bones by synovial fluids from patients with a loose joint prosthesis and from osteoarthritis patients," *Arthritis Research and Therapy*, vol. 9, no. 1, article R18, 2007.

[106] F. McQueen and E. Naredo, "The "disconnect" between synovitis and erosion in rheumatoid arthritis: a result of treatment or intrinsic to the disease process itself?" *Annals of the Rheumatic Diseases*, vol. 70, no. 2, pp. 241–244, 2011.

[107] E. Villeneuve and B. Haraoui, "Uncoupling of disease activity and structural damage. Does it matter clinically?" *Annals of the Rheumatic Diseases*, vol. 72, no. 1, pp. 1–2, 2013.

[108] E. M. Ruderman, "Defining the value of structural inhibition: is low-dose etanercept inferior to the standard dose?" *Rheumatology*, vol. 51, no. 12, Article ID kes263, pp. 2118–2119, 2012.

[109] E. M. Shanahan, M. Smith, L. Roberts-Thomson, A. Esterman, and M. Ahern, "Influence of rheumatoid arthritis on work

participation in Australia," *Internal Medicine Journal*, vol. 38, no. 3, pp. 166–173, 2008.

[110] A. Finckh, N. Bansback, C. A. Marra et al., "Treatment of very early rheumatoid arthritis with symptomatic therapy, disease-modifying antirheumatic drugs, or biologic agents: a cost-effectiveness analysis," *Annals of Internal Medicine*, vol. 151, no. 9, pp. 612–621, 2009.

[111] J. S. Smolen and D. Aletaha, "Forget personalised medicine and focus on abating disease activity," *Annals of the Rheumatic Diseases*, vol. 72, no. 1, pp. 3–6, 2013.

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*Research Article*

# IL-12 Inhibits Lipopolysaccharide Stimulated Osteoclastogenesis in Mice

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Lipopolysaccharide (LPS) is related to osteoclastogenesis in osteolytic diseases. Interleukin- (IL-) 12 is an inflammatory cytokine that plays a critical role in host defense. In this study, we investigated the effects of IL-12 on LPS-induced osteoclastogenesis. LPS was administered with or without IL-12 into the supracalvariae of mice, and alterations in the calvarial suture were evaluated histochemically. The number of osteoclasts in the calvarial suture and the mRNA level of tartrate-resistant acid phosphatase (TRAP), an osteoclast marker, were lower in mice administered LPS with IL-12 than in mice administered LPS alone. The serum level of tartrate-resistant acid phosphatase 5b (TRACP 5b), a bone resorption marker, was also lower in mice administered LPS with IL-12 than in mice administered LPS alone. These results revealed that IL-12 might inhibit LPS-induced osteoclastogenesis and bone resorption. In TdT-mediated dUTP-biotin nick end-labeling (TUNEL) assays, apoptotic changes in cells were recognized in the calvarial suture in mice administered LPS with IL-12. Furthermore, the mRNA levels of both Fas and FasL were increased in mice administered LPS with IL-12. Taken together, the findings demonstrate that LPS-induced osteoclastogenesis is inhibited by IL-12 and that this might arise through apoptotic changes in osteoclastogenesis-related cells induced by Fas/FasL interactions.

## 1. Introduction

Lipopolysaccharide (LPS) is a large molecule consisting of a lipid and a polysaccharide joined by a covalent bond [1–4]. It is found in the cell walls of gram-negative bacteria and acts as an endotoxin. It induces a series of proinflammatory cytokines and results in the occurrence of strong immune responses. Osteolytic diseases such as periodontitis, osteomyelitis, and arthritis are related to LPS-induced immune reactions [5, 6]. Through binding to Toll-like receptor-4 on the surface of target cells, LPS induces the production of proinflammatory cytokines such as tumor necrosis factor- (TNF-) $\alpha$, interleukin- (IL-) 1, and IL-6 [7–11].

Osteoclasts are multinucleated giant cells that originate from hematopoietic stem cells [12, 13]. They play important roles in bone resorption and remodeling in association with a series of transcription factors and cytokines. In osteolytic diseases, the formation and activity of osteoclasts are exceptionally stimulated. Receptor activator of necrosis factor-$\kappa$B ligand (RANKL) and macrophage colony-stimulating factor are known as major cytokines for osteoclastogenesis. TNF-$\alpha$ is also related to osteoclastogenesis [14–16].

IL-12 was reported to inhibit osteoclast formation in spleen cell cultures *in vitro* [17]. We previously found that IL-12 inhibited TNF-$\alpha$-mediated osteoclastogenesis by inducing apoptosis of bone marrow cells *in vitro* [18, 19]. The induction of apoptosis was mediated by the interaction of TNF-$\alpha$-induced Fas and IL-12-induced FasL [18, 19]. IL-12 was also shown to inhibit TNF-$\alpha$-mediated osteoclastogenesis in the calvarial suture and during mechanical tooth movement *in*

*vivo* [20, 21]. We reported that apoptotic changes were recognized histochemically when osteoclastogenesis was inhibited [20, 21]. Nagata et al. [22] confirmed that RANKL-induced osteoclastogenesis was inhibited by IL-12 and concluded that IL-12 might not be involved in cell death. Thus, several investigators have reported that IL-12 is related to inhibition of TNF-$\alpha$ or RANKL-induced osteoclastogenesis.

The aim of this study was to investigate the effects of IL-12 on bacterial LPS-induced osteoclastogenesis and bone resorption.

## 2. Materials and Methods

*2.1. Mice and Reagents.* Male 8-week-old C57BL6/J mice were purchased from SLC (Shizuoka, Japan) for use in this study. All animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Nagasaki University with approval of the Institutional Animal Care and Use Committee. LPS from *Escherichia coli* was purchased from Sigma (St. Louis, MO). Recombinant mouse IL-12 was purchased from R&D Systems (Minneapolis, MN).

*2.2. LPS-Induced Osteoclastogenesis In Vivo.* It has been reported that daily injections of LPS (100 $\mu$g/day) for 5 days into the supracalvariae of mice were sufficient for osteoclast induction *in vivo* [23]. In this study, mice were divided into four groups and received daily injections of LPS alone (100 $\mu$g/day), LPS with IL-12 (1.5 $\mu$g/day), IL-12 alone (1.5 $\mu$g/day), or phosphate-buffered saline (PBS) as a control. After 5 days of administration, the mice were euthanized and the calvariae were immediately removed. After overnight fixation with 4% paraformaldehyde at 4°C, the calvariae were demineralized in 10% EDTA for 4 days at 4°C. Paraffin-embedded samples were sectioned at 4 $\mu$m. To observe osteoclasts, sections of the calvariae were deparaffinized, stained for tartrate-resistant acid phosphatase (TRAP), and counterstained with hematoxylin. The numbers of TRAP-positive cells in the calvarial suture were counted.

*2.3. Microfocal Computed Tomography Assessment.* The calvariae were fixed with paraformaldehyde and evaluated by microfocal computed tomography (RmCT; Rigaku, Tokyo, Japan) to clarify the bone resorption pits and calvarial suture expansion. Images of the calvariae were used for calculation of the radiolucent areas with ImageJ software (National Institutes of Health, Bethesda, MD). The relative values for the radiolucent areas in the groups were normalized by the value in the PBS group.

*2.4. Serum Tartrate-Resistant Acid Phosphatase 5b (TRACP 5b) Assay.* Serum was obtained from blood samples collected from the heart chambers under anesthesia. The serum levels of TRACP 5b were determined using a Mouse TRAP Assay Kit (IDS, Tyne and Wear, UK), in accordance with the manufacturer's protocol.

*2.5. RNA Preparation and Real-Time RT-PCR Analysis.* To isolate total RNA, mouse calvariae were frozen in liquid nitrogen, ground, and processed using an RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized from 1 $\mu$g of total RNA using reverse transcriptase (Toyobo, Osaka, Japan) and random primers (Invitrogen) in a final volume of 20 $\mu$L. The expression levels of TRAP, Fas, FasL, and TNF-$\alpha$ mRNAs were quantified by real-time RT-PCR using an Mx3000P/Mx3005P real-time PCR system (Stratagene, La Jolla, CA). Reactions were performed in a 25 $\mu$L volume containing 2 $\mu$L of cDNA, 12.5 $\mu$L of SYBR Premix Ex Taq (Takara, Shiga, Japan), 10 $\mu$M primers, and 0.5 $\mu$L of ROX Reference Dye II. The primers used were as follows: GAPDH, 5′-ACCCAGAAGACTGTGGATGG-3′ and 5′-CACATTGGGGGTAGGAACAC-3′; TRAP, 5′-AACTTGCGACCATTGTTAGC-3′ and 5′-GGGGACCTTTCGTTGATGT-3′; Fas, 5′-TGGCAGAGGAGCCTAGTTGT-3′ and 5′-CACACCCAGGAACAGTCCTT-3′; FasL, 5′-ATCCCTCTGGAATGGGAAGA-3′ and 5′-CCATATCTGTCCAGTAGTGC-3′; TNF-$\alpha$, 5′-CTGTAGCCCACGTCGTAGC-3′ and 5′-TTGAGATCCATGCCGTTG-3′. The cycling conditions were as follows: initial denaturation at 95°C for 10 s; 45 cycles of amplification, each comprising a denaturation step at 95°C for 5 s and an annealing step at 60°C for 20 s. The relative expression levels of TRAP, Fas, FasL, and TNF-$\alpha$ mRNAs were normalized by the corresponding expression levels of GAPDH mRNA.

*2.6. Apoptosis Detection by the TdT-Mediated dUTP-Biotin Nick End-Labeling (TUNEL) Assay.* An ApopTag Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA) was used for TUNEL staining. Deparaffinized sections were pretreated with 20 $\mu$g/mL proteinase K for 15 min and then incubated with 3% hydrogen peroxide for 5 min at room temperature to quench endogenous peroxidase activity. Next, the sections were sequentially incubated with TdT enzyme for 1 h at 37°C and antidigoxigenin peroxidase, followed by development with a diaminobenzidine peroxidase substrate. The sections were counterstained with methyl green.

*2.7. Statistical Analysis.* All data are presented as means ± SD. Statistical analyses were performed using Scheffe's $F$ tests. Differences were considered significant when $P < 0.05$.

## 3. Results

*3.1. IL-12 Inhibits LPS-Induced Osteoclastogenesis in the Mouse Calvariae.* To analyze the effects of IL-12 on LPS-induced osteoclastogenesis *in vivo*, TRAP staining of paraffin-embedded sections was performed after LPS was administered with or without IL-12 into mouse supracalvariae. In the LPS alone group, many TRAP-positive cells were observed in the calvarial suture (Figure 1(a)). On the other hand, there were few TRAP-positive cells in the LPS with IL-12 group. The number of TRAP-positive cells, counted as osteoclasts, was significantly reduced in the LPS with IL-12 group compared with the LPS alone group (Figure 1(b)). The levels of TRAP

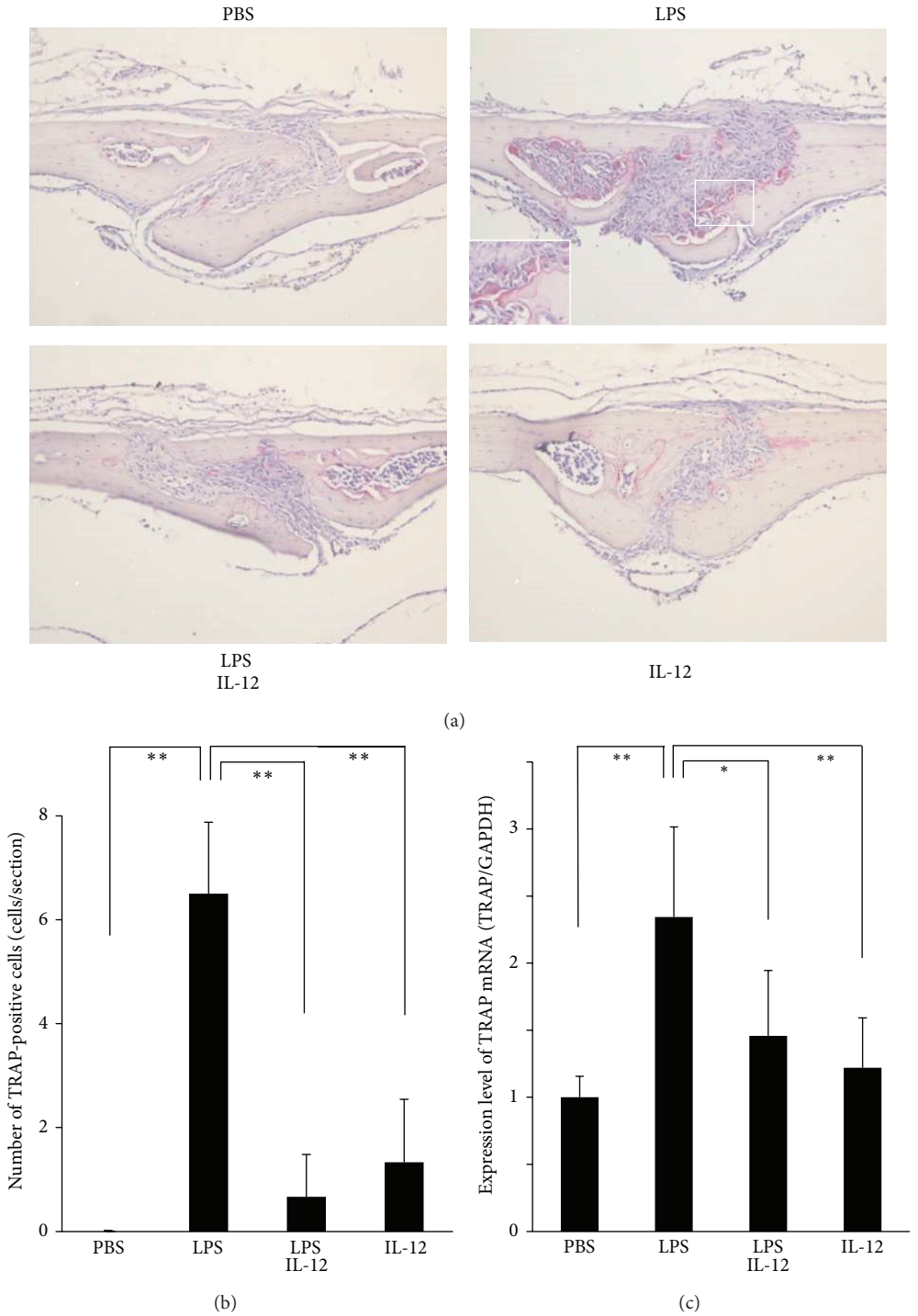


Figure 1: Osteoclastogenesis in the calvarial suture. (a) Histological sections of calvariae excised from mice after daily supracalvarial administrations of PBS, LPS alone (100 $\mu$g/day), LPS with IL-12 (1.5 $\mu$g/day), or IL-12 alone for 5 days. The sections were stained for TRAP activity. (b) Numbers of TRAP-positive cells in the calvarial suture. (c) TRAP mRNA levels in the mouse calvariae detected by real-time RT-PCR. Total RNA was isolated from the mouse calvariae after daily supracalvarial injections of PBS, LPS, LPS with IL-12, or IL-12 for 5 days. The TRAP mRNA levels were normalized by the corresponding GAPDH mRNA levels. Results are expressed as means ± SD ($N = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$). Differences were detected using Scheffe's $F$ tests.

mRNA in the calvariae were determined by real-time RT-PCR. The expression of TRAP was significantly higher in the LPS alone group than in the PBS group. Furthermore, the level of TRAP was significantly decreased in the LPS with IL-12 group compared with the LPS alone group (Figure 1(c)).

*3.2. IL-12 Inhibits LPS-Induced Bone Resorption in the Mouse Calvariae.* To investigate the effects of bone resorption by IL-12 on LPS-induced osteoclastogenesis, the radiolucent areas in calvariae were observed by RmCT after LPS was administered with or without IL-12 into mouse supracalvariae. In the LPS alone group, the radiolucent area was expanded on the mouse calvariae (Figures 2(a) and 2(b)). On the other hand, in the LPS with IL-12 group, the radiolucent area was decreased compared with the LPS alone group. Furthermore, the serum TRACP 5b levels were lower in the LPS with IL-12 group than in the LPS alone group (Figure 2(c)).

*3.3. Administration of LPS with IL-12 Induces Apoptosis in the Mouse Calvariae.* IL-12 was previously reported to induce apoptosis of osteoclast precursor cells in TNF-$\alpha$-induced osteoclast formation and inhibit osteoclast formation in TNF-$\alpha$-administered mice [18, 20]. Because osteoclastogenesis was decreased in the LPS with IL-12 group, we performed histological examinations with TUNEL staining to determine whether induction of apoptosis occurred in the calvarial suture. In the LPS alone group, few TUNEL-positive cells were observed. In comparison, many TUNEL-positive cells were observed in the LPS with IL-12 group (Figure 3).

*3.4. Administration of LPS and IL-12 Affects Expression of Fas and FasL.* To elucidate how apoptosis was induced in the mouse calvariae, the expression levels of Fas and FasL mRNAs were examined by real-time RT-PCR. The expression levels of Fas mRNA were significantly higher in the LPS alone and LPS with IL-12 groups than in the PBS group (Figure 4(a)). In addition, the expression levels of FasL mRNA were significantly higher in the LPS alone and LPS with IL-12 groups than in the PBS group (Figure 4(b)).

*3.5. LPS Induces Expression of TNF-α in the Mouse Calvariae.* The levels of TNF-$\alpha$ mRNA were examined by real-time RT-PCR to determine how apoptotic changes were induced in the calvarial suture. It is important to assess the levels of TNF-$\alpha$, because it is a key factor for osteoclastogenesis. The results indicated that the levels of TNF-$\alpha$ mRNA were increased in the LPS alone and LPS with IL-12 groups compared with the PBS group (Figure 5).

## 4. Discussion

In this study, we have demonstrated the effects of IL-12 on LPS-induced osteoclastogenesis in mouse calvariae *in vivo*. Previously, a number of investigators have examined LPS-mediated osteoclastogenesis [7, 23–25]. Among these reports, there were two *in vivo* studies in which LPS was administered especially into calvariae [23, 25]. The protocol, dose, and days of LPS administration were based on these reports. As shown by RmCT and histological images, daily injections of LPS (100 $\mu$g/day) for 5 days into the supracalvariae were sufficient for osteoclast induction in calvariae in this study. Loss of bone and expansion of the calvarial suture were observed after LPS administration.

Although a number of studies have investigated the functions of IL-12, there are few studies related to osteoclastogenesis. IL-12 is mainly produced by macrophages, dendritic cells, and B cells and induces cytotoxic properties of T cells and NK cells [26, 27]. One of these studies further showed that IL-12 plays a pivotal role in controlling innate and adaptive immunity against a variety of infections [26]. IL-12 particularly induces the production of interferon- (IFN-) $\gamma$, a potent activator of antimicrobial functions and tumor control, by T cells and NK cells. IL-12 can also induce the differentiation and proliferation of T-helper 1 (Th1) cells from Th0 cells [26]. Kerkar et al. [28] indicated that IL-12 triggers myeloid-derived cells sensitized for tumor destruction, while Eisenring et al. [29] showed that IL-12 induces tumor suppression by stimulating a subset of NKp46$^+$ lymphoid tissue-inducer cells. Thus, IL-12 is related to various immunological responses. Although some investigators have examined the expression of IL-12 during LPS-induced osteoclastogenesis [30, 31], no studies have clarified how IL-12 affects LPS-induced osteoclastogenesis. Therefore, we investigated the effects of IL-12 on LPS-induced osteoclastogenesis. LPS was administered with or without IL-12 into the mouse supracalvariae to evaluate how IL-12 affects LPS-induced osteoclastogenesis *in vivo*. In the LPS alone group, many osteoclasts and bone destruction spots were recognized in the calvarial suture. On the other hand, in the LPS with IL-12 group, osteoclasts and bone destruction spots were decreased. The levels of TRAP mRNA in the mouse calvariae were also decreased in the LPS with IL-12 group compared with the LPS alone group. These results suggested that IL-12 can inhibit LPS-mediated bone resorption.

LPS is known to induce proinflammatory cytokines [7–11]. TNF-$\alpha$, a proinflammatory cytokine, is related to osteoclastogenesis [14–16]. In the present study, when LPS was administered alone into the mouse calvariae, expression of TNF-$\alpha$ was increased at the mRNA level. TNF-$\alpha$ might be related to the loss of calvarial bone when LPS was administered alone. TNF-$\alpha$ was also increased when IL-12 was administered with LPS, compared with administration of PBS alone. Previous reports have shown that TNF-$\alpha$-induced osteoclastogenesis was inhibited by IL-12 *in vitro* and *in vivo* [18–21, 32, 33]. They concluded that osteoclastogenesis might be inhibited by apoptotic changes in osteoclast precursor cells and might be mediated by interactions between TNF-$\alpha$-upregulated Fas and IL-12-upregulated FasL. In this study, the expression level of Fas was increased in the LPS alone and LPS with IL-12 administered groups, and FasL was increased in the LPS with IL-12 and IL-12 alone administered groups. These findings indicated that the apoptotic changes in calvarial cells might be caused by interactions of TNF-$\alpha$ (induced by LPS) induced Fas and IL-12-induced FasL. Zhang et al. [34] showed that LPS increased Fas expression on memory B (mB) cells and caused mB cell apoptosis through the Fas/FasL pathway. In this study, LPS might, in part,

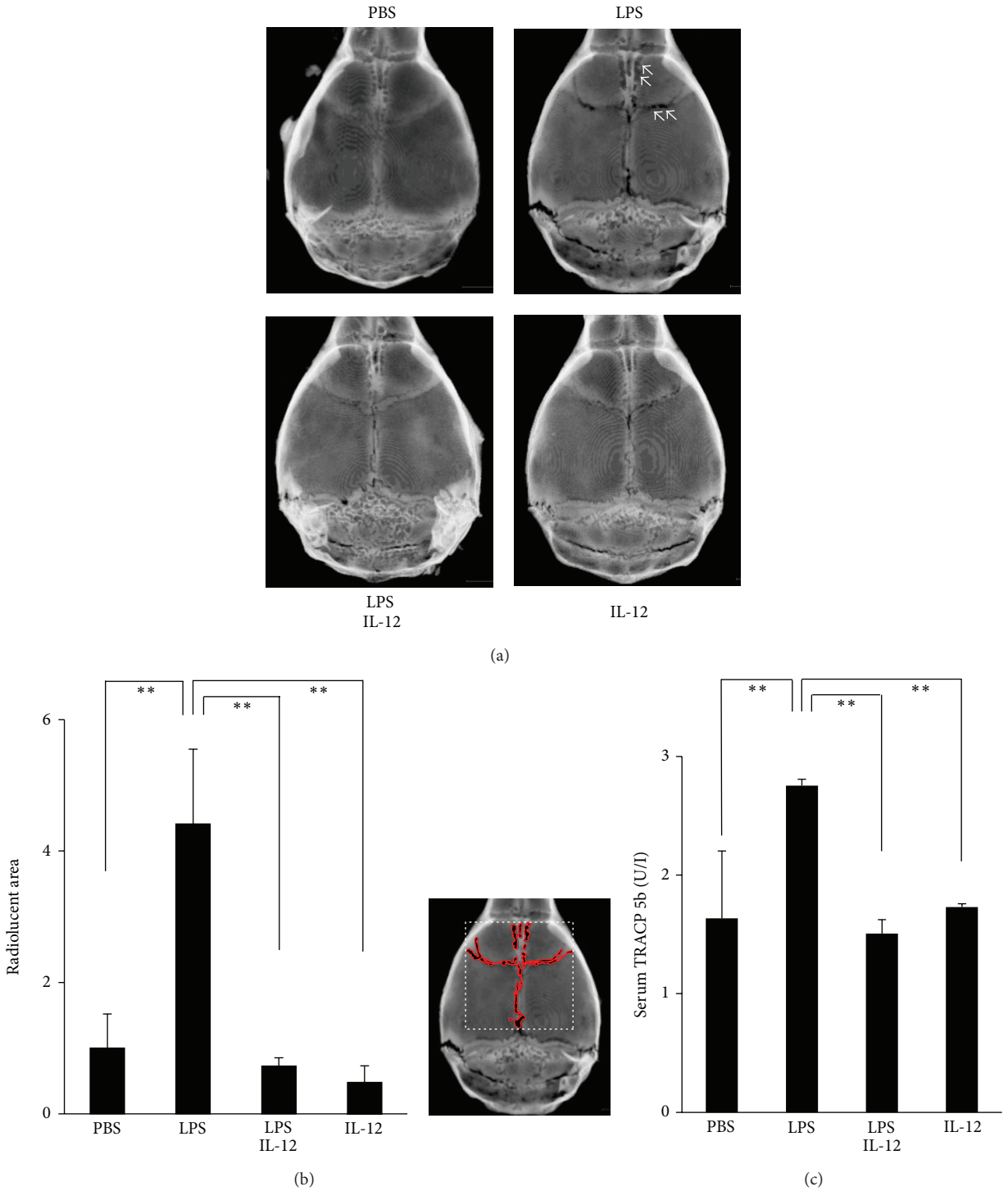


Figure 2: Radiolucent areas in the mouse calvariae. (a) Microfocal computed tomography reconstructed images of calvariae. Images of calvariae excised from mice after daily supracalvarial administrations of PBS, LPS alone (100 $\mu$g/day), LPS with IL-12 (1.5 $\mu$g/day), or IL-12 alone for 5 days. The arrows show the resorption lacunae. (b) Evaluation of the radiolucent areas on the calvariae. The radiolucent areas in the dotted boxed area shown in the right side of the graph were calculated. The red areas indicate the radiolucent areas. The relative values of the radiolucent areas in the groups were normalized by the corresponding values in the PBS group. (c) Serum levels of TRACP 5b. Circulating TRACP 5b levels were determined by ELISA. Results are expressed as means ± SD ($N = 9$; $^{**}P < 0.01$). Differences were detected using Scheffe's $F$ tests.

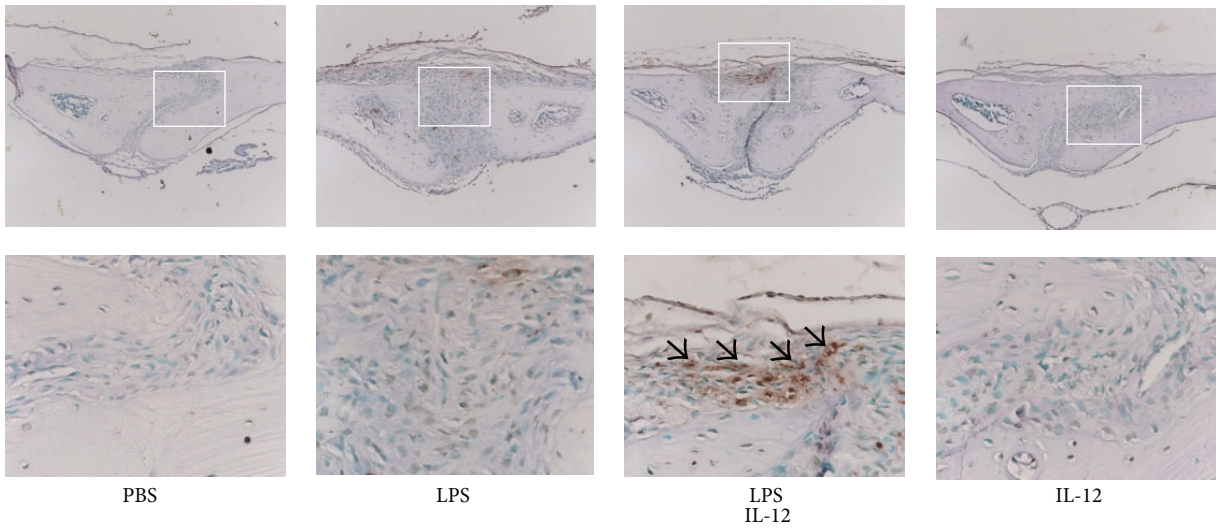


Figure 3: Induction of apoptosis by LPS and IL-12 in the mouse calvariae. Histological sections of calvariae excised from mice after daily supracalvarial injections of PBS, LPS alone (100 $\mu$g/day), LPS with IL-12, or LPS alone (1.5 $\mu$g/day) for 5 days were subjected to TUNEL staining to detect apoptotic cells. The lower panels show high-magnification images of the boxed areas in the upper panels. Apoptotic cells are indicated by arrows.

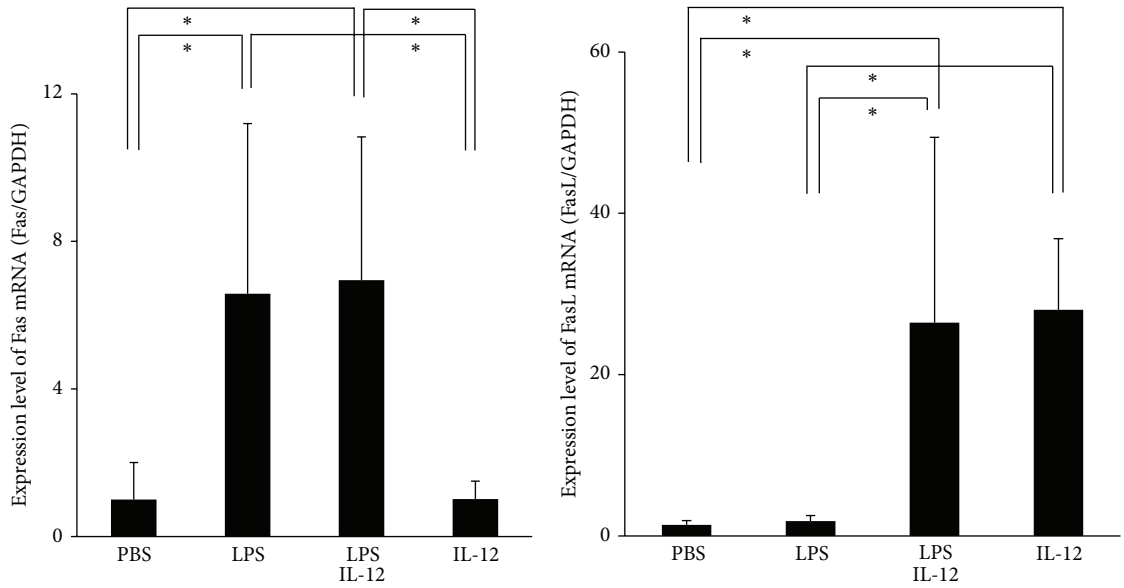


Figure 4: Fas and FasL mRNA levels in the mouse calvariae detected by real-time RT-PCR. Total RNA was isolated from mice calvariae after daily supracalvarial injections of PBS, LPS alone (100 $\mu$g/day), LPS with IL-12 (1.5 $\mu$g/day), or IL-12 alone for 5 days. (a) Fas mRNA levels. (b) FasL mRNA levels. The mRNA levels of Fas and FasL were normalized by the corresponding GAPDH mRNA levels. Results are expressed as means ± SD ($N = 6$; $^*P < 0.05$). Differences were detected using Scheffe's $F$ tests.

be directly related to Fas/FasL interactions. Yim et al. [35] reported that IL-12 has the ability to induce macrophage apoptosis by IFN-$\gamma$-induced nitric oxide synthesis. IL-12 is able to induce tumor apoptosis in mouse hepatocellular carcinoma *in vivo* [36]. The IL-12-related apoptotic changes in tumor cells are mediated by T lymphocytes, NK cells, and NKT cells. The authors mentioned the involvement of IFN-$\gamma$ in their report. Nagata et al. [22] previously demonstrated that RANKL-induced osteoclastogenesis is possibly inhibited by IFN-$\gamma$, which was induced by IL-12 *in vitro*. Thus, IFN-$\gamma$ is possibly also associated with the IL-12-mediated inhibition of osteoclastogenesis. However, IFN-$\gamma$ was not increased at the mRNA level in the groups administered IL-12 in this study (data not shown). Therefore, the inhibition of osteoclastogenesis observed when IL-12 was administered with LPS in this study might not be related to IFN-$\gamma$. Yang et al.

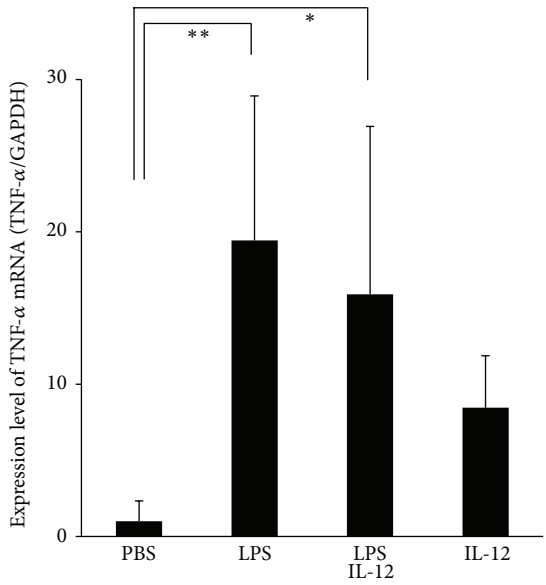


FIGURE 5: TNF-$\alpha$ mRNA levels in the mouse calvariae detected by real-time RT-PCR. Total RNA was isolated from mice calvariae after daily supracalvarial injections of PBS, LPS alone (100 $\mu$g/day), LPS with IL-12 (1.5 $\mu$g/day), or IL-12 alone for 5 days. The mRNA levels of TNF-$\alpha$ were normalized by the corresponding GAPDH mRNA levels. Results are expressed as means ± SD ($N = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$). Differences were detected using Scheffe's $F$ tests.

[37] reported that LPS induces osteoclastogenesis through the induction of RANKL expression in osteoblasts. It means that RANKL is related to LPS-induced osteoclastogenesis. It has also been reported previously that IL-12 inhibited RANKL-induced osteoclastogenesis via a nonapoptotic pathway [22]. Therefore, it is possible that IL-12 may also inhibit osteoclastogenesis mediated by LPS-induced RANKL expression. It is possible that several complicating factors could be responsible for the occurrence of apoptosis in osteoclastogenesis-related cells. Additionally, the identity of the apoptotic cells is also not clear at this stage. Further studies are required to clarify these points.

In summary, our study has demonstrated that IL-12 inhibits LPS-induced osteoclastogenesis *in vivo*. Furthermore, the mRNA levels of Fas and FasL were both increased in mice administered LPS with IL-12 and it might lead to apoptotic changes in osteoclastogenesis-related cells through Fas/FasL interactions.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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## References

[1] M. J. Osborn, S. M. Rosen, L. Rothfield, L. D. Zeleznick, and B. L. Horecker, "Lipopolysaccharide of the gram-negative cell wall," *Science*, vol. 145, no. 3634, pp. 783–789, 1964.

[2] J. Cohen, "The immunopathogenesis of sepsis," *Nature*, vol. 420, no. 6917, pp. 885–891, 2002.

[3] F. Laugerette, C. Vors, N. Peretti, and M. C. Michalski, "Complex links between dietary lipids, endogenous endotoxins and metabolic inflammation," *Biochimie*, vol. 93, no. 1, pp. 39–45, 2011.

[4] Y. Tan and J. C. Kagan, "A cross-disciplinary perspective on the innate immune responses to bacterial lipopolysaccharide," *Molecular Cell*, vol. 54, no. 2, pp. 212–223, 2014.

[5] M. A. Taubman, P. Valverde, X. Han, and T. Kawai, "Immune response: the key to bone resorption in periodontal disease," *Journal of Periodontology*, vol. 76, no. 11, supplement, pp. 2033–2041, 2005.

[6] M. C. Walsh, N. Kim, Y. Kadono et al., "Osteoimmunology: interplay between the immune system and bone metabolism," *Annual Review of Immunology*, vol. 24, pp. 33–63, 2006.

[7] Y. Abu-Amer, F. P. Ross, J. Edwards, and S. L. Teitelbaum, "Lipopolysaccharide-stimulated osteoclastogenesis is mediated by tumor necrosis factor via its P55 receptor," *The Journal of Clinical Investigation*, vol. 100, no. 6, pp. 1557–1565, 1997.

[8] N. Ueda, M. Koide, M. Ohguchi et al., "Involvement of prostaglandin $E_2$ and interleukin-1$\alpha$ in the differentiation and survival of osteoclasts induced by lipopolysaccharide from *Actinobacillus actinomycetemcomitans* Y4," *Journal of Periodontal Research*, vol. 33, no. 8, pp. 509–516, 1998.

[9] T. Kikuchi, T. Matsuguchi, N. Tsuboi et al., "Gene expression of osteoclast differentiation factor is induced by lipopolysaccharide in mouse osteoblasts via toll-like receptors," *The Journal of Immunology*, vol. 166, no. 5, pp. 3574–3579, 2001.

[10] K. Itoh, N. Udagawa, K. Kobayashi et al., "Lipopolysaccharide promotes the survival of osteoclasts via toll-like receptor 4, but cytokine production of osteoclasts in response to lipopolysaccharide is different from that of macrophages," *The Journal of Immunology*, vol. 170, no. 7, pp. 3688–3695, 2003.

[11] Z. Bar-Shavit, "Taking a toll on the bones: regulation of bone metabolism by innate immune regulators," *Autoimmunity*, vol. 41, no. 3, pp. 195–203, 2008.

[12] S. L. Teitelbaum, "Bone resorption by osteoclasts," *Science*, vol. 289, no. 5484, pp. 1504–1508, 2000.

[13] W. J. Boyle, W. S. Simonet, and D. L. Lacey, "Osteoclast differentiation and activation," *Nature*, vol. 423, no. 6937, pp. 337–342, 2003.

[14] Y. Azuma, K. Kaji, R. Katogi, S. Takeshita, and A. Kudo, "Tumor necrosis factor-$\alpha$ induces differentiation of and bone resorption by osteoclasts," *Journal of Biological Chemistry*, vol. 275, no. 7, pp. 4858–4864, 2000.

[15] K. Kobayashi, N. Takahashi, E. Jimi et al., "Tumor necrosis factor alpha stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction," *The Journal of Experimental Medicine*, vol. 191, no. 2, pp. 275–285, 2000.

[16] N. Kim, Y. Kadono, M. Takami et al., "Osteoclast differentiation independent of the TRANCE-RANK-TRAF6 axis," *The Journal of Experimental Medicine*, vol. 202, no. 5, pp. 589–595, 2005.

[17] N. J. Horwood, J. Elliott, T. J. Martin, and M. T. Gillespie, "IL-12 alone and in synergy with IL-18 inhibits osteoclast formation in

vitro," *The Journal of Immunology*, vol. 166, no. 8, pp. 4915–4921, 2001.

[18] H. Kitaura, N. Nagata, Y. Fujimura, H. Hotokezaka, N. Yoshida, and K. Nakayama, "Effect of IL-12 on TNF-$\alpha$-mediated osteoclast formation in bone marrow cells: apoptosis mediated by Fas/Fas ligand interaction," *The Journal of Immunology*, vol. 169, no. 9, pp. 4732–4738, 2002.

[19] H. Kitaura, M. Tatamiya, N. Nagata et al., "IL-18 induces apoptosis of adherent bone marrow cells in TNF-$\alpha$ mediated osteoclast formation in synergy with IL-12," *Immunology Letters*, vol. 107, no. 1, pp. 22–31, 2006.

[20] M. Yoshimatsu, H. Kitaura, Y. Fujimura et al., "IL-12 inhibits TNF-$\alpha$ induced osteoclastogenesis via a T cell-independent mechanism *in vivo*," *Bone*, vol. 45, no. 5, pp. 1010–1016, 2009.

[21] M. Yoshimatsu, H. Kitaura, Y. Fujimura et al., "Inhibitory effects of IL-12 on experimental tooth movement and root resorption in mice," *Archives of Oral Biology*, vol. 57, no. 1, pp. 36–43, 2012.

[22] N. Nagata, H. Kitaura, N. Yoshida, and K. Nakayama, "Inhibition of RANKL-induced osteoclast formation in mouse bone marrow cells by IL-12: involvement of IFN-$\gamma$ possibly induced from non-T cell population," *Bone*, vol. 33, no. 4, pp. 721–732, 2003.

[23] K. Kimura, H. Kitaura, T. Fujii, Z. W. Hakami, and T. Takano-Yamamoto, "Anti-c-Fms antibody inhibits lipopolysaccharide-induced osteoclastogenesis *in vivo*," *FEMS Immunology & Medical Microbiology*, vol. 64, no. 2, pp. 219–227, 2012.

[24] J. J. Zambon, J. Slots, K. Miyasaki et al., "Purification and characterization of the serotype *c* antigen from *Actinobacillus actinomycetemcomitans*," *Infection and Immunity*, vol. 44, no. 1, pp. 22–27, 1984.

[25] A. Hussain Mian, H. Saito, N. Alles, H. Shimokawa, K. Aoki, and K. Ohya, "Lipopolysaccharide-induced bone resorption is increased in TNF type 2 receptor-deficient mice *in vivo*," *Journal of Bone and Mineral Metabolism*, vol. 26, no. 5, pp. 469–477, 2008.

[26] G. Trinchieri, "Interleukin-12 and the regulation of innate resistance and adaptive immunity," *Nature Reviews Immunology*, vol. 3, no. 2, pp. 133–146, 2003.

[27] D. A. Vignali and V. K. Kuchroo, "IL-12 family cytokines: immunological playmakers," *Nature Immunology*, vol. 13, no. 8, pp. 722–728, 2012.

[28] S. P. Kerkar, R. S. Goldszmid, P. Muranski et al., "IL-12 triggers a programmatic change in dysfunctional myeloid-derived cells within mouse tumors," *Journal of Clinical Investigation*, vol. 121, no. 12, pp. 4746–4757, 2011.

[29] M. Eisenring, J. vom Berg, G. Kristiansen, E. Saller, and B. Becher, "IL-12 initiates tumor rejection via lymphoid tissue-inducer cells bearing the natural cytotoxicity receptor NKp46," *Nature Immunology*, vol. 11, no. 11, pp. 1030–1038, 2010.

[30] M. A. Islam, M. Pröll, M. Hölker et al., "Alveolar macrophage phagocytic activity is enhanced with LPS priming, and combined stimulation of LPS and lipoteichoic acid synergistically induce pro-inflammatory cytokines in pigs," *Innate Immunity*, vol. 19, no. 6, pp. 631–643, 2013.

[31] L. M. Shaddox, P. F. Gonçalves, A. Vovk et al., "LPS-induced inflammatory response after therapy of aggressive periodontitis," *Journal of Dental Research*, vol. 92, no. 8, pp. 702–708, 2013.

[32] H. Kitaura, K. Kimura, M. Ishida, H. Kohara, M. Yoshimatsu, and T. Takano-Yamamoto, "Immunological reaction in TNF-$\alpha$-mediated osteoclast formation and bone resorption *in vitro* and *in vivo*," *Clinical and Developmental Immunology*, vol. 2013, Article ID 181849, 8 pages, 2013.

[33] H. Kitaura, K. Kimura, M. Ishida et al., "Effect of cytokines on osteoclast formation and bone resorption during mechanical force loading of the periodontal membrane," *The Scientific World Journal*, vol. 2014, Article ID 617032, 7 pages, 2014.

[34] L. Zhang, Z. Luo, S. F. Sieg et al., "lasmacytoid dendritic cells mediate synergistic effects of HIV and LPS on $CD27^{+}IgD^{-}$ memory B cell apoptosis," *Journal of Virology*, vol. 88, no. 19, pp. 11430–11441, 2014.

[35] J. Y. Yim, S. J. Yang, J. M. Yim et al., "Lymphocyte-mediated macrophage apoptosis during IL-12 stimulation," *Cytokine*, vol. 64, no. 1, pp. 62–70, 2013.

[36] J. R. Rodriguez-Madoz, M. Zabala, M. Alfaro, J. Prieto, M. G. Kramer, and C. Smerdou, "Short-term intratumoral interleukin-12 expressed from an alphaviral vector is sufficient to induce an efficient antitumoral response against spontaneous hepatocellular carcinomas," *Human Gene Therapy*, vol. 25, no. 2, pp. 132–143, 2014.

[37] S. Yang, N. Takahashi, T. Yamashita et al., "Muramyl dipeptide enhances osteoclast formation induced by lipopolysaccharide, IL-1$\alpha$, and TNF-$\alpha$ through nucleotide-binding oligomerization domain 2-mediated signaling in osteoblasts," *The Journal of Immunology*, vol. 175, no. 3, pp. 1956–1964, 2005.

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*Research Article*

# The Alteration and Clinical Significance of Th22/Th17/Th1 Cells in Patients with Chronic Myeloid Leukemia

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T helper- (Th-) cell immunodeficiency plays important roles in tumor development and their effects in chronic myeloid leukemia (CML) remain unclear. In the present study, we mainly investigated the role of Th22, Th17, and Th1 cell and their related cytokines (IL-22, IL-17, and IFN-r) in the pathophysiology of CML. Bone marrow (BM) and peripheral blood (PB) were extracted from newly diagnosed (ND), chronic phase- (CP-) CML patients, and controls. Th subsets were examined by flow cytometry. Plasma IL-22, IL-17, and IFN-r concentrations were measured by ELISA. AHR and RORC mRNA expressions were examined by RT-PCR. The frequencies of Th22, Th17, and Th1 cells, along with the expression of specific transcription factors RORC and AHR, were significantly decreased in ND patients compared with healthy controls, while all these abnormality recovered in CP patients. In addition, there existed a significantly positive relationship between Th22 and Th17 cells in PB or BM. A significantly negative relationship was found between Th cells (Th22, Th17, or Th1) and BCR-ABL (%) IS or the number of PB white blood cells. All these results demonstrated that Th22, Th17, and Th1 cells might be important therapeutic targets in CML and could facilitate a better outcome for tumor immunotherapy.

## 1. Introduction

Chronic myeloid leukemia (CML) is a malignant hematopoietic stem cell disease characterized by the presence of Philadelphia chromosome, t(9:22)(q34:q11), resulting in the BCR-ABL fusion gene which encodes for a constitutively activated tyrosine kinase [1]. BCR-ABL gene is considered as the molecular basis of the pathogenesis of CML and as an effective indicator of diagnosis and prognosis [2]. Although the disease can be readily controlled by the introduction of ABL tyrosine kinase inhibitors (TKI), approximately one-third of patients show no response to this treatment, which seems to be associated with ABL mutation related drug resistance and the blast crisis [3, 4]. It is widely accepted that both genetic and immune factors play significant roles in the development of CML. Immune status in CML is very complex and ill-defined, and the roles of immune factors in CML have received increasing attention in recent years [5]. However, little is known about the immunopathological events, especially the abnormal T helper (Th) subsets, in the pathophysiology of CML.

Th cells play critical roles in the development and progression of inflammatory and autoimmune diseases and tumors. Th17 cells and Th22 cells are two newly described Th subsets, which have important roles in peripheral immune responses. Th17 is a unique $CD4^+$ Th subset characterized by production of interleukin-17 (IL-17). Th17 cells may have evolved for host protection against microbes that Th1 or Th2 immunity are not well suited for, such as extracellular bacteria and some fungi. IL-17 is a highly inflammatory cytokine with robust effects on stromal cells in many tissues [6]. Recent data in humans and mice suggest that Th17 cells play an important role in the pathogenesis of a diverse group of immune-mediated diseases as well as in tumor. However, the role of Th17 in cancer is still being intensively discussed, with conflicting reports related to the protumoral versus antitumoral effects of these cells [7]. Recently, a novel subset of $CD4^+$ Th cells, IL-22 producing T helper cells (Th22 cells), has been identified and

Table 1: The characteristics of subjects.

| | ND CML ($n = 31$) | CP-CML ($n = 32$) | Healthy control ($n = 33$) |
|---|---|---|---|
| Age (years) | 48.25 ± 14.99 | 46.22 ± 13.73 | 37.00 ± 10.28 |
| Gender (male/female) | 18/13 | 19/13 | 20/13 |
| WBC ($*10^9$/L) | 189.74 ± 116.37 | 6.10 ± 1.69 | 6.37 ± 2.52 |
| HB (g/L) | 103.93 ± 24.26 | 134.33 ± 17.55 | 128.25 ± 14.35 |
| PLT ($*10^9$/L) | 505.12 ± 323.68 | 209.16 ± 91.25 | 215.25 ± 115.64 |
| BCR-ABL (%) IS | 61.16 (27.51–183.9) | 0.056 (0.000–28.85) | |

ND: newly diagnosed; CP-CML: chronic phase-CML; WBC: white blood cell; RBC: red blood cell; HB: hemoglobin; PLT: platelet.

showed to challenge the classical Th1/Th2 paradigm [8, 9]. Th22 cells are inflammatory $CD4^+$ T cells that secrete IL-22 but do not express IL-17 or interferon-gamma (IFN-$\gamma$). Th22 cells have been shown to be important in the pathogenesis of many autoimmunity diseases. The discovery of Th17 and Th22 cells has opened up a new avenue for research into the etiology and treatment of a broad spectrum of diseases.

Recent studies have implicated the roles of Th17 and Th22 cells and their effector cytokines in the pathogenesis of several autoimmune diseases and solid tumors in humans, such as Crohn's disease, gastric cancer, and lung cancer [10]. Recent studies also reported that Th17 and Th22 cells may play vital roles in bone related diseases. Zhang et al. showed that the frequencies of both Th22 cells and Th17 cells were elevated in PB from patients with ankylosing spondylitis (AS) and rheumatoid arthritis (RA). These findings suggest that Th22 cells and Th17 cells may be implicated in the pathogenesis of AS and RA [11]. In another study, Benham et al. indicated that elevated frequencies of IL-17 and IL-22 producing $CD4^+$ T cells were a feature of psoriatic arthritis [12]. CML is a kind of myeloproliferative clonal disorder of stem cell; extramedullary disease during chronic phase and blast crisis has been documented. CML in chronic phase can very rarely cause destructive bone lesions both in adults and in children and osteolytic lesions in chronic phase of CML is usually considered an impending blast crisis [13].

However, little is known regarding the roles of Th17 and Th22 cells in hematological malignancy. To date, no previous study has reported data about the immunobiology of Th subsets especially Th22, Th17, and Th1 cells in CML.

In this study, we measured the frequencies of Th cells (Th1, Th17, and Th22), the expression of their transcription factors (RORC and AHR) and their related cytokines (IFN-$\gamma$, IL-17, and IL-22) in PB and BM of CML patients. We also evaluated their correlations with clinical pathological characteristics.

## 2. Materials and Methods

*2.1. Subjects and Ethics Statement.* A total of 63 CML patients (26 females and 37 males; age range, 20–85 years; mean age, 47.16 ± 14.25 years) were enrolled in this study. CML patients were diagnosed according to the World Health Organization (WHO) classification [14]. Thirty-one of them were ND CML patients (13 females and 18 males; age range, 20–85 years; mean age 48.25 ± 14.99 years), and thirty-two were CP-CML patients (13 females and 19 males; age range, 22–73 years; median age 46.22 ± 13.73 years). Twenty-two age-matched healthy PB individuals (9 females and 13 males; age range 19–52 years; median age, 37.13 ± 10.22 years) were included in the study. Eleven hematologically normal age-matched BM transplant donors (4 females, 7 males; age range 21–58 years; median age, 36.63 ± 10.94 years) were used as BM controls. All cases of CP-CML were treated with imatinib mesylate. The demographic and key clinical features of CML patients are listed in Table 1. Patients with diabetes, hypertension, cardiovascular diseases, pregnant, active or chronic infection, or connective tissue diseases were excluded. Enrollment occurred between October 2013 and September 2014 in the Department of Hematology of Qilu Hospital, Shandong University (Jinan, China). Our research was approved by the Medical Ethical Committee of Qilu Hospital, Shandong University. Informed consent was obtained from all patients before enrollment in the study in accordance with the Declaration of Helsinki.

*2.2. Flow Cytometric Analysis of Th22, Th17, and Th1 Cells.* Intracellular cytokines were studied by flow cytometry to reflex the cytokine-producing cells. Briefly, heparinized whole blood (100 $\mu$l) with an equal volume of Roswell Park Memorial Institute- (RPMI-) 1640 medium was incubated for 4 h at 37°C in 5% $CO_2$ in the presence of 2.5 ng/mL of phorbol myristate acetate (PMA), 1 mg/mL of ionomycin, and 1.7 mg/mL of monensin (all from Alexis Biochemicals, San Diego, CA, USA). PMA and ionomycin are pharmacologic T-cell activating agents that mimic signals generated by the T-cell receptor (TCR) complex and have the advantage of stimulating T cells of any antigen specificity. Monensin was used to block the intracellular transport mechanisms, thereby leading to an accumulation of cytokines in the cells. After incubation, the cells were stained with PE-CY5 conjugated anti-human CD4 monoclonal antibody at room temperature in the dark for 20 min. The cells were next stained with FITC-conjugated anti-interferon- (IFN-) $\gamma$ monoclonal antibody, Alexa Fluor 647 conjugated anti-IL-17A monoclonal antibody, and PE-conjugated anti-IL22 monoclonal antibody after fixation and permeabilization. All the antibodies were purchased from eBioscience, San Diego, CA, USA. Isotype controls were given to enable correct compensation and confirm antibody specificity. Fix-Perm reagents were from Invitrogen (Carlsbad, CA, USA). All samples were assayed using BD FACS Calibur Flow Cytometer. Data were analyzed with FlowJo 7.6.2.

*2.3. Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR).* The total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Approximately, 1 $\mu$g of total RNA from each sample was used to synthesize cDNA with Prime Script RT reagent Kit (Takara Bio Inc., Dalian, China). Reverse transcription reaction was done at 37°C for 15 min, followed by 85°C for 5 s. Real-time quantitative PCR was conducted using an ABI Prism 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions. The real-time PCR contained, in a final volume of 10 $\mu$L, 5 $\mu$L of 2x SYBR Green Real-time PCR Master Mix, 1 $\mu$L of cDNA, 3.2 $\mu$L of $ddH_2O$, and 0.4 $\mu$L of the forward and reverse primers. The primers were shown as follows: RORC Forward 5′-CAA TGG AAG TGG TGC TGG TTA G-3′, Reverse 5′-GGG AGT GGG AGA AGT CAA AGA T-3′; AHR Forward 5′-CAA ATC CTT CCA AGC GGC ATA-3′, Reverse 5′-CGC TGA GCC TAA GAA CTG AAA G-3′. All experiments were conducted in triplicate. The PCR products were analyzed by melt curve analysis and agarose gel electrophoresis to determine product size and to confirm that no byproducts were formed. The results were expressed relative to the number of GAPDH transcripts used as an internal control. GAPDH was analyzed using the following primers: Forward 5′-GCT CTC TGC TCC TCC TGT TC-3′ and Reverse 5′-GTT GAC TCC GAC CTT CAC CT-3′. Relative gene expression level (the amount of target, normalized to endogenous control gene) was calculated using the comparative Ct method formula $2^{-\Delta Ct}$.

*2.4. IFN-γ, IL-17A, and IL-22 Enzyme-Linked Immunosorbent Assay.* PB and BM were collected into heparin-anticoagulant vacutainer tubes. Plasma was obtained by centrifugation and stored at −80°C for determination of cytokines. The levels of IFN-$\gamma$ (Cat: BMS228), IL-17A (Cat: BMS2017), and IL-22 (Cat: BMS2047) were measured by enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions (eBioscience, San Diego, CA). The lower detection limits were as follows: IFN-$\gamma$, 0.99 pg/mL; IL-17, 0.5 pg/mL; IL-22, 5 pg/mL.

*2.5. Statistical Analysis.* Results were expressed as mean ± SD or median (range). Statistical significance of Th subset cells, plasma cytokines, and transcription factors was determined by ANOVA, and difference between two groups was determined by Newman–Keuls multiple comparison test ($q$ test) unless the data were not normally distributed, in which case Kruskal-Wallis test ($H$ test) and Nemenyi test were used. The Pearson or Spearman correlation test was used for correlation analysis depending on data distribution. All tests were performed by SPSS 13.0 system. $P$ value less than 0.05 was considered statistically significant.

## 3. Results

*3.1. Th22 Cells Were Decreased in ND CML Patients and Recovered after the Treatment with TKI.* We analyzed the frequency of PB or BM Th22 ($CD4^+$ $IL\text{-}22^+$, $IL\text{-}17^-$, and $IFN\gamma^-$) cells based on cytokine patterns after in vitro stimulation by PMA plus ionomycin in short-term cultures (Figure 1). As shown in Figures 2(a) and 2(b), the frequencies of PB or BM Th22 cells were significantly decreased in ND CML patients (0.42 ± 0.26% or 0.57 ± 0.38%; $^*P = 0.005$ or $^*P = 0.037$) compared to healthy controls (2.92 ± 1.65% or 1.85 ± 0.66%). Both PB and BM Th22 cells frequencies (3.53 ± 2.94%, 2.17 ± 1.17%; $^*P = 0.029$, $^*P = 0.009$, resp.) in CP-CML patients were statistically increased compared to ND CML patients. Although the PB and BM Th22 cells frequencies in CP-CML patients were higher than those in healthy controls, no statistical significance was observed. Meanwhile, we compared the Th22 cells between PB and B, and no difference was observed in ND or CP-CML patients. The levels of IL-22 in PB or BM of CML patients were measured by ELISA. PB IL-22 levels in CP (101.12 ± 18.29 pg/mL) patients were slightly higher than controls (83.14 ± 14.85 pg/mL; $^*P = 0.001$) (Figure 3(a)). As for the levels of BM IL-22, there was no significant difference between each group (ND: 96.32 ± 26.44 pg/mL; CP: 92.67 ± 17.95 pg/mL, and controls: 89.99 ± 24.77 pg/mL) (Figure 3(b)). No correlation was found between Th22 frequencies and the levels of IL-22 in CML patients.

*3.2. Th17 Cells Were Declined in ND CML Patients and Increased in CP-CML Patients.* Similar to the findings of Th22 cells, the frequencies of PB or BM Th17 ($CD4^+$ $IL\text{-}17^+$) cells (Figure 1) were profoundly decreased (Figures 2(d) and 2(e)) in ND CML patients (0.82 ± 0.80% or 0.99 ± 0.60%; $^*P = 0.029$ or $^*P = 0.008$) compared with healthy controls (3.52 ± 2.28% or 2.23 ± 0.66%). A significant increase was also observed in CP patients (5.56 ± 3.40% or 3.03 ± 1.36%; $^*P = 0.005$ or $^*P = 0.000$) compared with in ND patients. We also compared Th17 cells between PB and BM and no statistical significance was found in CML patients (Figure 2(f)). The levels of PB IL-17 (Figure 3(c)) in ND (1.45 ± 0.21 pg/mL, $P = 0.02$) or CP (1.39 ± 0.22 pg/mL, $^*P = 0.01$) patients were increased than controls (1.19 ± 0.17 pg/mL), while no significant difference was observed between ND and CP patients ($P = 0.437$). There was no significant difference between each BM group (ND: 1.56 ± 0.22 pg/m, CP: 1.42 ± 0.10 pg/mL, controls: 1.50 ± 0.61 pg/mL) (Figure 3(d)). No correlation was identified between Th17 frequency and IL-17 level.

*3.3. Abnormal Th1 Cells in CML Patients.* Compared with PB or BM Th1 ($CD4^+$ $IFN\text{-}\gamma^+$) cells frequencies in healthy controls (19.68 ± 7.38% or 17.61 ± 7.96%), there was an observable decrease in ND patients (3.15 ± 1.92% or 5.66 ± 4.72%; $^*P = 0.001$ or $^*P = 0.02$). Similarly, both PB and BM Th1 cells frequencies in CP patients (23.00 ± 6.1284%, 20.22 ± 9.49%, $^*P = 0.001$, $^*P = 0.01$, resp.) were significantly increased than in ND patients. Nevertheless, there was no difference between CP patients and healthy controls (Figures 2(g) and 2(h)). A statistical increase of BM IFN-$\gamma$ level was found in ND (4.21 ± 0.71 pg/mL) patients compared with in controls (3.34 ± 0.43 pg/mL, $^*P = 0.015$). In addition, no statistical significance was found between ND and CP-CML patients ($P = 0.203$). There was no significant

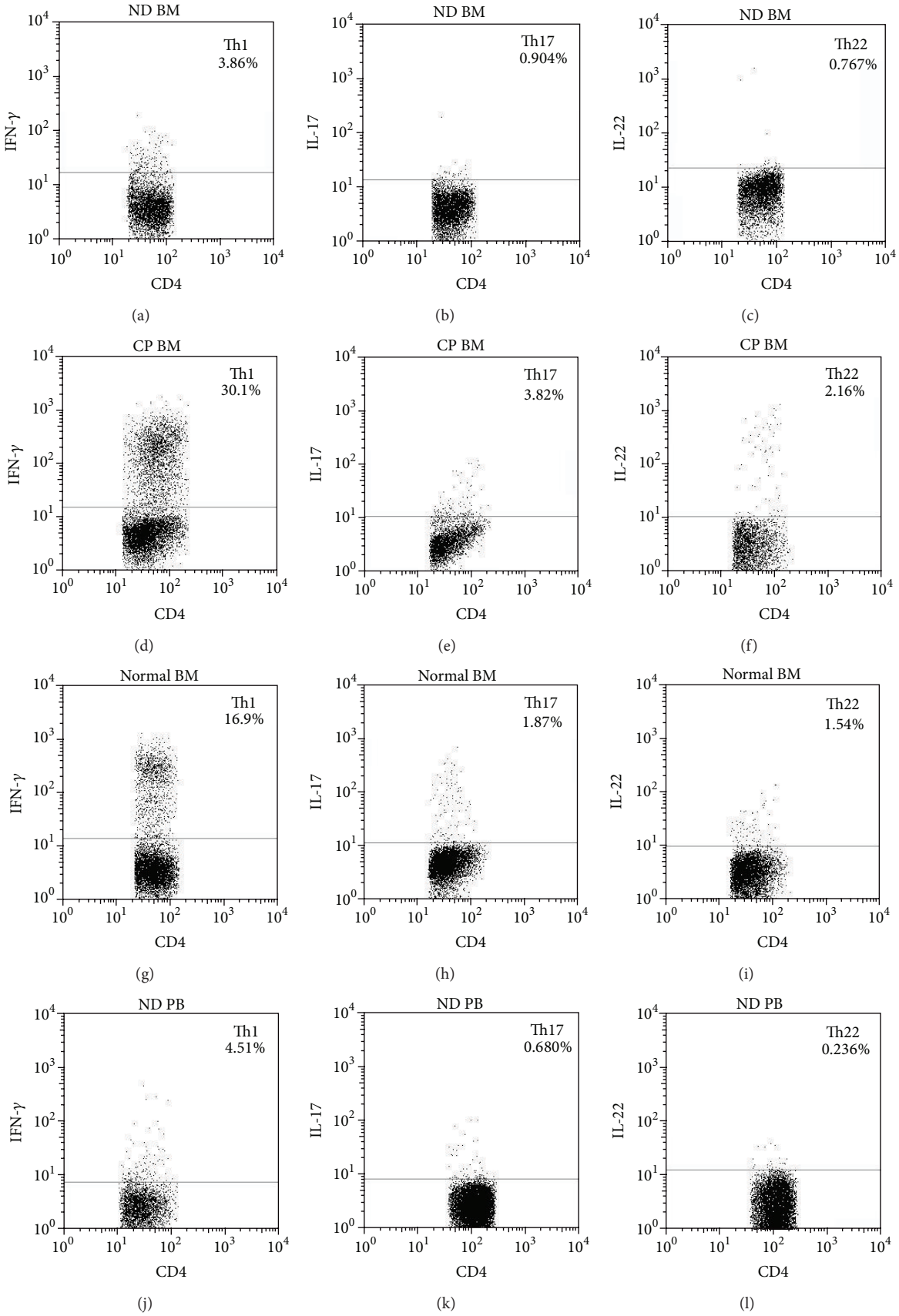


Figure 1: Continued.

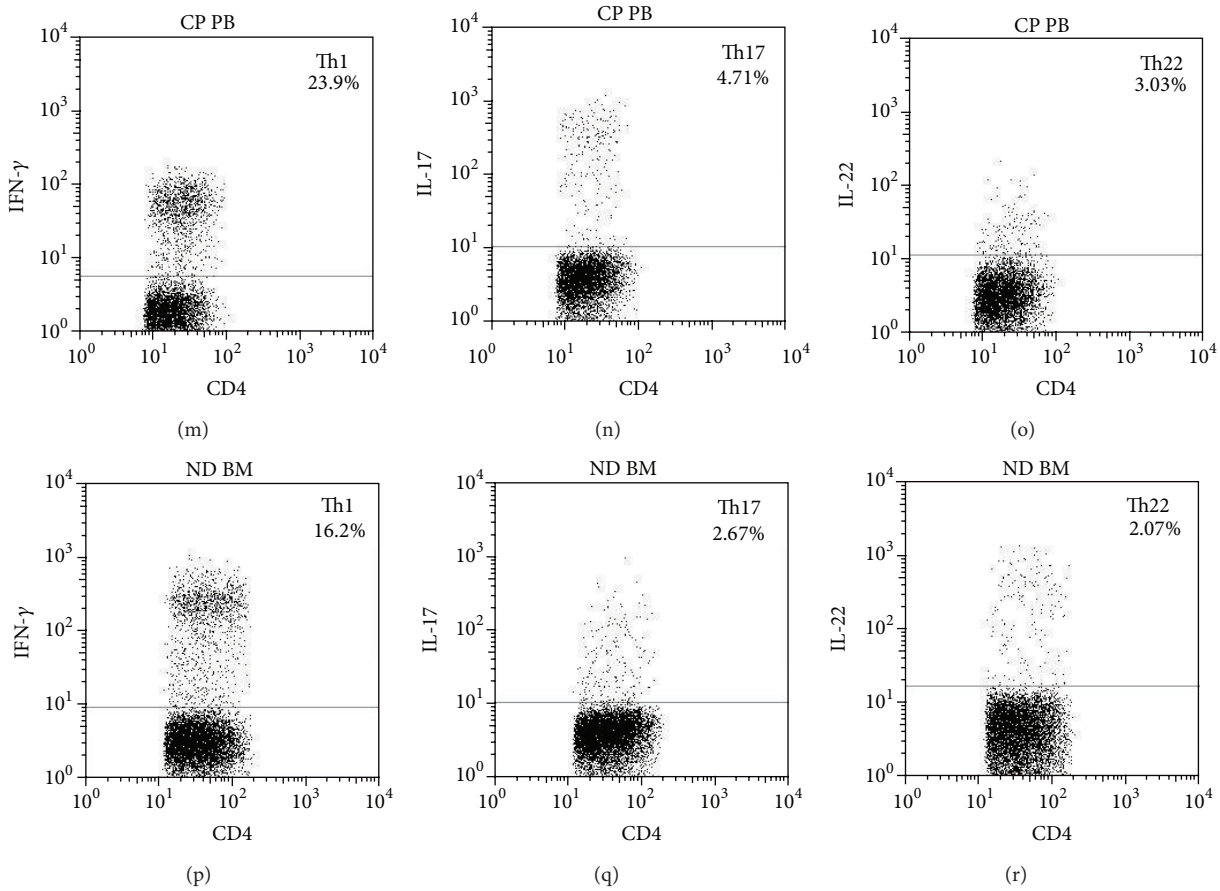


Figure 1: PB and BM percentages of Th1, Th17 and Th22 cells in representative ND patients, CP-CML patients, and healthy controls. (a, b, c) Representative FACS dot plots of Th1 ($CD4^+$ IFN-$r^+$), Th17 ($CD4^+$ IL-$17^+$), and Th22 ($CD4^+$ IL-$22^+$, IL-$17^-$, and IFN$\gamma^-$) cells in BM of ND CML patients. (d, e, f) Representative FACS dot plots of Th1, Th17, and Th22 cells in BM of CP-CML patients. (g, h, i,) Representative FACS dot plots of Th1, Th17, and Th22 cells in BM of healthy controls. (j, k, l) Representative FACS dot plots of Th1, Th17, and Th22 cells in PB of ND CML patients. (m, n, o) Representative FACS dot plots of Th1, Th17, and Th22 cells in PB of CP-CML patients. (p, q, r) Representative FACS dot plots of Th1, Th17, and Th22 cells in PB of healthy controls.

difference regarding PB plasma IFN-$\gamma$ between each group (ND: 3.30 ± 0.33 pg/mL, CP: 3.68 ± 1.26 pg/mL, and controls: 3.44 ± 0.64 pg/mL) (Figures 3(e) and 3(f)). No correlation was observed between Th1 frequency and IFN-$\gamma$ level.

*3.4. AHR and RORC Expression Levels Were Downregulated Accordingly in CML Patients.* AHR, the key transcription factor directing Th22 lineage commitment, was determined by RT-PCR. Our results demonstrated that AHR was significantly decreased in PB or BM of ND CML patients (0.2647 ± 0.3103 or 0.1653 ± 0.2083) compared with CP patients (0.8243 ± 0.4582 or 0.5892 ± 0.4755; $^*P = 0.000$ or $^*P = 0.000$) and healthy controls (0.5560 ± 0.2245 or 0.4368 ± 0.4194; $^*P = 0.017$ or $^*P = 0.024$). The mRNA levels of AHR were increased in PB of CP patients compared with healthy controls ($^*P = 0.012$), while no statistical significance of BM AHR mRNA levels was shown between CP patients and controls (Figures 4(a) and 4(c)).

We also found that RORC transcript, the key transcription factor directing Th17 lineage commitment, was significantly decreased in PB or BM of ND CML patients (0.0466 ± 0.0464 or 0.3267 ± 0.0599) compared with healthy controls (0.0922 ± 0.0728 or 0.0819 ± 0.0842; $^*P = 0.034$ or $^*P = 0.047$). It was lower in ND than CP patients (0.0862 ± 0.0617 or 0.1190 ± 0.0752; $^*P = 0.048$, $^*P = 0.000$). No significant difference of RORC mRNA level was observed between CP patients and healthy controls (Figures 4(b) and 4(d)).

*3.5. Th22 Cells Were Correlated with Th17 in CML Patients.* In CML patients, a significantly positive correlation was found between Th22 cells and Th17 cells both in PB and in BM ($R^2 = 0.717$, $^*P = 0.00$; $R^2 = 0.609$, $^*P = 0.00$, resp.). However, Th1 cells showed no significant correlations with Th22 or Th17 cells in CML patients (Figure 5).

*3.6. Clinical Relevance of Th22, Th17, and Th1 in CML Patients.* We analyzed the association between Th cells and peripheral white blood cell counts in CML patients. The results showed that there were significantly negative correlations between Th22, Th17, or Th1 cells and peripheral white blood cell counts ($R^2 = 0.243$, $^*P = 0.007$; $R^2 = 0.47$, $^*P = 0.000$; $R^2 = 0.481$,

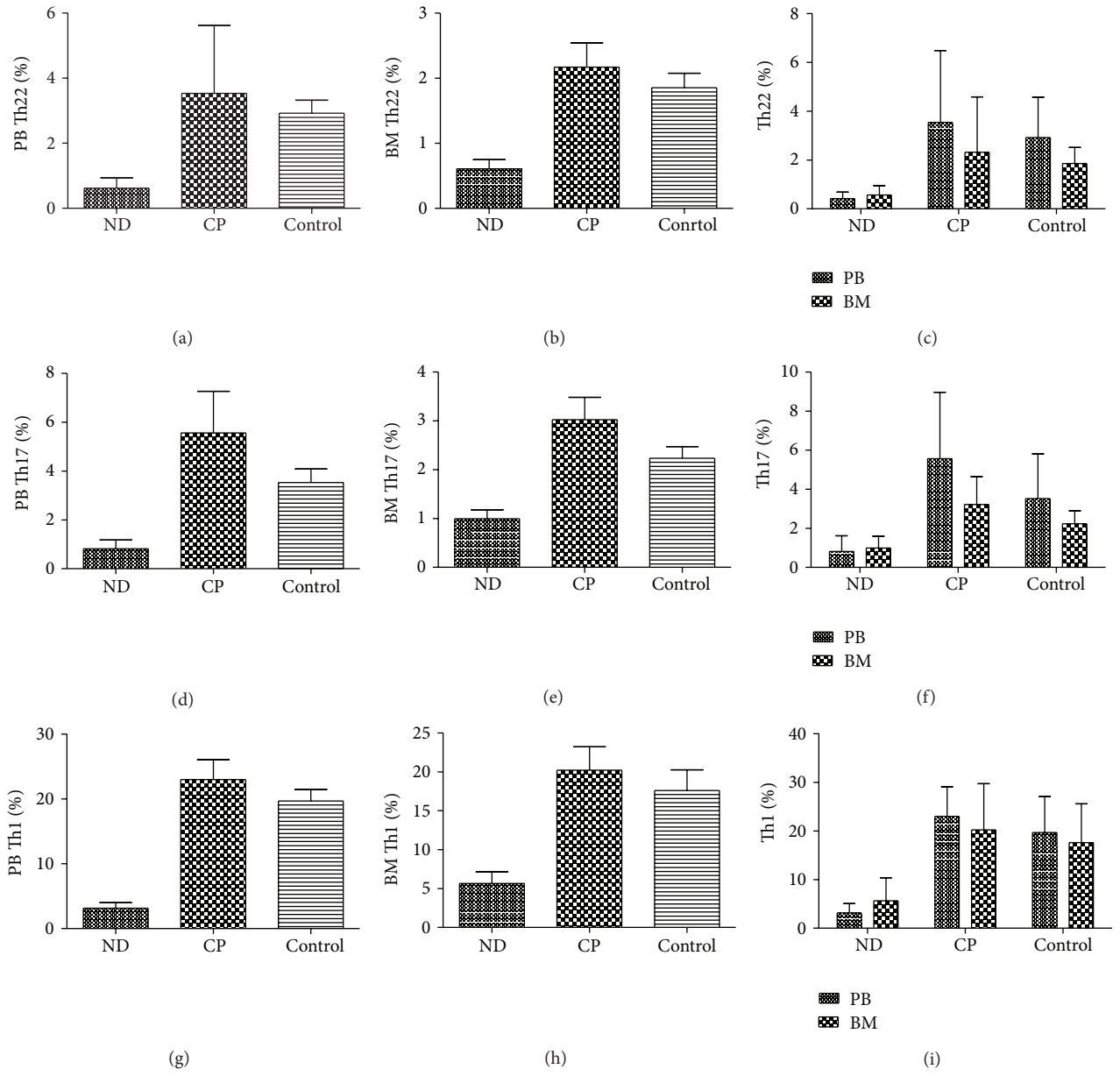


Figure 2: Percentages of Th17, Th22, and Th1 cells in CML patients and healthy controls. (a) Percentages of PB Th22 cells from CML patients and healthy controls. Significantly decreased percentages of Th22 cells were found in ND patients compared to CP patients ($^{*}P = 0.029$) or healthy controls ($^{*}P = 0.005$). (b) Percentages of BM Th22 cells from CML patients and healthy controls. Significantly decreased percentages of Th22 cells were found in ND patients compared to CP patients ($^{*}P = 0.009$) or healthy controls ($^{*}P = 0.037$). (c) Percentages of Th22 cells in PB or BM of ND patients, CP patients, and healthy controls. No significant differences were found between PB and BM. (d) Percentages of Th17 cells from CML patients and healthy controls. The circulating percentages of Th17 cells were profoundly decreased in ND patients compared to CP patients ($^{*}P = 0.005$) or healthy controls ($^{*}P = 0.029$). (e) The percentages of Th17 cells in BM of ND patients was proudly decreased compared to CP patients ($^{*}P = 0.000$) or healthy controls ($^{*}P = 0.008$). (f) There were no differences in the percentages of Th22 cells between PB or BM in ND patients, CP patients and healthy controls. (g) Percentages of Th1 cells from CML patients and healthy controls. The percentages of Th1 cells in PB of ND patients were proudly decreased compared to CP patients ($^{*}P = 0.001$) or healthy controls ($^{*}P = 0.001$). (h) The frequencies of Th1 cells in BM of ND patients, CP patients, and healthy controls. The frequency of Th1 cells in BM of ND patients was also significantly decreased compared to CP patients ($^{*}P = 0.01$) or healthy controls ($^{*}P = 0.02$). (i) The percentages of Th1 cells in PB or BM were not statistically different in ND patients, CP patients, and healthy controls.

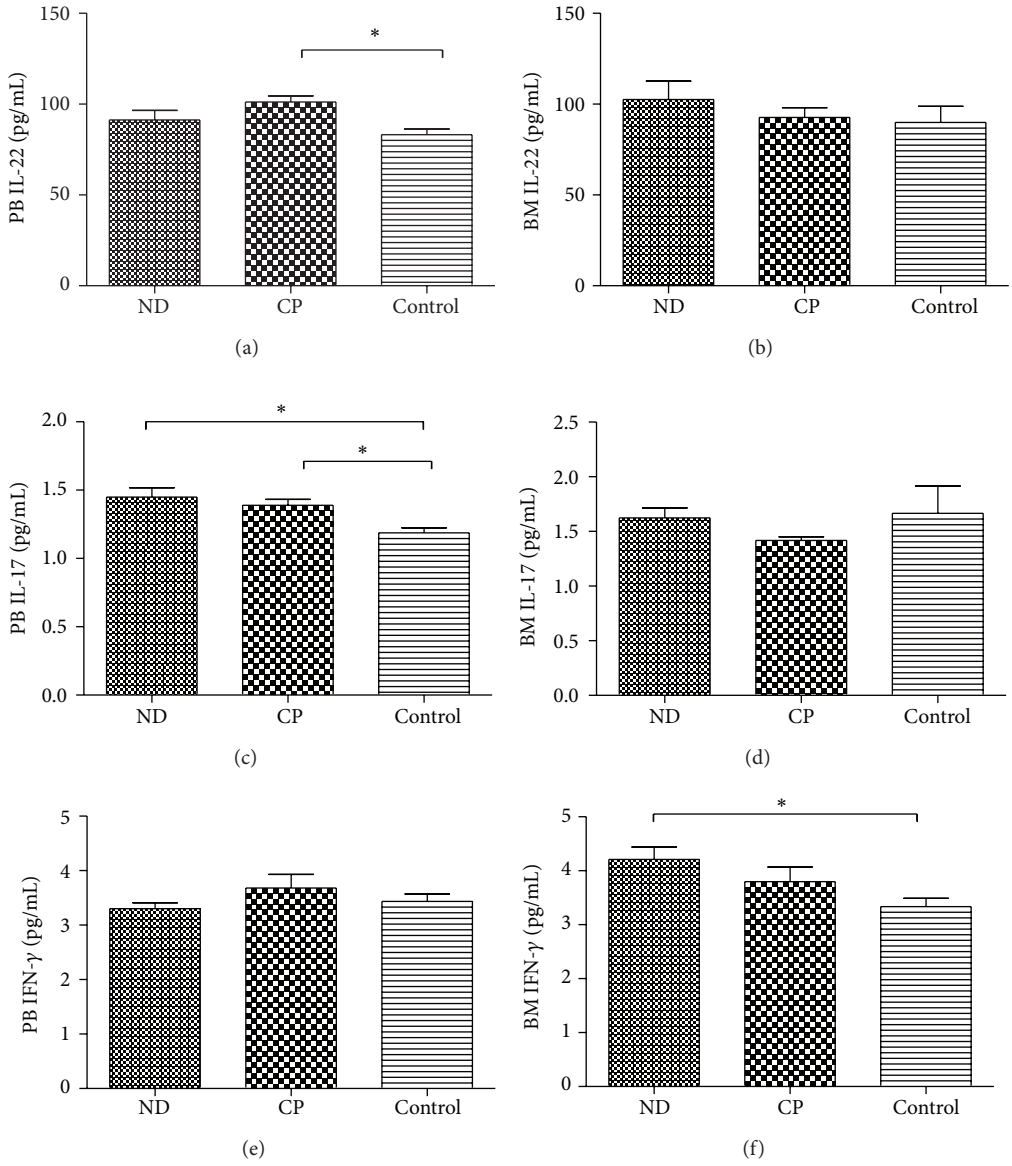


Figure 3: Concentrations of IL-22, IL-17, and IFN-$\gamma$ in PB or BM plasma of CML patients and healthy controls. (a) Concentrations of IL-22 in PB plasma from CP ($^*P = 0.001$) patients were increased compared to in controls. No significant difference was found between ND and CP patients ($P = 0.110$) or controls ($P = 0.194$). (b) Concentrations of IL-22 in BM plasma from ND patients, CP patients, and healthy controls. No significant difference was found between ND ($P = 0.575$) or CP ($P = 0.803$) patients and healthy controls. (c) Concentrations of IL-17 in PB plasma from ND patients, CP patients, and healthy controls. The concentrations of PB plasma IL-17 in ND ($^*P = 0.02$) or CP ($^*P = 0.01$) patients were significantly higher than in healthy controls. There was no significant difference between ND and CP patients ($P = 0.437$). (d) Concentrations of IL-17 in BM plasma from ND patients, CP patients, and healthy controls. There was no significant difference between ND ($P = 0.142$) or CP ($P = 0.645$) patients and healthy controls. (e) Concentrations of IFN-$\gamma$ in PB plasma from ND patients, CP patients, and healthy controls. No significant difference was found between ND ($P = 0.711$) or CP ($P = 0.380$) patients and healthy controls. (f) Concentrations of IFN-$\gamma$ in BM plasma from ND patients, CP patients, and healthy controls. The concentration of BM plasma IFN-$\gamma$ in ND patients ($^*P = 0.015$) was significantly higher than in healthy controls. There was no significant difference between ND and CP ($P = 0.203$) patients.

$^*P = 0.000$, resp.) (Figure 6). In addition, we also found statistical negative correlations between Th22, Th17, or Th1 cells and BCR-ABL (%) IS ($R^2 = 0.166$, $^*P = 0.028$; $R^2 = 0.321$, $^*P = 0.001$; $R^2 = 0.242$, $^*P = 0.007$, resp.) (Figure 7).

## 4. Discussion

CML represents 15% of newly diagnosed leukemia cases in adults in China [5]. Recent studies have shown that T-cell immunodeficiency is a common feature in cancer patients,

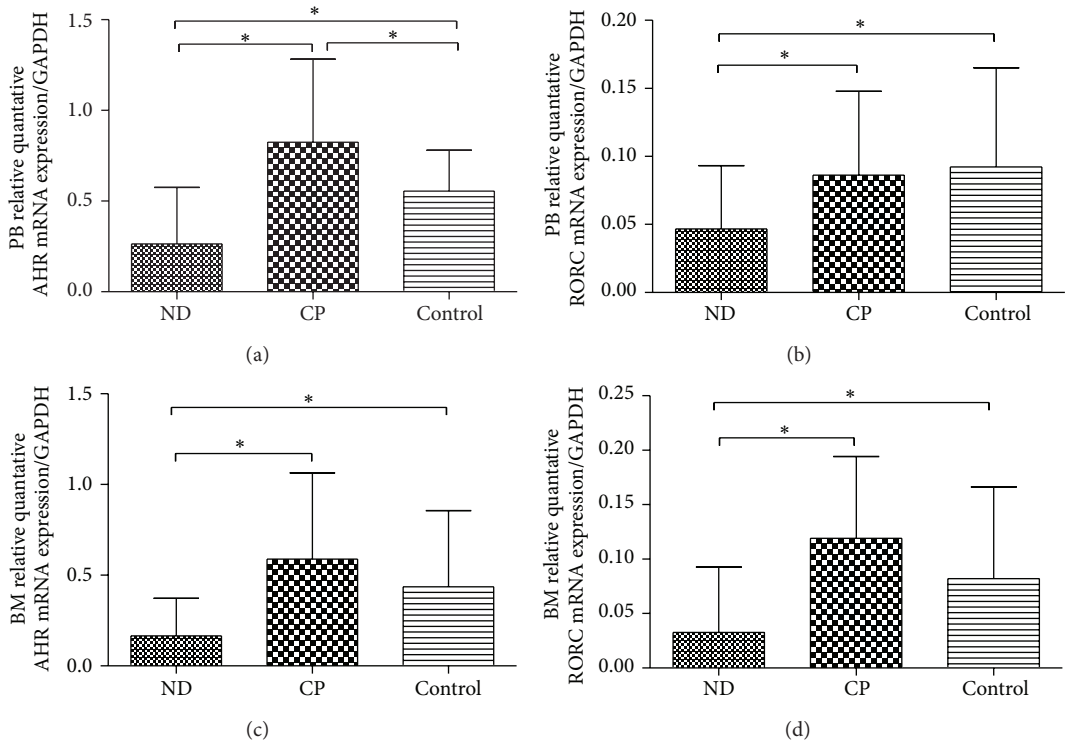


Figure 4: Expression of AHR and RORC mRNA in CML patients and healthy controls. (a) The expression of PB AHR mRNA was significantly decreased in ND patients compared to CP ($P = 0.000$) and healthy controls ($P = 0.017$). (b) The expression of PB RORC mRNA was significantly decreased in ND patients compared to CP ($P = 0.048$) and healthy controls ($P = 0.034$). (c) The expression of BM AHR mRNA was significantly decreased in ND patients compared to CP ($P = 0.000$) and healthy controls ($P = 0.024$). (d) The expression of BM RORC mRNA was significantly decreased in ND patients compared to CP ($P = 0.000$) and healthy controls ($P = 0.047$).

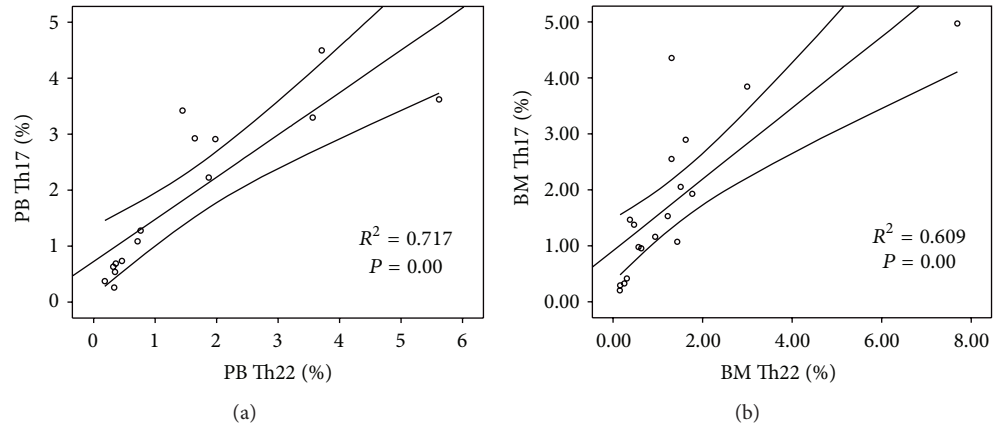


Figure 5: Correlation between the percentages of Th22 and Th17 cells in CML Patients. (a) Positive correlation was found between Th22 cells and Th17 cells in PB of CML patients. (b) Positive correlation was found between Th22 cells and Th17 cells in BM of CML patients.

which may relate to initiation and development of tumor [5] and immunotherapy may become a kind of effective method to control tumor growth and recurrence. Therefore, understanding the immune status especially the Th-cell immune function in patients with CML is very important and essential to make effective immunotherapy. However, to date, there are no sufficient data regarding the specific roles of Th cells in the development and progression of CML.

Th22 cells are distinct from other Th cells, such as Th1, Th2, and Th17, indicating that Th22 have an individual signature. IL-22, as the main effector cytokine of Th22, belongs to the IL-10 cytokine family [15]. Recent studies have implicated the role of Th22 and IL-22 in the pathogenesis of a variety of solid tumors and a limited number of hematological malignancies including AML [16], MDS [17] and ALL [18]. No data has been reported regarding the role of Th22 cells

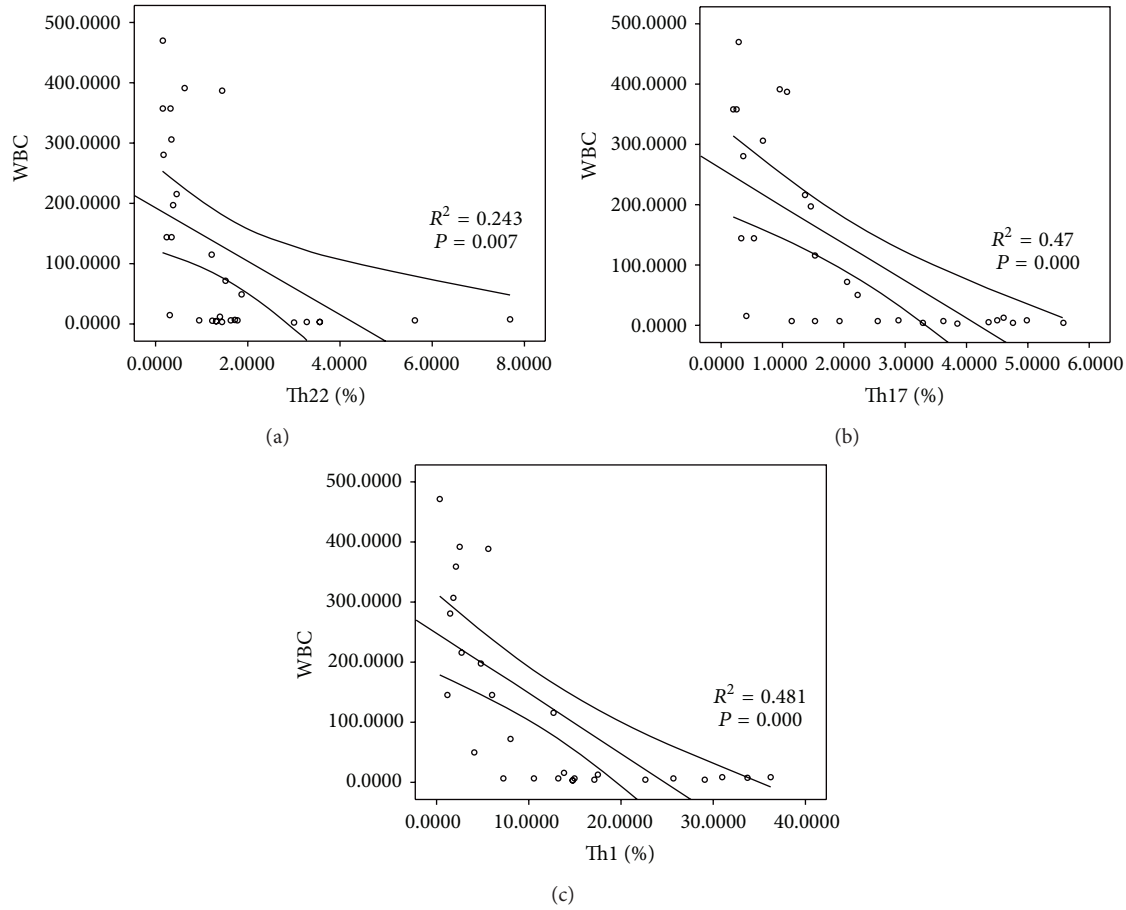


Figure 6: Clinical relevance between the frequencies of Th22, Th17, and Th1 cells and peripheral white blood cell number in CML patients. (a) Negative correlation was found in Th22 cell and peripheral white blood cell number. (b) Negative correlation was found in Th17 cell and peripheral white blood cell number. (c) Negative correlation was found in Th1 cell and peripheral white blood cell number.

and IL-22 in the pathogenesis of CML. Therefore, the present study aimed to determine the levels of Th22 cells and IL-22 and its specific transcription factor AHR in CML patients. Our results demonstrated that the percentages of PB and BM Th22 subset were significantly decreased in ND CML patients compared to healthy controls, while Th22 cells may attain a certain degree of recovery when the patients achieved complete remission. Moreover, we further investigated the expression of AHR, the key transcription factor directing Th22 lineage commitment, and found that AHR mRNA expression also significantly decreased in PB and BM of ND CML patients. All these results suggested that downregulation of Th22 cellular immunity and impaired Th22 immune function may contribute to the occurrence and development of CML.

Considering the involvement of IL-22 in the regulation of cell growth, proliferation, and cell cycle control, it is conceivable that IL-22 might play an acceleration or inhibition role during tumorigenesis [19]. However, in our study, the levels of IL-22 have been found comparable in each group of CML patients. And there is no connection between Th22 and IL-22. As we know that, in humans, adaptive immune cells of the immune system, such as Th22 and Th17, are the major T-cell subsets producing IL-22 [15, 20, 21]. IL-22 is also expressed by $CD8^+$ T cells [22, 23] and other innate lymphocytes, including NK22 subset of natural killer cells [24, 25], and $CD11c^+$cells [23, 26]. Our results may suggest that, in CML, Th22 cells may only account for a minor proportion in producing IL-22 and probably other kinds of cells made up this difference.

Although the role of Th17 in autoimmune diseases and infection has been relatively well documented, the impact of Th17 in cancer remains difficult to ascertain [7]. The current data regarding the role of Th17 cells in pathogenesis of cancer are scarce and controversial [27]. There are several researches about the roles of Th17 in hematological malignancies including multiple myeloma [28] and CLL [29], AML [16, 30], and non-Hodgkin lymphoma [31]. It has been reported that serum levels of IL-17 are increased in multiple myeloma patients and are correlated with disease prognosis, which suggested IL-17 as tumor-promoting factor in multiple myeloma. Th17 was found to be accumulated in the bone marrow of multiple myeloma [28]. Another study indicated that progression of CLL is associated with downregulation of IL-17-producing T cells, implying contribution of these subsets of T cells in the progression of CLL [29].

In the present study, similarly the results of Th22 cells, we showed that the frequency of Th17 was significantly decreased in ND CML patients compared to healthy controls.

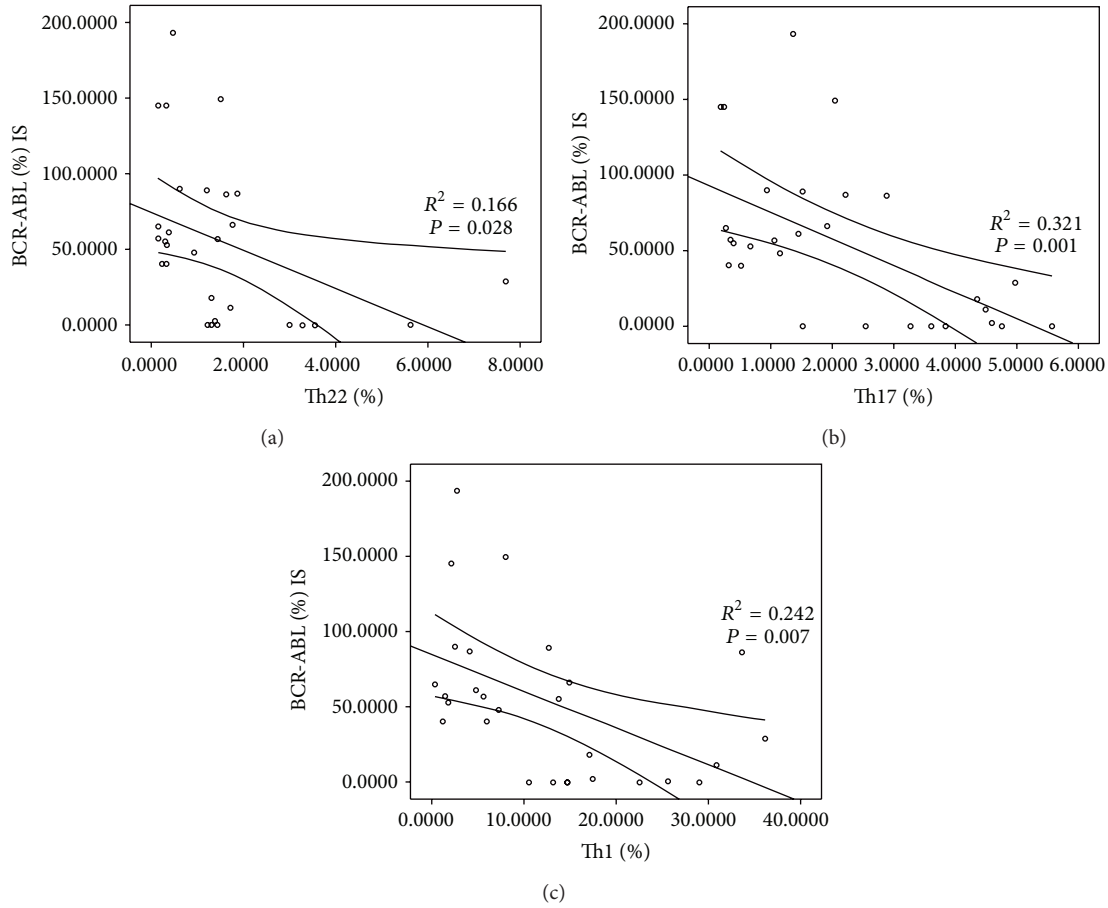


Figure 7: Clinical relevance between the frequencies of Th22, Th17, and Th1 cells and BCR-ABL (%) IS in CML patients. (a) Negative correlation was found in Th22 cell and BCR-ABL (%) IS. (b) Negative correlation was found in Th17 cell and BCR-ABL (%) IS. (c) Negative correlation was found in Th1 cell and BCR-ABL (%) IS.

The RORC expression was also dramatically reduced in ND patients. Moreover, both Th17 frequency and RORC expression all recovered to the normal level in CP-CML patients. These results may suggest that Th17 cells may play a protective role in CML pathogenesis and their potential implication in immunotherapy of this malignancy.

Th1 cells have been linked to the development of autoimmune inflammatory processes. Th1 cells secrete a large number of IFN-$\gamma$ and promote the cell-mediated immunity [32]. We also detected the frequencies of Th1 cells and related cytokine IFN-$\gamma$ in CML patients. In accordance with the variation of Th22 and Th17 cells, the immunobiology of Th1 cells was damaged in ND CML patients and recovered after the treatment with TKI.

In addition, there existed a positive correlation between Th22 and Th17 subsets both in PB and in BM of CML patients, implying that differentiation of Th22 and Th17 cells may be induced in an influential manner in CML. This coreduced frequencies of Th22 and Th17 cells suggested that these two T-cell subsets may play a synergistic role in leading to a dramatic immunodeficiency in CML patients.

We analyzed the association between Th cells and peripheral white blood cell counts or BCR-ABL (%) IS in CML patients. Our results showed that there was significantly negative correlation between Th22, Th17, or Th1 cells and peripheral white blood cell counts or BCR-ABL (%) IS. These data may suggest that the proportions of Th22, Th17, and Th1 cells are associated with tumor burden in CML patients and may be useful in evaluating therapeutic effects.

In conclusion, we firstly demonstrated that the frequencies of Th22, Th17, or Th1 cells and their specific transcription factor expression are significantly reduced in ND CML patients both in PB and in BM. Furthermore, these cells immunodeficiency may recover after the treatment with TKI. All these results indicate that these Th subsets may be involved in the pathogenesis of CML and may become effective therapeutic targets for CML patients.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Ping Chen and Min Wang contributed equally to this paper.

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## References

[1] F. Bresciani, R. Paoluzi, M. Benassi, C. Nervi, C. Casale, and E. Ziparo, "Cell kinetics and growth of squamous cell carcinomas in man," *Cancer Research*, vol. 34, no. 9, pp. 2405–2415, 1974.

[2] S. Faderl, M. Talpaz, Z. Estrov, S. O'Brien, R. Kurzrock, and H. M. Kantarjian, "The biology of chronic myeloid leukemia," *The New England Journal of Medicine*, vol. 341, no. 3, pp. 164–172, 1999.

[3] C. M. Lucas, L. Wang, G. M. Austin et al., "A population study of imatinib in chronic myeloid leukaemia demonstrates lower efficacy than in clinical trials," *Leukemia*, vol. 22, no. 10, pp. 1963–1966, 2008.

[4] H. de Lavallade, J. F. Apperley, J. S. Khorashad et al., "Imatinib for newly diagnosed patients with chronic myeloid leukemia: incidence of sustained responses in an intention-to-treat analysis," *Journal of Clinical Oncology*, vol. 26, no. 20, pp. 3358–3363, 2008.

[5] Y. Li, S. Geng, Q. Yin et al., "Decreased level of recent thymic emigrants in CD4+ and CD8+T cells from CML patients," *Journal of Translational Medicine*, vol. 8, article 47, 2010.

[6] L. A. Tesmer, S. K. Lundy, S. Sarkar, and D. A. Fox, "Th17 cells in human disease," *Immunological Reviews*, vol. 223, no. 1, pp. 87–113, 2008.

[7] D. Alizadeh, E. Katsanis, and N. Larmonier, "The multifaceted role of Th17 lymphocytes and their associated cytokines in cancer," *Clinical and Developmental Immunology*, vol. 2013, Article ID 957878, 11 pages, 2013.

[8] L. E. Harrington, R. D. Hatton, P. R. Mangan et al., "Interleukin 17-producing $CD4^+$ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages," *Nature Immunology*, vol. 6, no. 11, pp. 1123–1132, 2005.

[9] H. Park, Z. Li, X. O. Yang et al., "A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17," *Nature Immunology*, vol. 6, no. 11, pp. 1133–1141, 2005.

[10] S. Qin, S. Ma, X. Huang, D. Lu, Y. Zhou, and H. Jiang, "Th22 cells are associated with hepatocellular carcinoma development and progression," *Chinese Journal of Cancer Research*, vol. 26, no. 2, pp. 135–141, 2014.

[11] L. Zhang, Y.-G. Li, Y.-H. Li et al., "Increased frequencies of th22 cells as well as th17 cells in the peripheral blood of patients with ankylosing spondylitis and rheumatoid arthritis," *PLoS ONE*, vol. 7, no. 4, Article ID e31000, 2012.

[12] H. Benham, P. Norris, J. Goodall et al., "Th17 and Th22 cells in psoriatic arthritis and psoriasis," *Arthritis Research and Therapy*, vol. 15, no. 5, article R136, 2013.

[13] K. Prabhash, D. Loknath, K. G. Babu, A. G. Hasib, S. Biswas, and P. P. Bapsy, "Chronic myeloid leukemia with osteolytic bone involvement," *Journal of Association of Physicians of India*, vol. 51, pp. 740–741, 2003.

[14] J. W. Vardiman, "The World Health Organization (WHO) classification of tumors of the hematopoietic and lymphoid tissues: an overview with emphasis on the myeloid neoplasms," *Chemico-Biological Interactions*, vol. 184, no. 1-2, pp. 16–20, 2010.

[15] S. Trifari, C. D. Kaplan, E. H. Tran, N. K. Crellin, and H. Spits, "Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from $T_H$-17, $T_H1$ and $T_H2$ cells," *Nature Immunology*, vol. 10, no. 8, pp. 864–871, 2009.

[16] S. Yu, C. Liu, L. Zhang et al., "Elevated Th22 cells correlated with Th17 cells in peripheral blood of patients with acute myeloid leukemia," *International Journal of Molecular Sciences*, vol. 15, no. 2, pp. 1927–1945, 2014.

[17] L.-L. Shao, L. Zhang, Y. Hou et al., "Th22 Cells as Well as Th17 cells expand differentially in patients with early-stage and late-stage myelodysplastic syndrome," *PLoS ONE*, vol. 7, no. 12, Article ID e51339, 2012.

[18] T. Tian, Y. Sun, M. Li et al., "Increased Th22 cells as well as Th17 cells in patients with adult T-cell acute lymphoblastic leukemia," *Clinica Chimica Acta*, vol. 426, pp. 108–113, 2013.

[19] T. Tian, S. Yu, and D. Ma, "Th22 and related cytokines in inflammatory and autoimmune diseases," *Expert Opinion on Therapeutic Targets*, vol. 17, no. 2, pp. 113–125, 2013.

[20] T. Duhen, R. Geiger, D. Jarrossay, A. Lanzavecchia, and F. Sallusto, "Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells," *Nature Immunology*, vol. 10, no. 8, pp. 857–863, 2009.

[21] N. Zhang, H.-F. Pan, and D.-Q. Ye, "Th22 in inflammatory and autoimmune disease: prospects for therapeutic intervention," *Molecular and Cellular Biochemistry*, vol. 353, no. 1-2, pp. 41–46, 2011.

[22] K. E. Nograles, L. C. Zaba, A. Shemer et al., "IL-22-producing 'T22' T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing TH17 T cells," *The Journal of Allergy and Clinical Immunology*, vol. 123, no. 6, pp. 1244–1252, 2009.

[23] Y. Zheng, D. M. Danilenko, P. Valdez et al., "Interleukin-22, a TH17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis," *Nature*, vol. 445, no. 7128, pp. 648–651, 2007.

[24] M. Cella, A. Fuchs, W. Vermi et al., "A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity," *Nature*, vol. 457, no. 7230, pp. 722–725, 2009.

[25] L. A. Zenewicz, G. D. Yancopoulos, D. M. Valenzuela, A. J. Murphy, S. Stevens, and R. A. Flavell, "Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease," *Immunity*, vol. 29, no. 6, pp. 947–957, 2008.

[26] Y. Zheng, P. A. Valdez, D. M. Danilenko et al., "Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens," *Nature Medicine*, vol. 14, no. 3, pp. 282–289, 2008.

[27] W. Zou and N. P. Restifo, "$T_H17$ cells in tumour immunity and immunotherapy," *Nature Reviews Immunology*, vol. 10, no. 4, pp. 248–256, 2010.

[28] R. H. Prabhala, D. Pelluru, M. Fulciniti et al., "Elevated IL-17 produced by TH17 cells promotes myeloma cell growth and inhibits immune function in multiple myeloma," *Blood*, vol. 115, no. 26, pp. 5385–5392, 2010.

[29] F. Jadidi-Niaragh, G. Ghalamfarsa, A. Memarian et al., "Down-regulation of IL-17-producing T cells is associated with regulatory T cell expansion and disease progression in chronic lymphocytic leukemia," *Tumor Biology*, vol. 34, no. 2, pp. 929–940, 2013.

[30] C. Wu, S. Wang, F. Wang et al., "Increased frequencies of T helper type 17 cells in the peripheral blood of patients with acute myeloid leukaemia," *Clinical & Experimental Immunology*, vol. 158, no. 2, pp. 199–204, 2009.

[31] Z.-Z. Yang, A. J. Novak, S. C. Ziesmer, T. E. Witzig, and S. M. Ansell, "Malignant B cells skew the balance of regulatory T cells and T H17 cells in B-cell non-Hodgkin's lymphoma," *Cancer Research*, vol. 69, no. 13, pp. 5522–5530, 2009.

[32] P. Feng, R. Yan, X. Dai, X. Xie, H. Wen, and S. Yang, "The alteration and clinical significance of Th1/Th2/Th17/Treg cells in patients with multiple myeloma," *Inflammation*, 2014.

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*Review Article*

# Interactions between MSCs and Immune Cells: Implications for Bone Healing

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It is estimated that, of the 7.9 million fractures sustained in the United States each year, 5% to 20% result in delayed or impaired healing requiring therapeutic intervention. Following fracture injury, there is an initial inflammatory response that plays a crucial role in bone healing; however, prolonged inflammation is inhibitory for fracture repair. The precise spatial and temporal impact of immune cells and their cytokines on fracture healing remains obscure. Some cytokines are reported to be proosteogenic while others inhibit bone healing. Cell-based therapy utilizing mesenchymal stromal cells (MSCs) is an attractive option for augmenting the fracture repair process. Osteoprogenitor MSCs not only differentiate into bone, but they also exert modulatory effects on immune cells via a variety of mechanisms. In this paper, we review the current literature on both *in vitro* and *in vivo* studies on the role of the immune system in fracture repair, the use of MSCs in the enhancement of fracture healing, and interactions between MSCs and immune cells. Insight into this paradigm can provide valuable clues in identifying cellular and noncellular targets that can potentially be modulated to enhance both natural bone healing and bone repair augmented by the exogenous addition of MSCs.

## 1. Introduction

The normal process of fracture repair begins with an immediate inflammatory response as the innate immune system (macrophages, monocytes, neutrophils, and NK cells) responds with a variety of cytokines that recruit and activate several cell types, including osteoprogenitor mesenchymal stem cells (MSCs), to the site of injury [1, 2]. The adaptive immune response, primarily comprised of T and B lymphocytes, has important implications in the fracture healing process as well [3, 4]. For example, mice genetically deficient for adaptive immunity displayed accelerated bone healing. While some signals are mitogenic and proosteogenic, others function to inhibit osteogenesis and increase bone resorption, and it appears that a well-controlled, delicate balance of inflammatory factors is necessary for proper fracture repair [3–6]. Thus any process or systemic condition that alters this optimal inflammatory milieu, such as bone diseases like osteoporosis or severe trauma, steroid therapy, diabetes, or advanced age, can disrupt the normal fracture healing process, resulting in nonunions or delayed healing, pain, disfigurement, and loss of function. Approximately 5–15% of patients experience these complications and will require revision surgeries, prolonged hospitalization, and rehabilitation, all of which result in a high socioeconomic cost for society [7, 8].

Multipotent mesenchymal stromal cells (MSCs), also known as mesenchymal stem cells, have the capacity to differentiate into a variety of cell types (Figure 1), including adipocytes, chondrocytes, and osteocytes [9, 10]. Coupled with reports that allogeneic MSCs have immunoprivileged status and immunomodulatory properties, there has been considerable interest in exploring the use of these cells as a therapeutic option for bone repair. MSCs were initially isolated from bone marrow but are now known to exist in a wide range of tissues in the human adult, including brain, thymus, lung, liver, spleen, kidney, and dental pulp [11, 12]. MSCs have also been derived from embryonic tissues,

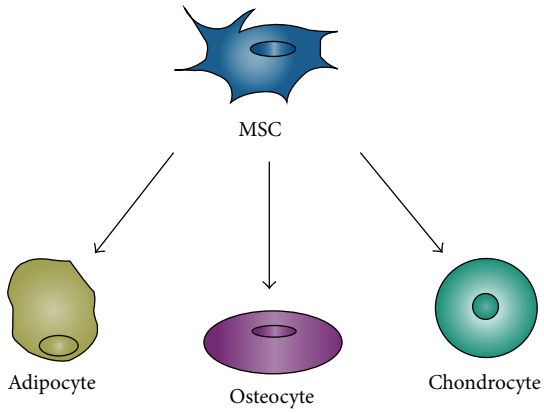


Figure 1: Multipotential differentiation of MSCs into adipogenic, osteogenic, and chondrogenic cell lineages. MSC = multipotent mesenchymal stromal cell.

such as Wharton's jelly and umbilical cord blood [13, 14]. Adipose-derived MSCs, in particular, pose an attractive option for cell-based therapy due to their relatively decreased morbidity during isolation and potential for expansion and differentiation [12].

MSCs are able to evade the host cell immune system due to their low expression of major histocompatibility complex (MHC) class I molecules and complete lack MHC class II molecules and other costimulatory molecules (CD40, CD40L, CD80, and CD86) required for immune cell stimulation [15–17]. Although the expression of MHC class I and II molecules can be upregulated by MSC exposure to inflammatory cytokines interferon-gamma (IFN-$\gamma$) and tumor necrosis factor-alpha (TNF-$\alpha$), they are still unable to induce an immunological response [18]. There is also evidence that MSCs are able to modulate the immune system by a variety of mechanisms, including the release of soluble factors. Allogeneic MSCs have been shown to suppress T cell proliferation and antigen presenting cell maturation, as well as inducing a regulatory T cell phenotype that further suppresses the immune response *in vitro* [19–21]. Several *in vivo* studies using animal models, however, have yielded conflicting results as to whether allogeneic MSCs are immunoprivileged and maintain the ability to differentiate and proliferate [22–24].

Similarly immune cells recruited to injured bone can modulate osteogenic differentiation of osteoprogenitors. We have shown that Th1 immune response represented by enhanced expression of IFN-$\gamma$ in the implants of allogeneic MSCs significantly inhibits expression of osteocalcin, Runx2, and alkaline phosphatase genes subsequently inhibiting bone formation [24]. Liu et al. have reported that combined action of IFN-$\gamma$ and TNF-$\alpha$ that are primarily produced by activated T cells can induce apoptosis of MSCs [25]. These findings from animal studies were endorsed by a recent finding in human patients that $CD8^+$ T cells in the circulation as well as in the fracture hematoma lead to delayed healing [26]. This continuous interaction between immune cells and MSCs during the bone repair process is one of the key factors that determine successful outcome of fracture healing. A new concept called "osteoimmunomodulation" is recently introduced which refers to alteration of immune response using various strategies to enhance bone repair [27]. It was reported that coating the magnesium scaffolds that are used very frequently for tissue engineering purposes, with $\beta$-tricalcium phosphate favored generation of M2 phenotype of macrophages which promoted osteogenic differentiation of MSCs [27]. M2 macrophages are known to suppress Th1 response and promote Th2 response. Another simple but very effective strategy was reported by Liu et al. [25]. Local delivery of aspirin inhibited IFN-$\gamma$ and TNF-$\alpha$ activities and promoted bone regeneration [25]. These osteoimmunomodulatory strategies may become leading therapeutic interventions to enhance bone regeneration in near future.

In this review, we discuss the current understanding of the interactions between MSCs and the immune system in the context of osteogenesis and fracture repair.

## 2. Clinical Trials on Enhancement of Fracture Healing through Exogenous Addition of MSCs

Although there are numerous *in vitro* and *in vivo* studies published to date on the use of MSCs for regenerative medicine purposes, clinical trials using MSC-based approaches are limited due to medical and regulatory reasons [55]. As of September 2014, ten clinical trials were in process or completed investigating either autologous or allogeneic MSCs for fracture repair (http://www.clinicaltrials.gov) [55–60]. Most of the clinical trials used autologous MSCs that were culture expanded [56, 57] or bone marrow aspirate, concentrated using centrifugation [60, 61]. Since MSCs were delivered with the intention to increase the pool of osteoprogenitor cells and not as agents to modulate immune cells, potential change induced by MSCs in the local microenvironment of immune cells was not considered in relation to bone healing. It is also not clear whether allogeneic MSCs are as effective as autologous MSCs since no clinical trial has compared allogeneic and autologous MSCs. Therefore, existing data from the clinical trials throws very little light on the relationship between MSCs-induced immunomodulation and successful fracture healing.

## 3. *In Vivo* Animal Studies Demonstrating the Integral Role of Immune Cells in the Regulation of Natural Fracture Healing as well as the Success of Bone Repair through Transplantation of MSCs

*3.1. Role of Immune Cells in Natural Fracture Healing.* The fracture healing process begins with the formation of a soft callus that is subsequently mineralized and remodeled [3, 4, 62]. Successful fracture healing can be defined by adequate callus mineralization and the regeneration of biomechanical competence [3, 4, 62]. In the early phase of healing, the innate immune response plays a key role in

the recruitment and activation of a variety of cell types that are critical in the fracture healing process, including MSCs [1, 2].

A study by Toben et al. investigated a standard closed femoral fracture in both wild-type (WT) and *recombination activating gene* knockout (*Rag1*$^{-}$/$^{-}$) mice that lack T and B cells [22]. It was found that fracture healing was significantly enhanced in *Rag1*$^{-}$/$^{-}$ mice suggesting detrimental functions of T and B lymphocytes on fracture healing. Higher numbers of lymphocytes were present during the repair process in the hematoma on day 3 and during formation of the hard callus on day 14 in the WT mice. Which of the two lymphocytes plays the predominant role in the regulation of bone repair remains a debate. It is reported that T cells are responsible for promoting bone resorption by induction of osteoclastogenesis via RANK-RANKL interactions with osteoclasts [63]. In *Rag1*$^{-}$/$^{-}$ mice, however, higher than normal numbers of osteoclasts were observed in the bone callus of these animals, even though they lacked the T lymphocytes to promote osteoclastogenesis [22]. The presumed reason for this increase in the number of osteoclasts is that they formed in response to the elevated osteoblast activity and bone formation in these animals [22].

Faster healing in these mice also correlated with lower levels of expression of TNF-$\alpha$, a proinflammatory cytokine, in the callus [22]. This may favor bone formation since TNF-$\alpha$ can have proapoptotic effects on osteoblasts, and increased levels have been implicated in animal models of rheumatoid arthritis and other bone diseases characterized by excessive bony destruction [63].

After the initial innate inflammatory response, there appears to be a shift from proinflammatory to anti-inflammatory cytokines. The lymphopenic *Rag1*$^{-}$/$^{-}$ mice demonstrated an earlier and significantly higher expression of anti-inflammatory interleukin-10 (IL-10) [22]. The central role of IL-10 in promoting bone growth and accelerating fracture healing is further supported by studies showing that IL-10 regulates bone resorption, and its absence leads to osteopenia, mechanistic fragility, and malunion [64–66].

Another study carried out with $\gamma/\delta$ T cell deficient mice ($\delta$ T cell receptor- [TCR-] knockout mice) demonstrated superior quality of bony union with more osseous and chondral elements and mature bone marrow early on in the repair process compared to wild type controls [6]. TCR-knockout mice produced significantly lower levels of inflammatory cytokines IL-2, IFN-$\gamma$, and IL-6, at the fracture site [6]. Overall, the T cell deficient mice demonstrated improved biomechanical strength and stability compared to control animals, as evidenced by quantitative increases in osseous and chondral elements, increased gene expression of type II collaged, BSP, and BMP-2 [6].

A more recent study showed increased levels of terminally differentiated CD8$^{+}$ effector memory T cells in the peripheral blood of humans with delayed fracture healing [26]. Furthermore, CD8$^{+}$ T cells, as well as their cytokines IFN-$\gamma$ and TNF-$\alpha$, were enriched in the fracture hematoma of these patients [26]. In addition, CD8$^{+}$ T cell-deficient mice demonstrated enhanced endogenous fracture healing, and a transfer of CD8$^{+}$ T cells impaired the regenerative process [26]. This data supports the integral role that the adaptive immune response has on the outcome of endogenous bone regeneration [26].

In contradiction with the notion that the T cells inhibit bone healing, Nam et al. reported that T and B cell deficient *Rag1*$^{-}$/$^{-}$ mice displayed impaired fracture healing in comparison with wild type mice and the lack of T cells in the *Rag1*$^{-}$/$^{-}$ mice correlated with delayed osteoblast maturation and decreased bone formation [67]. Additionally, the proinflammatory cytokine IL-17, which is produced by Th17 lymphocytes (Th17 cells), was shown to be a key mediator in osteogenesis of the fracture healing process [67].

These studies suggest that T cells are inhibitory to fracture healing. The inflammatory cytokines produced by T cells, IFN-$\gamma$ and TNF-$\alpha$, play an important role in the T cells inhibition of bone regeneration. Further studies are required to elucidate roles of different subtypes of T cells as well as B cells in fracture repair.

### 3.2. Role of Immune Cells in Enhancement of Bone Formation through Exogenous Addition of Allogeneic and Syngeneic MSCs

*3.2.1. Use of Syngeneic MSCs.* Liu et al. investigated the role of recipient T cells in MSCs-mediated osteogenesis in a calvarial defect in C57BL6 mice. This study demonstrated that proinflammatory T cells inhibited MSCs-induced bone formation via IFN-$\gamma$ and TNF-$\alpha$ release [64]. IFN-$\gamma$ induced downregulation of the runt-related transcription factor 2 (Runx2) pathway and enhanced TNF-$\alpha$ regulated MSC apoptosis (Figure 3) [64]. In addition, TNF-$\alpha$ was shown to convert IFN-$\gamma$ activated nonapoptotic Fas to a caspase-8/3-associated apoptotic signal in MSCs via inhibition of NF-$\kappa\beta$ signaling, leading to MSC apoptosis [64]. Furthermore, systemic infusion of T cells inhibitory Foxp3$^{+}$ regulatory T cells (Tregs) significantly reduced levels of TNF-$\alpha$ and IFN-$\gamma$ and resulted in improved MSCs-mediated bone regeneration and calvarial defect repair [64].

*3.2.2. Use of Allogeneic MSCs.* Since MSCs isolated from senior people, diseased individuals, and females possess inferior osteogenic potential, it is advantageous to use allogeneic MSCs isolated from young, healthy males to enhance bone repair in these populations. However, some of the studies conducted in animal models suggest that use of allogeneic MSCs is not feasible owing to the immune response of the recipient host to transplanted MSCs.

An early study demonstrated a greater proportion of host-derived CD8$^{+}$ T cells and NK cells infiltrate in the implants of MSCs implanted subcutaneously in allogeneic MHC-mismatched mice compared to syngeneic controls [68].

In another study by Nauta et al., bone marrow transplantation was performed with or without host or donor MSCs in allogeneic murine recipients [69]. The addition of host MSCs significantly increased the long-term engraftment associated with tolerance to host and donor antigens [69]. The infusion of donor MSCs, on the other hand, was associated with significantly increased rejection of allogeneic bone

marrow cells and the induction of a memory T cell response [69]. This suggests that although autologous MSCs promote bone marrow engraftment *in vivo*, allogeneic MSCs are not intrinsically immunoprivileged [69].

In a murine model of allogeneic heart transplantation, MSCs from MHC mismatched allogeneic donors were implanted at various doses with and without cyclosporine A administration [70]. MHC mismatched MSCs not only failed to prolong allograft survival, but tended to accelerate allograft rejection [70]. Subsequently, MSC injections were ineffective at prolonging allograft survival and may even contribute to rejection [70]. Furthermore, in this study the immunosuppressive effect of cyclosporine A was abrogated by allogeneic MSCs, indicating a potential interaction *in vivo* between allogeneic MSC and cyclosporine A activities which is generally not observed *in vitro*.

The immunosuppressive potential of MSCs *in vivo* was tested by examining their ability to construct ectopic bone in both syngeneic and allogeneic murine recipients [71]. MSCs derived from bone marrow, placenta, and umbilical tissue were implanted with demineralized bone matrix under the kidney capsule. Bone formation was observed in only the syngeneic hosts, whereas the allogeneic hosts experienced transplant rejection. This data supports the argument for strong immunogenicity of MSCs in allogeneic recipients *in vivo* [71].

Our group has shown that cloned MSCs isolated from Balb/c mice could not induce ectopic bone formation in allogeneic B6 mice but bone formation was observed in syngeneic Balb/c mice and allogeneic mice lacking T and B cells. Expression of osteogenic genes (alkaline phosphatase, osteocalcin, and Runx2) was severely inhibited in allogeneic implants in comparison with syngeneic setting [24]. We also demonstrated a significant increase in numbers of T and B lymphocytes and macrophages recruited to the site of MSC implants in allogeneic hosts compared to the syngeneic group. Additionally, MSCs were shown to induce a larger proportion of Treg cells in the syngeneic group compared to the allogeneic group [24]. The Th1 immune response seems to be responsible for inhibiting osteogenesis in the allogeneic hosts, as evidenced by significantly increased levels of IFN-$\gamma$, the signature cytokine for the Th1 immune response [24].

In a more recent study of allogeneic versus autogeneic MSC implantation in rhesus macaques, increased production of NK, B and T cell subsets, and allo-specific antibodies was detected in the peripheral blood of those animals that received injection of the allogeneic MSCs targeted to caudate nucleus of the brain [72]. The magnitude and nature of the immune response correlated with the degree of MHC class I and II mismatch between the donor and recipients [72]. However, secondary antigen challenge did not elicit a measureable immune response in those recipients of allogeneic MSCs. Thus it was concluded that MSCs are weakly immunogenic in MHC mismatched individuals, which has implications for durable engraftment [72].

In a rat model of knee meniscus regeneration, the effects of autogeneic and allogeneic transplantation of synovial MSCs into rats with anterior meniscus defects were investigated [73]. The autogeneic group demonstrated a greater degree of meniscus regeneration than major mismatched transplant recipients at four weeks after transplant [73]. The number of macrophages and $CD8^+$ T cells in the knee synovium was significantly lower in the autogeneic recipients compared to the allogeneic major mismatched group [73]. Results for the allogeneic minor mismatched recipients were comparable to the autogeneic group [73].

In complete disagreement with the studies mentioned above, several other investigations have obtained promising results on use of allogeneic MSCs. These animal studies suggest that allogeneic MSCs are immunoprivileged and it is possible to employ allogeneic MSCs for enhancement of bone repair.

In a study by Arinzeh et al., autogeneic and allogeneic MSCs were loaded onto a hollow cylinder of hydroxyapatite-tricalcium phosphate before being implanted into a critical-sized femoral defect of dogs. After radiographic, histological, and serum antibody evaluation at four, eight, and sixteen weeks, there were no adverse host responses detected at any time point [74]. Histological results between those defects filled with implants containing allogeneic MSCs and those filled with autologous MSCs were similar at 16 weeks, demonstrating callus formation across the length of the defect and lamellar bone in the pore of the implant at the host bone-implant interface [74]. Those implants filled with either autogeneic or allogeneic MSCs demonstrated a significantly greater amount of bone growth within the pore spaces than those implants that contained no MSCs [74].

In another study, autogeneic and allogeneic bone marrow MSCs were cultured in an osteogenesis-inducing medium and implanted into tibial shaft defects of mini-pigs [75]. There was no statistically significant difference in bone repair between the two groups [75]. There was a slight statistically significant increase in CD4 and CD8 T cells, as well as levels of IL-2 in both groups after transplantation, which likely indicates a traumatic inflammatory response [75]. This seemed to have no influence on the immunogenicity and osteogenic capacity of either autogeneic or allogeneic MSCs [75].

In a study of segmental radius defects in rabbits, bone marrow MSCs were culture expanded *in vitro*, and the defect was filled with hydroxyapatite alone, hydroxyapatite with autogeneic MSCs, or hydroxyapatite with allogeneic MSCs [76]. The groups with the addition of either autogeneic or allogeneic MSCs both demonstrated increased osteogenesis with superior quality cancellous bone and bone marrow formation as compared to the control group with hydroxyapatite alone [76]. No significant differences in results were observed between the autogeneic or allogeneic groups [76].

Similarly, allogeneic adipose-derived MSCs combined with demineralized bone matrix were shown to successfully regenerate ulnar bone defects in rabbits without generating an immunological response [77].

Yet another study yielded similar results using allogeneic peripheral blood derived MSCs or bone marrow derived MSCs combined with resorbable porous calcium phosphate substitute (Skelite) and implanted in bilateral critical-sized ulnar defect in rabbits [78]. Bone formation in the peripheral blood derived MSCs/Skelite group was comparable to

the bone marrow derived MSCs/Skelite group, and both groups showed significantly enhanced bone regeneration when compared to controls [78].

A study of human adipose-derived MSCs embedded in fibrin glue and then implanted in a critical-sized defect in immunocompetent rat mandibles demonstrated a significantly higher amount of ossification on radiographic examination when compared to controls [79]. The level of bone regeneration using the adipose-derived MSCs was shown to be comparable to the gold standard of autologous bone grafting [79]. Similarly, another study using human MSCs in a hydroxyapatite-tricalcium phosphate scaffold implanted into a critical-sized calvarial defect in nude mice resulted in enhanced osteogenesis when compared to controls with scaffold alone [80].

Another study evaluated ectopic bone formation induced by allogeneic MSCs that were seeded onto a $\beta$-tricalcium phosphate scaffold and implanted subcutaneously into dogs [30]. There was no significant difference found in the number of CD4 T cells, CD8, T cells, and CD4/CD8 T cell ratios between the recipients of allogeneic MSCs and those that received either scaffold alone or scaffold seeded with autogeneic MSCs [30]. Both the autogeneic and allogeneic implants yielded subcutaneous ectopic bone formation, unlike the control group with scaffold alone [30].

A study by Lee et al. tested the immunogenicity of allogeneic human umbilical cord blood-derived MSCs via repeated intravenous injection into a humanized immunocompromised mouse model [81]. The human MSCs did not elicit an immunological response in the form of T cell proliferation or increased IFN-$\gamma$ and TNF-$\alpha$ levels [81]. Additionally, mice that received intravenous injections of human peripheral blood mononuclear cells demonstrated lymphocyte infiltration in the lung and small intestine and reduced survival rates, while those that received MSCs demonstrated no such adverse events, suggesting the low immunogenicity of MSCs *in vivo* [81].

Similar to their effect on natural fracture healing, T cells, IFN-$\gamma$, and TNF-$\alpha$ inhibit bone formation induced by exogenously added MSCs. Treg cells that inhibit activities of T cells promote MSC-mediated bone formation. While more scientific studies addressing the controversial immunoprivileged status of MSCs are certainly needed, it is possible to draw a few inferences from existing published literature that can help to decide future direction of the research in this field. Intriguingly, out of seven studies [24, 68–73] that attest to the nonimmunoprivileged status of MSCs, six were performed in mice while only one study used a rhesus macaques model. On the other hand, out of nine studies [23, 30, 74–80] that demonstrated successful use of allogeneic (or xenogeneic) MSCs, six utilized large animal models (rabbits, pigs, and dogs) and one study used xenogeneic rats while two studies used immunocompromised xenogeneic mice. This observation suggests that mice are not good hosts to accept allogeneic MSCs in comparison with other animal models, but the mechanisms remain unknown at this time. Another interesting difference between two groups of studies was that all the studies that demonstrated successful use of allogeneic MSCs used a fracture model or bone defect model while all the studies demonstrating failure of allogeneic MSCs transplanted MSCs in tissues other than bone. It is necessary to investigate what factors in the inflammatory microenvironment at injured bone promote survival and differentiation of allogeneic MSCs.

## 4. *In Vitro* Studies on MSC Regulation of T Cells

It is widely believed that, upon transplantation, MSCs can evade the immune system of major histocompatibility complex- (MHC-) mismatched host since MSCs display low expression of MHC class I molecules and completely lack MHC class II molecules as well as other costimulatory molecules (CD40, CD40L, CD80, and CD86) required for immune cell stimulation. Although the expression of MHC class I and II molecules can be upregulated by MSC exposure to inflammatory cytokines interferon-gamma (IFN-$\gamma$) and tumor necrosis factor-alpha (TNF-$\alpha$), they are still unable to induce an immunological response [18].

MSCs possess significant and diverse immunomodulatory properties that affect both the innate and adaptive immune systems. With regard to the adaptive immune system, MSCs have been shown to have direct immunosuppressive properties by inhibiting the activation and proliferation of effector T cells (both $CD4^+$ and $CD8^+$) via cell-to-cell contact and the elaboration of various soluble factors [18]. MSCs can also induce generation and the proliferation of T-cell inhibitory regulatory T (Treg) cells [18]. Both the direct suppression of MSCs on effector T lymphocytes and the indirect suppression mediated by MSC induction of Treg proliferation have been well documented in *in vitro* studies, which will be reviewed later in this section. Of note, MSCs seem to require "licensing" or activation by exposure to inflammatory cytokines such as IFN-$\gamma$, TNF-$\alpha$, and interleukin- (IL-) 1$\beta$ prior to exerting their immunomodulatory effects [82–84].

Intriguingly, the abundance of mediators and proposed mechanisms suggests a complex interplay in which MSCs may be either immunosuppressive or immunogenic [82, 83]. The dominant effect seems to depend on the microenvironment of the cellular milieu as well as the ratio of MSCs to T lymphocytes. A high MSC to lymphocyte ratio is associated with an inhibitory effect on the immune response, whereas a low MSC to lymphocyte ratio is characterized by an enhanced proliferation of lymphocytes [85]. The immunomodulatory effects of MSCs on these T cell subsets also appear to occur in a dose-dependent fashion [85]. More recently, a new paradigm has been proposed in which MSCs can be polarized into two phenotypes based on the stimulation of specific toll-like receptors. TLR4 stimulation polarizes them into a proinflammatory phenotype whereas TLR3 stimulation of MSCs leads to immunosuppressive signature. The first proinflammatory and immunocompetent phenotype is denoted as MSC1, while MSC2 is used to denote MSCs possessing anti-inflammatory and immunosuppressive characteristics [29, 86].

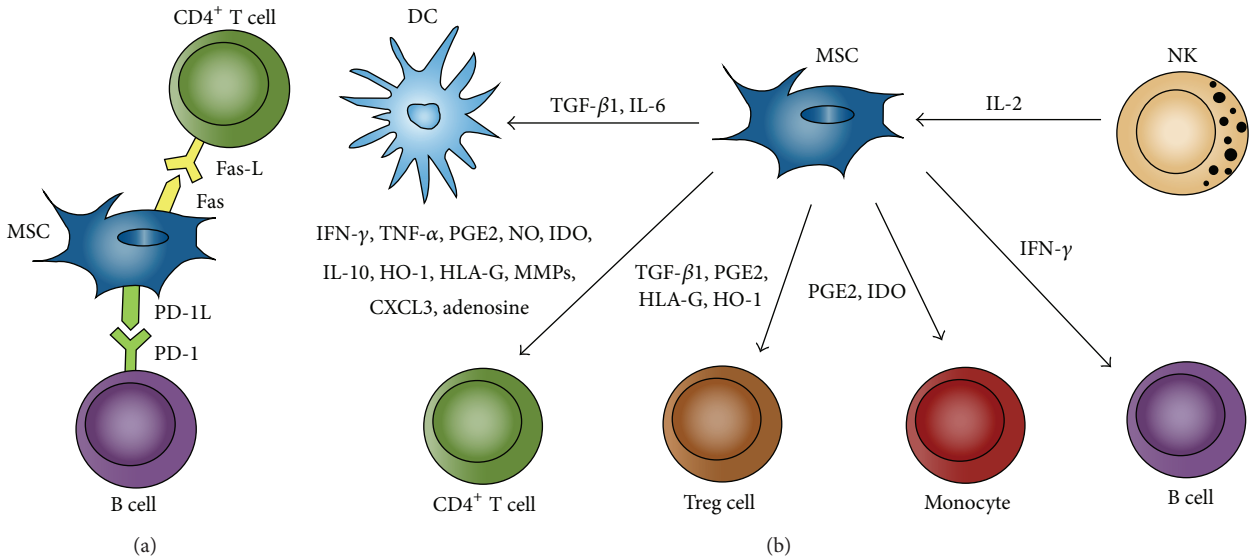


Figure 2: Mechanism of MSC modulation of immune cells. (a) Direct cell-cell contact, (b) soluble factors interactions.

*4.1. T Cell Differentiation and Function.* T helper (Th) cells are cytokine-producing $CD4^+$ T cells that may differentiate into either of the well-defined subsets Th1 and Th2, depending on the peptides presented to them by major histocompatibility complex (MHC) class II molecules on antigen presenting cells (APCs) [87]. The differentiation of Th1 cells is guided by interleukin- (IL-) 2, IL-12, and interferon-gamma (IFN-$\gamma$). The main effector cytokines of Th1 cells are IFN-$\gamma$ and tumor necrosis factor-beta (TNF-$\beta$). Th1 cells function to recruit macrophages, as well as induce the production of immunoglobulin (Ig) G by B cells. Th2 cell differentiation is guided by IL-4, and their main effector cytokines are IL-4, IL-5, and IL-13. The primary effector function of Th2 cells is to recruit eosinophils, basophils, and mast cells [87]. Th2 cells also mediate B cell antibody class switching to IgE and IgG. Cytotoxic T lymphocytes (CTLs) are $CD8^+$ T cells whose differentiation is guided by the presentation of an antigen by an MHC class I molecule upon an antigen presenting cell (APC), as well as costimulation by either CD80 or CD86 on the same APC. Once activated, IL-2 stimulates CTL proliferation. Th17 cells are a developmentally distinct type of T helper cell whose differentiation is guided by TGF-$\beta$, IL-6, and IL-21. The main effector cytokine of Th17 cells is IL-17, which plays an antimicrobial role at epithelial and mucosal barriers. Regulatory T cells (Tregs) are a subset of $CD4^+$ T lymphocytes that are characterized by the expression of cell surface receptor CD25, as well as the presence of high levels of transcription factor forkhead box P3 (Foxp3). Tregs function to modulate the immune system and maintain tolerance to self-antigens. The mechanism by which Tregs carry out their regulatory function is not well understood, though immunosuppressive cytokines TGF-$\beta$ and IL-10 are well implicated as role players [87].

*4.2. MSCs Inhibit T Lymphocyte Proliferation.* Both murine and human MSCs have been shown to inhibit the proliferation of stimulated T lymphocytes *in vitro* in both allogeneic and autologous settings [86]. The immunosuppressive effect of MSCs on allogeneic and autologous T lymphocyte proliferation is dependent on a high MSC to lymphocyte ratio and soluble factors [86]. Schurgers et al. demonstrated similar dose-dependent immunosuppressive effects of MSCs on anti-CD3-induced allogeneic T cell proliferation. However, MSCs did not show immunosuppressive effects *in vivo*. The authors demonstrated a role for inducible nitric oxide (iNOS), programmed death ligand-1 (PD-L1), and prostaglandin E2 (PGE2), but not indoleamine 2,3-dioxygenase (IDO), in T cell inhibition *in vitro* [88].

There are a variety of proposed mechanisms by which MSCs mediate this T cells inhibition (Table 1, Figure 2). Initial data demonstrated that MSCs do not induce T-cell apoptosis but instead inhibit proliferation by inducing T cell cycle arrest in the G0 phase [36, 89, 90]. However, a recent study demonstrated that MSCs also could induce transient T cell apoptosis mediated by the FAS ligand-(FASL-) dependent FAS pathway [34]. Additionally, MSC immunosuppression seems to be mediated in part by the activation of nuclear factor kappa B (NF-$\kappa$B) signaling in MSCs, and this pathway is activated by TNF-$\alpha$ generated by the TCR stimulation of allogeneic T cells [35, 91]. MSCs have been shown to inhibit the effects of CTLs by limiting their proliferation rather than their cytolytic activity. The mechanism by which MSCs exert this immunosuppressive effect on CTLs involves B7-H4, a negative costimulatory molecule that induces cell cycle arrest and inhibits the nuclear translocation of nuclear factor kappa beta (NF-$\kappa\beta$) [92, 93].

Human bone marrow MSCs have also been shown to inhibit antigen-dependent $CD4^+$ and $CD8^+$ T cell proliferation in an allogeneic setting *in vitro* [94]. The suppressive effect of MSCs on $CD4^+$ and $CD8^+$ T cells is due to inhibition of T cell proliferation, as opposed to effector function, as the cytotoxicity of T cells seems to be unaffected [95]. Human MSCs were shown to downregulate level of CD8 expression significantly on allogeneic $CD8^+$ T cells.

Table 1: List of mediators MSCs use to modulate proliferation and function of T cells.

| Mediator | Target Cells | Modulation | Reference |
|---|---|---|---|
| Inducible nitric oxide (iNOS) | T cells | Inhibition of proliferation induced by anti-CD3 antibody | [28] |
| Programmed death ligand-1 (PD-L1) | T cells | Inhibition of proliferation induced by anti-CD3 antibody | [29] |
| Prostaglandin E2 (PGE2) | T cells | Inhibition of proliferation induced by anti-CD3 antibody | [30–33] |
| B7-H4 (Negative co-stimulatory molecule) | CTLs | Induces cell cycle arrest | [34, 35] |
| Fas ligand (Fas L) | T cells | Transient T cell apoptosis | [36] |
| TGF-$\beta$, human leukocyte antigen-G5 (HLA-G5), Notch1 ligands, heme oxygenase-1 (HO-1) | $CD4^+$ T cells | Induction of Treg phenotype | [31, 37–41] |
| Chemokine receptor 6 (CCR6), and CD39 | Th17 cells | Induction of Treg phenotype | [42–44] |
| ICAM-1, VCAM-1 | T cells | Inhibition of proliferation through cell-cell contact | [45] |
| EP4 receptor, PD-L1, IL-10 | T cells | Inhibition of Th17 differentiation | [32, 46, 47] |
| Galectins | T cells | Inhibition of proliferation | [48–50] |
| Indoleamine dioxygenase (IDO) | Human T cells | Inhibition of proliferation | [51–53] |
| MMP-2, MMP-9 | Activated T cells | Cleavage of IL-2 receptor (CD25) on T cell surface leading to inhibition of proliferation | [54] |

The mechanism involved induction of a tolerogenic monocyte phenotype (lower expression of costimulatory molecules CD80 and CD86, higher expression of inhibitory receptors ILT-3 and ILT-4) representing an alternative mechanism for immunosuppression [96]. A more recent study confirmed that allogeneic MSCs inhibit the proliferation of $CD8^+$ T cells in a mixed lymphocyte reaction [31].

Conflicting data exists as to whether MSCs are susceptible to lysis by activated CTLs. MSCs were shown to be resistant to lysis by allogeneic effector CTLs, and this was associated with inefficient upregulation of CD25 surface molecules on activated cells, as well as a lack of IFN-$\gamma$ and TNF-$\alpha$ production by the CTLs [21]. Allogeneic MSCs were shown to be susceptible to lysis by $CD8^+$ CTLs, whereas autologous MSCs were resistant to $CD8^+$ CTL lysis [97]. Another study demonstrated that $CD8^+$ T cells were capable of HLA specific lysis of allogeneic BMSCs, and that this effect was augmented by exposure to IFN-$\gamma$ [37].

*4.3. MSCs Induce Treg Proliferation.* As a part of exerting their immunosuppressive effects, MSCs are able to induce the generation of classic $CD4^+CD25^+Foxp3^+$ Tregs. Numerous mediators and mechanisms have been proposed to be involved in MSC promotion of this classic Treg phenotype. Allogeneic MSCs have been shown to induce Foxp3 and CD25 expression in $CD4^+$ T cells through direct cell contact followed by production of MSC-derived TGF-$\beta$1 and PGE2 [38, 82]. Another study in which MSCs were shown to promote the generation of $CD4^+CD25^+Foxp3^+$ Tregs also supports the role of TGF-$\beta$1 in the mechanism of induction [46]. Selmani et al. demonstrated that human leukocyte antigen-G5 (HLA-G5) was required for MSC promotion of Tregs in an allogeneic setting [98]. Notch1 signaling has been implicated in the mechanism of MSC induction of Treg differentiation from allogeneic, activated $CD4^{(+)}$ T lymphocytes given that MSCs express the Notch1 ligands Jagged1, Jagged2, and Delta-Like (DLL) 1, 3, and 4 [39]. Luz-Crawford et al. demonstrated that MSCs were able to suppress the proliferation, activation, and differentiation of allogeneic Th1 and Th17 cells, and this immunosuppressive effect was associated with the induction of $CD4^+CD25^+Foxp3^+$ Treg cells [42]. Additionally, when MSCs were cocultured with allogeneic Tregs, MSCs seemed to augment the immunosuppressive capability of the Treg cells, and this effect was accompanied by an upregulation of the PD-1 receptor on Tregs via the production of IL-10 [43]. Yet another study demonstrated that MSC production of heme oxygenase-1 (HO-1) is involved in Treg induction [99].

In addition, MSCs have been shown to induce epigenetic changes at the promoter of the FOXP3 gene locus in allogeneic Th17 cells that led the acquisition by Th17 cells to inhibit the proliferative response of activated $CD4^+$ T cells *in vitro* [45]. In this same study, MSCs seemed able to promote the differentiation of proinflammatory Th17 cells into functional Tregs via chemokine receptor 6 (CCR6) [45]. Another study suggests that adenosine produced by MSCs could play a role in promoting the differentiation of Th17 cells into Tregs via the upregulation of CD39 [32].

*4.4. Direct Cell-to-Cell Contact.* Direct modulatory effects of MSCs on both autologous and allogeneic T lymphocytes via cell-to-cell contact have been well described *in vitro*, supported by the demonstration that MSCs express various integrins, intracellular adhesion molecules, and vascular cell adhesion proteins on their cells surface [16, 48]. Ren et al. provided further evidence for the necessity of cell-to-cell contact for the immunosuppressive effects of MSCs on T

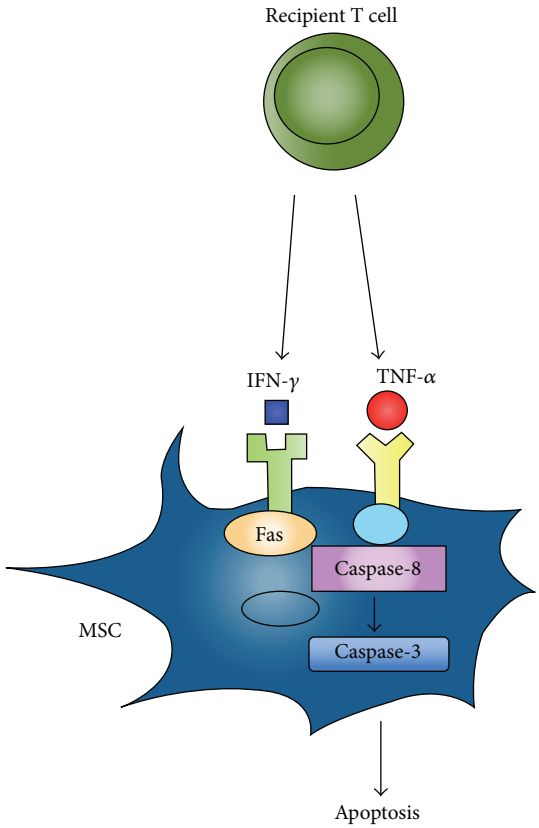


Figure 3: Immune cell modulation of MSCs. Combined action of IFN-$\gamma$ and TNF-$\alpha$ induces apoptosis of MSC.

lymphocytes [49]. The expression of cell-to-cell adhesion molecules ICAM-1 and VCAM-1 by MSCs was positively correlated with the immunosuppressive effects of MSCs towards various subtypes of T cells [49]. Furthermore, the genetic deletion or functional blocking of these adhesion molecules led to significant reversal of MSC immunosuppressive effects [49].

One study proposes that MSC inhibition of allogeneic Th17 cell differentiation is mediated by PGE2 via the EP4 receptor and is dependent on cell-cell contact [50]. Another study supports the notion that cell-to-cell contact is necessary for the inhibition of Th17 differentiation, and that this is mediated specifically by the upregulation of programmed death-1 (PD-1) ligand expression on IFN-$\gamma$ primed allogeneic MSCs [42].

Galectins are a family of cell surface proteins with a broad variety of functions, including the ability to bind neuropilin-1 (NP-1) on the surface of T cells and induce cell cycle arrest [51]. Allogeneic MSCs have been shown to constitutively express galectins, and these molecules help to mediate the immunosuppressive effect of MSCs [52]. Specifically, galectin-1 and galectin-3 were shown to inhibit T-cell proliferation, and genetic knockdown of these molecules resulted in a significant loss of immunomodulatory properties, specifically upon $CD4^+$ and $CD8^+$ T cell proliferation [51–53]. The effect of allogeneic MSCs on NK cells appeared to be unaffected by galectin-1 knockdown, however [53]. It was also found that the production of galectin-9 in allogeneic MSCs was strongly upregulated in the presence of proinflammatory cytokines IFN-$\gamma$ and TNF-$\alpha$, and this was associated with the antiproliferative effects that MSCs have on T cells [53].

*4.5. Mechanism of MSCs Inhibition of T Cells through Soluble Mediators.* Although it has been shown that cell-to-cell contact is necessary for MSC mediated immunosuppression, there are several experiments that have been performed demonstrating that both autologous and allogeneic MSCs also exert their immunomodulatory effects through the elaboration of soluble factors [48]. Given the plethora of mediators proposed, it is likely a combination of complex interplay between these factors and the specific inflammatory milieu that contributes to metabolic manipulation of the microenvironment and the overall immunomodulatory effects of MSCs [48].

It is important to recognize that there are some well-delineated differences between MSCs from different species. Notably, MSCs derived from mice produce nitric oxide (NO) via inducible nitric oxide synthase (iNOS) to suppress T cell proliferation [49]. NO has been shown to suppress the phosphorylation of signal transducer and activator of transcription-5 (STAT-5), which is a critical transcription factor for T cell activation and proliferation [49]. In contrast, human allogeneic MSCs exert this effect by the upregulation of indoleamine 2,3-dioxygenase (IDO), an enzyme involved in the catabolism of essential amino acid tryptophan to N-formylkynurenine [100]. Delarosa et al. demonstrated that human adipocyte-derived MSCs are activated by IFN-$\gamma$ to express functional IDO in allogeneic settings [101]. Furthermore, IDO expression upon IFN-$\gamma$ activation is essential for the immunosuppressive activity of allogeneic AMSCs, since IDO exerts its effects through the accumulation of tryptophan metabolites in the local microenvironment [101, 102].

*4.5.1. IFN-$\gamma$.* Interferon-gamma (IFN-$\gamma$) is an inflammatory cytokine that plays a critical role in licensing allogeneic MSCs to inhibit activated T cell proliferation, and this process is IDO-dependent [103]. IFN-$\gamma$ activates the synthesis of IDO and upregulates the expression of hepatocyte growth factor (HGF) and TGF-$\beta$ by allogeneic MSCs [104]. When compared to unprimed MSCs, MSCs pretreated with IFN-$\gamma$ and TNF-$\alpha$ were more effective at inhibiting T cell proliferation [47]. IFN-$\gamma$ also plays a role in allogeneic MSC suppression of T lymphocyte effector functions, namely through the inhibition of Th1 cytokines (IFN-$\gamma$, TNF-$\alpha$, and IL-2), and this process is mediated by the PD-1 ligand on MSCs [105].

*4.5.2. TNF-$\alpha$.* Tumor necrosis factor-alpha (TNF-$\alpha$) is another inflammatory cytokine that has been shown to augment the immunomodulatory properties of MSCs. Studies have shown that TNF-$\alpha$, along with IFN-$\gamma$, promotes the expression of HGF, PGE2, and COX-2 levels by allogeneic MSCs, contributing to the inhibition of proliferating T lymphocytes [106]. More recently, it has been demonstrated

that TNF-$\alpha$ released by activated T cells binds to TNF-R1 on allogeneic MSCs, activating the NF-$\kappa$B pathway and contributing the immunosuppressive properties of MSCs [35, 91].

*4.5.3. IL-10.* IL-10 is an anti-inflammatory cytokine produced by monocytes, Th2 cells, and Tregs. It functions to downregulate the expression of Th1 cytokines, MHC class II antigens, and macrophage costimulatory molecules. Allogeneic MSCs cocultured with either naïve or activated T cells have been shown to produce a significant amount of IL-10, and this was associated with significant suppression of T cell proliferation [100]. The addition of anti-IL-10 and anti-IL-10-receptor antibodies restored T cell proliferative capacity, providing further evidence for the critical role of IL-10 in allogeneic MSC immunosuppression of T lymphocyte proliferation [100]. Qu et al. demonstrated that allogeneic MSCs were able to inhibit Th17 differentiation *in vitro* via the secretion of IL-10 [107]. More recently, it has been shown that allogeneic MSCs cocultured with $CD4^+$ T cells led to increased secretion of IL-10 by T helper cells [108]. Allogeneic MSCs are able to inhibit Th17 cell differentiation [107]. Since Th17 differentiation was restored when IL-10 was specifically neutralized or the expression of IL-10 by MSCs was downregulated by RNA interference, it has been suggested that this effect is mediated by IL-10 secretion by MSCs [107].

*4.5.4. PGE2.* Prostaglandin E2 (PGE2) is an enzyme responsible for the metabolism of arachidonic acid and prostaglandin production [109]. PGE2 prevents the proliferation of T cells and inhibits production of cytokines such as TNF-$\alpha$ and IL-12 [33, 110]. It also downregulates MHC class II molecules on macrophage surfaces and skews the T helper differentiation towards a Th2 response with IL-4 and IL-5 production [28, 111]. High levels of PGE2 produced by allogeneic MSCs have been shown to inhibit the maturation of dendritic cells, as well as the proliferation of activated T cells and their subsequent proinflammatory cytokine production [40].

*4.5.5. HO-1.* Heme oxygenase-1 (HO-1) is an inducible enzyme that catalyzes the first and rate-limiting step in the degradation of heme into biliverdin, iron, and carbon monoxide [41]. The products of heme metabolism produced by HO-1 during inflammation are associated with antiapoptotic, antioxidative, and anti-inflammatory effects [41]. HO-1 has been implicated as having a role in the mechanism of allogeneic MSC induction of Treg proliferation and IL-10 production [99]. Once MSCs have been licensed by inflammatory factors in a mixed lymphocyte reaction, however, there was substantial downregulation of HO-1, yet Treg induction as well as IL-10 production by MSCs was not affected [99]. This suggests that HO-1 plays an initial role in MSC immunosuppressive effects *in vitro*, but this is taken over by other molecules after alloreactive priming [99].

*4.5.6. Nitric Oxide.* As mentioned earlier, NO has been shown to suppress the phosphorylation of signal transducer and activator of transcription-5 (STAT-5), which is a critical transcription factor for T cell activation and proliferation [49]. Another study demonstrated that NO production by allogeneic MSCs suppressed the proliferation of T lymphocytes via the inhibition of STAT5 phosphorylation, and that inhibitors of inducible NO synthase (iNOS) restored the proliferation of T cells [112]. The presence of cytokines TNF-$\alpha$ and IL-1$\beta$ was shown to provoke the expression of high levels of iNOS by MSCs [49].

*4.5.7. HLA-G.* MSCs have been shown to mediate their immunomodulatory effects via the production of soluble factor human leukocyte antigen-G (HLA-G) [54]. HLA-G secretion by allogeneic MSCs has been shown to suppress T cell proliferation in mixed lymphocyte reactions [54, 113]. Exogenous IL-10 was shown to stimulate HLA-G secretion and was shown to play a key role in allogeneic MSC inhibition of peripheral blood mononuclear cell response to phytohemagglutinin [44]. Another study demonstrated that HLA-G secretion by human allogeneic MSCs not only suppressed allogeneic T lymphocytes, but also induced the proliferation of $CD4^+CD25^+Foxp3^+$ Tregs [98]. This same study also demonstrated that MSCs inhibit cell-mediated lysis and IFN-$\gamma$ secretion by allogeneic NK cells [98].

*4.5.8. MMPs.* Matrix metalloproteinases (MMPs) derived from allogeneic MSCs, in particular MMP-2 and MMP-9, have been shown to cause the cleavage of IL-2 receptor $\alpha$ (CD25) from the surface of activated T cells and thus the suppression of IL-2 production and T cell proliferation [62, 114].

*4.5.9. Chemokines.* Chemokines CXCL1, 2, and 3 were shown to be induced in T cells cocultured with allogeneic MSCs. CXCL3, in particular, was associated with the inhibition of T cell proliferation and increased apoptosis [115].

*4.5.10. Adenosine.* Both human and murine allogeneic MSCs have been shown to generate adenosine, which inhibits the proliferation of T lymphocytes by acting through its receptor A(2a) (ADORA2A) [116, 117]. MSCs upregulate CD39 and increase adenosine production to suppress activated T-lymphocytes [116].

## 5. *In Vitro* Studies on MSC Regulation of Other Immune Cells

*5.1. Macrophages.* Macrophages differentiate from monocytes into one of two main phenotypes—immunogenic M1 macrophages and immunosuppressive M2 macrophages. Monocytes are stimulated towards the M1 phenotype by bacterial products, such as lipopolysaccharide (LPS), and inflammatory cytokines [118]. These M1 macrophages function in the phagocytosis of cellular debris and pathogens and secrete IFN-$\gamma$, TNF-$\alpha$, and IL-6, among other proinflammatory cytokines [118]. The M2 phenotype is induced by IL-4 and IL-13, secretes primarily IL-10, and functions in tissue repair [118]. Autologous and allogeneic MSCs have been shown

to significantly suppress the production of inflammatory cytokines TNF-$\alpha$, IL-6, IL-12p70, and IFN-$\gamma$ by macrophages, while increasing the production of anti-inflammatory IL-10 and IL-12p40 [119–121]. This process seemed to be mediated by PGE2 [119–121]. Additionally, both autologous and allogeneic MSCs seemed to inhibit the upregulation of CD86 and MHC class II expression in LPS-stimulated macrophages, impairing their immunogenic effects on $CD4^+$ T cell [103, 119]. More recent studies provide evidence that allogeneic MSCs promote the shifting of monocytes toward an anti-inflammatory M2 phenotype [122–124]. This M2 polarization induced by allogeneic MSCs may occur through the NF-$\kappa$B and STAT-3 pathways and involve IDO activity [103, 125]. Melief et al. suggest that the pathway involved in the shifting of monocytes towards the M2 phenotype is a necessary part of the ability of MSCs to induce Treg proliferation [122].

*5.2. Dendritic Cells.* Dendritic cells (DCs) are antigen presenting cells (APCs) that phagocytose and process antigens into peptides and present them via MHC molecules on their cell surface to prime T lymphocytes as part of the adaptive immune response [126]. DCs differentiate from monocytes and secrete IL-12, which aids in the differentiation of Th1 cells from naive $CD4^+$ T cells. Allogeneic MSCs have been shown to impair the maturation of DCs from monocytes or $CD34^+$ hematopoietic precursors, as well as their ability to secrete proinflammatory cytokines [127]. Additionally, allogeneic MSCs were shown to increase the release of anti-inflammatory IL-10, as well as inhibit the polarization of naïve $CD4^+$ lymphocytes into Th1 cells [128, 129]. Similar to the mechanisms of immunosuppression on T lymphocytes, allogeneic MSC mediated inhibition of DC function appears to be dependent on cell-to-cell contact [130]. One study suggests that TGF-$\beta$1 production and the downregulation of DC costimulatory molecules (such as CD80, CD86, and CD40) are responsible for the inhibitory effect of MSCs on DCs [131, 132]. DCs that have been cocultured with either autologous or allogeneic MSCs also display the ability to induce classic Treg differentiation from naïve T cells [131, 132]. One study suggests that allogeneic MSCs cocultured with monocyte-derived DCs secrete growth-regulated oncogene chemokines that drive the DCs towards a myeloid-derived suppressor cell-(MDSC-) like phenotype [133]. More recently, it has been suggested that MSCs mediate the upregulation of the gene SOCS1 via IL-6, which instructs DCs to acquire a tolerogenic phenotype with a significant increase in the production of IL-10 and the ability to induce Treg and Th2 differentiation [134].

*5.3. NK Cells.* Natural killer (NK) cells are a subset of cytotoxic lymphocytes that differentiate from the common lymphoid progenitor cell and help compose the immune response to viral-infected and tumor cells. NK cells may be activated by cytokines, such as IL-2, IL-12, IL-15, and IL-18, or by the recognition of cells that are missing MHC class I surface molecules [87]. Activation triggers the release of cytotoxic granules that induce cell lysis or apoptosis [87]. Allogeneic MSCs have been shown to inhibit resting NK cell proliferation induced by IL-2 but had a limited effect on active NK cell proliferation [135]. This same study demonstrated that IL-2-activated NK cells efficiently lyse autologous and allogeneic MSCs, but this lysis was inhibited when MSCs were exposed to IFN-$\gamma$, presumably due to the upregulation of HLA class I molecules on MSCs [135]. Another study demonstrated an inverse correlation between HLA class I expression on MSCs and lysis by NK cells [136]. A more recent study demonstrated that priming with Toll-like receptors (TLR), specifically TLR3, may play a role in decreasing allogeneic MSC susceptibility to IL-2-activated NK cell killing [137].

*5.4. B Cells.* B cells differentiate from the common lymphoid progenitor cells and function in the humoral immunity of the adaptive immune response by the production of antibodies. Early studies demonstrated that murine allogeneic MSCs had inhibitory effects on the proliferation, activation, and IgG secretion of B cells [138]. Allogeneic MSCs inhibit B cell proliferation by inducing cell cycle arrest in the G0/G1 phase and by the production of soluble factors [139, 140]. Allogeneic MSCs were also shown to modify the activation pattern of the extracellular response kinase 1/2 and the p38 mitogen-activated protein kinase pathways, which are both involved in B-cell viability, activation, and proliferation [140]. Another study suggests that MSCs mediate their inhibitory effects on B cells through maturation protein-1 expression [141]. Allogeneic MSC inhibition of B cell activation seems to be dependent on IFN-$\gamma$ and cell-to-cell contact via PD-1/PD-L1 interaction, in a similar fashion to MSC immunosuppression of T lymphocytes [142]. Contradictory data exists in which MSCs promote the proliferation and differentiation of B cells *in vitro* [143].

In summary, the immunosuppressive effects of allogeneic and autogeneic MSCs on immune cells are dependent on both the elaboration of soluble mediators as well as cell-to-cell contact. A high MSC to lymphocyte ratio also appears to be necessary to exert these effects, signifying a dose-dependent phenomenon.

The soluble mediators that function in the immunomodulatory role of MSCs with regard to the immune system have overlapping roles with the immunomodulation of bone cells, namely, osteoclasts and osteoblasts. Activated immune cells mediate increased bone turnover during inflammatory states; thus it seems plausible that the inhibitory effects of MSCs on these cells would promote an osteogenic state.

A key concern is that of negative regulation to prevent overimmunosuppression. In other words, great care should be taken to prevent complete suppression of the immune system, which could facilitate tumor formation or increased susceptibility to opportunistic infections.

It is likely a complex combination of synergism and antagonism among these various mechanisms that function to regulate the immune response. It is important to take into consideration that the aforementioned investigations were all conducted in *in vitro* settings, which may fail to include integral factors that are present in the *in vivo* milieu. Additionally, there are likely other unaccounted for factors that are specific to species, tissue, and experimental methods.

## 6. Role of Immune Cells and Cytokines in Modulating Osteogenic Differentiation of MSCs *In Vitro*

As discussed in Section 3, data from studies on human patients and experimental animals reveal that immune cells and cytokines produced by them, particularly T cells, IFN-$\gamma$, and TNF-$\alpha$ inhibit fracture healing and MSCs-induced bone formation. Treg cells and Th2 response seem to promote bone formation. However, the role of the Th2 response was reported in relation to ectopic bone formation and needs further validation in a fracture model. While IFN-$\gamma$ and TNF-$\alpha$ induced apoptosis of MSCs *in vitro*, which can explain inhibition of bone formation by these cytokines as recently reported, molecular mechanisms of immune cell regulation of bone formation remain largely unknown. Since the immune response is typically mounted sequentially—first the attack of innate immune cells (macrophages, monocytes, and NK cells), followed by the adaptive immune response (antigen presenting cells, $CD4^+$ T cells, $CD8^+$ T cells, and B cells)—and early responding cell types can alter response by T and B cells, it is necessary to understand how each cell type interacts with MSCs *in vitro*.

A study by Omar et al. demonstrated that human monocytes stimulated by either LPS or IL-4 communicate proosteogenic signals to allogeneic MSCs, as evidenced by the increased expression of run-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), and bone morphogenetic protein-2 (BMP-2) [144]. Since IL-4 stimulation primarily induces a Th2 response, this study suggests that the Th2 response would promote bone healing. Conditioned medium from cultures of human monocytes derived macrophages, however, was shown to suppress BMP-2-induced osteogenic differentiation of allogeneic MSCs, and this effect was associated with high levels of IL-1$\beta$ and TNF-$\alpha$ [145]. Several studies indicate an osteogenic role for monocytes and macrophages alike [146–148]. For example, Oncostatin M, a member of the IL-6 family of cytokines, produced by activated macrophages was identified as a key player in inducing osteoblast differentiation from allogeneic MSCs while also inhibiting adipogenesis [146, 147]. A more recent study provides supporting evidence that monocytes and macrophages induce osteogenic differentiation and proliferation of human allogeneic MSCs via the production of BMP-2 [148]. Nicolaidou et al. demonstrated that monocytes and macrophages potently induced human allogeneic MSC differentiation into osteoblasts, mediated by cell contact, the production of monocyte soluble factors, and the activation of MSC STAT3 signaling by monocyte production of OSM [147]. Another study demonstrated that LPS-stimulated monocytes induced osteogenesis from human allogeneic MSCs via exosomes that resulted in the increased expression of Runx2 and BMP-2 [149].

T cell subsets are reported to differently regulate osteogenic differentiation of human MSCs *in vitro*. Conditioned medium from human $CD4^+$ T cells but not from $CD8^+$ T cells was shown to significantly upregulate the expression of Runx2, osteocalcin, ALP, and bone sialoprotein of allogeneic MSCs, as well as increase the mineralization in osteogenic cultures of MSCs [150]. MSCs were shown to phagocytose apoptotic cells and this phagocytosis enhanced osteogenic differentiation of MSCs [151]. Apoptotic cells treated MSCs expressed CXCR4 and CXCR5 which might enable them to migrate towards inflamed sites such as fracture repair or arthritic joints. These MSCs also secreted IL-8, MCP-1, and RANTES that can induce chemotaxis of T cells [151].

## 7. Conclusion

Immune cells and the cytokines that they produce play an important role in bone healing. Along with growth factors, the cytokines also guide differentiation of osteoprogenitor MSCs. Although inflammation plays a key role in fracture repair, particularly during the initial and remodeling phases of healing, chronic exposure to lymphocytes and to inflammatory signaling have been shown to impair the fracture repair process. The role of various immune cells and their subtypes in bone healing is complex and not completely understood. Therefore, thorough understanding of the immune cells control of fracture healing and precise ways to control the immune cells will be necessary when modulating the inflammatory response as potential new therapy for bone tissue engineering. MSCs can be effectively used for this purpose since they possess abilities to modulate immune cells differentiation and functions in specific microenvironments.

With this in mind, we propose the following areas as key topics of future investigations in the field: investigating interaction between MSCs and immune cells, particularly T cells and their subtypes *in vitro* and *in vivo* [150, 151], developing noninvasive techniques for imaging trafficking and activation of immune cells [152], and investigating local and systemic delivery of immune cells modulating agents (Treg cells [153], cytokine specific antagonists [152], corticosteroids [154], and nonsteroidal anti-inflammatory drugs [155]) to enhance bone healing and to study mechanistic aspects of correlation between inhibition of specific immune cells activities and bone healing.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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## References

[1] E. Canalis, "Effect of growth factors on bone cell replication and differentiation," *Clinical Orthopaedics and Related Research*, vol. 193, pp. 246–263, 1985.

[2] H. M. Frost, "The biology of fracture healing. An overview for clinicians. Part I," *Clinical Orthopaedics and Related Research*, no. 248, pp. 283–293, 1989.

[3] P. Kolar, K. Schmidt-Bleek, H. Schell et al., "The early fracture hematoma and its potential role in fracture healing," *Tissue Engineering—Part B: Reviews*, vol. 16, no. 4, pp. 427–434, 2010.

[4] L. Claes, S. Recknagel, and A. Ignatius, "Fracture healing under healthy and inflammatory conditions," *Nature Reviews Rheumatology*, vol. 8, no. 3, pp. 133–143, 2012.

[5] K. Schmidt-Bleek, H. Schell, P. Kolar et al., "Cellular composition of the initial fracture hematoma compared to a muscle hematoma: a study in sheep," *Journal of Orthopaedic Research*, vol. 27, no. 9, pp. 1147–1151, 2009.

[6] N. T. Colburn, K. J. M. Zaal, F. Wang, and R. S. Tuan, "A role for $\gamma/\delta$ T cells in a mouse model of fracture healing," *Arthritis & Rheumatism*, vol. 60, no. 6, pp. 1694–1703, 2009.

[7] T. A. Einhorn and J. M. Lane, "Significant advances have been made in the way surgeons treat fractures," *Clinical Orthopaedics and Related Research*, no. 355, supplement, pp. S2–S3, 1998.

[8] C. Zeckey, P. Mommsen, H. Andruszkow et al., "The aseptic femoral and tibial shaft non-union in healthy patients—an analysis of the health-related quality of life and the socioeconomic outcome," *The Open Orthopaedics Journal*, vol. 5, no. 1, pp. 193–197, 2011.

[9] F. P. Barry and J. M. Murphy, "Mesenchymal stem cells: clinical applications and biological characterization," *The International Journal of Biochemistry & Cell Biology*, vol. 36, no. 4, pp. 568–584, 2004.

[10] M. F. Pittenger, A. M. Mackay, S. C. Beck et al., "Multilineage potential of adult human mesenchymal stem cells," *Science*, vol. 284, no. 5411, pp. 143–147, 1999.

[11] P. C. Demircan, A. E. Sariboyaci, Z. S. Unal, G. Gacar, C. Subasi, and E. Karaoz, "Immunoregulatory effects of human dental pulp-derived stem cells on T cells: comparison of transwell coculture and mixed lymphocyte reaction systems," *Cytotherapy*, vol. 13, no. 10, pp. 1205–1220, 2011.

[12] L. da Silva Meirelles, P. C. Chagastelles, and N. B. Nardi, "Mesenchymal stem cells reside in virtually all post-natal organs and tissues," *Journal of Cell Science*, vol. 119, no. 11, pp. 2204–2213, 2006.

[13] T. Tondreau, N. Meuleman, A. Delforge et al., "Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity," *Stem Cells*, vol. 23, no. 8, pp. 1105–1112, 2005.

[14] M. Najar, R. Rouas, G. Raicevic et al., "Mesenchymal stromal cells promote or suppress the proliferation of T lymphocytes from cord blood and peripheral blood: the importance of low cell ratio and role of interleukin-6," *Cytotherapy*, vol. 11, no. 5, pp. 570–583, 2009.

[15] M. Krampera, L. Cosmi, R. Angeli et al., "Role for interferon-$\gamma$ in the immunomodulatory activity of human bone marrow mesenchymal stem cells," *Stem Cells*, vol. 24, no. 2, pp. 386–398, 2006.

[16] M. K. Majumdar, M. Keane-Moore, D. Buyaner et al., "Characterization and functionality of cell surface molecules on human mesenchymal stem cells," *Journal of Biomedical Science*, vol. 10, no. 2, pp. 228–241, 2003.

[17] W. T. Tse, J. D. Pendleton, W. M. Beyer, M. C. Egalka, and E. C. Guinan, "Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation," *Transplantation*, vol. 75, no. 3, pp. 389–397, 2003.

[18] E. Klyushnenkova, J. D. Mosca, V. Zernetkina et al., "T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression," *Journal of Biomedical Science*, vol. 12, no. 1, pp. 47–57, 2005.

[19] S. Aggarwal and M. F. Pittenger, "Human mesenchymal stem cells modulate allogeneic immune cell responses," *Blood*, vol. 105, no. 4, pp. 1815–1822, 2005.

[20] S. Beyth, Z. Borovsky, D. Mevorach et al., "Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness," *Blood*, vol. 105, no. 5, pp. 2214–2219, 2005.

[21] I. Rasmusson, O. Ringdén, B. Sundberg, and K. le Blanc, "Mesenchymal stem cells inhibit lymphocyte proliferation by mitogens and alloantigens by different mechanisms," *Experimental Cell Research*, vol. 305, no. 1, pp. 33–41, 2005.

[22] D. Toben, I. Schroeder, T. El Khassawna et al., "Fracture healing is accelerated in the absence of the adaptive immune system," *Journal of Bone and Mineral Research*, vol. 26, no. 1, pp. 113–124, 2011.

[23] R. H. Lee, S. C. Hsu, J. Munoz et al., "A subset of human rapidly self-renewing marrow stromal cells preferentially engraft in mice," *Blood*, vol. 107, no. 5, pp. 2153–2161, 2006.

[24] A. S. Dighe, S. Yang, V. Madhu, G. Balian, and Q. Cui, "Interferon gamma and T cells inhibit osteogenesis induced by allogeneic mesenchymal stromal cells," *Journal of Orthopaedic Research*, vol. 31, no. 2, pp. 227–234, 2013.

[25] Y. Liu, L. Wang, T. Kikuiri et al., "Mesenchymal stem cell-based tissue regeneration is governed by recipient T lymphocytes via IFN-$\gamma$ and TNF-$\alpha$," *Nature Medicine*, vol. 17, no. 12, pp. 1594–1601, 2011.

[26] S. Reinke, S. Geissler, W. R. Taylor et al., "Terminally differentiated $CD8^+$ T cells negatively affect bone regeneration in humans," *Science Translational Medicine*, vol. 5, no. 177, Article ID 177ra36, 2013.

[27] Z. Chen, X. Mao, L. Tan et al., "Osteoimmunomodulatory properties of magnesium scaffolds coated with $\beta$-tricalcium phosphate," *Biomaterials*, vol. 35, no. 30, pp. 8553–8565, 2014.

[28] K. Katamura, N. Shintaku, Y. Yamauchi et al., "Prostaglandin E2 at priming of naive CD4+ T cells inhibits acquisition of ability to produce IFN-$\gamma$ and IL-2, but not IL-4 and IL-5," *The Journal of Immunology*, vol. 155, no. 10, pp. 4604–4612, 1995.

[29] A. M. Betancourt, "Induction of bone marrow-derived multipotent mesenchymal stromal cells into pro-inflammatory MSC1 and anti-inflammatory MSC2 phenotypes," in *Mesenchymal Stem Cells—Basics and Clinical Application II*, vol. 130 of *Advances in Biochemical Engineering/Biotechnology*, pp. 163–197, Springer, Berlin, Germany, 2013.

[30] F. Xie, L. Teng, Q. Wang et al., "Ectopic osteogenesis of allogeneic bone mesenchymal stem cells loading on beta-tricalcium phosphate in canines," *Plastic & Reconstructive Surgery*, vol. 133, no. 2, pp. 142e–153e, 2014.

[31] A. U. Engela, C. C. Baan, N. H. R. Litjens et al., "Mesenchymal stem cells control alloreactive $CD8^+CD28^-$ T cells," *Clinical and Experimental Immunology*, vol. 174, no. 3, pp. 449–458, 2013.

[32] M. Mandapathil, S. Lang, E. Gorelik, and T. L. Whiteside, "Isolation of functional human regulatory T cells (Treg) from the peripheral blood based on the CD39 expression," *Journal of Immunological Methods*, vol. 346, no. 1-2, pp. 55–63, 2009.

[33] T. C. T. M. van der Pouw Kraan, L. C. M. Boeije, R. J. T. Smeenk, J. Wijdenes, and L. A. Aarden, "Prostaglandin-E2 is a potent inhibitor of human interleukin 12 production," *Journal of Experimental Medicine*, vol. 181, no. 2, pp. 775–779, 1995.

[34] K. Akiyama, C. Chen, D. Wang et al., "Mesenchymal-stem-cell-induced immunoregulation involves FAS-ligand-/FAS-mediated T cell apoptosis," *Cell Stem Cell*, vol. 10, no. 5, pp. 544–555, 2012.

[35] A. Dorronsoro, I. Ferrin, J. M. Salcedo et al., "Human mesenchymal stromal cells modulate T-cell responses through TNF-$\alpha$-mediated activation of NF-$\kappa$B," *European Journal of Immunology*, vol. 44, no. 2, pp. 480–488, 2014.

[36] M. Krampera, S. Glennie, J. Dyson et al., "Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide," *Blood*, vol. 101, no. 9, pp. 3722–3729, 2003.

[37] M. R.-V. Rhijn, M. Khairoun, S. S. Korevaar et al., "Human bone marrow- and adipose tissue-derived mesenchymal stromal cells are immunosuppressive *in vitro* and in a humanized allograft rejection model," *Journal of Stem Cell Research & Therapy*, no. 1, supplement 6, Article ID 20780, 2013.

[38] K. English, J. M. Ryan, L. Tobin, M. J. Murphy, F. P. Barry, and B. P. Mahon, "Cell contact, prostaglandin $E_2$ and transforming growth factor beta 1 play non-redundant roles in human mesenchymal stem cell induction of $CD4^+CD25^{High}$forkhead box $P3^+$ regulatory T cells," *Clinical and Experimental Immunology*, vol. 156, no. 1, pp. 149–160, 2009.

[39] B. del Papa, P. Sportoletti, D. Cecchini et al., "Notch1 modulates mesenchymal stem cells mediated regulatory T-cell induction," *European Journal of Immunology*, vol. 43, no. 1, pp. 182–187, 2013.

[40] R. Yañez, A. Oviedo, M. Aldea, J. A. Bueren, and M. L. Lamana, "Prostaglandin E2 plays a key role in the immunosuppressive properties of adipose and bone marrow tissue-derived mesenchymal stromal cells," *Experimental Cell Research*, vol. 316, no. 19, pp. 3109–3123, 2010.

[41] H.-O. Pae and H.-T. Chung, "Heme oxygenase-1: its therapeutic roles in inflammatory diseases," *Immune Network*, vol. 9, no. 1, pp. 12–19, 2009.

[42] P. Luz-Crawford, M. Kurte, J. Bravo-Alegría et al., "Mesenchymal stem cells generate a $CD4^+CD25^+Foxp3^+$ regulatory T cell population during the differentiation process of Th1 and Th17 cells," *Stem Cell Research and Therapy*, vol. 4, no. 3, article 65, 2013.

[43] Z. Yan, Y. Zhuansun, R. Chen, J. Li, and P. Ran, "Immunomodulation of mesenchymal stromal cells on regulatory T cells and its possible mechanism," *Experimental Cell Research*, vol. 324, no. 1, pp. 65–74, 2014.

[44] R. Rizzo, D. Campioni, M. Stignani et al., "A functional role for soluble HLA-G antigens in immune modulation mediated by mesenchymal stromal cells," *Cytotherapy*, vol. 10, no. 4, pp. 364–375, 2008.

[45] S. Ghannam, J. Pène, G. Torcy-Moquet, C. Jorgensen, and H. Yssel, "Mesenchymal stem cells inhibit human Th17 cell differentiation and function and induce a T regulatory cell phenotype," *The Journal of Immunology*, vol. 185, no. 1, pp. 302–312, 2010.

[46] S. M. Melief, E. Schrama, M. H. Brugman et al., "Multipotent stromal cells induce human regulatory T cells through a novel pathway involving skewing of monocytes toward anti-inflammatory macrophages," *Stem Cells*, vol. 31, no. 9, pp. 1980–1991, 2013.

[47] J. Cuerquis, R. Romieu-Mourez, M. François et al., "Human mesenchymal stromal cells transiently increase cytokine production by activated T cells before suppressing T-cell proliferation: effect of interferon-$\gamma$ and tumor necrosis factor-$\alpha$ stimulation," *Cytotherapy*, vol. 16, no. 2, pp. 191–202, 2014.

[48] M. D. Nicola, C. Carlo-Stella, M. Magni et al., "Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli," *Blood*, vol. 99, no. 10, pp. 3838–3843, 2002.

[49] G. Ren, L. Zhang, X. Zhao et al., "Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide," *Cell Stem Cell*, vol. 2, no. 2, pp. 141–150, 2008.

[50] M. M. Duffy, T. Ritter, R. Ceredig, and M. D. Griffin, "Mesenchymal stem cell effects on T-cell effector pathways," *Stem Cell Research and Therapy*, vol. 2, no. 4, article 34, 2011.

[51] Y. Lepelletier, S. Lecourt, A. Renand et al., "Galectin-1 and semaphorin-3A are two soluble factors conferring t-cell immunosuppression to bone marrow mesenchymal stem cell," *Stem Cells and Development*, vol. 19, no. 7, pp. 1075–1079, 2010.

[52] M. Sioud, A. Mobergslien, A. Boudabous, and Y. Fløisand, "Mesenchymal stem cell-mediated T cell suppression occurs through secreted galectins," *International Journal of Oncology*, vol. 38, no. 2, pp. 385–390, 2011.

[53] F. Gieseke, A. Kruchen, N. Tzaribachev, F. Bentzien, M. Dominici, and I. Müller, "Proinflammatory stimuli induce galectin-9 in human mesenchymal stromal cells to suppress T-cell proliferation," *European Journal of Immunology*, vol. 43, no. 10, pp. 2741–2749, 2013.

[54] F. Montespan, F. Deschaseaux, L. Sensébé, E. D. Carosella, and N. Rouas-Freiss, "Osteodifferentiated mesenchymal stem cells from bone marrow and adipose tissue express HLA-G and display immunomodulatory properties in HLA-mismatched settings: implications in bone repair therapy," *Journal of Immunology Research*, vol. 2014, Article ID 230346, 10 pages, 2014.

[55] S. Beyth, J. Schroeder, and M. Liebergall, "Stem cells in bone diseases: current clinical practice," *The British Medical Bulletin*, vol. 99, no. 1, pp. 199–210, 2011.

[56] R. Quarto, M. Mastrogiacomo, R. Cancedda et al., "Repair of large bone defects with the use of autologous bone marrow stromal cells," *New England Journal of Medicine*, vol. 344, no. 5, pp. 385–386, 2001.

[57] H. Kitoh, T. Kitakoji, H. Tsuchiya, M. Katoh, and N. Ishiguro, "Transplantation of culture expanded bone marrow cells and platelet rich plasma in distraction osteogenesis of the long bones," *Bone*, vol. 40, no. 2, pp. 522–528, 2007.

[58] D. Dallari, L. Savarino, C. Stagni et al., "Enhanced tibial osteotomy healing with use of bone grafts supplemented with platelet gel or platelet gel and bone marrow stromal cells," *Journal of Bone and Joint Surgery—Series A*, vol. 89, no. 11, pp. 2413–2420, 2007.

[59] B. G. Ochs, U. Schmid, J. Rieth, A. Ateschrang, K. Weise, and U. Ochs, "Acetabular bone reconstruction in revision arthroplasty: a comparison of freeze-dried, irradiated and chemically-treated allograft vitalised with autologous marrow versus frozen non-irradiated allograft," *Journal of Bone and Joint Surgery—Series B*, vol. 90, no. 9, pp. 1164–1171, 2008.

[60] C. Hendrich, F. Engelmaier, G. Waertel, R. Krebs, and M. Jäger, "Safety of autologous bone marrow aspiration concentrate transplantation: initial experiences in 101 patients," *Orthopedic Reviews*, vol. 1, no. 2, article e32, 2009.

[61] M. Jäger, M. Herten, U. Fochtmann et al., "Bridging the gap: bone marrow aspiration concentrate reduces autologous bone grafting in osseous defects," *Journal of Orthopaedic Research*, vol. 29, no. 2, pp. 173–180, 2011.

[62] M.-J. Park, H.-S. Park, M.-L. Cho et al., "Transforming growth factor $\beta$-transduced mesenchymal stem cells ameliorate experimental autoimmune arthritis through reciprocal regulation of Treg/Th17 cells and osteoclastogenesis," *Arthritis and Rheumatism*, vol. 63, no. 6, pp. 1668–1680, 2011.

[63] M. Feldmann and R. N. Maini, "Anti-TNF$\alpha$ therapy of rheumatoid arthritis: what have we learned?" *Annual Review of Immunology*, vol. 19, pp. 163–196, 2001.

[64] H. Liu, J. Zhang, C.-Y. Liu, Y. Hayashi, and W. W.-Y. Kao, "Bone marrow mesenchymal stem cells can differentiate and assume corneal keratocyte phenotype," *Journal of Cellular and Molecular Medicine*, vol. 16, no. 5, pp. 1114–1124, 2012.

[65] H. Sasaki, L. Hou, A. Belani et al., "IL-10, but not IL-4, suppresses infection-stimulated bone resorption in vivo," *Journal of Immunology*, vol. 165, no. 7, pp. 3626–3630, 2000.

[66] R. Dresner-Pollak, N. Gelb, D. Rachmilewitz, F. Karmeli, and M. Weinreb, "Interleukin 10-deficient mice develop osteopenia, decreased bone formation, and mechanical fragility of long bones," *Gastroenterology*, vol. 127, no. 3, pp. 792–801, 2004.

[67] D. Nam, E. Mau, Y. Wang et al., "T-lymphocytes enable osteoblast maturation via IL-17F during the early phase of fracture repair," *PLoS ONE*, vol. 7, no. 6, Article ID e40044, 2012.

[68] N. Eliopoulos, J. Stagg, L. Lejeune, S. Pommey, and J. Galipeau, "Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice," *Blood*, vol. 106, no. 13, pp. 4057–4065, 2005.

[69] A. J. Nauta, G. Westerhuis, A. B. Kruisselbrink, E. G. A. Lurvink, R. Willemze, and W. E. Fibbe, "Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting," *Blood*, vol. 108, no. 6, pp. 2114–2120, 2006.

[70] S. Inoue, F. C. Popp, G. E. Koehl et al., "Immunomodulatory effects of mesenchymal stem cells in a rat organ transplant model," *Transplantation*, vol. 81, no. 11, pp. 1589–1595, 2006.

[71] T. B. Prigozhina, S. Khitrin, G. Elkin, O. Eizik, S. Morecki, and S. Slavin, "Mesenchymal stromal cells lose their immunosuppressive potential after allotransplantation," *Experimental Hematology*, vol. 36, no. 10, pp. 1370–1376, 2008.

[72] I. A. Isakova, C. Lanclos, J. Bruhn et al., "Allo-reactivity of mesenchymal stem cells in rhesus macaques is dose and haplotype dependent and limits durable cell engraftment in vivo," *PLoS ONE*, vol. 9, no. 1, Article ID e87238, 2014.

[73] M. Okuno, T. Muneta, H. Koga et al., "Meniscus regeneration by syngeneic, minor mismatched, and major mismatched transplantation of synovial mesenchymal stem cells in a rat model," *Journal of Orthopaedic Research*, vol. 32, no. 7, pp. 928–936, 2014.

[74] T. L. Arinzeh, S. J. Peter, M. P. Archambault et al., "Allogeneic mesenchymal stem cells regenerate bone in a critical-sized canine segmental defect," *The Journal of Bone and Joint Surgery—American Volume*, vol. 85-A, no. 10, pp. 1927–1935, 2003.

[75] S.-Q. Guo, J. Z. Xu, Q. M. Zou, and D. M. Jiang, "Immunological study of allogeneic mesenchymal stem cells during bone formation," *Journal of International Medical Research*, vol. 37, no. 6, pp. 1750–1759, 2009.

[76] R. K. Udehiya, H. P. Aithal, P. Kinjavdekar, A. M. Pawde, R. Singh, and G. Taru Sharma, "Comparison of autogenic and allogenic bone marrow derived mesenchymal stem cells for repair of segmental bone defects in rabbits," *Research in Veterinary Science*, vol. 94, no. 3, pp. 743–752, 2013.

[77] H. Gu, Z. Xiong, X. Yin et al., "Bone regeneration in a rabbit ulna defect model: use of allogeneic adipose-derivedstem cells with low immunogenicity," *Cell and Tissue Research*, vol. 358, no. 2, pp. 453–464, 2014.

[78] C. Wan, Q. He, and G. Li, "Allogenic peripheral blood derived mesenchymal stem cells (MSCs) enhance bone regeneration in rabbit ulna critical-sized bone defect model," *Journal of Orthopaedic Research*, vol. 24, no. 4, pp. 610–618, 2006.

[79] P. Streckbein, S. Jäckel, C. Y. Malik et al., "Reconstruction of critical-size mandibular defects in immunoincompetent rats with human adipose-derived stromal cells," *Journal of Cranio-Maxillofacial Surgery*, vol. 41, no. 6, pp. 496–503, 2013.

[80] J. Y. Im, W. K. Min, C. You, H. O. Kim, H. K. Jin, and J. S. Bae, "Bone regeneration of mouse critical-sized calvarial defects with human mesenchymal stem cells in scaffold," *Laboratory Animal Research*, vol. 29, no. 4, pp. 196–203, 2013.

[81] M. Lee, S. Y. Jeong, J. Ha et al., "Low immunogenicity of allogeneic human umbilical cord blood-derived mesenchymal stem cells in vitro and in vivo," *Biochemical and Biophysical Research Communications*, vol. 446, no. 4, pp. 983–989, 2014.

[82] K. English and K. J. Wood, "Mesenchymal stromal cells in transplantation rejection and tolerance," *Cold Spring Harbor perspectives in medicine*, vol. 3, no. 5, 2013.

[83] F. Benvenuto, S. Ferrari, E. Gerdoni et al., "Human mesenchymal stem cells promote survival of T cells in a quiescent state," *Stem Cells*, vol. 25, no. 7, pp. 1753–1760, 2007.

[84] G. Xu, Y. Zhang, L. Zhang, G. Ren, and Y. Shi, "The role of IL-6 in inhibition of lymphocyte apoptosis by mesenchymal stem cells," *Biochemical and Biophysical Research Communications*, vol. 361, no. 3, pp. 745–750, 2007.

[85] M. Najar, G. Raicevic, H. I. Boufker et al., "Adipose-Tissue-Derived and Wharton's jelly-derived mesenchymal stromal cells suppress lymphocyte responses by secreting leukemia inhibitory factor," *Tissue Engineering, Part A*, vol. 16, no. 11, pp. 3537–3546, 2010.

[86] C. Bocelli-Tyndall, L. Bracci, S. Schaeren et al., "Human bone marrow mesenchymal stem cells and chondrocytes promote and/or suppress the in vitro proliferation of lymphocytes stimulated by interleukins 2, 7 and 15," *Annals of the Rheumatic Diseases*, vol. 68, no. 8, pp. 1352–1359, 2009.

[87] D. D. Chaplin, "Overview of the immune response," *Journal of Allergy and Clinical Immunology*, vol. 125, no. 2, supplement 2, pp. S3–S23, 2010.

[88] E. Schurgers, H. Kelchtermans, T. Mitera, L. Geboes, and P. Matthys, "Discrepancy between the in vitro and in vivo effects of murine mesenchymal stem cells on T-cell proliferation and collagen-induced arthritis," *Arthritis Research and Therapy*, vol. 12, no. 1, article R31, 2010.

[89] E. Zappia, S. Casazza, E. Pedemonte et al., "Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy," *Blood*, vol. 106, no. 5, pp. 1755–1761, 2005.

[90] S. Glennie, I. Soeiro, P. J. Dyson, E. W.-F. Lam, and F. Dazzi, "Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells," *Blood*, vol. 105, no. 7, pp. 2821–2827, 2005.

[91] V. Pistoia and L. Raffaghello, "Unveiling the role of TNF-$\alpha$ in mesenchymal stromal cell-mediated immunosuppression," *European Journal of Immunology*, vol. 44, no. 2, pp. 352–356, 2014.

[92] L. Sensebé, M. Krampera, H. Schrezenmeier, P. Bourin, and R. Giordano, "Mesenchymal stem cells for clinical application," *Vox Sanguinis*, vol. 98, no. 2, pp. 93–107, 2010.

[93] Q. Xue, X.-Y. Luan, Y.-Z. Gu et al., "The negative co-signaling molecule b7-h4 is expressed by human bone marrow-derived mesenchymal stem cells and mediates its T-cell modulatory activity," *Stem Cells and Development*, vol. 19, no. 1, pp. 27–38, 2010.

[94] G. li Pira, F. Ivaldi, L. Bottone, R. Quarto, and F. Manca, "Human bone marrow stromal cells hamper specific interactions of CD4 and CD8 T lymphocytes with antigen-presenting cells," *Human Immunology*, vol. 67, no. 12, pp. 976–985, 2006.

[95] R. Ramasamy, C. K. Tong, H. F. Seow, S. Vidyadaran, and F. Dazzi, "The immunosuppressive effects of human bone marrow-derived mesenchymal stem cells target T cell proliferation but not its effector function," *Cellular Immunology*, vol. 251, no. 2, pp. 131–136, 2008.

[96] I. Hof-Nahor, L. Leshansky, S. Shivtiel et al., "Human mesenchymal stem cells shift $CD8^+$ T cells towards a suppressive phenotype by inducing tolerogenic monocytes," *Journal of Cell Science*, vol. 125, part 19, pp. 4640–4650, 2012.

[97] M. J. Crop, C. C. Baan, S. S. Korevaar, J. N. M. Ijzermans, W. Weimar, and M. J. Hoogduijn, "Human adipose tissue-derived mesenchymal stem cells induce explosive T-Cell proliferation," *Stem Cells and Development*, vol. 19, no. 12, pp. 1843–1853, 2010.

[98] Z. Selmani, A. Naji, I. Zidi et al., "Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce $CD4^+$ $CD25^{high}FOXP3^+$ regulatory T cells," *Stem Cells*, vol. 26, no. 1, pp. 212–222, 2008.

[99] D. Mougiakakos, R. Jitschin, C. C. Johansson, R. Okita, R. Kiessling, and K. Le Blanc, "The impact of inflammatory licensing on heme oxygenase-1-mediated induction of regulatory T cells by human mesenchymal stem cells," *Blood*, vol. 117, no. 18, pp. 4826–4835, 2011.

[100] S. H. Yang, M. J. Park, I. H. Yoon et al., "Soluble mediators from mesenchymal stem cells suppress T cell proliferation by inducing IL-10," *Experimental & Molecular Medicine*, vol. 41, no. 5, pp. 315–324, 2009.

[101] O. Delarosa, E. Lombardo, A. Beraza et al., "Requirement of IFN-$\gamma$-mediated indoleamine 2,3-dioxygenase expression in the modulation of lymphocyte proliferation by human adipose-derived stem cells," *Tissue Engineering—Part A*, vol. 15, no. 10, pp. 2795–2806, 2009.

[102] R. M. Roemeling-van, M. E. Reinders, M. Franquesa et al., "Human allogeneic bone marrow and adipose tissue derived mesenchymal stromal cells induce $CD8^+$ cytotoxic T cell reactivity," *Journal of Stem Cell Research & Therapy*, vol. 3, supplement 6, article 004, 2013.

[103] M. François, R. Romieu-Mourez, M. Li, and J. Galipeau, "Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation," *Molecular Therapy*, vol. 20, no. 1, pp. 187–195, 2012.

[104] J. M. Ryan, F. Barry, J. M. Murphy, and B. P. Mahon, "Interferon-$\gamma$ does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells," *Clinical and Experimental Immunology*, vol. 149, no. 4, article 34, pp. 353–363, 2007.

[105] R. Chinnadurai, I. B. Copland, S. R. Patel, and J. Galipeau, "IDO-independent suppression of T cell effector function by IFN-$\gamma$-licensed human mesenchymal stromal cells," *Journal of Immunology*, vol. 192, no. 4, pp. 1491–1501, 2014.

[106] K. English, F. P. Barry, C. P. Field-Corbett, and B. P. Mahon, "IFN-gamma and TNF-alpha differentially regulate immunomodulation by murine mesenchymal stem cells," *Immunology Letters*, vol. 110, no. 2, pp. 91–100, 2007.

[107] X. Qu, X. Liu, K. Cheng, R. Yang, and R. C. H. Zhao, "Mesenchymal stem cells inhibit Th17 cell differentiation by IL-10 secretion," *Experimental Hematology*, vol. 40, no. 9, pp. 761–770, 2012.

[108] E. Ivanova-Todorova, I. Bochev, R. Dimitrov et al., "Conditioned medium from adipose tissue-derived mesenchymal stem cells induces CD4+FOXP3+ cells and increases IL-10 secretion," *Journal of Biomedicine and Biotechnology*, vol. 2012, Article ID 295167, 8 pages, 2012.

[109] P. Needleman, J. Truk, B. A. Jakschik, A. R. Morrison, and J. B. Lefkowith, "Arachidonic acid metabolism," *Annual Review of Biochemistry*, vol. 55, no. 1, pp. 69–102, 1986.

[110] W. E. Scales, S. W. Chensue, I. Otterness, and S. L. Kunkel, "Regulation of monokine gene expression: prostaglandin $E_2$ suppresses tumor necrosis factor but not interleukin-1$\alpha$ or $\beta$-mRNA and cell-associated bioactivity," *Journal of Leukocyte Biology*, vol. 45, no. 5, pp. 416–421, 1989.

[111] D. S. Snyder, D. I. Beller, and E. R. Unanue, "Prostaglandins modulate macrophage Ia expression," *Nature*, vol. 299, no. 5879, pp. 163–165, 1982.

[112] K. Sato, K. Ozaki, I. Oh et al., "Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells," *Blood*, vol. 109, no. 1, pp. 228–234, 2007.

[113] A. Nasef, N. Mathieu, A. Chapel et al., "Immunosuppressive effects of mesenchymal stem cells: involvement of HLA-G," *Transplantation*, vol. 84, no. 2, pp. 231–237, 2007.

[114] M.-J. Park, J.-S. Shin, Y.-H. Kim et al., "Murine mesenchymal stem cells suppress T lymphocyte activation through IL-2 receptor $\alpha$ (CD25) cleavage by producing matrix metalloproteinases," *Stem Cell Reviews and Reports*, vol. 7, no. 2, pp. 381–393, 2011.

[115] Y.-S. Lee, K.-J. Won, S.-W. Park et al., "Mesenchymal stem cells regulate the proliferation of T cells via the growth-related oncogene/CXC chemokine receptor, CXCR2," *Cellular Immunology*, vol. 279, no. 1, pp. 1–11, 2012.

[116] F. Saldanha-Araujo, F. I. S. Ferreira, P. V. Palma et al., "Mesenchymal stromal cells up-regulate CD39 and increase adenosine production to suppress activated T-lymphocytes," *Stem Cell Research*, vol. 7, no. 1, pp. 66–74, 2011.

[117] C. Sattler, M. Steinsdoerfer, M. Offers et al., "Inhibition of T-cell proliferation by murine multipotent mesenchymal stromal cells is mediated by CD39 expression and adenosine generation," *Cell Transplantation*, vol. 20, no. 8, pp. 1221–1230, 2011.

[118] F. De Paoli, B. Staels, and G. Chinetti-Gbaguidi, "Macrophage phenotypes and their modulation in atherosclerosis," *Circulation Journal*, vol. 78, no. 8, pp. 1775–1781, 2014.

[119] J. Maggini, G. Mirkin, I. Bognanni et al., "Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile," *PLoS ONE*, vol. 5, no. 2, Article ID e9252, 2010.

[120] J. H. Ylöstalo, T. J. Bartosh, K. Coble, and D. J. Prockop, "Human mesenchymal stem/stromal cells cultured as spheroids are self-activated to produce prostaglandin E2 that directs stimulated macrophages into an anti-inflammatory phenotype," *Stem Cells*, vol. 30, no. 10, pp. 2283–2296, 2012.

[121] T. Asami, M. Ishii, H. Fujii et al., "Modulation of murine macrophage TLR7/8-mediated cytokine expression by mesenchymal stem cell-conditioned medium," *Mediators of Inflammation*, vol. 2013, Article ID 264260, 13 pages, 2013.

[122] S. M. Melief, S. B. Geutskens, W. E. Fibbe, and H. Roelofs, "Multipotent stromal cells skew monocytes towards an anti-inflammatory interleukin-10-producing phenotype by production of interleukin-6," *Haematologica*, vol. 98, no. 6, pp. 888–895, 2013.

[123] M. H. Abumaree, M. A. Al Jumah, B. Kalionis et al., "Human placental mesenchymal stem cells (pMSCs) play a role as immune suppressive cells by shifting macrophage differentiation from inflammatory M1 to anti-inflammatory M2 macrophages," *Stem Cell Reviews and Reports*, vol. 9, no. 5, pp. 620–641, 2013.

[124] D. I. Cho, M. R. Kim, H. Y. Jeong et al., "Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages," *Experimental & Molecular Medicine*, vol. 46, no. 1, article e70, 2014.

[125] S. Gao, F. Mao, B. Zhang et al., "Mouse bone marrow-derived mesenchymal stem cells induce macrophage M2 polarization through the nuclear factor-$\kappa$B and signal transducer and activator of transcription 3 pathways," *Experimental Biology and Medicine*, vol. 239, no. 3, pp. 366–375, 2014.

[126] G. M. Spaggiari and L. Moretta, "Cellular and molecular interactions of mesenchymal stem cells in innate immunity," *Immunology and Cell Biology*, vol. 91, no. 1, pp. 27–31, 2013.

[127] G. M. Spaggiari and L. Moretta, "Interactions between mesenchymal stem cells and dendritic cells," *Advances in Biochemical Engineering/Biotechnology*, vol. 130, pp. 199–208, 2013.

[128] R. Wehner, C. Taubert, T. Mende et al., "Engineered extracellular matrix components do not alter the immunomodulatory properties of mesenchymal stromal cells in vitro," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 7, no. 11, pp. 921–924, 2013.

[129] W.-H. Liu, J.-J. Liu, J. Wu et al., "Novel mechanism of inhibition of dendritic cells maturation by mesenchymal stem cells via interleukin-10 and the JAK1/STAT3 signaling pathway," *PLoS ONE*, vol. 8, no. 1, Article ID e55487, 2013.

[130] A. Aldinucci, L. Rizzetto, L. Pieri et al., "Inhibition of immune synapse by altered dendritic cell actin distribution: a new pathway of mesenchymal stem cell immune regulation," *Journal of Immunology*, vol. 185, no. 9, pp. 5102–5110, 2010.

[131] Z. G. Zhao, W. Xu, L. Sun, W. M. Li, Q. B. Li, and P. Zou, "The characteristics and immunoregulatory functions of regulatory dendritic cells induced by mesenchymal stem cells derived from bone marrow of patient with chronic myeloid leukaemia," *European Journal of Cancer*, vol. 48, no. 12, pp. 1884–1895, 2012.

[132] Y.-S. Choi, J.-A. Jeong, and D.-S. Lim, "Mesenchymal stem cell-mediated immature dendritic cells induce regulatory T cell-based immunosuppressive effect," *Immunological Investigations*, vol. 41, no. 2, pp. 214–229, 2012.

[133] H. W. Chen, H. Y. Chen, L. T. Wang et al., "Mesenchymal stem cells tune the development of monocyte-derived dendritic cells toward a myeloid-derived suppressive phenotype through growth-regulated oncogene chemokines," *Journal of Immunology*, vol. 190, no. 10, pp. 5065–5077, 2013.

[134] Y. Deng, S. Yi, G. Wang et al., "Umbilical cord-derived mesenchymal stem cells instruct dendritic cells to acquire tolerogenic phenotypes through the IL-6-mediated upregulation of SOCS1," *Stem Cells and Development*, vol. 23, no. 17, pp. 2080–2092, 2014.

[135] G. M. Spaggiari, A. Capobianco, S. Becchetti, M. C. Mingari, and L. Moretta, "Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation," *Blood*, vol. 107, no. 4, pp. 1484–1490, 2006.

[136] M. J. Crop, S. S. Korevaar, R. de Kuiper et al., "Human mesenchymal stem cells are susceptible to lysis by $CD8^+$ T cells and NK cells," *Cell Transplantation*, vol. 20, no. 10, pp. 1547–1559, 2011.

[137] M. Giuliani, A. Bennaceur-Griscelli, A. Nanbakhsh et al., "TLR ligands stimulation protects MSC from NK killing," *Stem Cells*, vol. 32, no. 1, pp. 290–300, 2014.

[138] W. Deng, Q. Han, L. Liao, S. You, H. Deng, and R. C. H. Zhao, "Effects of allogeneic bone marrow-derived mesenchymal stem cells on T and B lymphocytes from BXSB mice," *DNA and Cell Biology*, vol. 24, no. 7, pp. 458–463, 2005.

[139] A. Corcione, F. Benvenuto, E. Ferretti et al., "Human mesenchymal stem cells modulate B-cell functions," *Blood*, vol. 107, no. 1, pp. 367–372, 2006.

[140] S. Tabera, J. A. Perez-Simon, M. Diez-Campelo et al., "The effect of mesenchymal stem cells on the viability, proliferation and differentiation of B-lymphocytes," *Haematologica*, vol. 93, no. 9, pp. 1301–1309, 2008.

[141] S. Asari, S. Itakura, K. Ferreri et al., "Mesenchymal stem cells suppress B-cell terminal differentiation," *Experimental Hematology*, vol. 37, no. 5, pp. 604–615, 2009.

[142] F. Schena, C. Gambini, A. Gregorio et al., "Interferon-$\gamma$-dependent inhibition of B cell activation by bone marrow-derived mesenchymal stem cells in a murine model of systemic lupus erythematosus," *Arthritis & Rheumatism*, vol. 62, no. 9, pp. 2776–2786, 2010.

[143] E. Traggiai, S. Volpi, F. Schena et al., "Bone marrow-derived mesenchymal stem cells induce both polyclonal expansion and differentiation of B cells isolated from healthy donors and systemic lupus erythematosus patients," *Stem Cells*, vol. 26, no. 2, pp. 562–569, 2008.

[144] O. M. Omar, C. Granéli, K. Ekström et al., "The stimulation of an osteogenic response by classical monocyte activation," *Biomaterials*, vol. 32, no. 32, pp. 8190–8204, 2011.

[145] C. Chen, H. Uludag, Z. Wang, A. Rezansoff, and H. Jiang, "Macrophages inhibit migration, metabolic activity and osteogenic differentiation of human mesenchymal stem cells in vitro," *Cells Tissues Organs*, vol. 195, no. 6, pp. 473–483, 2012.

[146] P. Guihard, Y. Danger, B. Brounais et al., "Induction of osteogenesis in mesenchymal stem cells by activated monocytes/macrophages depends on oncostatin M signaling," *Stem Cells*, vol. 30, no. 4, pp. 762–772, 2012.

[147] V. Nicolaidou, M. M. Wong, A. N. Redpath et al., "Monocytes induce STAT3 activation in human mesenchymal stem cells to promote osteoblast formation," *PLoS ONE*, vol. 7, no. 7, Article ID e39871, 2012.

[148] R. P. Pirraco, R. L. Reis, and A. P. Marques, "Effect of monocytes/macrophages on the early osteogenic differentiation of hBMSCs," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 7, no. 5, pp. 392–400, 2013.

[149] K. Ekström, O. Omar, C. Granéli, X. Wang, F. Vazirisani, and P. Thomsen, "Monocyte exosomes stimulate the osteogenic gene expression of mesenchymal stem cells," *PLoS ONE*, vol. 8, no. 9, Article ID e75227, 2013.

[150] F. Grassi, L. Cattini, L. Gambari et al., "T cell subsets differently regulate osteogenic differentiation of human mesenchymal stromal cells *in vitro*," *Journal of Tissue Engineering and Regenerative Medicine*, 2013.

[151] G. H. W. Tso, H. K. W. Law, W. Tu, G. C. F. Chan, and Y. L. Lau, "Phagocytosis of apoptotic cells modulates mesenchymal stem cells osteogenic differentiation to enhance IL-17 and RANKL expression on CD4+ T cells," *Stem Cells*, vol. 28, no. 5, pp. 939–954, 2010.

[152] A. M. Kandahari, X. Yang, A. S. Dighe, D. Pan, and Q. Cui, "Recognition of immune response for the early diagnosis and treatment of osteoarthritis," *Journal of Immunology Research*, vol. 2014, Article ID 192415, 13 pages, 2014.

[153] X. Wang, L. Lu, and S. Jiang, "Regulatory T cells: customizing for the clinic," *Science Translational Medicine*, vol. 3, no. 83, Article ID 83ps19, 2011.

[154] P. M. Mountziaris, P. P. Spicer, F. K. Kasper, and A. G. Mikos, "Harnessing and modulating inflammation in strategies for bone regeneration," *Tissue Engineering, Part B: Reviews*, vol. 17, no. 6, pp. 393–402, 2011.

[155] J. Cottrell and J. P. O'Connor, "Effect of non-steroidal anti-inflammatory drugs on bone healing," *Pharmaceuticals*, vol. 3, no. 5, pp. 1668–1693, 2010.

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*Research Article*

# Human Myeloma Cell Lines Induce Osteoblast Downregulation of CD99 Which Is Involved in Osteoblast Formation and Activity

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CD99 is a transmembrane glycoprotein expressed in physiological conditions by cells of different tissues, including osteoblasts (OBs). High or low CD99 levels have been detected in various pathological conditions, and the supernatant of some carcinoma cell lines can modulate CD99 expression in OB-like cells. In the present work we demonstrate for the first time that two different human myeloma cell lines (H929 and U266) and, in a less degree, their conditioned media significantly downregulate CD99 expression in normal human OBs during the differentiation process. In the same experimental conditions the OBs display a less differentiated phenotype as demonstrated by the decreased expression of RUNX2 and Collagen I. On the contrary, when CD99 was activated by using a specific agonist antibody, the OBs become more active as demonstrated by the upregulation of Alkaline Phosphatase, Collagen I, RUNX2, and JUND expression. Furthermore, we demonstrate that the activation of CD99 is able to induce the phosphorylation of ERK 1/2 and AKT intracellular signal transduction molecules in the OBs.

## 1. Introduction

CD99 is a 32 kDa transmembrane glycoprotein, encoded by the MIC2 gene [1] which is located on the pseudoautosomal regions of both human X and Y chromosomes [2]. MIC2 gene encodes two distinct proteins produced by alternative splicing of the CD99 gene transcript [3] and, compared with the major wild-type full-length form, the minor splice variant form of CD99 has a relatively short intracytoplasmic fragment [4].

It is well known from the literature that CD99 can be expressed in both normal and pathological tissues. In normal tissues it is expressed in cortical thymocytes, pancreatic islet cells, granulose cells of ovary, Sertoli cells of testis, $CD34^+$ cells of bone marrow, and all leukocyte lineages [5].

Slightly less than a decade ago its linkage to human osteoblast (hOB) differentiation has emerged and MIC2 has been indicated under the control of the transcription factor RUNX2, which is essential for hOB differentiation [6, 7]. *In vitro* data have demonstrated CD99 expression in cell-adhesion structures of osteoblastic cell cultures, and *in vivo* its expression has been highly detected in hOBs adhering to each other and lining the bone surface in tissue samples [5]. Very recently, we have shown that during the differentiation process hOBs and bone marrow-mononuclear cells increased CD99 expression levels, suggesting its role in

osteoblastogenesis [8]. Accordingly, CD99 expression is high in lining cells and in mature hOBs [5, 8].

Quite variable CD99 levels have been demonstrated in pathological conditions since they have been detected either high or low. In particular, it is overexpressed in different sarcomas, such as Ewing's sarcoma [9, 10], synovial sarcoma [11], mesenchymal chondrosarcoma [12], and rhabdomyosarcoma [13], in lymphoblastic lymphoma/leukemia [14], and human breast cancer cells [15]. Conversely, CD99 is downregulated or completely lacking in pancreatic endocrine neoplasm [16], gastric adenocarcinoma [17], and osteosarcoma [5].

Literature evidences have also demonstrated that the supernatants from several carcinoma cell lines associated with osteolytic metastases (breast, colon, pancreatic, renal, and hepatoma cell lines) specifically downregulate CD99 on AHTO-7 cells (large T antigen transfected human trabecular OBs) [18]. Conversely, the conditioned media obtained from prostate cancer cell lines, which correlated with osteosclerotic lesions, induce an increase in CD99 expression [18]. A very recent paper performed on a large number of MM patients suggests CD99 as a new marker for risk stratification of disease severity [19]. However, no data are at present available on the effect of myeloma cells on OB CD99 expression and the possible implication of CD99 in the impairment of OB differentiation in multiple myeloma (MM), a hematological B cell malignancy associated with bone disease [20–27].

It is well established that myeloma cells, through a variety of cellular mechanisms, contribute to the onset of osteolytic bone lesions by altering bone remodeling as they induce both the increase of osteoclastic bone resorption and decrease of OB differentiation. With particular regard to the alteration of osteoblastic differentiation and function in MM bone disease it has been showed that, among the numerous cytokines, myeloma cells produce soluble molecules such as frizzled-related proteins-2 and -3 (sFRP-2 and -3) [27–29], Dickkopf-1 (DKK-1) [28], and sclerostin [25–27], responsible for the inhibition of the canonical Wingless-type (Wnt) signaling which is a crucial pathway for the correct OB differentiation and activity.

Not later than a few months ago, it was found that, under physiological conditions, OBs express increasing levels of CD99 during their differentiation [8] but regarding the influence of myeloma cells on the expression of this molecule currently lack experimental evidence. Therefore, having previously performed studies on the mechanisms altering OB differentiation contributing to osteolytic process in MM bone disease [25, 26], we here focused our interest on this issue. However, it should be considered into account that most information about CD99 activities derives from triggering CD99-mediated signaling events with agonistic CD99 monoclonal antibodies. By using these molecules it has been shown that CD99 is functionally implicated in the apoptosis of cells with immunological role (thymocytes, T-lymphocytes, Jurkat cell line, and normal and leukemia B cell precursors) [30–33], in triggering homotypic CD4+ CD8+ thymocytes aggregation [34], in inducing T cell migration to inflamed vascular endothelium [35], and in cell-to-cell contact and diapedesis of monocytes across endothelial cells by homophilic interactions between these two adjacent cells [36]. In addition to these effects, several evidences have also proved the role of CD99 in proliferation and activation of lymphocytes [37, 38] and regulation of MHC class I molecule transport from the Golgi complex to the cell surface [39].

Thus, on the basis of all these findings and the intriguing role of CD99 in osteogenesis and bone pathophysiology, in this paper we analyzed the influence of human myeloma cell lines (HMCLs) on CD99 expression by hOBs. We demonstrated that HMCLs display the ability to reduce the expression of CD99 in normal hOBs during the differentiation process. Furthermore, by using an anti-CD99 agonist monoclonal antibody, we demonstrated that the hOBs result more active in the expression of their differentiation parameters. These data suggest that CD99 can be important in the differentiation and activity of hOBs in physiological and pathological conditions.

## 2. Materials and Methods

*2.1. Human Osteoblasts.* Trabecular bone specimens, obtained from healthy subjects who undergo femur surgery following traumatological events, were cleaned off soft tissues, reduced to small fragments, and digested with 0.5 mg/mL *Clostridium histolyticum* neutral collagenase (Sigma Chemical Co., St. Louis, MO, USA) in minimum essential medium ($\alpha$-MEM) (Gibco Invitrogen, Milan, Italy) with gentle agitation for 30 minutes at 37°C. Bone fragments were then washed (three times) with phosphate-buffered saline (PBS) and cultured in $\alpha$-MEM supplemented with 10% fetal calf serum (FCS) (Gibco), 100 IU/mL penicillin (Gibco), 100 mg/mL streptomycin (Gibco), and 2.5 mg/mL amphotericin B (Gibco), at 37°C in a water-saturated atmosphere containing 5% $CO_2$. Cells were fed by medium replacement every 3 to 4 days. In these conditions, the hOBs resident in the explants proliferated and migrated to the culture substrate, reaching confluence within 3 to 4 weeks. Cells were then trypsinized and transferred to appropriate culture dishes for characterization and experiments.

Informed consent to the study was given according to the tenets of the Declaration of Helsinki. Approval was obtained from the Institutional Review Board of the Laboratory of Experimental Oncology, Rizzoli Orthopaedic Institute, Bologna, Italy (Protocol number 0021571 of June 28, 2013).

*2.2. Cell Culture Conditions and Cocultures.* H929 and U266 HMCLs were cultured in RPMI 1640 medium supplemented with 10% FCS and then lysed for protein extraction or used for coculture experiments.

Confluent hOBs were cultured in the presence or absence of $5 \times 10^3/cm^2$ HMCLs (H929 or U266) or their conditioned medium, in osteogenic medium consisting of $\alpha$-MEM medium supplemented with 10% FCS, 50 $\mu$g/mL ascorbic acid, and $10^{-8}$ M dexamethasone (all from Sigma), for 2, 7, 14, and 21 days before lysing them for protein extraction. In parallel, other hOBs were cultured in $\alpha$-MEM medium supplemented with 10% FCS and lysed for protein extraction after their adhesion (0 days of differentiation).

Moreover hOBs were cultured at a density of $1 \times 10^4$ cells/cm$^2$ in 96- or 48-well plates in $\alpha$-MEM medium supplemented with 10% FCS, in the presence or absence of 2 $\mu$g/mL anti-CD99 (DN-16) (Abcam, Cambridge Science Park) agonist monoclonal antibody or mouse IgG (Sigma) and after 24 and 48 hours or after 6 days of culture were analyzed, respectively, for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay or Alkaline Phosphatase- (ALP-) staining. These cells were also plated in 6 well plates and after reaching 70% of confluence were treated with 2 $\mu$g/mL of anti-CD99 agonist monoclonal antibody or mouse IgG or 100 ng/mL human TNF-related apoptosis-inducing ligand (h-TRAIL), for 4, 6, 12, and 24 hours and then lysed for protein extraction to evaluate apoptotic pathway. In addition, the stimulation with CD99 agonist antibody or mouse IgG was also performed for 2, 5, 10, and 20 minutes and these cells were then lysed for protein extraction to study AKT and ERK phosphorylation. hOBs were also treated with 100 nM Wortmannin (Calbiochem, Germany) for 30 minutes or with 10 $\mu$M PD98059 (Sigma) for 60 minutes, respectively, Phosphoinositide 3-Kinase (PI3K) and extracellular signal-regulated kinase-1 (ERK1) K inhibitors, and then stimulated with 2 $\mu$g/mL anti-CD99 agonist monoclonal antibody for 4 and 6 hours. Before the short times (2, 5, 10, and 20 minutes) stimulation, cells were starved for 12 hours with $\alpha$-MEM medium supplemented with 2% FCS and thus treated as previously described using the same medium.

All stimulation experiments were repeated for three times.

*2.3. Western Blot Analysis.* The protein levels of CD99 have been evaluated in H929 and U266. Additionally, CD99, Collagen I (COLLI), and RUNX2 protein levels have been also analyzed in hOBs cultured alone and cocultured with HMCLs or their conditioned medium. All the cells were solubilized with lysis buffer [50 mM Tris (tris(hydroxymethyl)aminomethane)-HCl (pH 8), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 1% NP40, and 1 mM phenylmethyl sulfonyl fluoride]. Moreover, COLLI, RUNX2, members of AP1 complex (FRA1, FRA2, and JUND), and mitogen-activated protein kinases (MAPKs) have been studied in hOBs after CD99 stimulation. COLLI, RUNX2, and JUND were also evaluated in hOBs treated for 30 minutes with 100 nM Wortmannin or for 60 minutes with 10 $\mu$M PD98059 and then stimulated for 4 and 6 hours with anti-CD99 agonist monoclonal antibody; these cells were solubilised with the lysis buffer previously described.

To detect the expression of caspases 3, 7, and 8 and Bid cleavage in hOBs after CD99 or hTRAIL stimulation, cells were lysed by incubation on ice for 30 min in lysis buffer containing 20 mM Tris-HCl (pH7.5), 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM $Na_3VO_4$, 50 mM NaF, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture.

Cell proteins (15 $\mu$g) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to nitrocellulose membranes (Hybond; Amersham Pharmacia, London, UK). The blots were probed overnight at 4°C with the appropriate primary antibody.

The following primary antibodies were used: monoclonal anti-COLLI, anti-p-ERK and anti-$\beta$-Actin, polyclonal anti-JUND, anti-FRA1, anti-FRA2, and anti-total-ERK (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA); monoclonal anti-p-AKT, anti-p-JNK, and anti-caspase-8, polyclonal anti-total-AKT, anti-p-P38, anti-caspase-3, anti-caspase-7, and anti-Bid (all from Cell Signaling, San Diego, CA, USA); monoclonal anti-CD99 (12E7) (Santa Cruz Biotechnology); and polyclonal anti-RUNX2 (Abnova, Taiwan).

After incubation with the appropriate fluorescent-dye-conjugated secondary antibody (LI-COR Biosciences GmbH, Bad Homburg, Germany), specific reactions were revealed with the LI-COR's Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE, USA).

*2.4. Alkaline Phosphatase.* ALP was histochemically assessed in hOBs treated and nontreated for 6 days with anti-CD99 agonist antibody or with mouse IgG, using Leukocyte Alkaline Phosphatase Kit, a commercial kit based on naphthol AS-BI and fast red violet LB (Sigma).

Cells were fixed with a citrate-acetone-formaldehyde fixative for 30″ at room temperature. After being gently rinsed with distilled water, cells were incubated for 15′ in dark with alkaline-dye mixture ($NaNO_2$, FRV-Alkaline Solution, Naphthol AS-BI Alkaline Solution) and finally washed with water.

The quantification and normalization of ALP histochemical staining was done counting ALP positive cells respect to total cells in three different fields (10x). Three different experiments were performed for ALP evaluation.

*2.5. Cell Viability Assay.* Mitochondrial dehydrogenases activity was determined by MTT assay. This assay is based on the ability of forming dye crystals to be developed only in living cells, providing an indication of the mitochondrial integrity and activity which, in turn, may be interpreted as a measure of cell viability. hOBs were cultured in 96-well tissue-culture plates as previously described. A part of the wells were used as control, while the others were treated with 2 $\mu$g/mL of anti-CD99 agonist antibody for 24 and 48 hours. The cell viability experiments were performed in the presence of 10% FCS. MTT 0.5 mg/mL were added to the culture media followed by 4 hours incubation at 37°C in a humidified 5% $CO_2$ atmosphere. The reaction was stopped by the addition of 150 $\mu$L of 0.04 N HCl in absolute isopropanol. The optical density was read at 570 nm using an automatic plate reader (550 Microplate Reader Bio-Rad Laboratories Inc., CA, USA). The results were compared to cells incubated under control conditions.

Cell viability was evaluated in three independent experiments.

*2.6. Statistical Analyses.* Statistical analyses were performed by Student's $t$-test with the Statistical Package for the Social

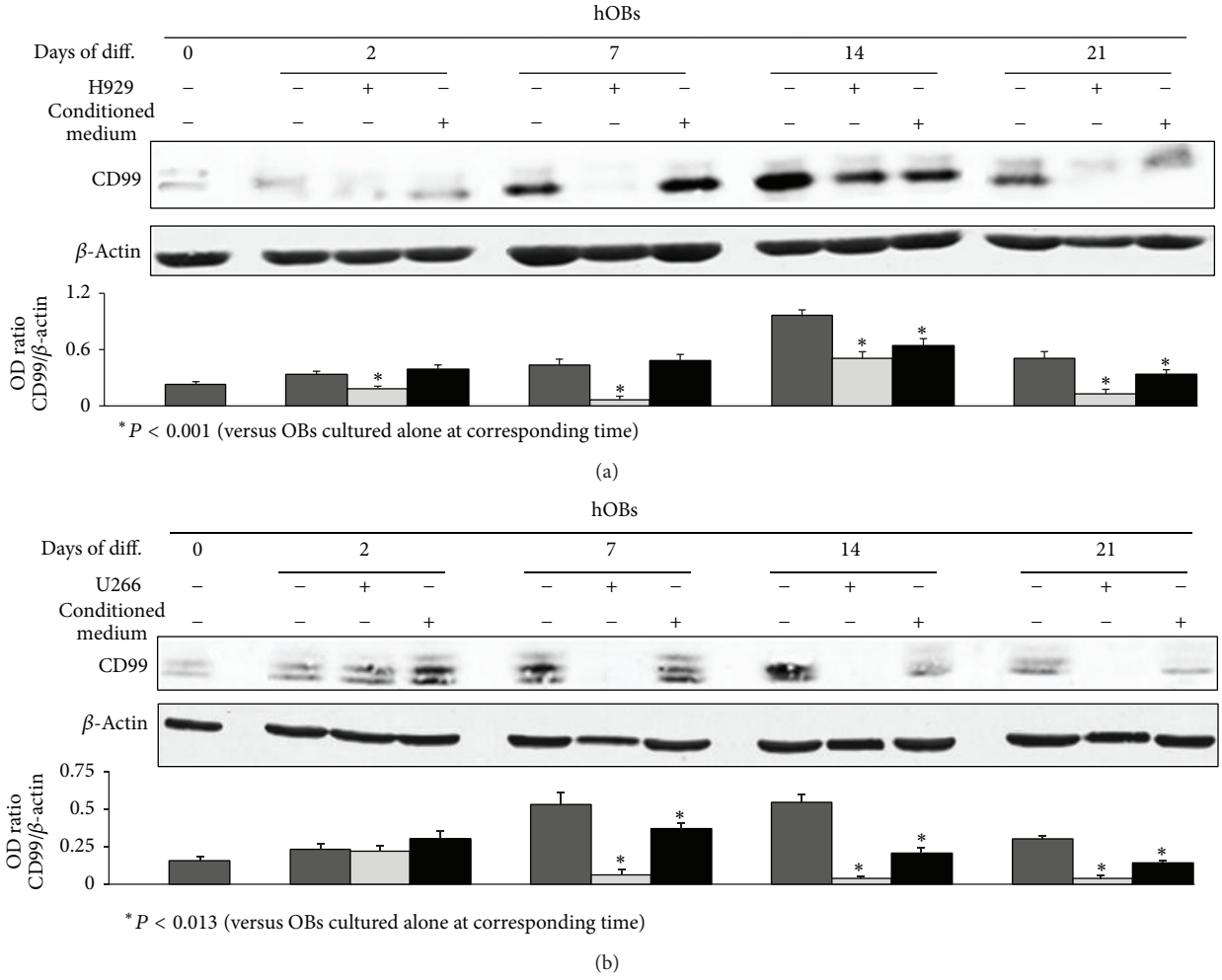


Figure 1: Human myeloma cell lines (HMCLs) inhibit CD99 expression on normal human osteoblasts (hOBs). Human undifferentiated osteoblasts or hOBs differentiated for 2, 7, 14, or 21 days (Days of diff.), were cultured in the presence or absence of H929 (a) or U266 (b) HMCLs or their conditioned medium, and then were analyzed for western blot analysis to detect the protein levels of CD99. The histograms represent the mean optical density (OD) of CD99 ratio normalized to the OD of $\beta$-Actin. Data are presented as mean ± SE. The figure shows one representative of three independent experiments.

Sciences (spssx/pc) software (SPSS, Chicago, IL, USA). The results were considered statistically significant for $P < 0.05$.

## 3. Results

*3.1. Effect of Human Myeloma Cell Lines on CD99 Expression during Osteoblast Differentiation.* It was shown that some carcinoma cell line-conditioned media downregulate CD99 on human AHTO-7 OBs [18]. Thus, after demonstrating that both H929 and U266 express very low protein levels of CD99 (data not shown), we studied the influence of HMCLs on CD99 expression by normal hOBs during their differentiation process.

At this purpose we performed cocultures between two different HMCLs (H929 and U266) and undifferentiated normal hOBs cultured in the presence of osteogenic medium from 2 up to 21 days of culture. By western blot analysis we showed that in the coculture system both HMCLs significantly ($P < 0.013$) inhibited hOB CD99 expression during the differentiation period reaching the maximum inhibition at the seventh day of culture (Figure 1). Although the inhibition induced by the two HMCLs was quite similar, the effect exerted by U266 was more pronounced and persistent throughout the whole differentiation period (Figure 1). In parallel, in the same experiment we cultured the hOBs in the presence of the conditioned media collected from the previously mentioned HMCLs to understand if the effect could be mediated by soluble factors. The conditioned medium of both HMCLs displayed a weaker but significant ($P < 0.013$) inhibition of CD99 expression in the late phase of hOB differentiation (14 and 21 days) compared to what was observed in the presence of the cells (Figure 1). These findings suggest that the inhibition on the CD99 expression by hOBs exerted

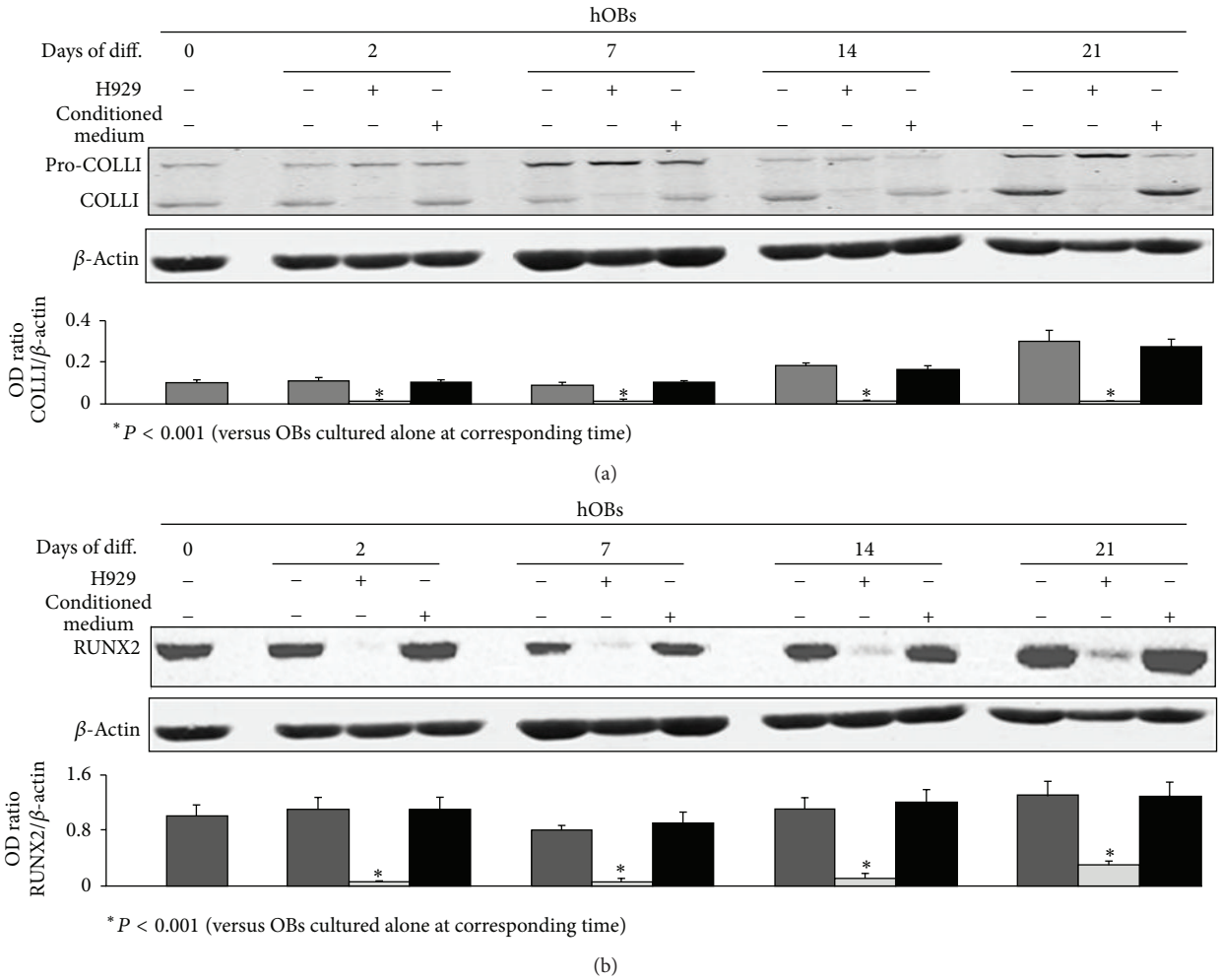


Figure 2: H929 inhibit Collagen I (COLLI) and RUNX2 expression on normal human osteoblasts (hOBs). Human undifferentiated osteoblasts or hOBs differentiated for 2, 7, 14, or 21 days (Days of diff.), were cultured in the presence or absence of H929 or their conditioned medium, and then were analyzed for western blot analysis to detect the protein levels of COLLI (a) or RUNX2 (b). The histograms represent the mean optical density (OD) of COLLI or RUNX2 ratio normalized to the OD of $\beta$-Actin. Data are presented as mean ± SE. The figure shows one representative of three independent experiments.

by HMCLs could be partially mediated by soluble factors and further enhanced by the presence of malignant cell lines.

Using the previously described coculture system, we also evaluated the effect of HMCLs or their conditioned medium on the expression of COLLI and RUNX2 in hOBs during their differentiation period. By western blot analysis we showed that both H929 (Figure 2) and U266 (Figure 3) significantly inhibited COLLI ($P < 0.001$) (Figures 2(a) and 3(a)) and RUNX2 ($P < 0.035$) (Figures 2(b) and 3(b)) protein levels during the entire differentiation period, whereas the conditioned medium of both HMCLs does not display any effect (Figures 2 and 3). Any toxic-or apoptotic-induced effect of HMCLs on OB cultures was excluded by MTT assay (data not shown).

*3.2. Effect of CD99 Activation on Normal Human Osteoblasts.* We have recently demonstrated that CD99 expression increases during normal hOB differentiation [8]; thus we here evaluated whether the activation of CD99 can have an impact on the activity of differentiated normal hOBs. In particular, we treated these cells with anti-CD99 agonist antibody to analyze ALP activity and COLLI expression compared to hOBs cultured in the absence of the agonist antibody as control condition. By using a histochemical assay, we demonstrated significantly ($P = 0.04$) higher ALP activity in hOBs treated for 6 days with 2 $\mu$g/mL of anti-CD99 agonist antibody compared to the control. In parallel, to exclude any nonspecific effect of the antibody, the cells were cultured for 6 days with mouse IgG and we did not find any difference (Figure 4(a)).

We have also evaluated the protein expression levels of COLLI, which is the most abundant protein produced by the hOBs [40, 41]. At this purpose, by western blot analyses we demonstrated that, in hOBs treated for 4 and 6 hours with 2 $\mu$g/mL of anti-CD99 agonist antibody, COLLI expression

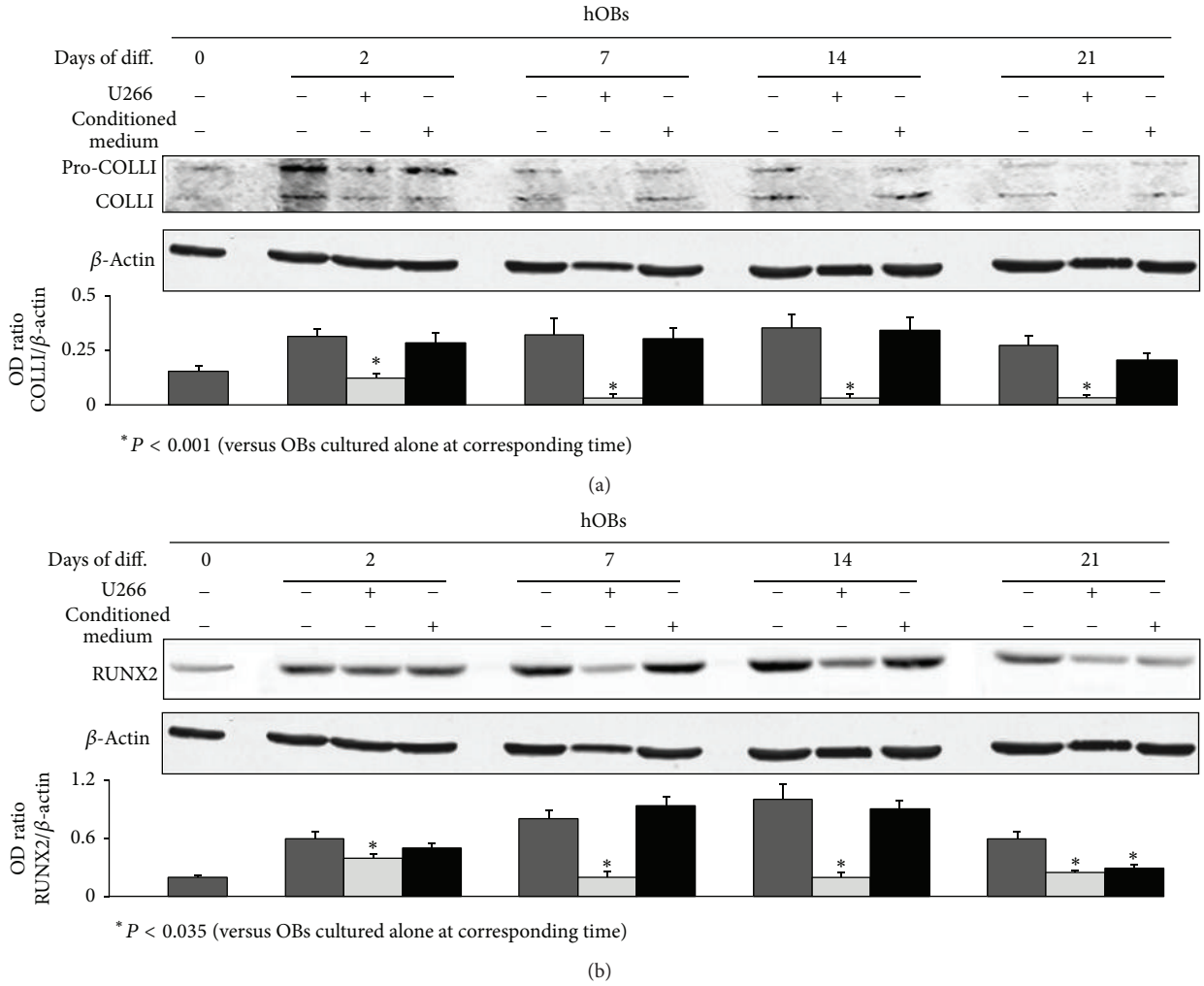


Figure 3: U266 inhibit Collagen I (COLLI) and RUNX2 expression on normal human osteoblasts (hOBs). Human undifferentiated osteoblasts or hOBs differentiated for 2, 7, 14, or 21 days (Days of diff.), were cultured in the presence or absence of U266 or their conditioned medium, and then were analyzed for western blot analysis to detect the protein levels of COLLI (a) or RUNX2 (b). The histograms represent the mean optical density (OD) of COLLI or RUNX2 ratio normalized to the OD of $\beta$-Actin. Data are presented as mean ± SE. The figure shows one representative of three independent experiments.

was significantly ($P < 0.001$) upregulated respect to untreated cells, and the treatment with the mouse IgG did not induce any effect (Figure 4(b)).

On the basis of these results, we evaluated whether the activation of CD99 could also affect the expression of transcription factors regulating hOB differentiation, such as RUNX2, and members of AP1 complex such as JUND, FRA1, and FRA2. hOBs treated for 4 and 6 hours with 2 $\mu$g/mL of anti-CD99 agonist antibody displayed significantly higher RUNX2 and JUND protein levels compared to the controls ($P < 0.012$ and $P < 0.001$, resp.) and no effect was exerted by mouse IgG (Figure 5). In addition, no effect was evidenced in FRA1 and FRA2 protein expression levels (data not shown).

To identify the intracellular signal transduction molecules involved in CD99 signaling pathway(s) in our hOB culture system, the expression of several signaling mediators was examined after CD99 activation. In particular, we demonstrated that the anti-CD99 agonist antibody significantly induces the phosphorylation of signaling components such as AKT and ERK1/2 ($P < 0.001$ and $P = 0.002$, resp.). As shown in Figure 6, the CD99 activation induces AKT phosphorylation after 2 minutes of treatment and ERK1/2 phosphorylation after 5 minutes of stimulation, whereas the usage of mouse IgG did not induce any effect. JNK and P38 were not phosphorylated after CD99 activation (data not shown). To evaluate if AKT and ERK phosphorylation was responsible for COLLI, RUNX2, and JUND modulation mediated by CD99 activation, we studied the expression of these molecules in the presence of PI3K and ERK1 K inhibitors. We demonstrated that after 4 and 6 hours (Figures 7(a) and 7(b)) of CD99 stimulation, both PI3K (involved in AKT phosphorylation) [15] and ERK1 K inhibitors significantly rescue COLLI ($P < 0.001$), RUNX2 ($P \leq 0.01$), and JUND ($P < 0.001$) protein levels in hOBs.

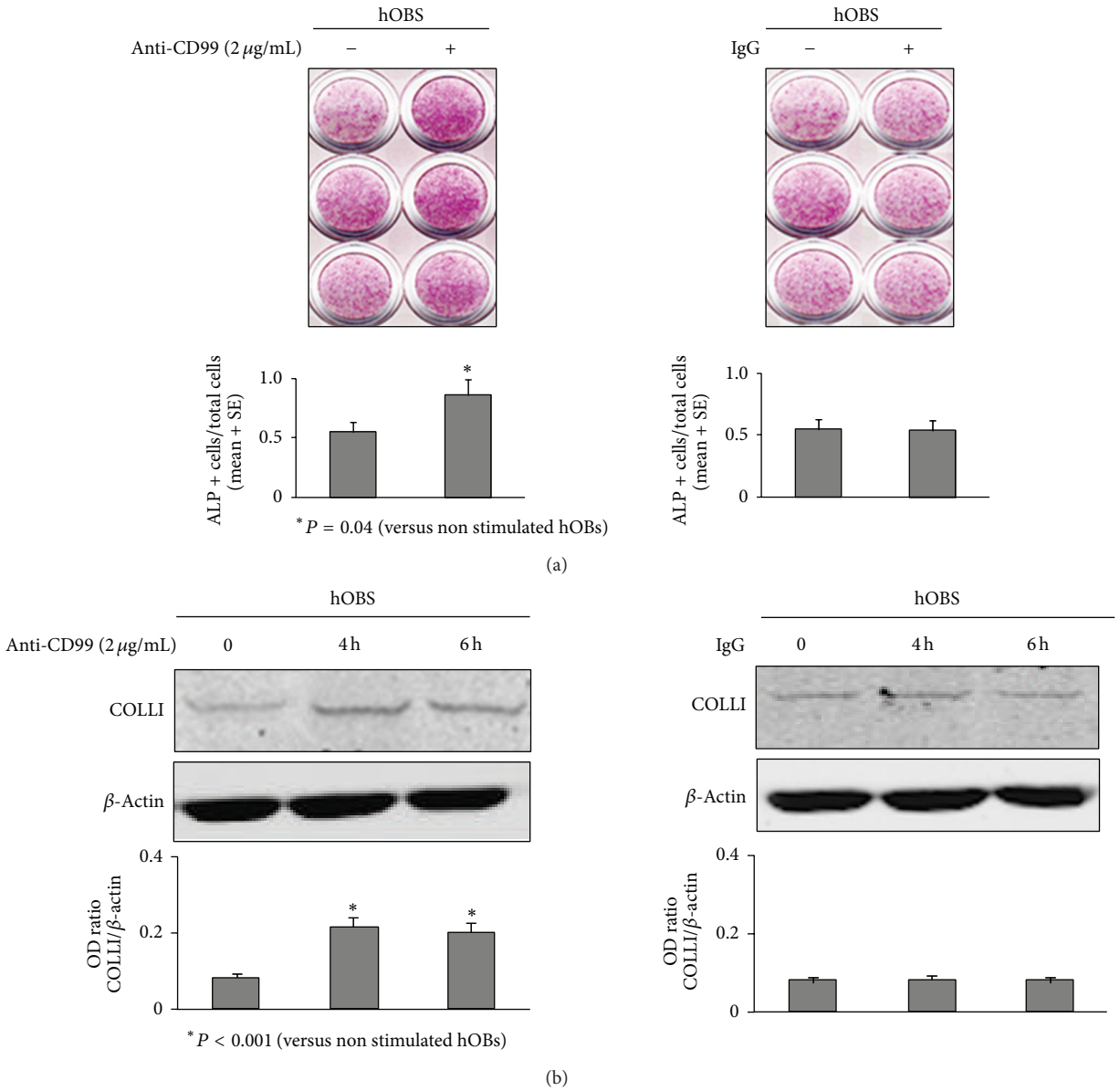


Figure 4: CD99 agonist monoclonal antibody increases Alkaline Phosphatase (ALP) activity and Collagen I (COLLI) expression in normal human osteoblasts (hOBs). (a) Histochemical staining for ALP in differentiated normal hOBs treated for 6 days with or without anti-CD99 agonist monoclonal antibody or mouse IgG. This experiment has been performed in triplicate. The histograms represent the number of ALP positive cells respect to total cells ± SE in three different fields (10x), of three independent experiments. (b) hOBs, treated with anti-CD99 agonist monoclonal antibody or with mouse IgG for 0, 4, and 6 hours (h), were lysed and analyzed by western blot analysis to detect the protein levels of COLLI. The histograms represent the mean optical density (OD) of COLLI ratio normalized to the OD of β-Actin. Data are presented as mean ± SE. The figure shows one of three independent experiments.

Finally, due to the knowledge that the activation of CD99 causes T-lymphocyte and thymocyte apoptosis [30, 31], in parallel to the previously described experiments, we investigate hOB sensitivity to CD99 activation induced apoptosis by analyzing cell viability through MTT assay. In particular, hOBs cultured in 96-well tissue-culture plates were treated for 24 and 48 hours with 2 μg/mL of anti-CD99 agonist antibody. As shown in Figure 8, we found that the activation of CD99 failed to exert any effect on cell viability. To support this finding, we further studied the expression of signaling molecules involved in the apoptosis caspase-cascade events, such as caspase-8 (the initial caspase) and caspase-3 or caspase-7 (the effector caspases) [42, 43] as well as Bid, a death agonist member of the Bcl2/Bcl-xL family [44]. By western blot analysis, we demonstrated that hOBs treated for 4, 6, 12, and 24 hours with 2 μg/mL of anti-CD99 agonist antibody do cause neither caspases 8, 3, and 7 fragmentation nor Bid cleavage (data not shown). In these experiments TRAIL stimulation, known to induce apoptosis in hOBs [45], was used as positive control (data not shown).

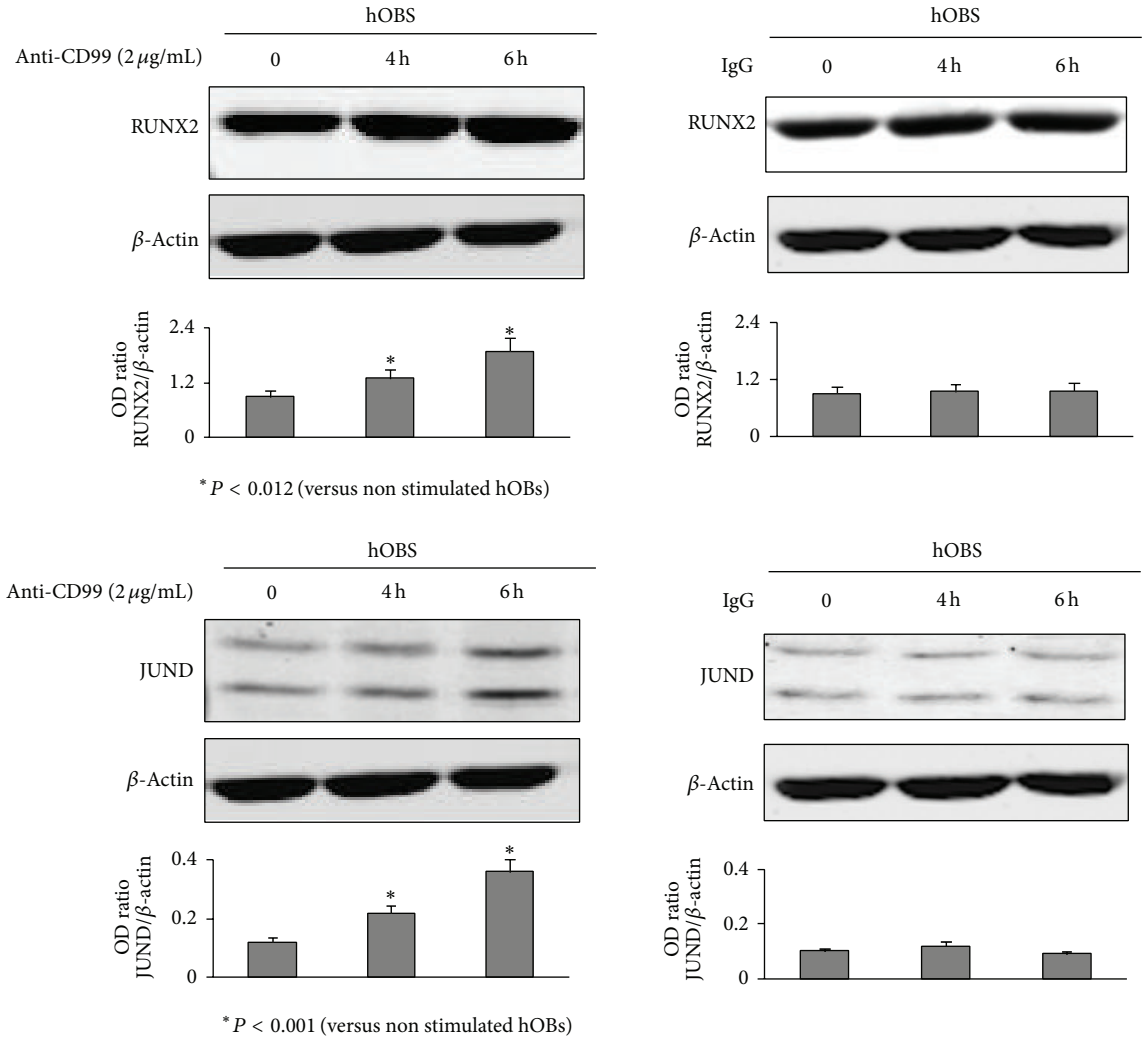


Figure 5: CD99 agonist monoclonal antibody increases RUNX2 and JUND expression in normal human osteoblasts (hOBs). hOBs, treated with anti-CD99 agonist monoclonal antibody or with mouse IgG for 0, 4 and 6 hours (h), were lysed and analyzed by western blot analysis to detect the protein levels of RUNX2 (upper panel) and JUND (lower panel) OB trascription factors. The histograms represent the mean optical density (OD) of RUNX2 or JUND ratio normalized to the OD of $\beta$-Actin. Data are presented as mean ± SE. The figure shows one representative of three independent experiments.

## 4. Discussion

In the present study we demonstrated that HMCLs or their conditioned media downregulate the expression of CD99 by hOBs during their differentiation process. Intriguingly, influenced by HMCLs, undifferentiated and differentiated hOBs, in addition to the reduced levels of CD99, display a less differentiated status. We further showed that CD99 is a critical molecule in the regulation of the physiological process of hOB differentiation and activity since the expression of ALP, COLLI, RUNX2 and JUND are upregulated by the activation of CD99 in hOBs. These findings suggested that the downregulated levels of CD99 could have a critical role in the well-known impairment of osteoblastogenesis and bone formation occurring in the osteolysis associated with MM.

Although CD99 is largely expressed in normal tissues [5] and recently linked to hOB differentiation, variable levels have been demonstrated in different pathological conditions. With particular regard to bone malignancies, a strong expression has been shown in Ewing's sarcoma whereas low levels have been detected in osteosarcoma. It has been shown that CD99-forced expression considerably affects osteosarcoma cell behavior reversing their cell malignancy by regulating critical biological processes required for metastases [5]. New data also provide evidence that when CD99 is restored in osteosarcoma cells, the molecule favors terminally differentiated phenotype [8].

It is worth noting that the supernatants from different tumors associated with osteolytic lesions or osteosclerotic metastasis can vary CD99 expression in osteoblastic cells [18]. However, no data are at present available in the literature regarding the possible modulation of CD99 expression in hOBs exerted by cells of MM, a neoplasm associated with osteolytic bone disease which is due not only to increased

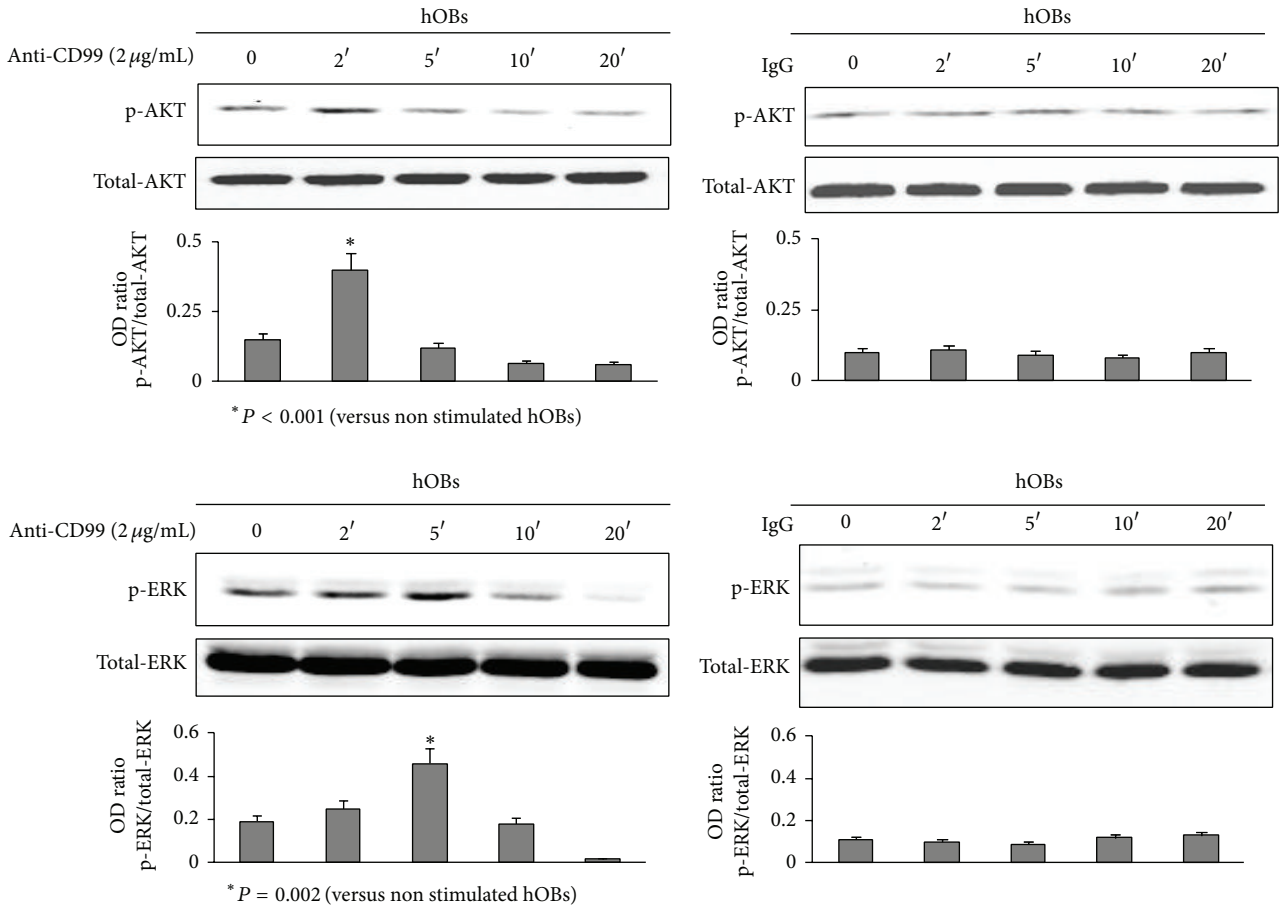


Figure 6: CD99 agonist monoclonal antibody induces AKT and ERK phosphorylation in normal human osteoblasts (hOBs). hOBs, treated with anti-CD99 agonist monoclonal antibody or with mouse IgG for 0, 2, 5, 10, and 20 minutes, were lysed and analyzed by western blot analysis to detect the protein levels of phosphorylated AKT (p-AKT, upper panel) and ERK (p-ERK, lower panel). The histograms represent the mean optical density (OD) of p-AKT or p-ERK ratio normalized to the OD of total-AKT or total-ERK, respectively. Data are presented as mean ± SE. The figure shows one representative of three independent experiments.

osteoclast activity but also to alteration of OB differentiation and function.

Increasing evidence demonstrate that MM cells can impair OB formation and activity through different cellular mechanisms including both secretion of soluble factors [25–29] and cellular contact, such as the interaction with stromal or OB cells [24, 27]. Therefore, based on the new findings demonstrating that differentiated OBs express high CD99 levels we first studied whether myeloma cells could influence the expression of this molecule and next if CD99 could be critical in the differentiation of hOBs.

In this work we found that HMCLs, H929 and U266, which weakly express CD99, induce a significant inhibition of CD99 expression by normal hOBs during their differentiation process. We also demonstrated that this inhibition, although significant, is less evident in the presence of the conditioned medium of both HMCLs indicating that the cell contact and, in a less way, the possible release of soluble molecules in the media induce CD99 reduction in hOBs.

On the basis of recent data demonstrating a high expression of CD99 in differentiated hOBs, we hypothesized that HMCLs through the reduction of CD99 could contribute to OB impairment.

Indeed, these latter cells cocultured with the two HMCLs express less RUNX2 and COLLI amount in favor of the hypothesis that the modulation of CD99 by myeloma cells, in addition to other mechanism(s), could take part in the induction of a less differentiated OB phenotype. In addition, a further support for this hypothesis comes from a very recent paper showing that CD99 expression in extramedullary biopsies of MM patients correlates with longer overall survival suggesting CD99 a new marker for risk stratification of disease severity [19]. These findings let us the possibility to consider the correlation between this *in vivo* study with our *in vitro* data in which the downregulation of CD99 could be involved in the alteration of osteoblastic differentiation and activity taking part in the onset of osteolysis in MM.

To prove that the reduction of CD99 we found could be directly responsible for the less differentiated status of OBs, it would be successful the use of a specific neutralizing antibody. However, a CD99 neutralizing antibody neither is at present available commercially nor is produced by some

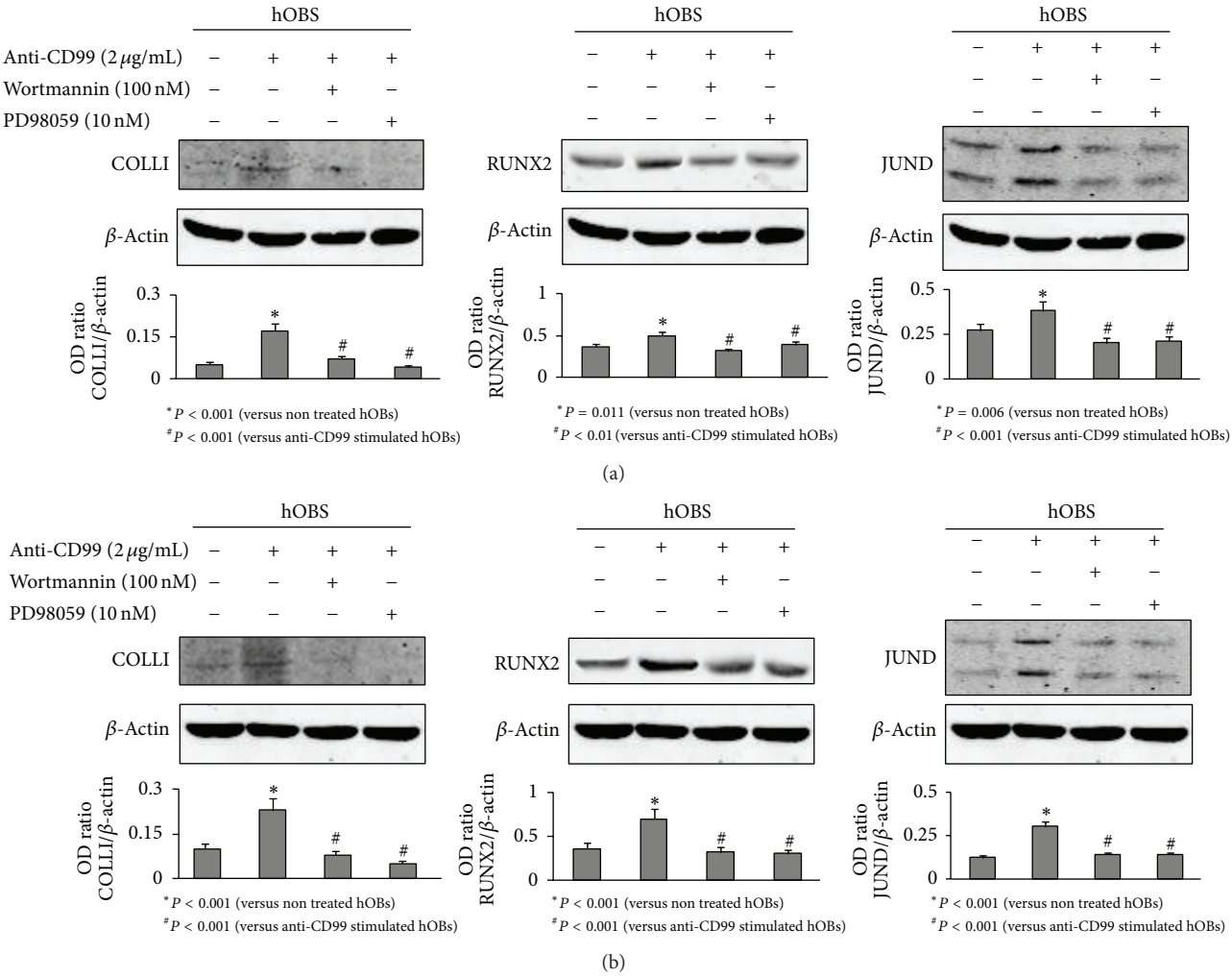


Figure 7: PI3K and ERK1 K inhibitors rescue Collagen I (COLLI), RUNX2, and JUND upregulation induced by CD99 agonist monoclonal antibody in normal human osteoblasts (hOBs). hOBs pretreated for 30 or 60 minutes with PI3K (Wortmannin) or ERK1 K (PD 98059) inhibitors, respectively, were stimulated for 4 (a) or 6 hours (h) (b) with anti-CD99 agonist monoclonal antibody and then lysed and analyzed by western blotting to detect the protein levels of COLLI, RUNX2, and JUND. The histograms represent the mean optical density (OD) of COLLI, RUNX2, or JUND ratio normalized to the OD of $\beta$-Actin. Data are presented as mean ± SE. The figure shows one representative of three independent experiments.

investigators. Thus, to overcome the difficulty of proving direct evidence that the CD99 reduction could take part in the impairment of osteoblastogenesis and bone formation occurring in the osteolytic process associated with MM, we investigated whether the activation of CD99 is able to modulate the activity of hOBs. At this purpose we performed different experiments on hOBs in the presence of a specific agonist antibody, widely used by other authors to achieve information on CD99 activities [32, 38, 39].

We here demonstrated that in hOBs the activation of CD99 stimulates the activity of ALP, the most widely recognized biochemical marker for hOB, and doubled the levels of COLLI, the most abundant organic component of bone matrix [46–48]. Such data point out that, by forcing the function of CD99, hOBs result more active in the expression of their differentiation parameters. This is in agreement with our previous demonstration showing that whenever CD99 expression was regained by osteosarcoma cells, they reactivate the terminal osteoblastic differentiation program [8]. Now, we also provide evidence that in hOBs CD99 activation contributes to increase the protein levels of RUNX2, the master transcription factor for OB differentiation which is central in triggering the expression of major bone matrix protein genes including the COLLI [49]. In parallel, we further demonstrated high levels of JUND, member of AP1 heterodimeric complex, crucial regulator of osteogenic genes that acts as coregulator of RUNX2 itself [50, 51]. We further found that in hOBs CD99 activation induces AKT and ERK phosphorylation, consistent with data demonstrating the ability of CD99 stimulation to induce MAPKs and protein kinase C activation [52, 53] and findings showing that, in human breast cancer cells, CD99 promotes SRC, AKT, ERK, and JNK activation, thus increasing JUND and FOSB AP-1 transcription factors expression [15]. In line with

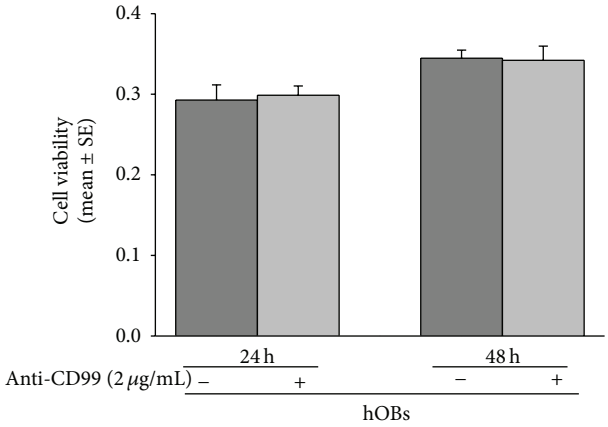


Figure 8: CD99 agonist monoclonal antibody does not induce the reduction of normal human osteoblast (hOBs) viability. Cell viability, evaluated by MTT assay, in hOBs treated for 24 or 48 h with anti-CD99 agonist monoclonal antibody. Results are expressed as values of optical density at 570 nm ± SE of three independent experiments performed in triplicate.

the recent demonstration that CD99-restored expression in osteosarcoma cell correlated with ERK 1/2, RUNX2, and AP-1 activation [8], we here show that PI3K and ERK1 K inhibitors rescue COLLI, RUNX2 and JUND OB protein levels. Therefore, we can here assess that CD99 activation, through the phosphorylation of AKT and ERK 1/2, increases RUNX2 and JUND transcription factors as well as COLLI levels, thereby playing a significant role in the activation of normal hOBs.

## 5. Conclusions

Our results highlight an important role of CD99 in the differentiation and activity of hOBs in physiological and pathological conditions. HMCLs induce a reduction of CD99 expression in hOBs which display a less differentiated phenotype, suggesting a possible contribution of this molecule in the impairment of osteoblastogenesis occurring in MM bone disease. Indeed, by forcing the function of CD99 the hOBs result more active in the expression of their differentiation parameters.

## Conflict of Interests

The authors declare no conflict of interests.

## Authors' Contribution

Silvia Colucci and Maria Grano contributed equally to the work as senior authors.

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## References

[1] R. Levy, J. Dilley, R. I. Fox, and R. Warnke, "A human thymus-leukemia antigen defined by hybridoma monoclonal antibodies," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 76, no. 12, pp. 6552–6556, 1979.

[2] N. A. Ellis, T.-Z. Ye, S. Patton, J. German, P. N. Goodfellow, and P. Weller, "Cloning of *PBDX*, an *MIC2*-related gene that spans the pseudoautosomal boundary on chromosome Xp," *Nature Genetics*, vol. 6, no. 4, pp. 394–400, 1994.

[3] J.-H. Hahn, M. K. Kim, E. Y. Choi et al., "CD99 (MIC2) regulates the LFA-1/1CAM-1-mediated adhesion of lymphocytes, and its gene encodes both positive and negative regulators of cellular adhesion," *Journal of Immunology*, vol. 159, no. 5, pp. 2250–2258, 1997.

[4] H.-Y. Kim, Y. M. Kim, Y.-K. Shin, S.-H. Park, and W. Lee, "Solution structure of the cytoplasmic domain of human CD99 Type I," *Molecules and Cells*, vol. 18, no. 1, pp. 24–29, 2004.

[5] M. C. Manara, G. Bernard, P.-L. Lollini et al., "CD99 acts as an oncosuppressor in osteosarcoma," *Molecular Biology of the Cell*, vol. 17, no. 4, pp. 1910–1921, 2006.

[6] K. Bertaux, O. Broux, C. Chauveau, J. Jeanfils, and J.-C. Devedjian, "Identification of CBFA1-regulated genes on SaOs-2 cells," *Journal of Bone and Mineral Metabolism*, vol. 23, no. 2, pp. 114–122, 2005.

[7] J. B. Lian, A. Javed, S. K. Zaidi et al., "Regulatory controls for osteoblast growth and differentiation: role of Runx/Cbfa/AML factors," *Critical Reviews in Eukaryotic Gene Expression*, vol. 14, no. 1-2, pp. 1–41, 2004.

[8] M. Sciandra, M. T. Marino, M. C. Manara et al., "CD99 drives terminal differentiation of osteosarcoma cells by acting as a spatial regulator of ERK 1/2," *Journal of Bone and Mineral Research*, vol. 29, no. 5, pp. 1295–1309, 2014.

[9] I. M. Ambros, P. F. Ambros, S. Strehl, H. Kovar, H. Gadner, and M. Salzer-Kuntschik, "MIC2 is a specific marker for Ewing's sarcoma and peripheral primitive neuroectodermal tumors: evidence for a common histogenesis of Ewing's sarcoma and peripheral primitive neuroectodermal tumors from MIC2 expression and specific chromosome aberration," *Cancer*, vol. 67, no. 7, pp. 1886–1893, 1991.

[10] K. Scotlandi, M. Serra, M. C. Manara et al., "Immunostaining of the p30/32(MIC2) antigen and molecular detection of EWS rearrangements for the diagnosis of Ewing's sarcoma and peripheral neuroectodermal tumor," *Human Pathology*, vol. 27, no. 4, pp. 408–416, 1996.

[11] C. Fisher, "Synovial sarcoma," *Annals of Diagnostic Pathology*, vol. 2, no. 6, pp. 401–421, 1998.

[12] R. E. Brown and J. L. Boyle, "Mesenchymal chondrosarcoma: molecular characterization by a proteomic approach, with morphogenic and therapeutic implications," *Annals of Clinical and Laboratory Science*, vol. 33, no. 2, pp. 131–141, 2003.

[13] P. Ramani, D. Rampling, and M. Link, "Immunocytochemical study of 12E7 in small round-cell tumours of childhood: an assessment of its sensitivity and specificity," *Histopathology*, vol. 23, no. 6, pp. 557–561, 1993.

[14] T. Terada, "TDT (-), KIT (+), CD34 (+), CD99 (+) precursor T lymphoblastic leukemia/lymphoma," *International Journal of Clinical and Experimental Pathology*, vol. 5, no. 2, pp. 167–170, 2012.

[15] H.-J. Byun, I.-K. Hong, E. Kim et al., "A splice variant of CD99 increases motility and MMP-9 expression of human breast cancer cells through the AKT-, ERK-, and JNK-dependent AP-1 activation signaling pathways," *The Journal of Biological Chemistry*, vol. 281, no. 46, pp. 34833–34847, 2006.

[16] A. Maitra, D. E. Hansel, P. Argani et al., "Global expression analysis of well-differentiated pancreatic endocrine neoplasms using oligonucleotide microarrays," *Clinical Cancer Research*, vol. 9, no. 16, pp. 5988–5995, 2003.

[17] K. C. Jung, W. S. Park, Y. M. Bae et al., "Immunoreactivity of CD99 in stomach cancer," *Journal of Korean Medical Science*, vol. 17, no. 4, pp. 483–489, 2002.

[18] G. Hamilton, R. Mallinger, H. Millesi, A. Engel, G. Baumgartner, and M. Raderer, "Modulation of CD99/MIC2 expression of human AHTO-7 osteoblasts by carcinoma cell line-conditioned media," *Anticancer Research*, vol. 21, no. 6A, pp. 3909–3913, 2001.

[19] S. J. Shin, H. Lee, G. Jung et al., "Expression of CD99 in multiple myeloma: a clinicopathologic and immunohistochemical study of 170 cases," *The Korean Journal of Pathology*, vol. 48, no. 3, pp. 209–216, 2014.

[20] G. Brunetti, A. Oranger, G. Mori et al., "The formation of osteoclasts in multiple myeloma bone disease patients involves the secretion of soluble decoy receptor 3," *Annals of the New York Academy of Sciences*, vol. 1192, pp. 298–302, 2010.

[21] S. Colucci, G. Brunetti, G. Mori et al., "Soluble decoy receptor 3 modulates the survival and formation of osteoclasts from multiple myeloma bone disease patients," *Leukemia*, vol. 23, no. 11, pp. 2139–2146, 2009.

[22] G. Brunetti, S. Colucci, R. Rizzi et al., "The role of OPG/TRAIL complex in multiple myeloma," *Annals of the New York Academy of Sciences*, vol. 1068, no. 1, pp. 334–340, 2006.

[23] S. Colucci, G. Brunetti, R. Rizzi et al., "T cells support osteoclastogenesis in an in vitro model derived from human multiple myeloma bone disease: the role of the OPG/TRAIL interaction," *Blood*, vol. 104, no. 12, pp. 3722–3730, 2004.

[24] N. Giuliani, S. Colla, F. Morandi et al., "Myeloma cells block RUNX2/CBFA1 activity in human bone marrow osteoblast progenitors and inhibit osteoblast formation and differentiation," *Blood*, vol. 106, no. 7, pp. 2472–2483, 2005.

[25] S. Colucci, G. Brunetti, A. Oranger et al., "Myeloma cells suppress osteoblasts through sclerostin secretion," *Blood Cancer Journal*, vol. 1, article e27, 2011.

[26] G. Brunetti, A. Oranger, G. Mori et al., "Sclerostin is overexpressed by plasma cells from multiple myeloma patients," *Annals of the New York Academy of Sciences*, vol. 1237, no. 1, pp. 19–23, 2011.

[27] A. Oranger, C. Carbone, M. Izzo, and M. Grano, "Cellular mechanisms of multiple myeloma bone disease," *Clinical and Developmental Immunology*, vol. 2013, Article ID 289458, 11 pages, 2013.

[28] N. Giuliani, F. Morandi, S. Tagliaferri et al., "Production of Wnt inhibitors by myeloma cells: potential effects on canonical Wnt pathway in the bone microenvironment," *Cancer Research*, vol. 67, no. 16, pp. 7665–7674, 2007.

[29] T. Oshima, M. Abe, J. Asano et al., "Myeloma cells suppress bone formation by secreting a soluble Wnt inhibitor, sFRP-2," *Blood*, vol. 106, no. 9, pp. 3160–3165, 2005.

[30] G. Bernard, J.-P. Breittmayer, M. de Matteis et al., "Apoptosis of immature thymocytes mediated by E2/CD99," *The Journal of Immunology*, vol. 158, no. 6, pp. 2543–2550, 1997.

[31] R. D. Pettersen, G. Bernard, M. K. Olafsen, M. Pourtein, and S. O. Lie, "CD99 signals caspase-independent T cell death," *The Journal of Immunology*, vol. 166, no. 8, pp. 4931–4942, 2001.

[32] K. C. Jung, N. H. Kim, W. S. Park, S. H. Park, and Y. Bae, "The CD99 signal enhances Fas-mediated apoptosis in the human leukemic cell line, Jurkat," *FEBS Letters*, vol. 554, no. 3, pp. 478–484, 2003.

[33] Z. Husak, D. Printz, A. Schumich, U. Pötschger, and M. N. Dworzak, "Death induction by CD99 ligation in TEL/AML1-positive acute lymphoblastic leukemia and normal B cell precursors," *Journal of Leukocyte Biology*, vol. 88, no. 2, pp. 405–412, 2010.

[34] G. Bernard, D. Zoccola, M. Deckert, J.-P. Breittmayer, C. Aussel, and A. Bernard, "The E2 molecule (CD99) specifically triggers homotypic aggregation of CD4+ CD8+ thymocytes," *The Journal of Immunology*, vol. 154, no. 1, pp. 26–32, 1995.

[35] G. Bernard, V. Raimondi, I. Alberti et al., "CD99 (E2) upregulates $\alpha 4\beta 1$-dependent T cell adhesion to inflamed vascular endothelium under flow conditions," *European Journal of Immunology*, vol. 30, no. 10, pp. 3061–3065, 2000.

[36] A. R. Schenkel, Z. Mamdouh, X. Chen, R. M. Liebman, and W. A. Muller, "CD99 plays a major role in the migration of monocytes through endothelial junctions," *Nature Immunology*, vol. 3, no. 2, pp. 143–150, 2002.

[37] M. Waclavicek, O. Majdic, T. Stulnig et al., "CD99 engagement on human peripheral blood T cells results in TCR/CD3- dependent cellular activation and allows for Th1-restricted cytokine production," *Journal of Immunology*, vol. 161, no. 9, pp. 4671–4678, 1998.

[38] D. Wingett, K. Forcier, and C. P. Nielson, "A role for CD99 in T cell activation," *Cellular Immunology*, vol. 193, no. 1, pp. 17–23, 1999.

[39] I.-S. Lee, C.-S. Park, and K. Ahn, "CD99 regulates the transport of MHC class I molecules from the Golgi complex to the cell surface," *Journal of Immunology*, vol. 166, no. 2, pp. 787–794, 2001.

[40] S. Gronthos, M. Mankani, J. Brahim, P. G. Robey, and S. Shi, "Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 25, pp. 13625–13630, 2000.

[41] M. Q. Hassan, J. A. R. Gordon, M. M. Beloti et al., "A network connecting Runx2, SATB2, and the miR-23a~27a~24-2 cluster regulates the osteoblast differentiation program," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 107, no. 46, pp. 19879–19884, 2010.

[42] K. M. Boatright and G. S. Salvesen, "Mechanisms of caspase activation," *Current Opinion in Cell Biology*, vol. 15, no. 6, pp. 725–731, 2003.

[43] Q. Bao and Y. Shi, "Apoptosome: a platform for the activation of initiator caspases," *Cell Death & Differentiation*, vol. 14, no. 1, pp. 56–65, 2007.

[44] K. Wang, X.-M. Yin, D. T. Chao, C. L. Milliman, and S. J. Korsmeyer, "BID: a novel BH3 domain-only death agonist," *Genes and Development*, vol. 10, no. 22, pp. 2859–2869, 1996.

[45] G. Brunetti, A. Oranger, C. Carbone et al., "Osteoblasts display different responsiveness to TRAIL-induced apoptosis during their differentiation process," *Cell Biochemistry and Biophysics*, vol. 67, no. 3, pp. 1127–1136, 2013.

[46] V. Kartsogiannis and K. W. Ng, "Cell lines and primary cell cultures in the study of bone cell biology," *Molecular and Cellular Endocrinology*, vol. 228, no. 1-2, pp. 79–102, 2004.

[47] E. Son, H. Do, H.-M. Joo, and S. Pyo, "Induction of alkaline phosphatase activity by l-ascorbic acid in human osteoblastic cells: a potential role for CK2 and Ikaros," *Nutrition*, vol. 23, no. 10, pp. 745–753, 2007.

[48] O. Wanachewin, K. Boonmaleerat, P. Pothacharoen, V. Reutrakul, and P. Kongtawelert, "Sesamin stimulates osteoblast differentiation through p38 and ERK1/2 MAPK signaling pathways," *BMC Complementary and Alternative Medicine*, vol. 12, article 71, 2012.

[49] T. Komori, "Regulation of skeletal development by the Runx family of transcription factors," *Journal of Cellular Biochemistry*, vol. 95, no. 3, pp. 445–453, 2005.

[50] P. J. Marie, "Transcription factors controlling osteoblastogenesis," *Archives of Biochemistry and Biophysics*, vol. 473, no. 2, pp. 98–105, 2008.

[51] T. Komori, "Regulation of osteoblast differentiation by transcription factors," *Journal of Cellular Biochemistry*, vol. 99, no. 5, pp. 1233–1239, 2006.

[52] M.-J. Hahn, S. S. Yoon, H. W. Sohn, H. G. Song, S. H. Park, and T. J. Kim, "Differential activation of MAP kinase family members triggered by CD99 engagement," *FEBS Letters*, vol. 470, no. 3, pp. 350–354, 2000.

[53] W. Kasinrerk, N. Tokrasinwit, S. Moonsom, and H. Stockinger, "CD99 monoclonal antibody induce homotypic adhesion of Jurkat cells through protein tyrosine kinase and protein kinase C-dependent pathway," *Immunology Letters*, vol. 71, no. 1, pp. 33–41, 2000.

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*Research Article*

# Muramyl Dipeptide Enhances Lipopolysaccharide-Induced Osteoclast Formation and Bone Resorption through Increased RANKL Expression in Stromal Cells

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Lipopolysaccharide (LPS) is bacterial cell wall component capable of inducing osteoclast formation and pathological bone resorption. Muramyl dipeptide (MDP), the minimal essential structural unit responsible for the immunological activity of peptidoglycans, is ubiquitously expressed by bacterium. In this study, we investigated the effect of MDP in LPS-induced osteoclast formation and bone resorption. LPS was administered with or without MDP into the supracalvariae of mice. The number of osteoclasts, the level of mRNA for cathepsin K and tartrate-resistant acid phosphatase (TRAP), the ratio of the bone destruction area, the level of tartrate-resistant acid phosphatase form 5b (TRACP 5b), and C-terminal telopeptides fragments of type I collagen as a marker of bone resorption in mice administrated both LPS and MDP were higher than those in mice administrated LPS or MDP alone. On the other hand, MDP had no effect on osteoclastogenesis in parathyroid hormone administrated mice. MDP enhanced LPS-induced receptor activator of NF-$\kappa$B ligand (RANKL) expression and Toll-like receptor 4 (TLR4) expression *in vivo* and in stromal cells *in vitro*. MDP also enhanced LPS-induced mitogen-activated protein kinase (MAPK) signaling, including ERK, p38, and JNK, in stromal cells. These results suggest that MDP might play an important role in pathological bone resorption in bacterial infection diseases.

## 1. Introduction

Lipopolysaccharide (LPS) is a major component of the cell wall of Gram-negative bacteria and is a well-known potent inducer of inflammation and inflammatory bone loss [1–5]. LPS is known to induce the production of many local factors, including proinflammatory cytokines, such as TNF-$\alpha$ and IL-1, from macrophages or other cells involved in mediating the inflammatory response in tissues [6]. There is reason to suggest that osteoclast recruitment could be central to diseases involving bone erosion, such as rheumatoid arthritis [7], periprosthetic bone loss [8], postmenopausal osteoporosis, [9] and periodontal disease [2]. Osteoclasts derived from bone marrow cells regulate bone resorption and remodeling [10]. Such osteoclast formation and activation require the expression of two factors: receptor activator of NF-$\kappa$B ligand (RANKL) and macrophage colony stimulating factor (M-CSF) [11]. Furthermore, tumor necrosis factor- (TNF-) $\alpha$ has also been reported to induce osteoclast formation *in vitro* [12–14] and *in vivo* [15, 16]. These inflammatory cytokines have been linked with LPS-induced osteoclast formation and bone destruction *in vivo* and *in vitro* [2, 17–20].

Peptidoglycan (PGN) is another major component of bacterial cell membranes. Muramyl dipeptide (MDP), the minimal essential structural unit responsible for the immunological activity of PGNs, is distributed ubiquitously

in the cell walls of both Gram-negative and Gram-positive bacteria. It has been reported that MDP can enhance the production of TNF-$\alpha$ when injected into mice [21] and can cause lethal shock in mice challenged with LPS [22]. In addition, MDP has been shown to synergistically enhance LPS-induced proinflammatory cytokine production in human monocyte cells [23]. MDP alone cannot induce osteoclast formation in mouse cocultures of primary osteoblasts and hematopoietic cells; however, it can enhance osteoclast formation induced by LPS, IL-1$\alpha$, and TNF-$\alpha$ but not by 1$\alpha$,25-dihydroxy-vitamin-$D_3$ (1$\alpha$,25$(OH)_2D_3$) or prostaglandin-E2 (PGE2). Indeed, it has been shown that MDP can upregulate RANKL expression in osteoblasts treated with LPS or TNF-$\alpha$ but not those treated with 1$\alpha$,25$(OH)_2D_3$ [24].

In this study, we show that MDP enhances LPS-induced osteoclast formation *in vivo* and increases the expression of RANKL *in vivo* and in stromal cell cultures *in vitro*. MDP also enhances the LPS-induced expression of TLR4—a signal transducing receptor for LPS—both *in vivo* and in stromal cells *in vitro*. Finally, MDP enhances LPS-induced MAPK signaling pathways in stromal cells.

## 2. Material and Methods

*2.1. Mice and Reagents.* Two- to 10-week-old male C57BL6/J mice were purchased from CLEA Japan (Tokyo, Japan) for use in this study. All animal procedures were in accordance with Tohoku University regulations. *Escherichia coli* LPS was purchased from Sigma-Aldrich (St. Louis, MO). MDP (Peptide Institute, Inc., Osaka, Japan) was purchased from Sigma-Aldrich. The following mouse antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA): polyclonal anti-phospho-p44/42ERK, anti-phospho-JNK, anti-phospho-p38, anti-$\beta$-Actin, and anti-rabbit IgG horseradish peroxidase- (HRP-) linked antibodies.

*2.2. Preparation for Histological Observation.* Mice calvariae were injected daily for 5 days with PBS, LPS alone (10 $\mu$g/day or 100 $\mu$g/day, referred to as low or high, resp.), MDP (100 $\mu$g/day) alone, or LPS (10 $\mu$g/day) and MDP (100 $\mu$g/day) (LPS + MDP). The mice were then sacrificed, and the calvariae were immediately harvested and fixed overnight in 4% paraformaldehyde at 4°C. Samples were then demineralized in 14% ethylene-diaminetetraacetic acid for 3 days at 4°C. The sections were stained for TRAP activity and counterstained with hematoxylin for analysis of osteoclast formation. Osteoclasts were counted at the sagittal suture. To cancel out any variations, the calvariae were divided into three sections by the coronal plane. Osteoclasts in five sagittal sutures per section were counted and averaged. In addition, the percentage of interface of bone marrow space covered by osteoclasts was histomorphometrically determined in specimens derived from each sample.

*2.3. Serum Tartrate-Resistant Acid Phosphatase 5b (TRACP 5b) Assay and Serum C-Terminal Telopeptide Fragments of Type I Collagen Cross-Links (CTX) Assay.* Serum was obtained from mice after 5 days of daily LPS administration with or without MDP. The levels of TRACP 5b were determined using a Mouse TRAP Assay kit (IDS, Tyne and Wear, UK). TRACP 5b levels were measured at 405 nm using an absorption microplate reader (model 550; Bio-Rad, Richmond, CA). The levels of C-terminal telopeptide fragments of type I collagen were determined using a Mouse CTX Assay kit (IDS, Tyne and Wear, UK). C-terminal telopeptide fragments of type I collagen levels were measured at 450 nm using an absorption microplate reader (model 550; Bio-Rad, Richmond, CA).

*2.4. RNA Preparation and Real-Time Reverse-Transcription Polymerase Chain Reaction (RT-PCR) Analysis In Vitro and In Vivo.* For *in vitro* experiments, bone marrow cells from the femora and tibiae of mice were flushed with culture medium. The harvested cells were incubated in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) containing 10% fetal bovine serum, 100 IU/mL penicillin G (Life Technologies, Carlsbad, CA), and 100 $\mu$g/mL streptomycin (Life Technologies). After 2 weeks of culture, cells were washed with PBS to remove floating cells. Adherent cells from these cultures were used as bone marrow stromal cells in this study. Adherent bone marrow stromal cells were incubated in culture medium supplemented with high or low LPS alone, LPS + MDP, or MDP alone. After 3 days of culture, total RNA was isolated from adherent cells using an RNeasy mini kit (Qiagen, Valencia, CA).

For *in vivo* experiments, harvested calvariae were frozen in liquid nitrogen, ground using a Micro Smash MS-100R (TOMY SEIKO, Tokyo, Japan), and then centrifuged in 800 $\mu$L of TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA was isolated from these samples using an RNeasy minikit (Qiagen). All cDNA was synthesized from 2 $\mu$g of total RNA using reverse transcriptase and oligo-dT primers (Invitrogen) in a reaction volume of 20 $\mu$L. The mRNA expression levels of TRAP, cathepsin K, RANKL, and TLR4 were quantified by real-time RT-PCR using a Thermal Cycler Dice Real Time System (Takara, Shiga, Japan). Reactions were performed in a 25 $\mu$L volume containing 2 $\mu$L of cDNA, 12.5 $\mu$L of SYBR Premix Ex Taq (Takara), and 25 pmol/$\mu$L primers. The cycling conditions were as follows: 95°C for 10 s for initial denaturation followed by 45 cycles of amplification, with each cycle consisting of a denaturation step at 95°C for 5 s and an annealing step at 60°C for 30 s. Gene expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The following primers were used: for GAPDH, 5′-GGTGGAGCCAAAAGGGTCA-3′ and 5′-GGGGGCTAAGCAGTTG-GT-3′; cathepsin K, 5′-GCAGAGGTTGTACTATGA-3′ and 5′-GCAGGCGTT-GTTCTTATT-3′; TRAP, 5′-AACTTGCGACCATTGTTA-3′ and 5′-GGGGACCTTTCGTTGATGT-3′; RANKL, 5′-CCTGAGGCCAGCCATTT-3′ and 5′-CTTGGCCCAGCC-T-3′; and TLR4, 5′-CACTGTTCTTCTCCTGCCTGAC-3′ and 5′-TGGTTGAAGAAGGAATGTCATC-3′.

*2.5. Measurement of Bone Destruction.* Calvariae were harvested and the soft tissues were carefully removed. Calvariae were then fixed in PBS-buffered formaldehyde (4%) for 3 days at 4°C and then washed with PBS for radiological

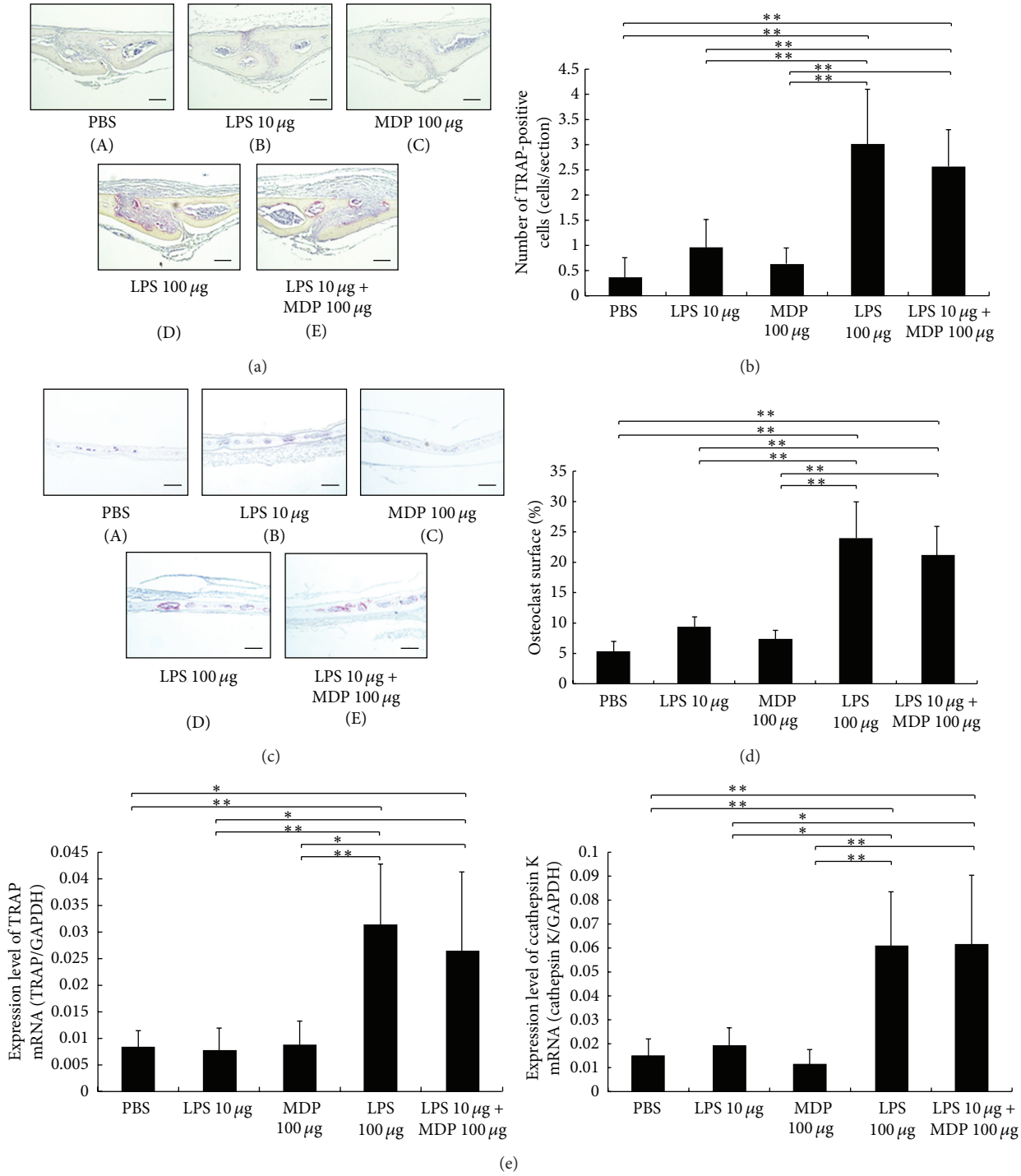



Figure 1: Effects of muramyl dipeptide (MDP) on lipopolysaccharide- (LPS-) induced osteoclast formation *in vivo*. (a) Histological sections of sutures of calvariae were obtained from mice after 5 days of daily supracalvarial injections of one of the following: PBS (A), 10 $\mu$g/day LPS (B), 100 $\mu$g/day MDP (C), 100 $\mu$g/day LPS (D), or 10 $\mu$g/day LPS + 100 $\mu$g/day MDP (E). Sections were stained with tartrate-resistant acid phosphatase (TRAP) staining and counterstained with hematoxylin. Cells that stained red are considered to be TRAP-positive. Scale bars = 50 $\mu$m. (b) The number of TRAP-positive cells with three or more nuclei in the calvariae ($n = 4$; $^{**}P < 0.01$). (c) Histological sections of calvariae were obtained from mice after 5 days of daily supracalvarial injections of one of the following: PBS (A), 10 $\mu$g/day LPS (B), 100 $\mu$g/day MDP (C), 100 $\mu$g/day LPS (D), or 10 $\mu$g/day LPS + 100 $\mu$g/day MDP (E). Scale bars = 100 $\mu$m. (d) The percentage of bone/marrow interface covered by osteoclasts was histomorphometrically determined in specimens ($n = 4$; $^{**}P < 0.01$). (e) TRAP and cathepsin K mRNA levels in mouse calvariae detected using real-time RT-PCR. Total RNA from mouse calvariae was isolated after 5 days of daily supracalvarial injections of PBS, LPS (10 $\mu$g/day), MDP (100 $\mu$g/day), LPS (100 $\mu$g/day), or LPS (10 $\mu$g/day) + MDP (100 $\mu$g/day). mRNA levels for TRAP and cathepsin K were normalized to GAPDH. Results are expressed as the mean ± SD ($n = 4$; $^{**}P < 0.01$; $^{*}P < 0.05$). Differences were determined using Scheffe's $F$ test.

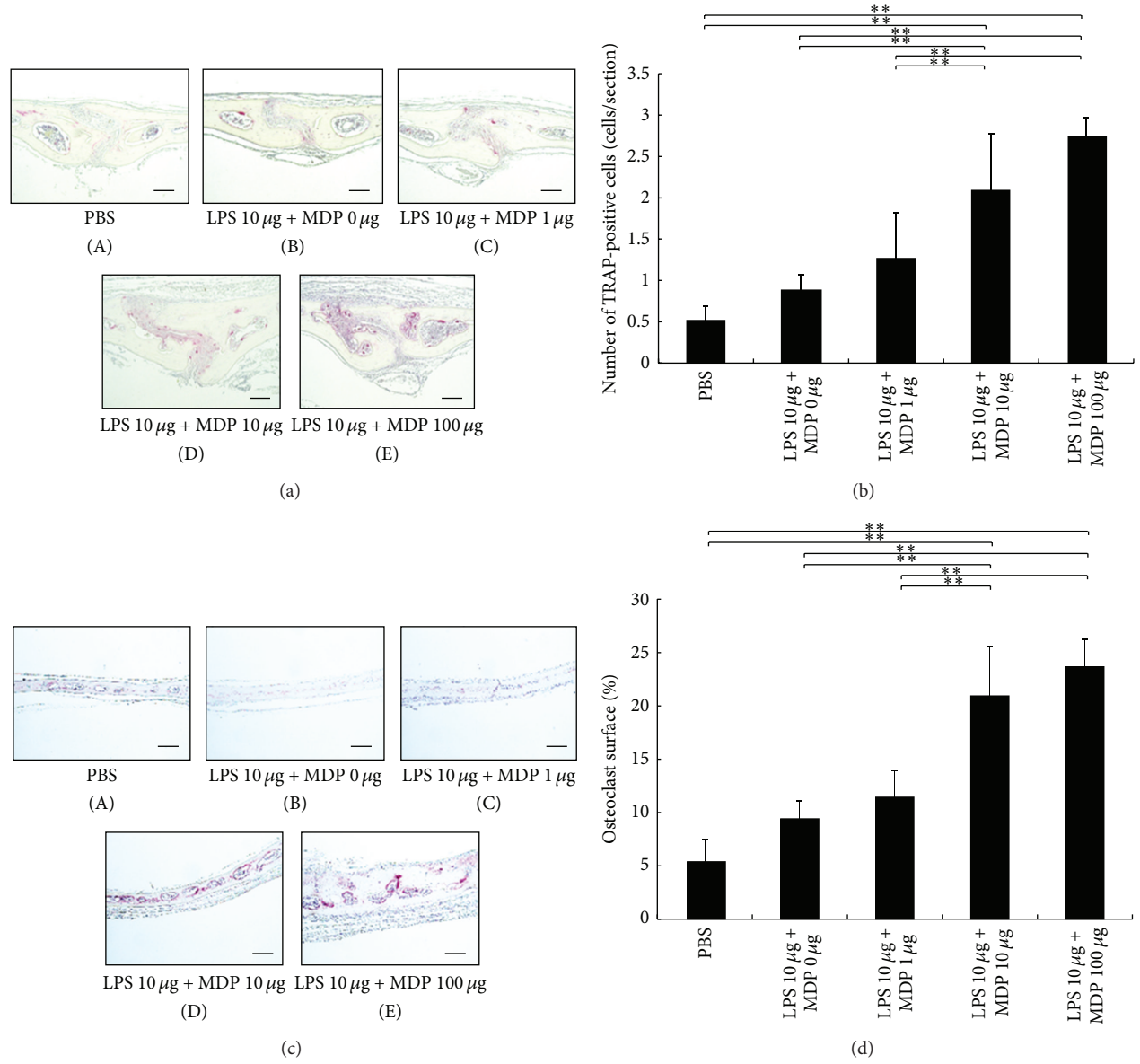



Figure 2: Osteoclast formation is dependent on muramyl dipeptide (MDP) concentration. (a) Osteoclast number in the sutures following treatment with a low concentration of lipopolysaccharide (LPS) (10 $\mu$g/day) with increasing concentrations of MDP for 5 days. Sections were stained with tartrate-resistant acid phosphatase (TRAP) staining and counterstained with hematoxylin. Cells that stained red are considered to be TRAP-positive. Scale bars = 50 $\mu$m. (b) Number of TRAP-positive cells with three or more nuclei in the calvariae ($n = 4$; $^{**}P < 0.01$). (c) Osteoclast number in the bone/marrow interface following treatment with a low concentration of lipopolysaccharide (LPS) (10 $\mu$g/day) with increasing concentrations of MDP for 5 days. Sections were stained with tartrate-resistant acid phosphatase (TRAP) staining and counterstained with hematoxylin. Cells that stained red are considered to be TRAP-positive. Scale bars = 100 $\mu$m. (d) The percentage of bone/marrow interface covered by osteoclasts was histomorphometrically determined in specimens ($n = 4$; $^{**}P < 0.01$). Differences were detected using Scheffe's $F$ test.

analysis. Microfocus computed tomography (ScanXmate-E090; Comscan, Kanagawa, Japan) was used to assay the bone resorption pits in the calvariae, and TRI/3D-BON64 software (RATOC System Engineering, Tokyo, Japan) was used to build three-dimensional reconstruction images of the calvariae. The ratio of bone destruction to total area was calculated using ImageJ (NIH, Bethesda, MD).

*2.6. Immunoblotting for Analysis of MAPK Signaling.* Stromal cells were cultured in serum-free DMEM for 3 h before treatment with LPS and/or MDP for the various durations, as indicated. Treated cells were washed twice with ice-cold PBS and then lysed in lysis buffer (Cell Signaling Technology) containing a protease inhibitor mixture. Cell lysates (30 $\mu$g) were boiled in the presence of lithium dodecyl sulfate

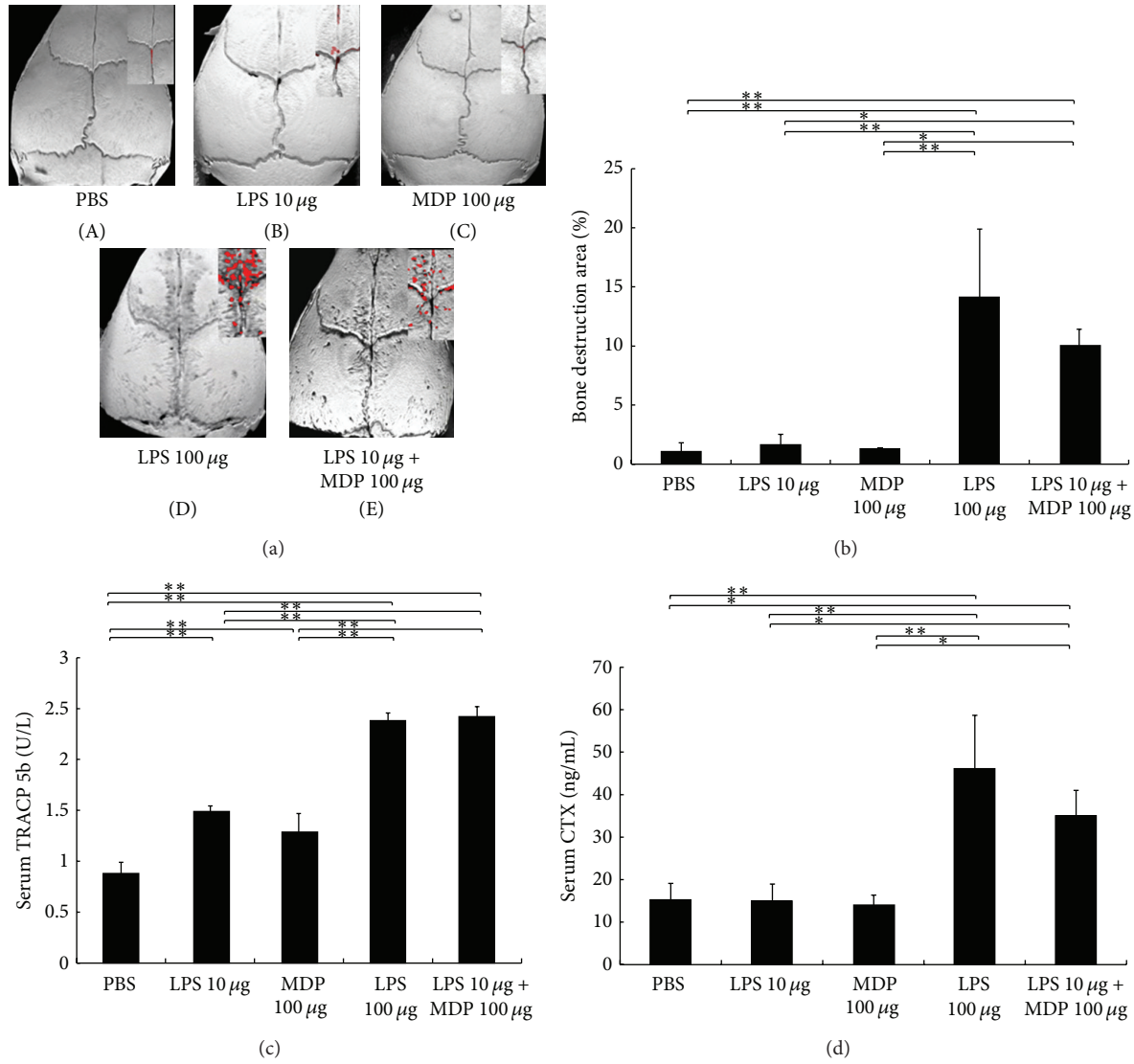



Figure 3: Muramyl dipeptide (MDP) enhances lipopolysaccharide- (LPS-) induced bone destruction in mouse calvariae. (a) Microfocus computed tomography reconstruction images of mouse calvariae harvested after 5 days of daily administration of PBS (A), 10 $\mu$g/day LPS (B), 100 $\mu$g/day MDP (C), 100 $\mu$g/day LPS (D), or 10 $\mu$g/day LPS + 100 $\mu$g/day MDP (E). Red areas indicate larger areas of bone destruction. (b) Ratio of bone destruction area to total area. Results are expressed as the mean ± SD ($n = 4$; $^{**}P < 0.01$; $^{*}P < 0.05$). Differences were determined using Scheffe's $F$ test. (c) Levels of TRACP 5b in mouse serum *in vivo*. Serum was obtained from mice after five days of daily administration into the calvariae. Circulating levels of tartrate-resistant acid phosphatase (TRACP 5b) were determined by enzyme-linked immunosorbent assay (ELISA). Results are expressed as the mean ± SD ($n = 4$; $^{**}P < 0.01$). (d) Levels of C-terminal telopeptide fragments of type I collagen in mouse serum *in vivo*. Circulating levels of C-terminal telopeptide fragments of type I collagen were determined by Mouse CTX Assay kit. Results are expressed as the mean ± SD ($n = 4$; $^{**}P < 0.01$; $^{*}P < 0.05$). Differences were determined using Scheffe's $F$ test.

sample buffer (Life Technologies) for 5 min and subjected to SDS polyacrylamide gel electrophoresis using 4–15% Mini-PROTEAN TGX gels (Bio-Rad, Hercules, CA). Proteins were transferred to nitrocellulose membranes using Trans-Blot Turbo (Bio-Rad) and incubated in blocking solution (5% bovine serum albumin in Tris-buffered saline containing 0.05% Tween-20) for 1 h to reduce nonspecific binding. Membranes were then exposed to primary antibodies for 1 h at 4°C, washed four times, and then incubated with anti-rabbit IgG HRP-conjugated secondary antibody for 30 min. Membranes were again washed extensively and then incubated with enhanced chemiluminescence detection using Supersignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Wilmington, DE).

*2.7. Statistical Analysis.* All data are expressed as the mean ± SD. Statistical analyses were performed using Scheffe's $F$ test.

## 3. Results

*3.1. MDP Enhances LPS-Induced Osteoclastogenesis in Mouse Calvariae.* LPS was administered with or without MDP into

the supracalvariae of mice to analyze the effect of MDP on LPS-induced osteoclastogenesis *in vivo*. In the high LPS (100 $\mu$g/day) group and the LPS + MDP group, numerous osteoclasts were observed. In comparison, significantly fewer osteoclasts were observed in the low LPS (10 $\mu$g/day), MDP alone, or PBS groups (Figures 1(a), 1(b), 1(c), and 1(d)).

Real-time RT-PCR was undertaken to analyze cathepsin K and TRAP mRNA levels—two markers of osteoclasts. We found that both cathepsin K and TRAP mRNA were significantly higher in the LPS + MDP group and the high LPS group as compared with the low LPS group (Figure 1(e)).

*3.2. Concentration-Dependent Increase in Osteoclastogenesis.* To further analyze the effect of MDP on LPS-induced osteoclast formation *in vivo*, LPS (10 $\mu$g/day) was injected into mouse calvariae with increasing concentrations of MDP (0, 1, 10, and 100 $\mu$g). We found that higher MDP concentrations led to an increase in osteoclast number in a dose-dependent manner (Figure 2).

*3.3. MDP Enhances LPS-Induced Bone Destruction in Supracalvariae.* We next used microfocus computed tomography to assess the degree of bone destruction observed in the calvariae of mice administered with LPS (Figure 3(a)). We found significantly more bone destruction in the high LPS group as compared with the PBS group. In addition, bone destruction in the LPS + MDP group was higher than that in the low LPS group (Figure 3(b)). This increased bone destruction was corroborated by the TRACP 5b serum analysis, where we found that TRACP 5b was increased in the high LPS group as compared with that in the PBS, low LPS, and MDP only groups. Moreover, TRACP 5b serum levels were higher in the LPS + MDP group than in the PBS, low LPS, and MDP only groups (Figure 3(c)). C-terminal telopeptide fragments of type I collagen serum levels were also higher in the LPS + MDP group than in the PBS, low LPS, and MDP only groups (Figure 3(d)).

*3.4. MDP Enhances LPS-Induced RANKL Expression In Vivo.* RANKL was related to the osteoclast formation. We found that RANKL mRNA was elevated in the high LPS and LPS + MDP groups as compared with PBS, low LPS, and MDP alone groups. MDP was thus able to enhance LPS-induced RANKL expression *in vivo* (Figure 4).

*3.5. MDP Enhances LPS-Induced RANKL Expression in Stromal Cells.* Bone marrow stromal cells were cultured for 3 days in the presence of LPS with or without MDP to ascertain the effect of these two additives on RANKL expression in stromal cell cultures *in vitro*. We found elevated RANKL mRNA expression in the high LPS group as compared with the PBS, low LPS, and MDP alone groups. Similarly, RANKL mRNA was significantly higher in the LPS + MDP group as compared with the PBS and low LPS groups (Figure 5).

*3.6. Effect of MDP on Parathyroid Hormone- (PTH-) Induced Osteoclastogenesis in Mouse Calvariae.* PTH stimulates RANKL expression by osteoblasts and thus indirectly stimulates osteoclastogenesis. We therefore sought to ascertain if

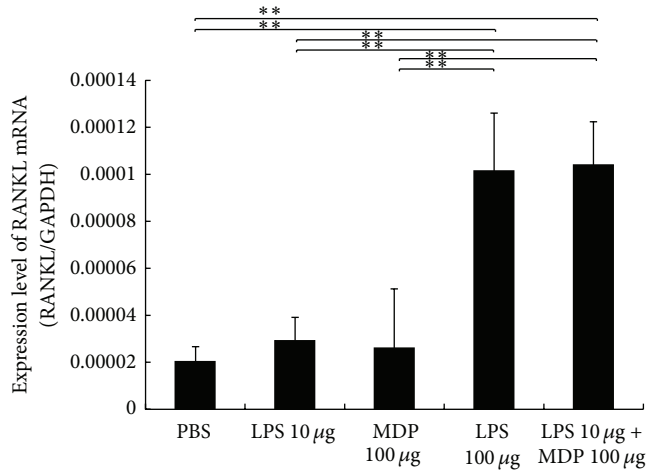



Figure 4: Enhancing lipopolysaccharide- (LPS-) induced receptor activator of NF-$\kappa$B ligand (RANKL) expression by muramyl dipeptide (MDP) *in vivo*. Total RNA from mouse calvariae was isolated after 5 days of daily supracalvarial injections of PBS, LPS (10 $\mu$g/day), MDP (100 $\mu$g/day), LPS (100 $\mu$g/day), or LPS (10 $\mu$g/day) + MDP (100 $\mu$g/day). mRNA levels for RANKL were normalized to those of GAPDH. Results are expressed as mean ± SD ($n = 4$; $^{**}P < 0.01$). Differences were determined using Scheffe's $F$ test.

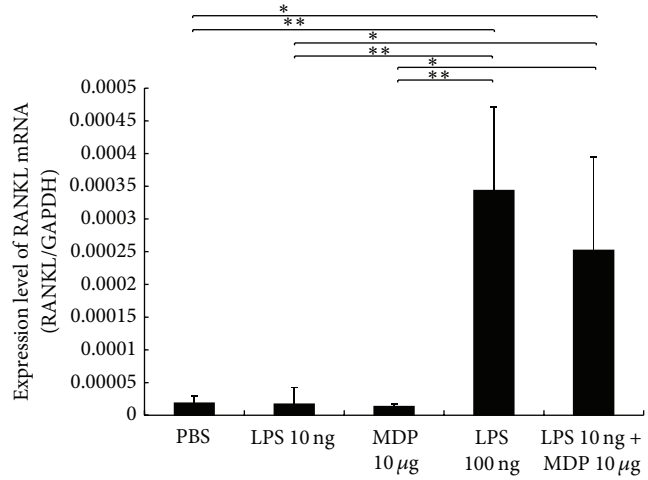
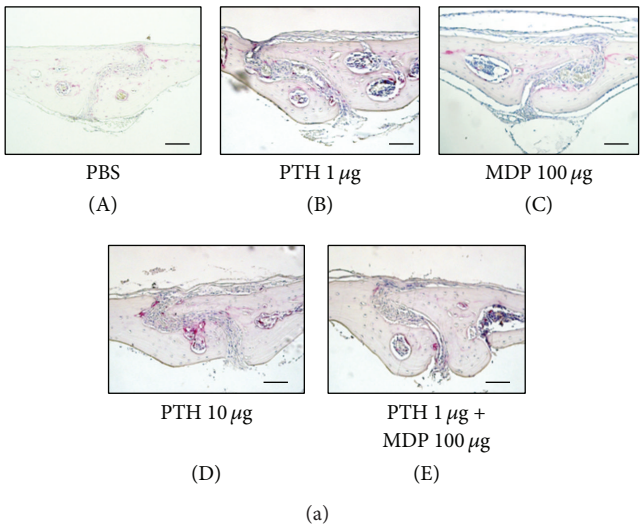
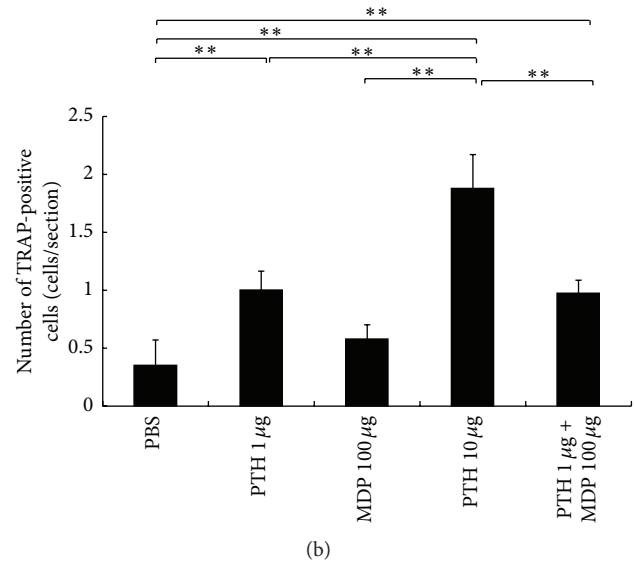



Figure 5: Enhancing lipopolysaccharide- (LPS-) induced receptor activator of NF-$\kappa$B ligand (RANKL) expression by muramyl dipeptide (MDP) in stromal cells *in vitro*. Total RNA from bone marrow stromal cells was isolated after 4 days of incubation in culture medium supplemented with 10 ng/mL LPS, 100 ng/mL LPS, or 10 ng/mL LPS and 10 $\mu$g/mL MDP. mRNA levels for RANKL were normalized to those of GAPDH. Results are expressed as mean ± SD ($n = 4$; $^{**}P < 0.01$; $^{*}P < 0.05$). Differences were determined using Scheffe's $F$ test.

PTH could similarly be enhanced by MDP. PTH (100 $\mu$g/day) was administered with or without MDP into mouse supracalvaria to analyze the effect of MDP on PTH-induced osteoclastogenesis *in vivo*. We observed numerous osteoclasts with the higher concentration of PTH (10 $\mu$g/day), which was significantly diminished in mice treated with low-dose




(a)

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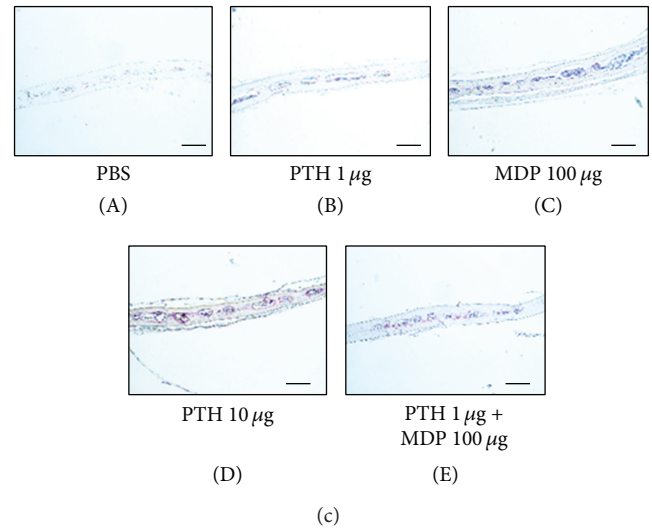


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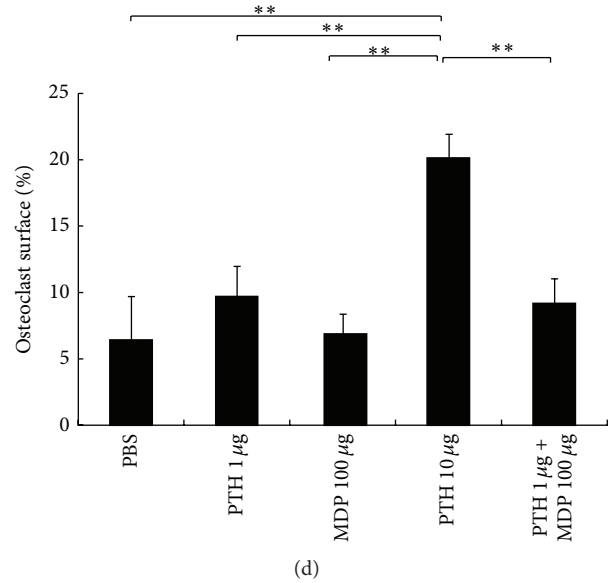
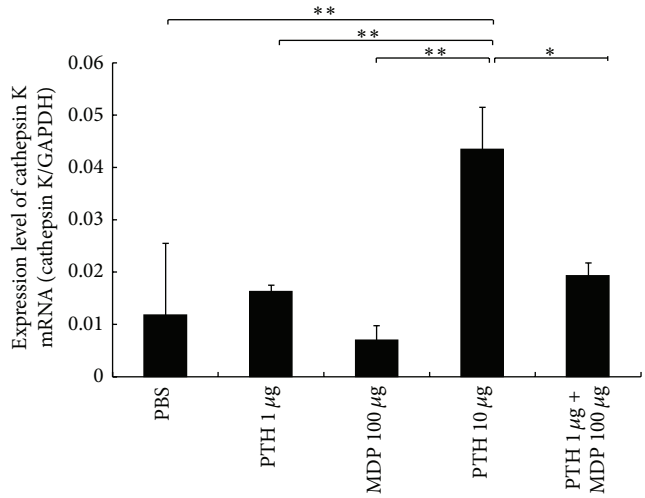
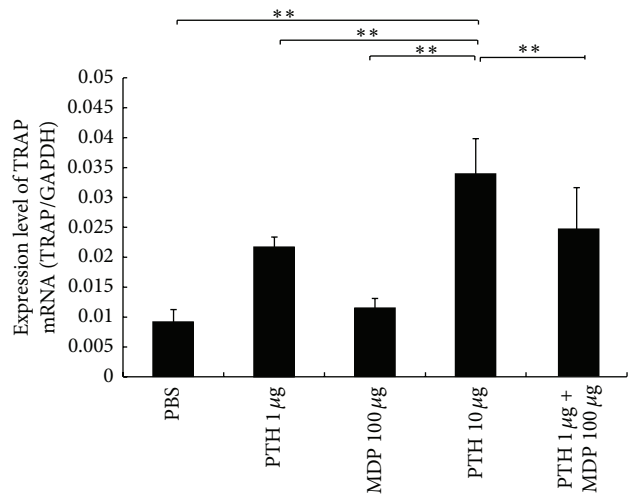


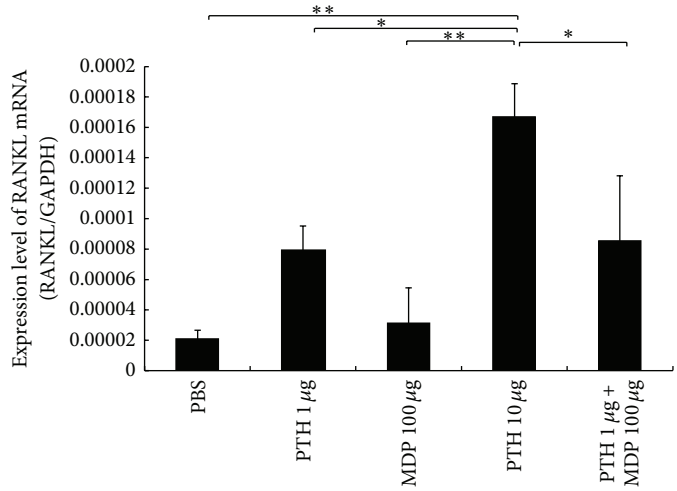

(d)




(e)

Figure 6: Continued.




(f)

Figure 6: Effects of muramyl dipeptide (MDP) on parathyroid hormone- (PTH-) induced osteoclast formation *in vivo*. (a) Histological sections of sutures of calvariae were obtained from mice after 5 days of daily supracalvarial administration with PBS (A), 1 $\mu$g/day PTH (B), 100 $\mu$g/day MDP (C), 10 $\mu$g/day PTH (D), or 1 $\mu$g/day PTH and 100 $\mu$g/day MDP (E). Sections were stained with tartrate-resistant acid phosphatase (TRAP) staining and counterstained with hematoxylin. Cells that stained red are considered to be TRAP-positive. Scale bars = 50 $\mu$m. (b) Number of TRAP-positive cells with three or more nuclei in the calvariae ($n = 4$; $^{**}P < 0.01$). (c) Histological sections of calvariae were obtained from mice after 5 days of daily supracalvarial administration with PBS (A), 1 $\mu$g/day PTH (B), 100 $\mu$g/day MDP (C), 10 $\mu$g/day PTH (D), or 1 $\mu$g/day PTH and 100 $\mu$g/day MDP (E). Sections were stained with tartrate-resistant acid phosphatase (TRAP) staining and counterstained with hematoxylin. Cells that stained red are considered to be TRAP-positive. Scale bars = 100 $\mu$m. (d) The percentage of bone/marrow interface covered by osteoclasts was histomorphometrically determined in specimens ($n = 4$; $^{**}P < 0.01$). (e) TRAP and cathepsin K mRNA levels in mouse calvariae were detected using real-time RT-PCR. Total RNA from mouse calvariae was isolated after 5 days of daily supracalvarial injections, as in (a). RNA levels for TRAP and cathepsin K were normalized to those of GAPDH. Results are expressed as the mean ± SD ($n = 4$; $^{**}P < 0.01$; $^{*}P < 0.05$). (f) Expression levels of RANKL mRNA in mouse calvariae *in vivo*. Total RNA from mouse calvariae was isolated after 5 days of daily supracalvarial injections, as in (a). mRNA levels for RANKL were normalized to those of GAPDH. Results are expressed as the mean ± SD ($n = 4$; $^{**}P < 0.01$; $^{*}P < 0.05$). Differences were determined using Scheffe's $F$ test.

PTH (1 $\mu$g/day), PTH (1 $\mu$g/day) + MDP, MDP alone, or PBS (Figures 6(a), 6(b), 6(c), and 6(d)). Cathepsin K and TRAP mRNA levels were significantly increased in the high PTH group as compared with the PTH + MDP, PBS, low PTH, and MDP alone groups (Figure 6(e)).

*3.7. Effect of MDP on RANKL Expression in PTH-Administered Mice.* Mice calvariae were injected daily for 5 days with PTH (1 $\mu$g) + MDP (100 $\mu$g) in a 100 $\mu$L volume of PBS or separately with high PTH (10 $\mu$g), low PTH (1 $\mu$g), MDP (100 $\mu$g), or PBS alone to ascertain the effect of these compounds on RANKL. We found that RANKL mRNA was higher in the high PTH group than in the PTH + MDP, PBS, low PTH, or MDP alone groups (Figure 6(f)).

*3.8. MDP Enhanced LPS-Induced TLR4 Expression In Vivo.* We next determined the effect of MDP on LPS- and PTH-induced TLR4 expression, a receptor for LPS. We found that TLR4 mRNA expression levels were higher in the high LPS and LPS + MDP groups than in the PBS, low LPS, and MDP alone groups. On the other hand, PTH did not induce TLR4 mRNA and MDP did not enhance TLR4 mRNA in the presence of PTH (Figure 7).

*3.9. MDP Enhanced LPS-Induced TLR4 Expression in Stromal Cells.* Bone marrow stromal cells were cultured for 3 days in LPS or PTH with or without MDP. In these cultures, we show that TLR4 mRNA with high LPS (100 ng/mL) was higher than that in the PBS, low LPS (10 ng/mL), or MDP alone groups. In addition, TLR4 mRNA expression in the LPS (10 ng/mL) + MDP group was significantly higher than that in the PBS and low LPS (10 ng/mL) groups. As seen in the *in vivo* analysis, PTH was also unable to induce TLR4 mRNA in stromal cells and this could not be recovered with the coadministration of MDP (Figure 8).

*3.10. MDP Enhanced LPS-Induced MAPK Signaling Pathway in Stromal Cells.* Finally, we sought to explore the molecular mechanisms through which MDP enhances LPS-activated signaling. We showed that LPS activated ERK, P38, and JNK in mouse bone marrow stromal cells after 15 min incubation. MDP alone was unable to induce phosphorylation of any of the kinases; however, MDP enhanced LPS-induced phosphorylation of all three kinases after just 15 min of incubation (Figure 9).

## 4. Discussion

In this study, we evaluated the effect of MDP in LPS-induced osteoclast formation and bone resorption *in vivo*. To our knowledge, this is the first time that this analysis has been reported. We found that MDP enhances LPS-induced

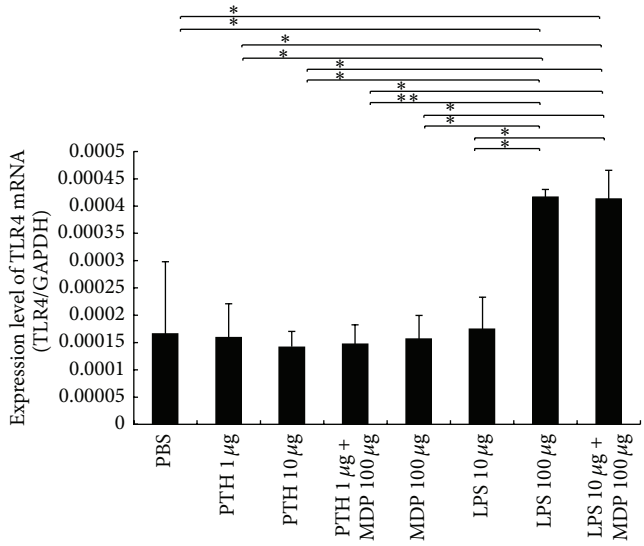



Figure 7: Enhancing lipopolysaccharide- (LPS-) induced Toll-like receptor (TLR4) expression using muramyl dipeptide (MDP) *in vivo*. Total RNA from mouse calvariae was isolated after 5 days of daily supracalvarial injections of PBS, PTH (1 $\mu$g/day), PTH (10 $\mu$g/day), PTH (1 $\mu$g/day) + MDP (100 $\mu$g/day), MDP (100 $\mu$g/day), LPS (10 $\mu$g/day), LPS (100 $\mu$g/day), or LPS (10 $\mu$g/day) + MDP (100 $\mu$g/day). mRNA levels for TLR4 were normalized to those of GAPDH. Results are expressed as the mean ± SD ($n = 4$; $^{**}P < 0.01$; $^{*}P < 0.05$). Differences were determined using Scheffe's $F$ test.

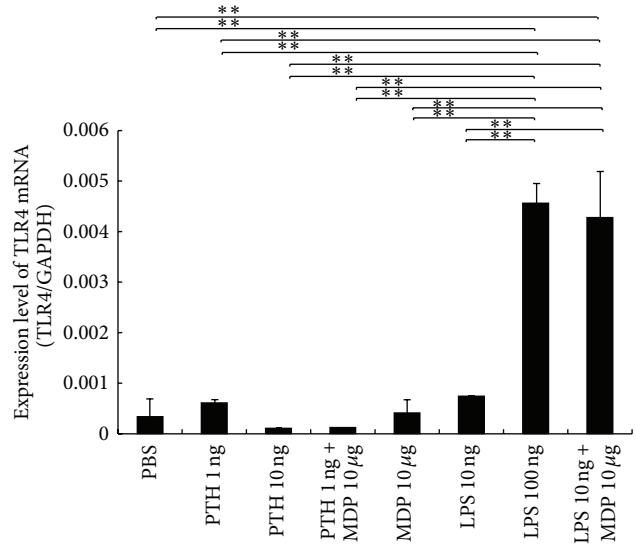



Figure 8: Enhancing lipopolysaccharide- (LPS-) induced Toll-like receptor (TLR4) expression using muramyl dipeptide (MDP) *in vitro*. Total RNA from bone marrow stromal cells was isolated after 4 days of incubation in culture medium supplemented with 1 ng/mL PTH, 10 ng/mL PTH, 1 ng/mL PTH and 10 $\mu$g/mL MDP, 10 $\mu$g/mL MDP, 100 ng/mL LPS, or 10 ng/mL LPS + 10 $\mu$g/mL MDP. mRNA levels for TLR4 were normalized to those of GAPDH. Results are expressed as the mean ± SD ($n = 4$; $^{**}P < 0.01$). Differences were determined using Scheffe's $F$ test.

osteoclast formation and bone resorption and also enhances LPS-induced RANKL and TLR4 expression *in vivo* and in stromal cell *in vitro*. Furthermore, MDP enhanced LPS-induced phosphorylation of ERK, p38, and JNK kinases in stromal cells, although MDP alone could not induce their activity.

It has been reported that LPS can induce osteoclast formation and bone resorption in certain clinical conditions, such as periodontal diseases [2, 25]. We have previously shown that osteoclasts can be induced in calvariae [26] and in periodontal membrane tissues [27] in the presence of LPS. Yang et al. [24] showed that MDP enhances LPS-induced osteoclast formation when cocultured with osteoblasts *in vitro*. In the present study, we evaluated whether MDP could enhance LPS-induced osteoclast formation and bone resorption *in vivo*. First, we analyzed the amount of LPS required for osteoclast formation. We found that a daily injection of 100 $\mu$g/day for 5 days was sufficient to induce osteoclasts *in vivo*, but not with injections of 10 $\mu$g/day for 5 days. Next, to analyze the effect of MDP on LPS-induced osteoclastogenesis *in vivo*, the lower concentration of LPS was administered with or without MDP into mouse supracalvaria. We found increased numbers of osteoclasts and an elevated expression of osteoclast markers (cathepsin K and TRAP) with high LPS (100 $\mu$g/day) and with low LPS (10 $\mu$g/day) plus MDP but not with low LPS (10 $\mu$g/day) or MDP alone or with the vehicle, PBS. These results suggest that MDP can enhance LPS-induced osteoclast formation *in vivo*.

Furthermore, we evaluated whether MDP could enhance LPS-induced bone resorption. Bone destruction was observed using microfocus computed tomography images. Serum TRACP 5b levels with LPS (10 $\mu$g) plus MDP were higher than that in the LPS only group. These results suggest that MDP enhances LPS-induced bone resorption. Kishimoto et al. [28] investigated the effect of PGN on LPS-induced osteoclast formation and bone resorption and found that PGN significantly induced osteoclast formation and bone resorption in mice coinjected with LPS. MDP is the minimal essential structural unit responsible for the immunological activity of PGN [29]. Thus, it is likely that MDP might be the key component in LPS-induced osteoclast formation and bone resorption as mediated by PGN.

LPS has also been reported to stimulate osteoblast production/secretion of RANKL [30]. In the present study, we, too, found elevated RANKL mRNA levels in the high-dose LPS group as compared with the control groups both *in vivo* and *in vitro*, indicating that LPS induced RANKL expression in stromal cells. Yang et al. also examined osteoblasts cultured in the presence of LPS with or without MDP. They showed that MDP stimulated the LPS-induced expression of RANKL mRNA [24]. Our results with stromal cells support these previous findings. However, we showed that MDP alone could not induce RANKL expression either *in vitro* or *in vivo*, suggesting that MDP enhances the effect of LPS.

PTH stimulates RANKL expression by osteoblasts and thus promotes osteoclastogenesis [31–35]. We also evaluated whether MDP could enhance PTH-induced osteoclast

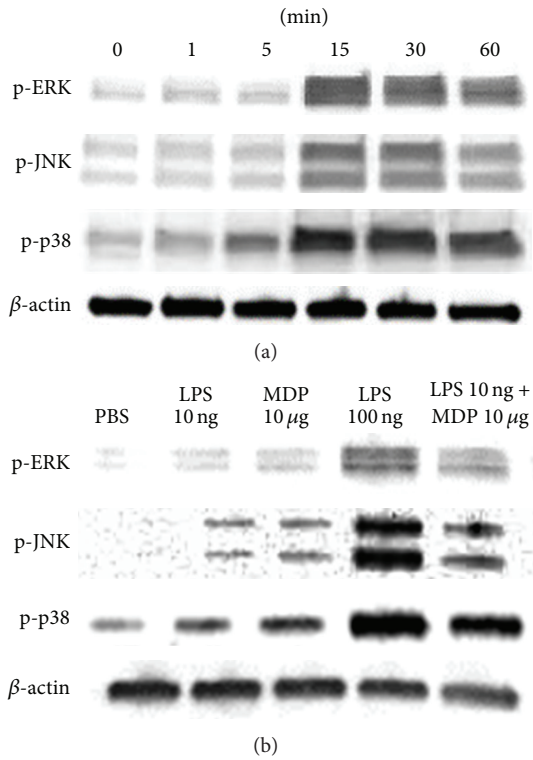



Figure 9: Effect of muramyl dipeptide (MDP) on lipopolysaccharide- (LPS-) induced mitogen-activated protein kinase (MAPK) signaling in mouse stromal cell *in vitro*. (a) Stromal cells were stimulated using LPS (100 ng/mL) as indicated. Cells were then lysed and analyzed by western blotting. (b) Stromal cells were stimulated using PBS, LPS (10 ng/mL), MDP (10 $\mu$g/mL), LPS (100 ng/mL), or LPS (10 ng/mL) + MDP (10 $\mu$g/mL) for 15 min. Cells were then lysed and analyzed by western blotting.

formation and bone resorption. In the present study, PTH induced osteoclast formation and bone resorption in mouse calvariae. However, MDP could not enhance PTH-induced osteoclast formation and bone resorption. The results suggested that although MDP affects LPS-induced signaling it cannot affect PTH-induced signaling.

TLR4 induces the natural host defense system by rapidly triggering proinflammatory processes [36–38]. LPS is recognized by TLR4 on the cell surface [39, 40]. In this study, we found that LPS enhances TLR4 expression in mouse calvariae and in stromal cell culture. PTH, however, could not enhance TLR4 expression. We hypothesize that this phenomenon might increase the sensitivity of LPS in cells. Furthermore, we found that MDP could enhance LPS-induced TLR4 expression *in vivo* and in stromal cells. These results provide further support for the premise that MDP enhances LPS signaling, and its signaling through TLR4 may be how MDP enhances the effects of LPS.

Cyclooxygenase- (COX-) 2 and PGE2 are reportedly increased in dental pulp fibroblasts by costimulation with NOD1 or NOD2 ligands and TLR2 or TLR4 ligands. Furthermore, the production of IL-1$\beta$, IL-6, and IL-8 in these fibroblasts is accelerated by costimulation with these ligand combinations through the increased expression of TRAF6 [41]. It has been reported that MDP synergistically enhances osteoclast induction by LPS, IL-1$\alpha$, and TNF-$\alpha$ through increased RANKL expression in osteoblasts [24]. Bandow et al. [42] and Nakao et al. [43] have also shown that LPS activates the phosphorylation of ERK, P38, and JNK in osteoblasts. We corroborated these results, showing that LPS activates all three kinases in mouse bone marrow stromal cells. Yang et al. [24] also showed that LPS stimulated ERK1/2 phosphorylation in osteoblasts and that this could be enhanced by MDP. However, they did not check the effect of LPS on other MAPKs, such as p38 and JNK. We found that MDP enhanced the phosphorylation of ERK, p38, and JNK that was induced by LPS in stromal cells. Yet, MDP alone was unable to activate MAPKs. Although these results provide some insight into the signaling pathways activated by LPS, the exact mechanism by which MDP enhances LPS signaling is unclear, and further studies are needed to clarify this point.

## 5. Conclusions

We found that MDP enhances LPS-induced osteoclast formation, as measured by increased RANKL and TLR4 expression *in vivo* and *in vitro*. Our findings suggest that MDP might play an important role in pathological bone resorption in diseases with associated bacterial infections, such as periodontitis.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Acknowledgments

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## References

[1] P. Orcel, M. Feuga, J. Bielakoff, and M. C. de Vernejoul, "Local bone injections of LPS and M-CSF increase bone resorption by different pathways *in vivo* in rats," *The American Journal of Physiology—Endocrinology and Metabolism*, vol. 264, no. 3, part 1, pp. E391–E397, 1993.

[2] Y. Abu-Amer, F. P. Ross, J. Edwards, and S. L. Teitelbaum, "Lipopolysaccharide-stimulated osteoclastogenesis is mediated by tumor necrosis factor via its P55 receptor," *The Journal of Clinical Investigation*, vol. 100, no. 6, pp. 1557–1565, 1997.

[3] Y. Sakuma, K. Tanaka, M. Suda et al., "Crucial involvement of the EP4 subtype of prostaglandin E receptor in osteoclast formation by proinflammatory cytokines and lipopolysaccharide," *Journal of Bone and Mineral Research*, vol. 15, no. 2, pp. 218–227, 2000.

[4] A. L. Dumitrescu, S. A. El-Aleem, B. Morales-Aza, and L. F. Donaldson, "A model of periodontitis in the rat: effect of lipopolysaccharide on bone resorption, osteoclast activity, and local peptidergic innervation," *Journal of Clinical Periodontology*, vol. 31, no. 8, pp. 596–603, 2004.

[5] Y.-H. Chung, E.-J. Chang, S.-J. Kim et al., "Lipopolysaccharide from *Prevotella nigrescens* stimulates osteoclastogenesis in cocultures of bone marrow mononuclear cells and primary osteoblasts," *Journal of Periodontal Research*, vol. 41, no. 4, pp. 288–296, 2006.

[6] N. Bostanci, R. P. Allaker, G. N. Belibasakis et al., "*Porphyromonas gingivalis* antagonises *Campylobacter rectus* induced cytokine production by human monocytes," *Cytokine*, vol. 39, no. 2, pp. 147–156, 2007.

[7] K. Redlich, S. Hayer, R. Ricci et al., "Osteoclasts are essential for TNF-$\alpha$-mediated joint destruction," *The Journal of Clinical Investigation*, vol. 110, no. 10, pp. 1419–1427, 2002.

[8] K. D. Merkel, J. M. Erdmann, K. P. McHugh, Y. Abu-Amer, F. P. Ross, and S. L. Teitelbaum, "Tumor necrosis factor-$\alpha$ mediates orthopedic implant osteolysis," *The American Journal of Pathology*, vol. 154, no. 1, pp. 203–210, 1999.

[9] R. B. Kimble, S. Srivastava, F. P. Ross, A. Matayoshi, and R. Pacifici, "Estrogen deficiency increases the ability of stromal cells to support murine osteoclastogenesis via an interleukin-1- and tumor necrosis factor-mediated stimulation of macrophage colony-stimulating factor production," *The Journal of Biological Chemistry*, vol. 271, no. 46, pp. 28890–28897, 1996.

[10] S. L. Teitelbaum, "Bone resorption by osteoclasts," *Science*, vol. 289, no. 5484, pp. 1504–1508, 2000.

[11] S. L. Teitelbaum, "Osteoclasts: what do they do and how do they do it?" *The American Journal of Pathology*, vol. 170, no. 2, pp. 427–435, 2007.

[12] Y. Azuma, K. Kaji, R. Katogi, S. Takeshita, and A. Kudo, "Tumor necrosis factor-$\alpha$ induces differentiation of and bone resorption by osteoclasts," *Journal of Biological Chemistry*, vol. 275, no. 7, pp. 4858–4864, 2000.

[13] K. Kobayashi, N. Takahashi, E. Jimi et al., "Tumor necrosis factor $\alpha$ stimulates osteoclast differentiation by a mechanism independent of the ODF/RANKL-RANK interaction," *The Journal of Experimental Medicine*, vol. 191, no. 2, pp. 275–285, 2000.

[14] K. Fuller, C. Murphy, B. Kirstein, S. W. Fox, and T. J. Chambers, "TNFalpha potently activates osteoclasts, through a direct action independent of and strongly synergistic with RANKL," *Endocrinology*, vol. 143, no. 3, pp. 1108–1118, 2002.

[15] H. Kitaura, M. S. Sands, K. Aya et al., "Marrow stromal cells and osteoclast precursors differentially contribute to TNF-$\alpha$-induced osteoclastogenesis *in vivo*," *The Journal of Immunology*, vol. 173, no. 8, pp. 4838–4846, 2004.

[16] H. Kitaura, P. Zhou, H.-J. Kim, D. V. Novack, F. P. Ross, and S. L. Teitelbaum, "M-CSF mediates TNF-induced inflammatory osteolysis," *Journal of Clinical Investigation*, vol. 115, no. 12, pp. 3418–3427, 2005.

[17] C.-Y. Chiang, G. Kyritsis, D. T. Graves, and S. Amar, "Interleukin-1 and tumor necrosis factor activities partially account for calvarial bone resorption induced by local injection of lipopolysaccharide," *Infection and Immunity*, vol. 67, no. 8, pp. 4231–4236, 1999.

[18] W. Zou and Z. Bar-Shavit, "Dual modulation of osteoclast differentiation by lipopolysaccharide," *Journal of Bone and Mineral Research*, vol. 17, no. 7, pp. 1211–1218, 2002.

[19] G. P. Garlet, C. R. Cardoso, T. A. Silva et al., "Cytokine pattern determines the progression of experimental periodontal disease induced by *Actinobacillus actinomycetemcomitans* through the modulation of MMPs, RANKL, and their physiological inhibitors," *Oral Microbiology and Immunology*, vol. 21, no. 1, pp. 12–20, 2006.

[20] M. Mörmann, M. Thederan, I. Nackchbandi, T. Giese, C. Wagner, and G. M. Hänsch, "Lipopolysaccharides (LPS) induce the differentiation of human monocytes to osteoclasts in a tumour necrosis factor (TNF) $\alpha$-dependent manner: a link between infection and pathological bone resorption," *Molecular Immunology*, vol. 45, no. 12, pp. 3330–3337, 2008.

[21] H. Takada, S. Yokoyama, and S. Yang, "Enhancement of endotoxin activity by muramyldipeptide," *Journal of Endotoxin Research*, vol. 8, no. 5, pp. 337–342, 2002.

[22] H. Takada and C. Galanos, "Enhancement of endotoxin lethality and generation of anaphylactoid reactions by lipopolysaccharides in muramyl-dipeptide-treated mice," *Infection and Immunity*, vol. 55, no. 2, pp. 409–413, 1987.

[23] S. Yang, R. Tamai, S. Akashi et al., "Synergistic effect of muramyldipeptide with lipopolysaccharide or lipoteichoic acid to induce inflammatory cytokines in human monocytic cells in culture," *Infection and Immunity*, vol. 69, no. 4, pp. 2045–2053, 2001.

[24] S. Yang, N. Takahashi, T. Yamashita et al., "Muramyl dipeptide enhances osteoclast formation induced by lipopolysaccharide, IL-1$\alpha$, and TNF-$\alpha$ through nucleotide-binding oligomerization domain 2-mediated signaling in osteoblasts," *Journal of Immunology*, vol. 175, no. 3, pp. 1956–1964, 2005.

[25] J. Slots and R. J. Genco, "Black-pigmented *Bacteroides* species, *Capnocytophaga* species, and *Actinobacillus actinomycetemcomitans* in human periodontal disease: virulence factors in colonization, survival, and tissue destruction," *Journal of Dental Research*, vol. 63, no. 3, pp. 412–421, 1984.

[26] K. Kimura, H. Kitaura, T. Fujii, Z. W. Hakami, and T. Takano-Yamamoto, "Anti-c-Fms antibody inhibits lipopolysaccharide-induced osteoclastogenesis *in vivo*," *FEMS Immunology and Medical Microbiology*, vol. 64, no. 2, pp. 219–227, 2012.

[27] K. Kimura, H. Kitaura, T. Fujii, M. Ishida, Z. W. Hakami, and T. Takano-Yamamoto, "An anti-c-Fms antibody inhibits osteoclastogenesis in a mouse periodontitis model," *Oral Diseases*, vol. 20, no. 3, pp. 319–324, 2014.

[28] T. Kishimoto, T. Kaneko, T. Ukai et al., "Peptidoglycan and lipopolysaccharide synergistically enhance bone resorption and osteoclastogenesis," *Journal of Periodontal Research*, vol. 47, no. 4, pp. 446–454, 2012.

[29] J. J. Oppenheim, A. Togawa, L. Chedid, and S. Mizel, "Components of mycobacteria and muramyl dipeptide with adjuvant activity induce lymphocyte activating factor," *Cellular Immunology*, vol. 50, no. 1, pp. 71–81, 1980.

[30] T. Kikuchi, T. Matsuguchi, N. Tsuboi et al., "Gene expression of osteoclast differentiation factor is induced by lipopolysaccharide in mouse osteoblasts via Toll-like receptors," *The Journal of Immunology*, vol. 166, no. 5, pp. 3574–3579, 2001.

[31] N. J. Horwood, J. Elliott, T. J. Martin, and M. T. Gillespie, "Osteotropic agents regulate the expression of osteoclast differentiation factor and osteoprotegerin in osteoblastic stromal cells," *Endocrinology*, vol. 139, no. 11, pp. 4743–4746, 1998.

[32] K. Itoh, N. Udagawa, K. Matsuzaki et al., "Importance of membrane- or matrix-associated forms of M-CSF and RANKL/ODF in osteoclastogenesis supported by SaOS-4/3 cells expressing recombinant PTH/PTHrP receptors," *Journal of Bone and Mineral Research*, vol. 15, no. 9, pp. 1766–1775, 2000.

[33] M. Kanzawa, T. Sugimoto, T. Kobayashi, A. Kobayashi, and K. Chihara, "Association between parathyroid hormone (PTH)/PTH-related peptide receptor gene polymorphism and the extent of bone mass reduction in primary hyperparathyroidism," *Hormone and Metabolic Research*, vol. 32, no. 9, pp. 355–358, 2000.

[34] S.-K. Lee and J. A. Lorenzo, "Parathyroid hormone stimulates TRANCE and inhibits osteoprotegerin messenger ribonucleic acid expression in murine bone marrow cultures: correlation with osteoclast-like cell formation," *Endocrinology*, vol. 140, no. 8, pp. 3552–3561, 1999.

[35] K. Tsukii, N. Shima, S.-I. Mochizuki et al., "Osteoclast differentiation factor mediates an essential signal for bone resorption induced by 1$\alpha$,25-dihydroxyvitamin $D_3$, prostaglandin $E_2$, or parathyroid hormone in the microenvironment of bone," *Biochemical and Biophysical Research Communications*, vol. 246, no. 2, pp. 337–341, 1998.

[36] B. Beutler, "TLR4 as the mammalian endotoxin sensor," *Current Topics in Microbiology and Immunology*, vol. 270, pp. 109–120, 2002.

[37] B. Beutler, Z. Jiang, P. Georgel et al., "Genetic analysis of host resistance: toll-like receptor signaling and immunity at large," *Annual Review of Immunology*, vol. 24, pp. 353–389, 2006.

[38] E. M. Pålsson-McDermott and L. A. J. O'Neill, "Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4," *Immunology*, vol. 113, no. 2, pp. 153–162, 2004.

[39] J. C. Chow, D. W. Young, D. T. Golenbock, W. J. Christ, and F. Gusovsky, "Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction," *The Journal of Biological Chemistry*, vol. 274, no. 16, pp. 10689–10692, 1999.

[40] G. L. Su, R. D. Klein, A. Aminlari et al., "Kupffer cell activation by lipopolysaccharide in rats: role for lipopolysaccharide binding protein and toll-like receptor 4," *Hepatology*, vol. 31, no. 4, pp. 932–936, 2000.

[41] L. Tang, X.-D. Zhou, Q. Wang et al., "Expression of TRAF6 and pro-inflammatory cytokines through activation of TLR2, TLR4, NOD1, and NOD2 in human periodontal ligament fibroblasts," *Archives of Oral Biology*, vol. 56, no. 10, pp. 1064–1072, 2011.

[42] K. Bandow, A. Maeda, K. Kakimoto et al., "Molecular mechanisms of the inhibitory effect of lipopolysaccharide (LPS) on osteoblast differentiation," *Biochemical and Biophysical Research Communications*, vol. 402, no. 4, pp. 755–761, 2010.

[43] J. Nakao, Y. Fujii, J. Kusuyama et al., "Low-intensity pulsed ultrasound (LIPUS) inhibits LPS-induced inflammatory responses of osteoblasts through TLR4-MyD88 dissociation," *Bone*, vol. 58, pp. 17–25, 2014.

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*Review Article*

# Bone-Immune Cell Crosstalk: Bone Diseases

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Bone diseases are associated with great morbidity; thus, the understanding of the mechanisms leading to their development represents a great challenge to improve bone health. Recent reports suggest that a large number of molecules produced by immune cells affect bone cell activity. However, the mechanisms are incompletely understood. This review aims to shed new lights into the mechanisms of bone diseases involving immune cells. In particular, we focused our attention on the major pathogenic mechanism underlying periodontal disease, psoriatic arthritis, postmenopausal osteoporosis, glucocorticoid-induced osteoporosis, metastatic solid tumors, and multiple myeloma.

## 1. Introduction

Bone is an active tissue that undergoes continuous remodelling by two distinct processes, bone formation and bone resorption [1]. These events are strongly linked and tightly regulated to maintain skeletal homeostasis [2]. The bone cells responsible for the dual processes include the bone resorbing cells, that is, the osteoclasts (OCs), which are differentiated cells derived from hematopoietic cells of the monocyte-macrophage lineage and bone forming cells, that is, the osteoblasts (OBs), which are of mesenchymal origin. Alteration of the differentiation/activity of OCs as well as OBs leads to bone diseases. The close relationship between the bone and the immune system has been increasingly recognized, in particular during pathological conditions in which activation of both systems occurs [3]. It is known that inflammation increase leads to an augment in the immune function, which culminates in an increased production of tumour necrosis factor (TNF) or receptor activator of NF-kB ligand (RANKL) by activated T cells, that has been linked to bone loss associated diseases (inflammatory and autoimmune disease, postmenopausal osteoporosis). Different studies have been performed to identify the T cell subset involved in osteoclastogenesis. In general, T cells could be classified as effector-cytotoxic T population (CD8+ cells) and helper T cells (CD4+ cells). CD4+ T cells, upon activation and expansion, develop into diverse T helper (Th) cell subsets secreting signature cytokine profiles and mediating distinct effector functions [4]. Until recently, T cells were divided into Th1 or Th2 cells, depending on the cytokines they produced (with Th1 producing IFN-gamma and IL-2 and Th2 producing primarily IL-4/IL-5/IL-10). Regulatory T cells (Tregs, CD4+CD25+Foxp3+) potently inhibit the function of effector T cells [4]. A third subset of IL-17-producing effector T helper cells, called Th17 cells, has been more recently discovered and characterized. Th17 cells produce IL-17, IL-17F, and IL-22, thereby inducing a massive tissue reaction owing to the broad distribution of the IL-17 and IL-22 receptors. Th17 cells support OC formation mostly through the expression of IL-17, which is recognized to induce RANK expression on OC precursors as well as RANKL production by cells supporting OC formation [4, 5]. IL-17 also makes possible local inflammation through the recruitment and the activation of immune cells, leading to the release of proinflammatory molecules, as IL-1 and TNF$\alpha$ [4]. These proinflammatory molecules increase RANKL expression and synergize with

RANKL signalling to maximize OC formation. A relatively high expression of RANKL on Th17 cells may also participate in the enhanced osteoclastogenesis. Collectively, Th17 cells can be considered an osteoclastogenic Th subset; however, they are not the only ones. In fact, activated T cells, expressing high RANKL levels, have the ability to directly induce OC differentiation by acting on OC precursor cells [6].

However, because T cells/immune cells also secrete a variety of cytokines and express membrane-bound factors other than RANKL, which could support OC formation, mainly in pathological condition; this issue might be further explored, together with the mechanisms that could modulate their expression.

We describe recent efforts highlighting the prominent role of immune system in the alteration of bone remodelling, thus favouring the development of many bone diseases, such as periodontal disease (PD), psoriatic arthritis (PsA), postmenopausal osteoporosis, glucocorticoid-induced osteoporosis (GIO), metastatic solid tumors, and multiple myeloma (MM).

*Periodontal Disease*. PD is a common complex infection of the oral cavity that specifically affects the gingiva, the periodontal ligament, and the alveolar bone. It is characterized by an inflammatory response to bacteria present in the gingival pocket [7] and may remain confined to the gingiva or may progress to extreme periodontal destruction with the loss of the alveolar bone. PD is the main cause of tooth loss among adults and is associated with important alteration in facial aesthetics and defeat of masticatory and phonetics function [8]. It is also well recognized that the presence of only pathogenic bacteria is insufficient to PD. Progression of this disease occurs due to a combination of factors, including the presence of periodontopathic bacteria, high levels of proinflammatory cytokines (IL-1, TNF$\alpha$, IL-4, IL-6, IL-8, and IL-11), prostaglandin E2 ($PGE_2$), low levels of anti-inflammatory cytokines including IL-10, transforming growth factor (TGF-$\beta$), and retinoic acid [9]. Genetic factors increase the susceptibility of some individuals in developing this inflammatory disease. It has been supported by reports of familial aggregation of severe forms of the disease [10], and twin studies [11]. Recent candidate gene studies for periodontal disease have focused on genes related to host immunity and inflammatory response such as cytokines, cell-surface receptors, chemokines, enzymes, and antigen recognition. Histological examination of periodontitis lesions reveals that the granulocytes appear to play a key role in the maintenance of the periodontal health. These cells are present in the junctional epithelium in large numbers and they isolate tissues from the bacteria action; thus, severe forms of periodontitis frequently affect patients with diseases such as leukocyte adhesion deficiency and neutropenia. The failure of granulocytes to transmigrate into the endothelium results in an increase on the inflammatory response and reduces the protective response against periodontal pathogens. In the presence of active disease, the epithelial migration causes a deep periodontal pocket resulting in bacterial invasion, inflammation, and destruction of the connective tissue, with subsequent bone loss and possible tooth loss. Langerhans cells and dendritic cells of bone marrow origin, that are located within the epithelium, are a connecting link with acquired immunity. The adaptive immune response is activated when the epithelial barrier, with its innate system, is penetrated. The dendritic cells participate to the innate inflammatory response and moreover they capture and present antigens to B and T cells of the acquired immune system [12]. Activated CD4 T helper cells produce subsets of cytokines with different immune responses: Th1 and Th2 cells, respectively, associated with cellular and humoral immunity [13]. The recently described Th17 and Treg cells have antagonistic roles as effector and suppressive cells [8]. B cells differentiate into plasma cells producing specific antibodies. Thus, tissues affected by periodontitis become colonized with both lymphocytes subtypes with B cells being more represented than T cells. In a not progressive lesion, IFN-$\gamma$ increases the phagocytic activity of both neutrophils and macrophages and hence contains the infection. In case of a reduced innate immune response, a consequent weak Th1 response may not contain infection. Moreover, activated mast cells determine a Th2 response, B cell activation, and antibody production. The antibodies can control the infection or, as in the case of production of IgG2 in large amount, the lesion will persist. Sustained B cell activation may lead to IL-1 secretion and periodontal disease progression. Th17 cells have been identified in the periodontal tissues. IL-17 mainly produced by Th17 has been shown to stimulate epithelial, endothelial, and fibroblastic cells to produce IL-6, IL-8, and $PGE_2$, thus sustaining the disease progression. In addition, IL-17 induces RANKL production by osteoblasts stimulating bone resorption. It has been demonstrated that *periodontitis bacteria* induce a significant increase in the production of IL-17 [14]. According to recent studies, IL-17 significantly enhances RANKL and inhibits osteoprotegerin (OPG) expression in human periodontal ligament cells [15]. It has been hypothesized that Th17 cells may be involved in Th1 modulation and enhanced inflammatory mediators' production by gingival fibroblasts in periodontal disease. Circulating T cells express high levels of RANKL and spontaneously promote osteoclastogenesis in patients [16]. Th1 and Th17 cells, as well as B cells, increase RANKL expression [17]. Other studies demonstrated that also B cells produce RANKL in response to periodontal pathogen stimulation [17]. Contrarily, Treg cells decrease RANKL secretion whereas TGF-$\beta$ stimulates Treg cell differentiation. This process is supported by retinoic acid and counteracted by IL-6 and IL-1. In chronic inflammatory disease as PD, retinoic acid levels are suppressed and Treg activity is inhibited in favor of Th17 pathogenic effect [18]. In PD, proinflammatory cytokines overcome anti-inflammatory ones, and Th17 cells surmount Treg: this inflammatory state determines the destruction of connective tissue and alveolar bone.

*Psoriatic Arthritis*. Psoriasis is a chronic inflammatory disease of the skin; a considerable part of patients with psoriasis develops an inflammatory arthritis characterized by increased bone remodeling with osteolysis called PsA [19].

The mechanisms responsible for the development of the PsA should be better explained, but the immune system plays the main role in the pathogenesis of this disorder, that has to

be considered as a chronic inflammation. Therefore, patients with psoriasis have elevated levels of circulating neutrophils. The Th cells play an important role; in particular, Th1 and Th17 are involved in the pathogenesis of the disease. PsA is characterized by T and B cell infiltrates and neoangiogenesis in the synovial membrane and by the overexpression of inflammatory cytokines. PsA synovitis is indicated by hyperplasia of the synovial lining cells and mononuclear cell infiltration. Moreover, ectopic lymphoid neogenesis appears. Fibroblasts and T cells in PsA synovial fluid induce osteoclastogenesis and bone resorption, mediated by RANKL, TNF-$\alpha$, and IL-7 [37]. Inflammatory cytokine set such as TNF-$\alpha$, IL-1$\beta$, IL-10, IFN-$\gamma$, IL-12, IL-15, IL-17, and IL-18 were highly expressed in synovial fluid of PsA patients, while fibroblasts isolated from their skin and joints secreted IL-1 and IL-6; some of these cytokines have also recognized osteoclastogenic features.

Both T cell suppression and TNF-$\alpha$ inhibitors are effective in humans in the treatment of psoriasis. In PsA patients, there is a great increase in the number of peripheral blood Th17 cells. Thus, recent studies indicated that Th17 cells [38] are the cells most significantly involved in psoriasis. Like Th1 and Th2 cells, Th17 cells appear to be evolved in inducing acquired immune responses against microorganisms, such as bacteria. Abnormal Th17 responses are believed to play a significant role in the onset of various autoimmune diseases. Moreover, IL-23 is indispensable for Th17 effector functions in immune disorders and maintenance of Th17 cells.

Orphan nuclear receptor ROR$\gamma$t (retinoid-related orphan receptor gamma t) has been identified as specific Th17 transcription factor [39]. ROR$\gamma$t is involved in the production of IL-23 receptor (IL23R) which is expressed by monocytes, Th1, Th0, Th17, NK, and dendritic cells. IL23R has an important role in stimulating Th17 cells. IL-23 receptors promote IL-17 transcription and Th17 cell differentiation via enforced ROR$\gamma$t expression. IL-23 acts on cells that have been differentiated into Th17 cells, potentiating ROR$\gamma$t activity, and participates in maintenance and proliferation of Th17 cells.

Clinical trials studying the effects of anti-IL-17 and anti-IL-23 neutralizing antibodies in PsA patients are in progress [29], while the first results seemed to be not as impressive as those for TNF-$\alpha$ inhibitor therapy, a recent clinical trial indicated that Brodalumab, an IL-17RA inhibitor, determined a significant improvement, when administered for 12 weeks to PsA patients [36]. Moreover, new studies demonstrated that Ustekinumab, a monoclonal antibody against both IL-12 and IL-23 cytokines, interfering, respectively, with Th1 and Th17 activity, improved significantly PsA symptoms, although similar efficacy of TNF-$\alpha$ inhibitors needs about 52 weeks of treatment to be achieved [33]. Although immune responses mediated by IL-17 and IL-23 are not as evident as those with TNF-$\alpha$, Th17 cells appear to play an important role in PsA.

The activation of natural immunity in PsA stimulates Th17 and Th1 cells, which sustain autoimmune pathology. There is an interesting report regarding the relationship of PsA with microbial infection that is suggestive of PsA pathogenesis [40].

The observation of the lack of symptoms improvement in PsA patients underwent to HIV infection and thus to CD4+ reduction, suggested that Th cells cooperate in the pathogenesis with CD8+ cells [41]. Probably, CD8+T cells potentiates the production of cytokines in the synovial membrane, and the cytokines induce fibroblast proliferation promoting fibrosis [42–44], that probably contribute to joint stiffness and ankylosis [45].

*Postmenopausal Osteoporosis.* Postmenopausal osteoporosis is a systemic skeletal disorder characterized by reduced bone mineral density and microarchitectural deterioration of bone tissue resulting in fragility and susceptibility to fractures [46] and uncoupling of osteoblast-mediated bone formation and osteoclast-mediated bone resorption. Postmenopausal osteoporosis stems from the cessation of ovarian function at menopause and from genetic and nongenetic factors which heighten and prolong the rapid phase of bone loss characteristic of the early postmenopausal period. OC activity increases after menopause; these cells may be considered as cells at the crossroad between immune system and bone as their precursors circulate within the mononuclear fraction of peripheral blood [47–53] and they interact with other immune cells as T cells [54].

OC precursors increase during estrogen deficiency [47] and in condition characterized by increased bone turnover as bone metastases [50, 55] or inflammatory diseases [55–57].

Estrogens act on OC formation and activity both directly and indirectly, in particular their action is mediated through the influence on immune system [54]. In particular, estrogen loss upregulates OC formation and activity through an increased production of proosteoclastogenic cytokines by bone marrow cells [58], OBs [59], and immune cells [47, 60].

Proinflammatory and proosteoclastogenic cytokines as macrophage colony stimulating factor (M-CSF) and RANKL are increased during estrogen deficiency [61, 62].

Additional inflammatory cytokines are responsible for the upregulation of OC formation observed during estrogen deficiency; some of these molecules have a well-established role in osteoclastogenesis and bone loss, while others have not. Among these molecules, the most involved ones in estrogen deficiency bone loss appear to be TNF-$\alpha$, IL-1, IL-7, and IL-17 [1, 63–70]. A key role of T cell-produced TNF$\alpha$ has been demonstrated also in bone metastasis [51, 71].

Estrogens are key regulators of immune function as demonstrated both in animals and in humans [72, 73]. Despite some inverse reports [74, 75], the main body of literature firmly supports the essential role of activated T cells in regulating bone loss induced by estrogen deficiency [47, 69, 72, 76–79].

In humans, we have demonstrated a fundamental role for T cells in postmenopausal bone loss. In particular, we showed that osteoclastogenesis from peripheral blood precursors occurs only in the presence of T cells and that T cells are more active than in healthy post- and premenopausal controls [47]. T cells from osteoporotic patients produce more RANKL and TNF-$\alpha$, thus inducing OC formation and activity [47]. It has also been demonstrated that hormone replacement therapy decreases osteoclastogenic cytokine production in postmenopausal women. RANKL expression on lymphocytes and marrow stromal cells is significantly elevated during estrogen deficiency in humans and correlates directly with

increases in bone resorption markers and inversely with serum estrogen levels [77].

Estrogen loss promotes T cell activation by increasing antigen presentation [76, 80] and increases thymus output of T cells into peripheral blood [68]. Estrogen loss expands the proliferation and lifespan of bone marrow T cells [76, 78] increasing expression of class II transactivator (CIITA), a transcriptional coactivator acting on MHCII promoter [76, 81, 82].

Estrogen deficiency increases the number of activated CD40L-expressing T cells that promote the expression of M-CSF and RANKL by stromal cells and downregulates the production of OPG. The net result is a significant increase in the rate of osteoclastogenesis [83, 84]. This mechanism was also described in bone loss due to increased PTH levels [85, 86]. It is known that the CD40/CD40L system is crucial for T cell activation and several functions of the immune system. It promotes macrophage activation and differentiation, antibody isotype switching, and the adequate organization of immunological memory in B cells.

Also, the Th17 cells have been implicated in ovariectomy-induced bone loss; these cells increased after ovariectomy and stimulate osteoclastogenesis through IL-17 production [69]. This effect is reversed by treatment with estradiol. IL-17 increases OB production of proosteoclastogenic cytokines as TNF$\alpha$, IL-6, and RANKL; these effects are antagonized by estradiol.

Activated T cells have also been suggested to inhibit osteoclastogenesis by diverting early OC precursors towards dendritic cells differentiation [87]. Indeed T cells have the capacity to generate both osteoclastogenic cytokines such as RANKL and TNF-$\alpha$ [47], as well as antiosteoclastogenic factors such as IL-4. It has also been suggested that the effects of activated T cells on osteoclastogenesis *in vitro* depend on the manner in which they are activated [88]. The net effect of T cells on OC formation may consequently represent the prevailing balance of anti- and proosteoclastogenic T cell cytokine secretion. However, in humans, T cells seem to be proosteoclastogenic in different diseases including estrogen deficiency [47, 56, 66, 89–91].

Taken together, these observations demonstrate the causal relation among estrogen deprivation, T cell activation, increased cytokines production, and bone demineralization.

Also another type of immune cell the B cell has recently been studied as directly implicated in the regulation of bone resorption and may be directly involved in the pathogenesis of postmenopausal osteoporosis. Recent data have shown that B cells are the dominant producers of OPG in the bone microenvironment *in vivo* [79]. In fact, B cell KO mice have an osteoporotic phenotype with enhanced osteoclastic bone resorption and reconstitution with B cells by adoptive transfer, completely rescued mice from development of osteoporosis, and normalizing OPG production [79].

In human and animal B cells, OPG production can be significantly upregulated by the activation of CD40 [79]. In line with these data, both CD40 and CD40L KO mice displayed an osteoporotic phenotype and a significant deficiency in bone marrow OPG concentrations [79].

Thus, the emerging data suggest that the B lineage, rather than the OB lineage, is likely the major source of OPG in the bone microenvironment and that T cell signalling to B cells, through the costimulatory molecules CD40L and CD40, plays an important role in regulating basal OC formation and in regulating bone homeostasis.

On the other hand, it has been recently demonstrated that activated B cells overexpress RANKL, contributing to bone resorption [92, 93] and that ovariectomy in mice increases the number of RANKL-expressing B lymphocytes in the bone marrow [94].

A recent paper shows that mice lacking RANKL in B cells were partially protected from the ovariectomy-induced loss of cancellous bone [60]. The role of B-lymphocytes has also been evaluated in disease characterized by focal bone loss as in periodontal inflammation [66, 69, 95] and rheumatoid arthritis [93]. In rheumatoid arthritis, a recent paper suggests that B cells depletion ameliorates the suppressed bone turnover [96].

Taken together, these data suggest that B-lymphocyte involvement in the adaptive immune response contributes to bone resorption by the upregulation of RANKL expression through Toll-like receptor pathways and aligns with the known ability of T cells to produce RANKL in the presence of immune stimulus and to increase osteoclastogenesis. The effect of estrogen deficiency on B cell modulation may be one of the mechanisms through which menopause affects bone metabolism.

Thus, the involvement of T and B cells in the control of bone turnover may provide a novel explanation for the propensity to osteopenia and osteoporosis development after the cessation of ovarian function.

*Glucocorticoid-Induced Osteoporosis*. GIO is the most frequent origin of secondary osteoporosis in adults due to the direct effects of glucocorticoids (GCs) on bone cells [97]. GCs primarily affect trabecular bone, whereas the cortical bone mass is reduced to a lower and slower extent. Thus, fractures of the vertebrae are more recurrent. GC exposure determines a rapid and early phase of bone loss, which is the consequence of bone resorption exacerbation. This phase is followed by a more chronic and progressive phase in which bone mass declines because of impaired OB activity. GCs augment RANKL expression and reduce OPG levels in stromal and osteoblastic cells leading to the initial phase of rapid bone loss. Further, GCs increase MCSF expression as well as receptor subunits for osteoclastogenic cytokines of the gp130 family. However, the main pathophysiological mechanism of GIO is the impaired bone formation, due to reduced OB formation and activity [97, 98]. GCs impair on OBs the synthesis of type I collagen, the major protein in bone matrix. GCs may also influence osteocyte metabolism and function, modifying the elastic modulus adjacent to the osteocyte lacunae leading to reduced mineral to matrix ratios in the same areas with an enlargement of the lacunar size. Besides the GC direct actions on bone cells, GC extraskeletal effects on calcium metabolism have been reported. In particular, GCs decrease renal tubular calcium reabsorption and

calcium absorption from the gastrointestinal tract is reduced by mechanisms that oppose vitamin D action [97].

GCs also impair bone metabolism during the growth. In particular, in animal models, GC administration during growth is the cause of decreased bone formation and resorption, reductions in the age-dependent increases in trabecular bone mineral and trabecular thickness, and reductions in linear growth and accrual of cortical thickness in the femur [99].

A decrease of bone mineral density (BMD) has been reported in numerous pediatric diseases that require GCs, both as long term replacement therapy, such as 21-hydroxylase deficiency (21-OHD), and as treatment of acute phase, such as asthma, systemic lupus erythematosus, juvenile rheumatoid arthritis, inflammatory bowel disease, organ transplantation, and steroid sensitive nephrotic syndrome [22]. In particular, in 21-OHD patients on chronic GC therapy, the high osteoclastogenic potential of peripheral blood mononuclear cells has been reported [100]. It is supported by both the presence of circulating OC precursors and RANKL released by T cells [100]. Further, high dickkopf-1 (DKK1) levels, a secreted antagonist of the Wnt/$\beta$-catenin pathway, have been demonstrated in sera and circulating monocytes, T cells, and neutrophils from 21-OHD patients [28].

*Multiple Myeloma*. MM is a haematological malignancies, characterized by the clonal proliferation of plasma cells in the bone marrow [101]. A major number of mechanisms have been proposed to explain the increased formation and activity of the bone resorbing cells, the OCs in MM bone disease, whereas few mechanisms have been identified to explain the impairment of the bone forming cells, the OBs. In particular, MM cells produce different cytokines that directly or indirectly affect the bone cell activity, such as IL-6, MIP-1alpha, IL-3, DKK1, and sclerostin [101–103]. The proposed mechanism is that MM cells adhere to bone marrow stromal cells (BMSCs) and induce the secretion of numerous proosteoclastogenic and antiosteoblastogenic cytokines. The adhesion involved integrins such as CTLA4-1 and VLA-4 expressed by MM cells and VCAM-1 expressed on BMSCs [101].

Moreover, it has previously demonstrated an important role of T cells in supporting the formation and survival of OCs from peripheral blood mononuclear cells (PBMCs) isolated from MM patients with osteolysis, through the expression of high levels of RANKL and decoy receptor 3 (DcR3) [104, 105]. Interestingly, Giuliani et al. showed that malignant human myeloma cells stimulate RANKL expression in T cells [66]. Additionally, other authors demonstrated the high expression levels of IL-17 in T cells from MM patients [30–32]. IL-17 plays a key function in the progression of bone disease in MM, since the levels of IL-17 are higher in the more advanced bone disease. IL-17 is also able to increase RANKL expression on BMSCs, thus determining osteoclastogenesis increase and consequently the development of bone lesions [31]. The amount of Th17 in the bone marrow positively correlated with the number of osteolytic lesions [31] as well as the clinical tumor stage [106]. Very recently, the involvement of LIGHT/TNFSF14 has been reported in MM-bone disease [21]. LIGHT is a newly identified member of the TNF superfamily, expressed by activated leukocytes [21]. Recent literature data linked the high serum levels of LIGHT with the bone loss associated with rheumatoid arthritis [107]. Higher expression levels of LIGHT were found in CD8+ T cells, monocytes, and neutrophils from osteolytic MM patients with respect to the same cells from asymptomatic MM patients as well as monogammopaty of undetermined significant (MGUS) and healthy subjects. Further, LIGHT inhibition significantly reduces OC formation from PBMCs of osteolytic MM patients and stimulates OB differentiation in cultures derived from MM bone marrow mononuclear cells, as demonstrated by the increase of colony forming units of OBs and by the upregulation of osterix transcription factor, bone sialoprotein, and osteocalcin bone matrix proteins.

*Bone Metastatic Tumors*. The skeleton is the predominant metastatic site for many cancers, including breast, prostate, and lung cancers [108, 109]. Tumor invasion into bone is associated with dramatic skeletal related events (SRE) such as fractures, bone pain, hypercalcemia, and spinal cord compression [110]. The current model for the pathophysiology of bone metastasis centers on the interaction between tumor cells and OCs and is known as the "tumor/bone bone vicious cycle." Tumor cells secrete a plethora of factors and cytokines that can directly stimulate OC activation. Once mature OCs start to resorb the bone, they release bone-stored factors, such as TGF-$\beta$, that further stimulate tumor cell recruitment and proliferation [111]. Animal studies have shown that antiresorptive therapies protect from SRE and reduce tumor burden. Thus, antiresorptive agents, such as zoledronic acid (ZOL) and the anti-RANKL monoclonal antibody (Ab), denosumab, are widely used in the clinic in patients with bone metastasis [23, 24, 108]. Despite reducing tumor-associated bone complications, recent meta-analysis studies show controversial results on the antitumor effects of OC blockade in breast cancer patients with bone metastasis [112, 113]. A significant fraction of breast cancer patients with bone metastases shows progression in their bone disease while they are on potent antiresorptive agent treatment [114–116]. A recent study suggested the existence of a preosteolytic early phase of bone metastasis that is independent of OC activation [117]. Considering the complexity of the bone microenvironment, serving as home to hematopoietic stem cells and their progeny, which constitute the immune system, it is logical to consider the interactions between tumor cells and immune cells as potentially important regulators of bone metastasis beyond the OC.

Presence of activated CD4+ and CD8+ T cells has been observed in the bone marrow of untreated patients with breast cancer [118]. CD8+ T cells have the capacity to specifically identify and eliminate tumor cells via recognition of tumor-specific antigens. Activated CD4+ T cells can further facilitate the development of cytotoxic CD8+ T cells by secreting numerous cytokines, including Interferon $\gamma$ (IFN). Interferon $\gamma$ (IFN) exerts antiproliferative [119], proapoptotic [120], and angiostatic [121] effects resulting in the killing of a proportion of the tumor. Thus, presence of CD4+ and CD8+ T cells at tumor site is a good prognostic indicator. However, whether T cells modulate bone metastatic dissemination and/or tumor growth in the bone microenvironment is not

totally clear. In a recent report, Bidwell et al. demonstrated that silencing of IFN regulatory factor (Irf)7, a transcription factor controlling the induction of IFN genes, in breast cancer cells promotes bone metastases through escaping from immune control [122]. Importantly, an association with low expression of Irf7 signatures in primary breast tumors and higher number of bone metastatic events has been observed [122]. This finding is a strong indication that the immune system can modulate metastatic dissemination to bone in breast cancer patients.

Using animal models with established T cell immune deficiencies, we have also demonstrated that CD4+ and CD8+ T cell populations exert antitumor effects in the context of bone metastases [123]. We found that depletion of both CD4+ or CD8+ T cell subsets can reduce the antitumor effects ZOL in animals with bone metastases. Importantly, ZOL treatment is still highly effective in suppressing tumor-induced bone loss [123]. Conversely, T cell activation induced by administration of anti-CTLA4 Ab can significantly reduce bone tumor burden [123]. These observations have important clinical implications and suggest that reduced T cell numbers or impaired T cell activation might be the cause for the failure of ZOL to reduce tumor burden and increase survival in breast cancer patients.

Developing neoplasms can also acquire the ability to escape CD8+ T cell cytotoxicity by promoting expansion of Th2-polarized CD4+ T helper and regulatory T cells, as well as immune suppressor cells of myeloid origin reviewed in [124–126]. Monteiro et al. recently found that CD4+ T cells isolated from bone marrow of tumor bearing mice are potent stimulators of osteoclastogenesis [126]. This subset of tumor-specific CD4+ T cells has the ability to promote OC activation and induce osteolytic bone disease even before seeding of tumor cells in the bone microenvironment. Importantly, when tumor-specific CD4+ T cells are adoptively transferred into mice orthotopically injected with 4T1 tumor cells, tumor colonization to bone, but not to other metastatic sites, is increased. Whether this particular population of CD4+ T cells is increasing tumor bone metastases by affecting the OCs or also by inducing an immune suppressive environment needs to be established.

The bone microenvironment is particularly enriched in a highly heterogeneous population of immature myeloid progenitor cells that have the ability to exert immune suppressive effects in the presence of a tumor. This immature myeloid population, herein referred to as myeloid derived suppressor cells (MDSCs), represents 30–40% of the total bone marrow cells of naïve mice and is further expanded up to 60–70% of total marrow cells depending on the tumor type [127]. Circulating MDSCs are detected in the blood of patients with various types of cancer [128]. In response to factors secreted by a tumor, MDSCs leave the bone marrow and are found in high numbers in circulation, spleen, and tumor sites where they induce suppression of cytotoxic T cells [129]. MDSCs exert their proneoplastic effects through the release of small soluble oxidizers, by altering T cell/antigen recognition, and depletion of essential amino acids from the local extracellular environment, all ultimately leading to T cell suppression [130–133]. In addition, MDSCs can induce the expansion of regulatory T cells, a subtype of T cells exerting immune suppressive functions. Furthermore, direct effects of MDSCs on tumor proliferation through overproduction of cytokines and angiogenic factors have also been proposed [134].

A correlation between high MDSC numbers, advanced stage of malignancy, and poor prognosis has been observed. We have recently shown that increased bone metastasis in PLC$\gamma$2−/− mice is due to suppression of antitumor T cells responses. Although PLC$\gamma$2 is not expressed by T cells, we found that PLC$\gamma$2−/− mice have increased MDSC numbers with more potent immune suppressive effects than WT [135]. Downregulation of PLC$\gamma$2 activation also occurs in the MDSCs of patients with advanced pancreatic cancer [135].

Recent evidence also indicates that MDSCs participate in the preparation of premetastatic niches where they create a favorable environment for subsequent tumor colonization [136, 137]. Accumulation of MDSCs in bone marrow has been observed during early stages of MM [138]. A role for MDSCs in promoting tumor growth in bone through the OCs has also been proposed. Zhuang et al. discovered that MDSCs from mice injected with MM cells have increased osteoclastogenic potential. Importantly, coinjection of tumor-challenged MDSCs together with MM cells leads to increased tumor burden and osteolytic lesions, an effect that is inhibited by administration of ZOL [139]. Similarly, Sawant et al., using an immune competent model of breast cancer bone metastases, showed that MDSCs isolated from the tumor bone microenvironment differentiate into resorbing OCs *in vitro*. Remarkably, MDSCs isolated from tumor-free mice or tumor-bearing animals without bone metastases lack the ability to undergo OC differentiation [140]. This important observation suggests that there are intrinsic differences between MDSCs, depending on the tumor location. Why MDSCs from mice bearing bone metastases have the ability to differentiate into OCs might depend on the proosteoclastogenic rich cytokine milieu that characterizes the tumor bone microenvironment. However, it is unlikely that the bone tumor promoting effects of this subset of MDSCs is primarily dependent on their ability to differentiate into OCs. PLC$\gamma$2−/− mice display increased bone metastatic dissemination and higher MDSC numbers, but deletion of PLC$\gamma$2−/− also impairs the OC differentiation process [123, 135]. Thus, in the context of PLC$\gamma$2−/− deficiency, MDSCs are more likely to support tumor growth in bone by suppressing T cell activity. All together, these studies indicate that MDSCs are central players in the tumor/bone vicious cycle either through suppression of antitumor T cell responses or through differentiation into resorbing OCs.

Unfortunately to date there is no curative treatment for bone metastasis. Tumor cells that reach the bone environment are usually resistant to the current antitumor therapeutic approaches. The only options for these patients are palliative treatments to reduce bone pain and prevent additional bone destruction. More studies are needed to exploit the importance of antitumor and tumor promoting immune responses in patients with bone metastases and whether manipulation of T cell-MDSC interactions could offer therapeutic advantages to maximize the antitumor effects of OC blockade.

Table 1: Established and possible novel therapeutic targets in the different bone diseases.

(a)

| Established therapeutic targets | Pathologies | References |
|---|---|---|
| TNF-$\alpha$ | PsA | [20] |
| RANKL | PsA, osteoporosis, MM, and bone metastatic tumors | [22–27] |
| IL-17 | PsA | [29] |
| IL-23 | PsA | [29, 33] |
| IL-17RA | PsA | [36] |

(b)

| Possible novel therapeutic targets | Pathologies | References |
|---|---|---|
| LIGHT | MM | [21] |
| DKK1 | Bone metastasis and GIO | [28] |
| IL-17 | MM | [30–32] |
| MDSC targeting | Bone metastasis | [34, 35] |

## 2. Conclusions

The reviewed mechanisms underlying the bone disease clearly highlighted the key involvement of the cells with an immunological role. Further, it is also clear that numerous pathways are common to the different diseases, whereas others are disease-specific. Thus, these recent findings represent an important issue, leading to the identification of new therapeutic targets, mainly biological drugs, which in the last years are in strong development (Table 1).

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## References


[1] L. J. Raggatt and N. C. Partridge, "Cellular and molecular mechanisms of bone remodeling," *The Journal of Biological Chemistry*, vol. 285, no. 33, pp. 25103–25108.

[2] R. Tamma and A. Zallone, "Osteoblast and osteoclast crosstalks: from OAF to Ephrin," *Inflammation and Allergy—Drug Targets*, vol. 11, no. 3, pp. 196–200, 2012.

[3] G. Mori, P. D'Amelio, R. Faccio, and G. Brunetti, "The interplay between the bone and the immune system," *Clinical and Developmental Immunology*, vol. 2013, Article ID 720504, 16 pages, 2013.

[4] K. Sato, A. Suematsu, K. Okamoto et al., "Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction," *Journal of Experimental Medicine*, vol. 203, no. 12, pp. 2673–2682, 2006.

[5] I. E. Adamopoulos, C. C. Chao, R. Geissler et al., "Interleukin-17A upregulates receptor activator of NF-kappaB on osteoclast precursors," *Arthritis Research and Therapy*, vol. 12, no. 1, article R29, 2010.

[6] K. Sato and H. Takayanagi, "Osteoclasts, rheumatoid arthritis, and osteoimmunology," *Current Opinion in Rheumatology*, vol. 18, no. 4, pp. 419–426, 2006.

[7] A. B. Berezow and R. P. Darveau, "Microbial shift and periodontitis," *Periodontology 2000*, vol. 55, no. 1, pp. 36–47, 2011.

[8] P. E. Petersen and H. Ogawa, "Strengthening the prevention of periodontal disease: the WHO approach," *Journal of Periodontology*, vol. 76, no. 12, pp. 2187–2193, 2005.

[9] E. Gemmell and G. J. Seymour, "Immunoregulatory control of Th1/Th2 cytokine profiles in periodontal disease," *Periodontology 2000*, vol. 35, pp. 21–41, 2004.

[10] L. Saxén, "Heredity of juvenile periodontitis," *Journal of Clinical Periodontology*, vol. 7, no. 4, pp. 276–288, 1980.

[11] B. S. Michalowicz, D. Aeppli, J. G. Virag et al., "Periodontal findings in adult twins," *Journal of Periodontology*, vol. 62, no. 5, pp. 293–299, 1991.

[12] C. W. Cutler and R. Jotwani, "Antigen-presentation and the role of dendritic cells in periodontitis," *Periodontology 2000*, vol. 35, pp. 135–157, 2004.

[13] K. M. Murphy and S. L. Reiner, "The lineage decisions of helper T cells," *Nature Reviews Immunology*, vol. 2, no. 12, pp. 933–944, 2002.

[14] T. Oda, H. Yoshie, and K. Yamazaki, "*Porphyromonas gingivalis* antigen preferentially stimulates T cells to express IL-17 but not receptor activator of NF-$\kappa$B ligand *in vitro*," *Oral Microbiology and Immunology*, vol. 18, no. 1, pp. 30–36, 2003.

[15] D. Lin, L. Li, Y. Sun et al., "IL-17 regulates the expressions of RANKL and OPG in human periodontal ligament cells via TRAF6/TBK1-JNK/NF-$\kappa$B pathways," *Immunology*, 2014.

[16] G. Brunetti, S. Colucci, P. Pignataro et al., "T cells support osteoclastogenesis in an in vitro model derived from human periodontitis patients," *Journal of Periodontology*, vol. 76, no. 10, pp. 1675–1680, 2005.

[17] T. Kawai, T. Matsuyama, Y. Hosokawa et al., "B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease," *The American Journal of Pathology*, vol. 169, no. 3, pp. 987–998, 2006.

[18] C. T. Weaver and R. D. Hatton, "Interplay between the TH 17 and TReg cell lineages: a co-evolutionary perspective," *Nature Reviews Immunology*, vol. 9, no. 12, pp. 883–889, 2009.

[19] A. Menter, A. Gottlieb, S. R. Feldman et al., "Guidelines of care for the management of psoriasis and psoriatic arthritis. Section 1. Overview of psoriasis and guidelines of care for the treatment of psoriasis with biologics," *Journal of the American Academy of Dermatology*, vol. 58, no. 5, pp. 826–850, 2008.

[20] M. L. Acosta Felquer, L. C. Coates, E. R. Soriano et al., "Drug therapies for peripheral joint disease in psoriatic arthritis: a systematic review," *The Journal of Rheumatology*, vol. 41, no. 11, pp. 2277–2285, 2014.

[21] G. Brunetti, R. Rizzi, A. Oranger et al., "LIGHT/TNFSF14 increases osteoclastogenesis and decreases osteoblastogenesis in multiple myeloma-bone disease," *Oncotarget*, vol. 5, no. 24, pp. 12950–12967, 2014.

[22] M. F. Faienza, V. Luce, A. Lonero et al., "Treatment of osteoporosis in children with glucocorticoid-treated diseases," *Expert Review of Endocrinology & Metabolism*, vol. 9, no. 5, pp. 525–534, 2014.

[23] G. A. Clines and T. A. Guise, "Molecular mechanisms and treatment of bone metastasis," *Expert Reviews in Molecular Medicine*, vol. 10, article e7, 2008.

[24] T. A. Guise, "Molecular mechanisms of osteolytic bone metastases," *Cancer*, vol. 88, no. 12, pp. 2892–2898, 2000.

[25] M. Mazzantini and O. di Munno, "Glucocorticoid-induced osteoporosis: 2013 update," *Reumatismo*, vol. 66, no. 2, pp. 144–152, 2014.

[26] H. K. Fizazi, A. Lipton, X. Mariette et al., "Randomized phase II trial of denosumab in patients with bone metastases from prostate cancer, breast cancer, or other neoplasms after intravenous bisphosphonates," *Journal of Clinical Oncology*, vol. 27, no. 10, pp. 1564–1571, 2009.

[27] S. P. Iyer, J. T. Beck, A. K. Stewart et al., "Phase IB multicentre dose-determination study of BHQ880 in combination with anti-myeloma therapy and zoledronic acid in patients with relapsed or refractory multiple myeloma and prior skeletal-related events," *British Journal of Haematology*, vol. 167, no. 3, pp. 366–375, 2014.

[28] G. Brunetti, M. F. Faienza, L. Piacente et al., "High dickkopf-1 levels in sera and leukocytes from children with 21-hydroxylase deficiency on chronic glucocorticoid treatment," *The American Journal of Physiology—Endocrinology and Metabolism*, vol. 304, no. 5, pp. E546–E554, 2013.

[29] A. Gottlieb, A. Menter, A. Mendelsohn et al., "Ustekinumab, a human interleukin 12/23 monoclonal antibody, for psoriatic arthritis: randomised, double-blind, placebo-controlled, cross-over trial," *The Lancet*, vol. 373, no. 9664, pp. 633–640, 2009.

[30] K. M. Dhodapkar, S. Barbuto, P. Matthews et al., "Dendritic cells mediate the induction of polyfunctional human IL17-producing cells (Th17-1 cells) enriched in the bone marrow of patients with myeloma," *Blood*, vol. 112, no. 7, pp. 2878–2885, 2008.

[31] K. Noonan, L. Marchionni, J. Anderson, D. Pardoll, G. D. Roodman, and I. Borrello, "A novel role of IL-17-producing lymphocytes in mediating lytic bone disease in multiple myeloma," *Blood*, vol. 116, no. 18, pp. 3554–3563, 2010.

[32] R. H. Prabhala, D. Pelluru, M. Fulciniti et al., "Elevated IL-17 produced by TH17 cells promotes myeloma cell growth and inhibits immune function in multiple myeloma," *Blood*, vol. 115, no. 26, pp. 5385–5392, 2010.

[33] P. Davari, M. S. Leo, F. Kamangar et al., "Ustekinumab for the treatment of psoriatic arthritis: an update," *Journal of Clinical, Cosmetic and Investigational Dermatology*, vol. 7, pp. 243–249, 2014.

[34] E. Suzuki, V. Kapoor, A. S. Jassar, L. R. Kaiser, and S. M. Albelda, "Gemcitabine selectively eliminates splenic Gr-$1^+$/$CD11b^+$ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity," *Clinical Cancer Research*, vol. 11, no. 18, pp. 6713–6721, 2005.

[35] H.-J. Ko, Y.-J. Kim, Y.-S. Kim et al., "A combination of chemoimmunotherapies can efficiently break self-tolerance and induce antitumor immunity in a tolerogenic murine tumor model," *Cancer Research*, vol. 67, no. 15, pp. 7477–7486, 2007.

[36] P. J. Mease, M. C. Genovese, M. W. Greenwald et al., "Brodalumab, an anti-IL17RA monoclonal antibody, in psoriatic arthritis," *The New England Journal of Medicine*, vol. 370, no. 24, pp. 2295–2306, 2014.

[37] S. Colucci, G. Brunetti, F. P. Cantatore et al., "Lymphocytes and synovial fluid fibroblasts support osteoclastogenesis through RANKL, TNF$\alpha$, and IL-7 in an in vitro model derived from human psoriatic arthritis," *Journal of Pathology*, vol. 212, no. 1, pp. 47–55, 2007.

[38] C. Infante-Duarte, H. F. Horton, M. C. Byrne, and T. Kamradt, "Microbial lipopeptides induce the production of IL-17 in Th cells," *The Journal of Immunology*, vol. 165, no. 11, pp. 6107–6115, 2000.

[39] I. I. Ivanov, B. S. McKenzie, L. Zhou et al., "The orphan nuclear recepto RORγt directs the differentiation program of pro-inflammatory IL-$17^+$ T helper cells," *Cell*, vol. 126, no. 6, pp. 1121–1133, 2006.

[40] A. Batycka-Baran, J. Maj, R. Wolf, and J. C. Szepietowski, "The new insight into the role of antimicrobial proteins-alarmins in the immunopathogenesis of psoriasis," *Journal of Immunology Research*, vol. 2014, Article ID 628289, 10 pages, 2014.

[41] O. FitzGerald and R. Winchester, "Psoriatic arthritis: from pathogenesis to therapy," *Arthritis Research and Therapy*, vol. 11, no. 1, article 214, 2009.

[42] L. R. Espinoza, J. L. Aguilar, C. G. Espinoza, M. L. Cuellar, E. Scopelitis, and L. H. Silveira, "Fibroblast function in psoriatic arthritis. I. Alteration of cell kinetics and growth factor respon ses," *The Journal of Rheumatology*, vol. 21, no. 8, pp. 1502–1506, 1994.

[43] L. R. Espinoza, C. G. Espinoza, M. L. Cuellar, E. Scopelitis, L. H. Silveira, and G. R. Grotendorst, "Fibroblast function in psoriatic arthritis. II. Increased expression of $\beta$ platelet derived growth factor receptors and increased production of growth factor and cytokines," *The Journal of Rheumatology*, vol. 21, no. 8, pp. 1507–1511, 1994.

[44] D. D. Gladman, "Toward unraveling the mystery of psoriatic arthritis," *Arthritis and Rheumatism*, vol. 36, no. 7, pp. 881–884, 1993.

[45] B. Menon, N. J. Gullick, G. J. Walter et al., "Interleukin-17+CD8+ T cells are enriched in the joints of patients with psoriatic arthritis and correlate with disease activity and joint damage progression," *Arthritis and Rheumatology*, vol. 66, no. 5, pp. 1272–1281, 2014.

[46] "Osteoporosis. National Institutes of Health Consensus Development Conference Statement," *National Institutes of Health Consensus Development Conference Statement*, vol. 5, no. 3, p. 6, 1984.

[47] P. D'Amelio, A. Grimaldi, S. di Bella et al., "Estrogen deficiency increases osteoclastogenesis up-regulating T cells activity: a key mechanism in osteoporosis," *Bone*, vol. 43, no. 1, pp. 92–100, 2008.

[48] P. D'Amelio, M. A. Cristofaro, A. Grimaldi et al., "The role of circulating bone cell precursors in fracture healing," *Calcified Tissue International*, vol. 86, no. 6, pp. 463–469, 2010.

[49] P. D'Amelio, A. Grimaldi, M. A. Cristofaro et al., "Alendronate reduces osteoclast precursors in osteoporosis," *Osteoporosis International*, vol. 21, no. 10, pp. 1741–1750, 2010.

[50] L. D'amico, M. A. Satolli, C. Mecca et al., "Bone metastases in gastric cancer follow a RANKL-independent mechanism," *Oncology Reports*, vol. 29, no. 4, pp. 1453–1458, 2013.

[51] I. Roato, P. D'Amelio, E. Gorassini et al., "Osteoclasts are active in bone forming metastases of prostate cancer patients," *PLoS ONE*, vol. 3, no. 11, Article ID e3627, 2008.

[52] A. E. Oostlander, V. Everts, T. Schoenmaker et al., "T cell-mediated increased osteoclast formation from peripheral blood as a mechanism for crohn's disease-associated bone loss," *Journal of Cellular Biochemistry*, vol. 113, no. 1, pp. 260–268, 2012.

[53] M. Reed, R. J. Baker, A. B. Mehta, and D. A. Hughes, "Enhanced differentiation of osteoclasts from mononuclear precursors

in patients with Gaucher disease," *Blood Cells, Molecules, & Diseases*, vol. 51, no. 3, pp. 185–194, 2013.

[54] R. Pacifici, "The immune system and bone," *Archives of Biochemistry and Biophysics*, vol. 503, no. 1, pp. 41–53, 2010.

[55] I. Roato, E. Gorassini, G. Brunetti et al., "IL-7 modulates osteoclastogenesis in patients affected by solid tumors," *Annals of the New York Academy of Sciences*, vol. 1117, pp. 377–384, 2007.

[56] A. E. Oostlander, V. Everts, T. Schoenmaker et al., "T cell-mediated increased osteoclast formation from peripheral blood as a mechanism for Crohn's disease-associated bone loss," *Journal of Cellular Biochemistry*, vol. 113, no. 1, pp. 260–268, 2012.

[57] Y. Xue, L. Jiang, Q. Cheng et al., "Adipokines in psoriatic arthritis patients: the correlations with osteoclast precursors and bone erosions," *PLoS ONE*, vol. 7, no. 10, Article ID e46740, 2012.

[58] P. D'Amelio, I. Roato, L. Damico et al., "Bone and bone marrow pro-osteoclastogenic cytokines are up-regulated in osteoporosis fragility fractures," *Osteoporosis International*, vol. 22, no. 11, pp. 2869–2877, 2011.

[59] N. Udagawa, N. Takahashi, E. Jimi et al., "Osteoblasts/stromal cells stimulate osteoclast activation through expression of osteoclast differentiation factor/RANKL but not macrophage colony-stimulating factor," *Bone*, vol. 25, no. 5, pp. 517–523, 1999.

[60] M. Onal, J. Xiong, X. Chen et al., "Receptor activator of nuclear factor $\kappa$B ligand (RANKL) protein expression by B lymphocytes contributes to ovariectomy-induced bone loss," *The Journal of Biological Chemistry*, vol. 287, no. 35, pp. 29851–29860, 2012.

[61] L. C. Hofbauer, S. Khosla, C. R. Dunstan, D. L. Lacey, W. J. Boyle, and B. L. Riggs, "The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption," *Journal of Bone and Mineral Research*, vol. 15, no. 1, pp. 2–12, 2000.

[62] Y. Y. Kong, H. Yoshida, I. Sarosi et al., "OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis," *Nature*, vol. 397, no. 6717, pp. 315–323, 1999.

[63] H. Hotokezaka, E. Sakai, N. Ohara et al., "Molecular analysis of RANKL-independent cell fusion of osteoclast-like cells induced by TNF-alpha, lipopolysaccharide, or peptidoglycan," *Journal of Cellular Biochemistry*, vol. 101, no. 1, pp. 122–134, 2007.

[64] S. Wei, H. Kitaura, P. Zhou, F. P. Ross, and S. L. Teitelbaum, "IL-1 mediates TNF-induced osteoclastogenesis," *The Journal of Clinical Investigation*, vol. 115, no. 2, pp. 282–290, 2005.

[65] N. Charatcharoenwitthaya, S. Khosla, E. J. Atkinson, L. K. McCready, and B. L. Riggs, "Effect of blockade of TNF-$\alpha$ and interleukin-1 action on bone resorption in early postmenopausal women," *Journal of Bone and Mineral Research*, vol. 22, no. 5, pp. 723–729, 2007.

[66] N. Giuliani, S. Colla, R. Sala et al., "Human myeloma cells stimulate the receptor activator of nuclear factor-$\kappa$B ligand (RANKL) in T lymphocytes: a potential role in multiple myeloma bone disease," *Blood*, vol. 100, no. 13, pp. 4615–4621, 2002.

[67] G. Toraldo, C. Roggia, W.-P. Qian, R. Pacific, and M. N. Weitzmann, "IL-7 induces bone loss *in vivo* by induction of receptor activator of nuclear factor $\kappa$B ligand and tumor necrosis factor $\alpha$ from T cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 1, pp. 125–130, 2003.

[68] M. R. Ryan, R. Shepherd, J. K. Leavey et al., "An IL-7-dependent rebound in thymic T cell output contributes to the bone loss induced by estrogen deficiency," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 46, pp. 16735–16740, 2005.

[69] A. M. Tyagi, K. Srivastava, M. N. Mansoori, R. Trivedi, N. Chattopadhyay, and D. Singh, "Estrogen deficiency induces the differentiation of IL-17 secreting Th17 cells: a new candidate in the pathogenesis of osteoporosis," *PloS ONE*, vol. 7, no. 9, Article ID e44552, 2012.

[70] R. Zhao, "Immune regulation of bone loss by Th17 cells in oestrogen-deficient osteoporosis," *European Journal of Clinical Investigation*, vol. 43, no. 11, pp. 1195–1202, 2013.

[71] L. Das Roy, L. B. Pathangey, T. L. Tinder, J. L. Schettini, H. E. Gruber, and P. Mukherjee, "Breast cancer-associated metastasis is significantly increased in a model of autoimmune arthritis," *Breast Cancer Research*, vol. 11, no. 4, article R56, 2009.

[72] H. Forsblad d'Elia and H. Carlsten, "The impact of hormone replacement therapy on humoral and cell-mediated immune responses in vivo in post-menopausal women with rheumatoid arthritis," *Scandinavian Journal of Immunology*, vol. 68, no. 6, pp. 661–667, 2008.

[73] S. Kumru, A. Godekmerdan, and B. Yilmaz, "Immune effects of surgical menopause and estrogen replacement therapy in perimenopausal women," *Journal of Reproductive Immunology*, vol. 63, no. 1, pp. 31–38, 2004.

[74] A. Anginot, R. Dacquin, M. Mazzorana, and P. Jurdic, "Lymphocytes and the Dap12 adaptor are key regulators of osteoclast activation associated with gonadal failure," *PLoS ONE*, vol. 2, no. 7, Article ID e585, 2007.

[75] S.-K. Lee, Y. Kadono, F. Okada et al., "T lymphocyte-deficient mice lose trabecular bone mass with ovariectomy," *Journal of Bone and Mineral Research*, vol. 21, no. 11, pp. 1704–1712, 2006.

[76] S. Cenci, G. Toraldo, M. N. Weitzmann et al., "Estrogen deficiency induces bone loss by increasing T cell proliferation and lifespan through IFN-$\gamma$-induced class II transactivator," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 18, pp. 10405–10410, 2003.

[77] G. Eghbali-Fatourechi, S. Khosla, A. Sanyal, W. J. Boyle, D. L. Lacey, and B. L. Riggs, "Role of RANK ligand in mediating increased bone resorption in early postmenopausal women," *The Journal of Clinical Investigation*, vol. 111, no. 8, pp. 1221–1230, 2003.

[78] Y. Gao, F. Grassi, M. R. Ryan et al., "IFN-$\gamma$ stimulates osteoclast formation and bone loss in vivo via antigen-driven T cell activation," *The Journal of Clinical Investigation*, vol. 117, no. 1, pp. 122–132, 2007.

[79] Y. Li, G. Toraldo, A. Li et al., "B cells and T cells are critical for the preservation of bone homeostasis and attainment of peak bone mass in vivo," *Blood*, vol. 109, no. 9, pp. 3839–3848, 2007.

[80] J. Adamski, Z. Ma, S. Nozell, and E. N. Benveniste, "17beta-estradiol inhibits class ii major histocompatibility complex (MHC) expression: influence on histone modifications and CBP recruitment to the class II MHC promoter," *Molecular Endocrinology*, vol. 18, no. 8, pp. 1963–1974, 2004.

[81] J. H. Kim, K. Kim, B. U. Youn, H. M. Jin, and N. Kim, "MHC class II transactivator negatively regulates RANKL-mediated osteoclast differentiation by downregulating NFATc1 and OSCAR," *Cellular Signalling*, vol. 22, no. 9, pp. 1341–1349, 2010.

[82] R. B. Mueller, A. Skapenko, M. Grunke et al., "Regulation of myeloid cell function and major histocompatibility complex class II expression by tumor necrosis factor," *Arthritis and Rheumatism*, vol. 52, no. 2, pp. 451–460, 2005.

[83] J.-Y. Li, H. Tawfeek, B. Bedi et al., "Ovariectomy disregulates osteoblast and osteoclast formation through the T-cell receptor CD40 ligand," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 2, pp. 768–773, 2011.

[84] M. Yokoyama, T. Ukai, E. R. Ayon Haro, T. Kishimoto, Y. Yoshinaga, and Y. Hara, "Membrane-bound CD40 ligand on T cells from mice injected with lipopolysaccharide accelerates lipopolysaccharide-induced osteoclastogenesis," *Journal of Periodontal Research*, vol. 46, no. 4, pp. 464–474, 2011.

[85] B. Bedi, J.-Y. Li, F. Grassi, H. Tawfeek, M. N. Weitzmann, and R. Pacifici, "Inhibition of antigen presentation and T cell costimulation blocks PTH-induced bone loss," *Annals of the New York Academy of Sciences*, vol. 1192, pp. 215–221, 2010.

[86] Y. Gao, X. Wu, M. Terauchi et al., "T cells potentiate PTH-induced cortical bone loss through CD40L signaling," *Cell Metabolism*, vol. 8, no. 2, pp. 132–145, 2008.

[87] D. Grčević, I. K. Lukić, N. Kovačić, S. Ivčević, V. Katavić, and A. Marušić, "Activated T lymphocytes suppress osteoclastogenesis by diverting early monocyte/macrophage progenitor lineage commitment towards dendritic cell differentiation through down-regulation of receptor activator of nuclear factor-kappaB and c-Fos," *Clinical and Experimental Immunology*, vol. 146, no. 1, pp. 146–158, 2006.

[88] N. Wyzga, S. Varghese, S. Wikel, E. Canalis, and F. A. Sylvester, "Effects of activated T cells on osteoclastogenesis depend on how they are activated," *Bone*, vol. 35, no. 3, pp. 614–620, 2004.

[89] Y. Y. Kong, U. Feige, I. Sarosi et al., "Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand," *Nature*, vol. 402, pp. 304–309, 1999.

[90] I. Roato, D. Caldo, L. D'Amico et al., "Osteoclastogenesis in peripheral blood mononuclear cell cultures of periprosthetic osteolysis patients and the phenotype of T cells localized in periprosthetic tissues," *Biomaterials*, vol. 31, no. 29, pp. 7519–7525, 2010.

[91] N. Giuliani, F. Morandi, S. Tagliaferri et al., "Interleukin-3 (IL-3) is overexpressed by T lymphocytes in multiple myeloma patients," *Blood*, vol. 107, no. 2, pp. 841–842, 2006.

[92] X. Han, X. Lin, A. R. Seliger, J. Eastcott, T. Kawai, and M. A. Taubman, "Expression of receptor activator of nuclear factor-kappaB ligand by B cells in response to oral bacteria," *Oral Microbiology and Immunology*, vol. 23, no. 3, pp. 190–196, 2009.

[93] L. Yeo, K.-M. Toellner, M. Salmon et al., "Cytokine mRNA profiling identifies B cells as a major source of RANKL in rheumatoid arthritis," *Annals of the Rheumatic Diseases*, vol. 70, no. 11, pp. 2022–2028, 2011.

[94] M. Kanematsu, T. Sato, H. Takai, K. Watanabe, K. Ikeda, and Y. Yamada, "Prostaglandin E-2 induces expression of receptor activator of nuclear factor-B ligand/osteoprotegrin ligand on pre-B cells: implications for accelerated osteoclastogenesis in estrogen deficiency," *Journal of Bone and Mineral Research*, vol. 15, no. 7, pp. 1321–1329, 2000.

[95] X. Han, X. Lin, X. Yu et al., "*Porphyromonas gingivalis* infection-associated periodontal bone resorption is dependent on receptor activator of NF-$\kappa$B ligand," *Infection and Immunity*, vol. 81, no. 5, pp. 1502–1509, 2013.

[96] G. Wheater, V. E. Hogan, Y. K. O. Teng et al., "Suppression of bone turnover by B-cell depletion in patients with rheumatoid arthritis," *Osteoporosis International*, vol. 22, no. 12, pp. 3067–3072, 2011.

[97] G. Mazziotti, A. Angeli, J. P. Bilezikian, E. Canalis, and A. Giustina, "Glucocorticoid-induced osteoporosis: an update," *Trends in Endocrinology and Metabolism*, vol. 17, no. 4, pp. 144–149, 2006.

[98] R. S. Weinstein, "Clinical practice. Glucocorticoid-induced bone disease," *The New England Journal of Medicine*, vol. 365, pp. 62–70, 2011.

[99] S. Ikeda, Y. Morishita, H. Tsutsumi et al., "Reductions in bone turnover, mineral, and structure associated with mechanical properties of lumbar vertebra and femur in glucocorticoid-treated growing minipigs," *Bone*, vol. 33, no. 5, pp. 779–787, 2003.

[100] M. F. Faienza, G. Brunetti, S. Colucci et al., "Osteoclastogenesis in children with 21-hydroxylase deficiency on long-term glucocorticoid therapy: the role of receptor activator of nuclear factor-$\kappa$B ligand/osteoprotegerin imbalance," *Journal of Clinical Endocrinology and Metabolism*, vol. 94, no. 7, pp. 2269–2276, 2009.

[101] G. D. Roodman, "Pathogenesis of myeloma bone disease," *Leukemia*, vol. 23, no. 3, pp. 435–441, 2009.

[102] S. Colucci, G. Brunetti, A. Oranger et al., "Myeloma cells suppress osteoblasts through sclerostin secretion," *Blood Cancer Journal*, vol. 1, no. 6, article e27, 2011.

[103] A. Oranger, G. Brunetti, C. Carbone et al., "Human myeloma cell lines induce osteoblast downregulation of CD99 which is involved in osteoblast formation and activity," *Journal of Immunology Research*. In press.

[104] G. Brunetti, S. Colucci, R. Rizzi et al., "The role of OPG/TRAIL complex in multiple myeloma," *Annals of the New York Academy of Sciences*, vol. 1068, no. 1, pp. 334–340, 2006.

[105] S. Colucci, G. Brunetti, G. Mori et al., "Soluble decoy receptor 3 modulates the survival and formation of osteoclasts from multiple myeloma bone disease patients," *Leukemia*, vol. 23, no. 11, pp. 2139–2146, 2009.

[106] C.-J. Shen, Z.-H. Yuan, Y.-X. Liu, and G.-Y. Hu, "Increased numbers of T helper 17 cells and the correlation with clinicopathological characteristics in multiple myeloma," *Journal of International Medical Research*, vol. 40, no. 2, pp. 556–564, 2012.

[107] J. R. Edwards, S. G. Sun, R. Locklin et al., "LIGHT (TNFSF14), a novel mediator of bone resorption, is elevated in rheumatoid arthritis," *Arthritis and Rheumatism*, vol. 54, no. 5, pp. 1451–1462, 2006.

[108] G. R. Mundy, "Metastasis to bone: causes, consequences and therapeutic opportunities," *Nature Reviews Cancer*, vol. 2, no. 8, pp. 584–593, 2002.

[109] K. N. Weilbaecher, T. A. Guise, and L. K. McCauley, "Cancer to bone: a fatal attraction," *Nature Reviews Cancer*, vol. 11, no. 6, pp. 411–425, 2011.

[110] M. Clemons, K. A. Gelmon, K. I. Pritchard, and A. H. G. Paterson, "Bone-targeted agents and skeletal-related events in breast cancer patients with bone metastases: the state of the art," *Current Oncology*, vol. 19, no. 5, pp. 259–268, 2012.

[111] S.-M. Käkönen, K. S. Selander, J. M. Chirgwin et al., "Transforming growth factor-$\beta$ stimulates parathyroid hormone-related protein and osteolytic metastases via Smad and mitogen-activated protein kinase signaling pathways," *The Journal of Biological Chemistry*, vol. 277, no. 27, pp. 24571–24578, 2002.

[112] A. Valachis, N. P. Polyzos, R. E. Coleman et al., "Adjuvant therapy with zoledronic acid in patients with breast cancer: a systematic review and meta-analysis," *The Oncologist*, vol. 18, no. 4, pp. 353–361, 2013.

[113] M. He, W. Fan, and X. Zhang, "Adjuvant zoledronic acid therapy for patients with early stage breast cancer: an updated systematic review and meta-analysis," *Journal of Hematology and Oncology*, vol. 6, no. 1, article 80, 2013.

[114] L. S. Rosen, D. Gordon, M. Kaminski et al., "Zoledronic acid versus pamidronate in the treatment of skeletal metastases in patients with breast cancer or osteolytic lesions of multiple myeloma: a phase III, double-blind, comparative trial," *Cancer Journal*, vol. 7, no. 5, pp. 377–387, 2001.

[115] L. S. Rosen, D. Gordon, S. Tchekmedyian et al., "Zoledronic acid versus placebo in the treatment of skeletal metastases in patients with lung cancer and other solid tumors: a phase III, double-blind, randomized trial—the zoledronic acid lung cancer and other solid tumors study group," *Journal of Clinical Oncology*, vol. 21, no. 16, pp. 3150–3157, 2003.

[116] A. T. Stopeck, A. Lipton, J.-J. Body et al., "Denosumab compared with zoledronic acid for the treatment of bone metastases in patients with advanced breast cancer: a randomized, double-blind study," *Journal of Clinical Oncology*, vol. 28, no. 35, pp. 5132–5139, 2010.

[117] X. H.-F. Zhang, X. Jin, S. Malladi et al., "Selection of bone metastasis seeds by mesenchymal signals in the primary tumor stroma," *Cell*, vol. 154, no. 5, pp. 1060–1073, 2013.

[118] M. Feuerer, M. Rocha, L. Bai et al., "Enrichment of memory T cells and other profound immunological changes in the bone marrow from untreated breast cancer patients," *International Journal of Cancer*, vol. 92, no. 1, pp. 96–105, 2001.

[119] J. F. Bromberg, C. M. Horvath, Z. Wen, R. D. Schreiber, and J. E. Darnell Jr., "Transcriptionally active Stat1 is required for the antiproliferative effects of both interferon $\alpha$ and interferon $\gamma$," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 93, no. 15, pp. 7673–7678, 1996.

[120] S. Kumar, "The Bcl-2 family of proteins and activation of the ICE-CED-3 family of proteases: a balancing act in apoptosis?" *Cell Death and Differentiation*, vol. 4, no. 1, pp. 2–3, 1997.

[121] Z. Qin and T. Blankenstein, "$CD4^+$ T cell—mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells," *Immunity*, vol. 12, no. 6, pp. 677–686, 2000.

[122] B. N. Bidwell, C. Y. Slaney, N. P. Withana et al., "Silencing of Irf7 pathways in breast cancer cells promotes bone metastasis through immune escape," *Nature Medicine*, vol. 18, no. 8, pp. 1224–1231, 2012.

[123] L. D'Amico and I. Roato, "The impact of immune system in regulating bone metastasis formation by osteotropic tumors," *Journal of Immunology Research*. In press.

[124] M. J. Smyth, G. P. Dunn, and R. D. Schreiber, "Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity," *Advances in Immunology*, vol. 90, pp. 1–50, 2006.

[125] D. G. DeNardo and L. M. Coussens, "Inflammation and breast cancer. Balancing immune response: crosstalk between adaptive and innate immune cells during breast cancer progression," *Breast Cancer Research*, vol. 9, no. 4, article 212, 2007.

[126] A. C. Monteiro, A. C. Leal, T. Gonçalves-Silva et al., "T cells induce pre-metastatic osteolytic disease and help bone metastases establishment in a mouse model of metastatic breast cancer," *PLoS ONE*, vol. 8, no. 7, Article ID e68171, 2013.

[127] J. Sceneay, M. T. Chow, A. Chen et al., "Primary tumor hypoxia recruits $CD11b^+/Ly6C^{med}/Ly6G^+$ immune suppressor cells and compromises NK cell cytotoxicity in the premetastatic niche," *Cancer Research*, vol. 72, no. 16, pp. 3906–3911, 2012.

[128] B. Almand, J. I. Clark, E. Nikitina et al., "Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer," *The Journal of Immunology*, vol. 166, no. 1, pp. 678–689, 2001.

[129] I. Marigo, L. Dolcetti, P. Serafini, P. Zanovello, and V. Bronte, "Tumor-induced tolerance and immune suppression by myeloid derived suppressor cells," *Immunological Reviews*, vol. 222, no. 1, pp. 162–179, 2008.

[130] S. Kusmartsev and D. I. Gabrilovich, "Inhibition of myeloid cell differentiation in cancer: the role of reactive oxygen species," *Journal of Leukocyte Biology*, vol. 74, no. 2, pp. 186–196, 2003.

[131] P. Liu, J. R. Keller, M. Ortiz et al., "Bcl11a is essential for normal lymphoid development," *Nature Immunology*, vol. 4, no. 6, pp. 525–532, 2003.

[132] A. Mazzoni, V. Bronte, A. Visintin et al., "Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism," *The Journal of Immunology*, vol. 168, no. 2, pp. 689–695, 2002.

[133] S. Kusmartsev, Y. Nefedova, D. Yoder, and D. I. Gabrilovich, "Antigen-specific inhibition of $CD8^+$ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species," *The Journal of Immunology*, vol. 172, no. 2, pp. 989–999, 2004.

[134] S. Kusmartsev and D. I. Gabrilovich, "Effect of tumor-derived cytokines and growth factors on differentiation and immune suppressive features of myeloid cells in cancer," *Cancer and Metastasis Reviews*, vol. 25, no. 3, pp. 323–331, 2006.

[135] A.-H. Capietto, S. Kim, D. E. Sanford et al., "Down-regulation of PLC$\gamma$2-$\beta$-catenin pathway promotes activation and expansion of myeloid-derived suppressor cells in cancer," *Journal of Experimental Medicine*, vol. 210, no. 11, pp. 2257–2271, 2013.

[136] D. Gao, N. Joshi, H. Choi et al., "Myeloid progenitor cells in the premetastatic lung promote metastases by inducing mesenchymal to epithelial transition," *Cancer Research*, vol. 72, no. 6, pp. 1384–1394, 2012.

[137] M. L. Ortiz, L. Lu, I. Ramachandran, and D. I. Gabrilovich, "Myeloid-derived suppressor cells in the development of lung cancer," *Cancer Immunology Research*, vol. 2, no. 1, pp. 50–58, 2014.

[138] I. R. Ramachandran, A. Martner, A. Pisklakova et al., "Myeloid-derived suppressor cells regulate growth of multiple myeloma by inhibiting T cells in bone marrow," *The Journal of Immunology*, vol. 190, no. 7, pp. 3815–3823, 2013.

[139] J. Zhuang, J. Zhang, S. T. Lwin et al., "Osteoclasts in multiple myeloma are derived from $Gr\text{-}1^+CD11b^+$myeloid-derived suppressor cells," *PLoS ONE*, vol. 7, no. 11, Article ID e48871, 2012.

[140] A. Sawant, J. Deshane, J. Jules et al., "Myeloid-derived suppressor cells function as novel osteoclast progenitors enhancing bone loss in breast cancer," *Cancer Research*, vol. 73, no. 2, pp. 672–682, 2013.