UPDATES ON OSTEOIMMUNOLOGY: WHAT'S NEW ON THE CROSSTALK BETWEEN BONE AND IMMUNE CELLS

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EDITORIAL
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Editorial: Updates on Osteoimmunology: What's New on the Crosstalk Between Bone and Immune Cells

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Editorial on the Research Topic

Updates on Osteoimmunology: What's New on the Crosstalk Between Bone and Immune Cells

Osteoimmunology is an important field of bone research, it deepens the crosstalk between bone, and immune cells in both physiological and pathological conditions (1). The relative mechanisms were reported in the papers of this special issue, grouped into different categories: general mechanisms, pathological conditions, and potential therapeutics.

OSTEOIMMUNOLOGY GENERAL MECHANISMS

Ponzetti and Rucci reviewed in detail this topic. They reported that many cytokines that are considered immune-related, such as Interleukins, Tumor Necrosis Factor (TNF)-α, and Receptor-Activator of Nuclear factor Kappa B Ligand (RANKL), have all been demonstrated to be critical in osteoblast and osteoclast biology. On the other hand, bone cells, crucial for bone remodeling, actually regulate immune cells, and thus the endosteal niche. Both osteoclasts and osteoblasts take part to this niche, either by supporting engraftment, or mobilization of Hematopoietic Stem Cells (HSCs).

Interestingly, Lombardi et al. explored the effect of physical activity on bone metabolism, taking into account not only the direct role of biomechanical load, but also focusing on the role of immunomodulation. Although the majority of the studies point to the inflammation-mediated effects of physical activity, it has been shown that it regulates immune function through the inflammasomes, and through the secretion of myokines and adipokines. Although it is known that regular physical activity is effective in improving and maintaining bone mass, there is no consensus on the best kind of exercise to prescribe.

Additionally, Xiao and Xiao described autophagy involvement in osteoimmunology. Autophagy is a cytologic essential event for dysfunctional organelles degradation thus involved in cell survival; the process is also responsible for immune cell polarization (2). Bone cells use autophagy for their main functions, in particular osteoclast differentiation, polarization and adhesion to bone matrix depend on autophagy (3); osteoblasts through their phagosomes secrete calcium phosphate crystals in extracellular matrix supporting osteoid matrix mineralization (4). Differently, autophagy inhibits osteocytes oxidative stress consequences, neutralizing damaged mitochondria that could induce apoptosis (5). The authors concluded

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that autophagy, driving the inflammatory process in the immune cells, have important consequences on bone tissue not only in physiological remodeling but also in the diseases.

Interestingly, Fischer et al. revised the role of Foxp3+ regulatory T cells, a particular subset of CD4+ T cells, in bone and hematopoietic homeostasis (6, 7). Foxp3+ Treg cells play a crucial role in preserving immune homeostasis, but may also control regenerative and metabolic processes, osteoclast activity, and differentiation of HSCs. Foxp3+ Treg cells affect lympho-haematopoiesis through indirect mechanisms, i.e., by acting on osteoclast differentiation and activity, which determines changes in the niche size.

In the review by Metzger and Narayanan the authors synthetized the role of osteocytes in immune mediated bone pathological events (8). Aging, changes the canalicular communication between osteocytes impairing their communication features, moreover, in old mice, osteocytes increase the production of inflammatory cytokines, increasing bone resorption. Pro-inflammatory signals can directly determine osteocytes apoptosis affecting bone turnover, or can stimulate osteocytes in the production of anti-osteoblastic or pro-osteoclastic cytokines stimulating bone loss.

OSTEOIMMUNOLOGY AND PATHOLOGICAL CONDITIONS

Osteoimmunology key mechanisms have been also highlighted in different pathological conditions (9–12), and in this special issue on obesity, osteopetrosis, osteolytic solid tumors, and multiple myeloma.

Childhood obesity is one of the main health problems worldwide. The excess of adipose tissue leads to inflammation, oxidative stress, apoptosis, and endothelial dysfunctions (13). Severe comorbidities are associated with obesity, most of them developing already in childhood (14). Among these, low bone mineral density, osteoporosis, and fracture risk have been observed in subjects with various degree of obesity. Faienza et al., reviewed the mechanisms involved in obesity-related bone fragility. The authors reported that the accumulation of adipose tissue positively regulates the function of osteoclasts, the bone reabsorbing cells, by up-regulating the production of several cytokines as RANKL, LIGHT, TRAIL, TNFα, and Monocyte Chemotactic Protein-1 (MCP1). In addition, in obese subjects the osteoblastogenesis is inhibited, thereby the result is an enhanced bone resorption.

Osteopetrosis is a rare bone disease characterized by increased bone mass due to defects in osteoclast function or formation (15). Penna et al. deal with this interesting topic reviewing the clinical and genetic heterogeneity of the disease and underscoring the importance of the research in order to develop further innovative treatment for this rare and often lethal disease. Treatment of these patients if often limited by the presence of neurodegeneration, however hematopoietic stem cell transplantation can be successfully applied in several patients, this treatment is effective only during the first months of life, hence a precocious molecular diagnosis is fundamental.

The research article of Zhang et al. reported an analysis on the single-nucleotide polymorphisms (SNPs) and expression of NLRP3 inflammasome related genes, NF-κB, NLRP3, IL-1β, IL-18, Caspase-1, and ASC in 308 acute lymphoblastic leukemia (ALL) patients and 300 healthy controls. Inflammasomes are large cytosolic multiprotein complexes that activate the caspase-1-mediated innate immune responses. The results of this study demonstrated an involvement of NLRP3 inflammasome-related SNPs, especially NF-κB-94ins/del ATTG and CARD8 (rs2043211) genotype, in ALL. Thus, this genetic variant of NLRP3 inflammasome could be a new biomarker and potential targets for ALL. Furthermore, IL-1β (rs16944) and IL-18 (rs1946518) polymorphism seem to predict ALL prognosis. Notably, ALL patients developed osteonecrosis (16), thus is fundamental the identification of new markers as well as therapeutic targets.

Bone is a preferred metastatic site for several cancer and cancer cells may remain dormant for years within bone before growing, thanks to their ability to overcome the immune system (17). Roato and Vitale deal with the interesting and under-investigated topic of bone metastatic niche, the relation with immune system and the role of Natural Killer (NK) cells in the control of bone metastases growth. They suggest that the stimulation of NK cells activity by the use of cytokine combinations and blocking of their checkpoint receptors can be crucial in increasing the opportunity for new therapeutic options in the treatment of bone metastatic disease.

Harmer et al. highlighted the role of interleukin 6 (IL-6) in multiple myeloma, a hematological malignancy of the plasma cells (18). About 80% of patients display the osteolytic bone disease at diagnosis, and different mechanisms have been demonstrated to orchestrate the negative process, such as IL-6 (18). It is an inflammatory cytokine that affects osteoclast differentiation and plasma cell proliferation. In multiple myeloma, IL-6 is responsible of bone homing, osteolytic process, progression as well as drug resistance. The review also reported current and potential therapeutic approaches against IL-6.

OSTEOIMMUNOLOGY AND POTENTIAL THERAPEUTICS

In the research paper by Massaccesi et al. the authors demonstrated Vitamin E effects on ultrahigh molecular weight polyethylene (UHMWPE). UHMWPE are currently used for joint prothesis, they determine the reduction of wear particles formation with a consequent decrease of free radicals' generation. Bone remodeling activity in sites close to UHMWPE prosthetic devices, can be affected by oxidative stress. Thus, oxidative stress can induce either osteoclast differentiation or osteoblast apoptosis leading to bone resorption. Vitamin E, being a powerful antioxidant molecule, could be proposed in joint prosthetic devices to decrease prothesis wear and at the same time inhibit the influences of the oxidation-induced osteolysis promoting osteoblast survival and osteoblasts apoptosis. The authors concluded that Vitamin E-Stabilization on UHMWPE could be used to increase the life of joint prothesis.

Kishikawa et al. studied the role of Docosahexaenoic acid (DHA) in inflammation-induced osteoclast formation. DHA, an n-3 fatty acid, is an important structural component of the cell membrane, regulates osteoclast differentiation, and activity and has a potent anti-inflammatory effect through G protein-coupled receptor 120 (GPR120), a functional receptor for n-3 fatty acids (19). Kishikawa et al. displayed that both RANKL- and TNF-α-induced osteoclastogenesis was inhibited by DHA. The authors also reported that the number of osteoclasts, bone resorption pits and the level of resorption marker were significantly lower in LPS+DHA-co-administered mice than LPS-administered mice. However, this DHA-induced inhibition was not found in LPS-, DHA-, and selective GPR120 antagonist AH7614-co-administered mice. Thus, the authors concluded that DHA inhibits LPS-induced osteoclast formation and activity *in vivo* via GPR120 by inhibiting LPS-induced TNF-α secretion by macrophages together with the direct inhibition of osteoclastogenesis.

Nicolin et al. focused on the current knowledge on the role of plant-derived polyphenols in suppressing osteoclast differentiation and bone resorption. Polyphenols are natural molecules derived from plants isolated and characterized in the fruits, and vegetables (20). Some of these bioactive compounds have bone anabolic action, by inhibiting bone resorption. Probably, polyphenols could affect bone metabolism through impairment of cytokines which are involved in promoting osteoclast differentiation and resorption. Furthermore, these compounds have antioxidant characteristics, since they can act as scavengers of reactive oxygen species (ROS). However, further studies are needed on the application of these compounds as therapeutic alternative to current therapies (21) in bone diseases.

In conclusion, all the articles dispensed an overview of the mechanisms regulating bone immune cell crosstalk in physiological and pathological conditions, thus identifying possible alternative therapeutic targets.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Interleukin-6 Interweaves the Bone Marrow Microenvironment, Bone Loss, and Multiple Myeloma

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The immune system is strongly linked to the maintenance of healthy bone. Inflammatory cytokines, specifically, are crucial to skeletal homeostasis and any dysregulation can result in detrimental health complications. Interleukins, such as interleukin 6 (IL-6), act as osteoclast differentiation modulators and as such, must be carefully monitored and regulated. IL-6 encourages osteoclastogenesis when bound to progenitors and can cause excessive osteoclastic activity and osteolysis when overly abundant. Numerous bone diseases are tied to IL-6 overexpression, including rheumatoid arthritis, osteoporosis, and bone-metastatic cancers. In the latter, IL-6 can be released with growth factors into the bone marrow microenvironment (BMM) during osteolysis from bone matrix or from cancer cells and osteoblasts in an inflammatory response to cancer cells. Thus, IL-6 helps create an ideal microenvironment for oncogenesis and metastasis. Multiple myeloma (MM) is a blood cancer that homes to the BMM and is strongly tied to overexpression of IL-6 and bone loss. The roles of IL-6 in the progression of MM are discussed in this review, including roles in bone homing, cancer-associated bone loss, disease progression and drug resistance. MM disease progression often includes the development of drug-resistant clones, and patients commonly struggle with reoccurrence. As such, therapeutics that specifically target the microenvironment, rather than the cancer itself, are ideal and IL-6, and its myriad of downstream signaling partners, are model targets. Lastly, current and potential therapeutic interventions involving IL-6 and connected signaling molecules are discussed in this review.

Keywords: multiple myeloma, IL-6, interleukin 6, bone marrow, MSCs

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INTRODUCTION

For many years, the skeletal system was regarded purely as a means for structural support and movement; however, abundant data have since been collected that reveal a complex bi-directional interaction between bone cells and surrounding bone marrow (BM) cells. BM is composed of a heterogeneous cell population including adipocytes, chondrocytes, endothelial cells, immune cells, fibroblasts, and two multipotent stem cell populations: mesenchymal stem cells (MSCs) and haematopoietic stem cells (HSCs). MSCs can differentiate into chondrocytes, bone marrow adipocytes (BMAs), and osteoblasts (bone-forming cells), while HSCs give rise to both the myeloid and lymphoid immune cell populations. The myeloid cell precursors are of particular interest as they can give rise to

both innate monocyte/macrophage immune cells, but can also fuse and differentiate to form osteoclasts, the cells responsible for bone resorption.

BM cells are involved in activating and intensifying numerous signaling pathways that contribute to diseased states. The instigation of these signaling pathways can be coupled to the secretion of soluble factors by bone and stromal cells, alike. Such soluble factors include interleukin 6 (IL-6), insulin-like growth factor-1 (IGF-1), stromal cell-derived factor-1 (SDF-1), tumor necrosis factor alpha (TNF-α), interleukin-8 (IL-8), interleukin-17 (IL-17) and vascular endothelial growth factor (VEGF), which play roles in inflammation, immunosuppression, tumorigenesis, and osteolysis and thus contribute to many disease states. A number of these molecules activate NF-κB signaling, which is key in the BMM, as it binds to and activates important transcription factors and signaling cascades.

The NF-κB molecule associates with STAT3, which then induces the expression of wound healing and cancer gene products (1), such as anti-apoptotic proteins. Furthermore, STAT3 signaling results in the release of inflammatory molecules, such as IL-6 (2), SDF-1 (3), TNF-α (4), interleukin-1 (IL-1) and (IL-6) (5), which are all known to stimulate NF-κB signaling, thus creating a positive feedback loop and increased downstream inflammatory cytokine production (2). This vicious circle of NF-κB signaling can be detrimental to bone due to chronic inflammation and damage, yet it remains key in the maintenance of healthy bone, as it facilitates osteoclast differentiation and survival (6) and thus bone homeostasis.

Receptor activator of nuclear factor kappa-B ligand (RANKL) is a molecule expressed on osteoblasts which can facilitate the fusion of myeloid progenitors into osteoclasts (7). When RANKL binds to progenitor cells, it promotes NF-κB signaling, blockade of TNF receptor associated factor-3 (TRAF-3), promotion of NF-κB inducing kinase (NIK), and a consequential translocation of RelB and p52 (NF-κB2) into cell nuclei (8), which promotes signaling for osteoclastogenesis (8). MAPK pathways, particularly those involving p38, feed into this system, as they are both activated by NF-κB, but furthermore, are known to result in the production of RANKL. IL-1, for example, can stimulate RANKL via p38 in stromal cells thus promoting osteoclastogenesis (9). The extent of osteoclastogenesis is regulated by an equilibrium between RANKL and its inhibitor, osteoprotegerin (OPG).

The regulation of these bone signaling pathways is key in the limitation of bone disease. Inflammatory cytokines can disrupt ratios of RANKL:OPG and can result in excessive osteoclastogenesis. IL-6, is tied to excessive promotion of RANKL and inhibition of OPG and thus has been linked to bone osteolysis (10), osteoporosis (11), rheumatoid arthritis (11) and other bone-related pathologies. Osteolysis has further implications as it allows for the release of growth factors which promote the homing and survival of bone-metastatic cancers (12), such as prostate (13), breast (14) neuroblastoma (15), acute myeloid leukemia (AML) (16) and MM (17, 18).

MM is a blood cancer which epitomizes the crosstalk between the immune and bone systems. It is a commonly refractory, and thus recurrent, pathology characterized by uncontrollable clonal expansion of plasma cells. Myeloma cells grow in the BMM where they disrupt the delicate balance between bone growth and resorption through the secretion of factors that directly and indirectly promote osteoclasts and inhibit osteoblasts. This interaction between myeloma cells and the BMM, and the potential to activate dormant immune cells to kill myeloma cells in the BM, demonstrate exciting areas of osteoimmunology under scrutiny. It is the hope that a better understanding of the signals in the BM may allow for the identification of better targets and development of better combination therapies for MM and other bone disease. This review focuses on just one signaling pathway, IL-6, and its ability to promote both diseased and healthy states within the BMM. Since MM is strongly tied to IL-6, it will be used frequently as a disease example to emphasize the role of IL-6 in bone osteolysis, bone-metastatic cancer and general bone disease.

IL-6 AND BONE REMODELING

Healthy bone is maintained by continuous bone resorption and regrowth, which is held at equilibrium through numerous signaling cascades (19, 20). Osteoclasts secrete acidic collagenases that break down matrices and form resorption pits. Osteoblasts line the border of mineralized bone and produce and deposit collagen, osteocalcin, and osteopontin to form an osteoid matrix. Osteoblasts then calcify osteoid through deposition of calcium phosphate, calcium carbonate and hydroxyapatite. Osteocytes, or other systemic signals, control the balance of bone resorption and formation. Osteocytes, the most abundant cell type in bone, are mechanosensing cells that produce signaling molecules in response to changes in pressure, sheer stress, or BMM chemical signals. Sclerostin is a signaling molecule that is synthesized and secreted by osteocytes, and acts as a WNT inhibitor causing inhibited osteoblast differentiation and supporting adipogenesis (21). Osteocytes also secrete Dentin-Matrix-acidic-Phosphoprotein-1(DMP-1), Matrix-Extracellular-Phospho-glycoprotein (MEPE), RANKL, Fibroblast-Growth-Factor-23 (FGF-23) and Phosphate-regulating-neutral-Endopeptidase-X-linked (PHEX), all of which play roles in regulating bone mineralisation, cell fate or phosphate homeostasis (21, 22).

Inflammatory cytokines, such as IL-6 and IL-11, can modulate skeletal homeostasis and osteoclast differentiation. When IL-6 binds receptors on pre-osteoclasts, they promote osteoclastogenesis (23), resulting in increased levels of bone resorption (**Figure 1**). Along with its direct roles on osteoclastogenesis, IL-6 also alters bone remodeling; when activated by IL-6, osteoblasts induce JAK/STAT3 pathways and ultimately secrete pro-osteoclast mediators, including RANKL, IL-1, parathyroid hormone related protein (PTHrP), and prostaglandin E2 (PGE2) (12, 24–27). PGE2 and PTHrP are particularly interesting factors as both, along with parathyroid hormone (PTH) and active vitamin D [1,25(OH)2D3], have been shown to stimulate IL-6 and RANKL production in osteoblasts within the BMM (28, 29). Thus, PGE2 and PTHrP reside both upstream and downstream of IL-6 signaling, facilitating

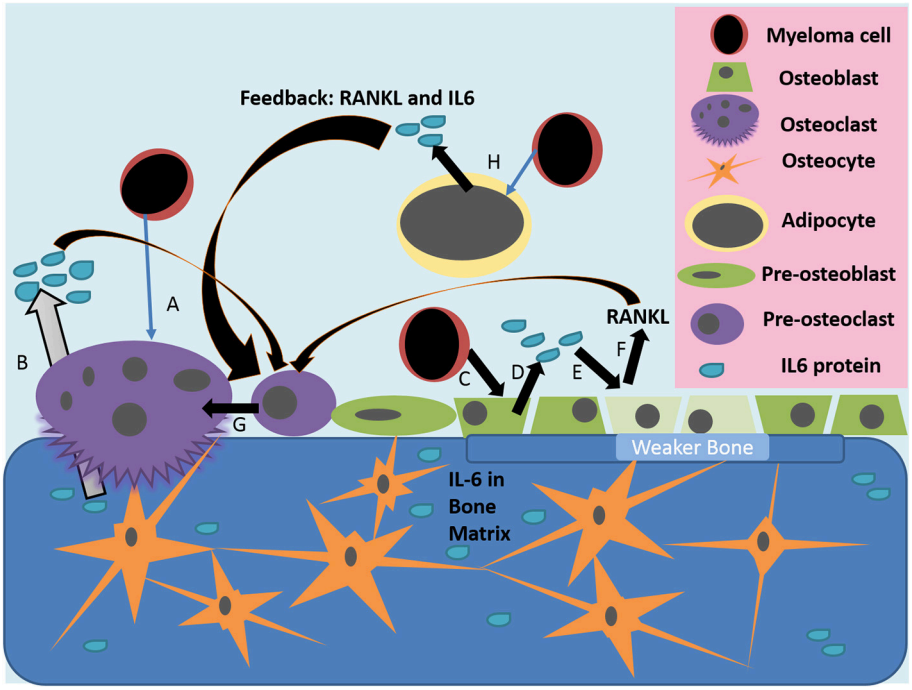

FIGURE 1 | Actions of IL-6 in the diseased bone marrow microenvironment. (A) Myeloma cells or other inflammatory triggers can induce osteoclast differentiation and osteolytic activity, which releases growth factors and IL-6 stored in the bone matrix (B). (C) Myeloma cells, or other inflammatory mediators, activate the release of IL-6 from osteoblasts (D); IL-6 then inhibits the activity of osteoblasts and induces their production of RANKL (E). (F,G) IL-6 from many sources, as well as RANKL from osteoblasts, induce pre-osteoclasts to differentiate into mature osteoclasts, which then resorb bone to induce greater release of stored growth factors, creating a vicious cycle. (H) Myeloma cells also alter bone marrow adipocytes to make a more supportive niche for tumor cells and to increase osteoclastic activity through IL-6 and other molecules.

a positive feedback loop that can exacerbate the detrimental pro-osteoclastic mechanisms seen in many diseased states. IL-6 can promote PTHrP secretion via TNF-α, which itself enhances osteoclastogenesis, bone loss and hypercalcaemia (30). The pathways that use osteoblastic mediation require the soluble IL-6 receptor (sIL-6R), as osteoblasts ordinarily express only low levels of the endogenous receptor (12). Soluble IL-6R forms as a result of cleavage or alternate splicing of IL-6R and is found in circulation in low numbers due to secretion from cells (31), commonly CD4 T cells (32). Therefore, it is necessary for IL-6 to meet and bind to sIL-6R within circulation, and then once bound, fuse with the osteoblastic membrane to trigger IL-6-inducible signaling pathways. Consequently, high levels of both IL-6 and sIL-6R are markers for osteolytic disorders, such as rheumatoid arthritis (10).

IL-6 also exacerbates osteolysis through inhibition of osteoblast differentiation, further disrupting the balance of healthy bone turnover. Both IL-6 and sIL-6R can cause a decrease in osteoblastic differentiation by reducing expression of genes involved in osteoblastic differentiation including alkaline phosphatase (ALP), Runx2 and osteocalcin (33). IL-6/sIL-6R signaling can also reduce the ability of osteoblasts to mineralise bone (**Figure 1**). This was determined to be a result of MEK/ERK and PI3K/AKT2 pathways; inhibiting these pathways increased expression of Runx2 and other mature osteoblastic phenotypes (33). Similarly, c-Src (a proto-oncogenic tyrosine kinase) has been shown to activate STAT3 and stimulate IL-6 within immature osteoblasts (34). This stimulation can induce expression of insulin-like growth factor 5 (IGFP5)—a molecule thought to act in a paracrine fashion to inhibit osteoclastogenesis (34). Overall, IL-6 has been shown to have anti-osteogenic and pro-osteoclastic effects leading to a net inhibition of bone.

IL-6 AND NON-CANCEROUS BONE DISEASE

Regulation of IL-6 plays a crucial role in bone maintenance and remodeling. Commonly, when chronic injury and/or inflammation occurs, it instigates overexpression of IL-6 within the BMM resulting in pro-osteoclastic pathways, osteopenia, osteoporosis, and an increased fracture risk (12). In fact, diseases characterized by excessive bone loss commonly coincide with IL-6 and RANKL overproduction (10). Numerous signaling molecules interact with IL-6 to accelerate bone disease including PGE2/COX2, PTH, 1,25(OH)2D3, estrogen, and VEGF, as described below.

PGE2 results from metabolism of the fatty acid arachidonic acid via cyclooxygenase-2 (COX-2). Overexpression of COX-2 is driven by IL-6 signaling and results in increased production PGE2 (35). PGE2 interacts with G-coupled receptors EP1, EP2, and EP4, which activate tissue-specific downstream signaling

pathways (36). Inflammatory conditions such as rheumatoid arthritis and osteoarthritis appear are tied PGE2-induced inflammation (37). Mast cells (38), Th17 cells (39) and dendrites (40) can all be stimulated by PGE2 via EP2 and EP4, resulting in the release of pro-inflammatory cytokines, e.g., IL-6 (41). PGE2 is also linked to osteoclastogenesis and breakdown of juxta-articular-bone and cartilage (42). PGE2 further disrupts bone homeostasis due to its ability to inhibit OPG secretion by osteoblasts (27). Use of anti-IL-6 antibodies in osteoblastic and monocyte/macrophage-like cell lines demonstrated increased OPG secretion through alleviation of PGE2-driven inhibition (27), thus concluding that PGE2 increases IL-6 signaling, driving osteoclastogenesis in part through OPG inhibition.

Similar to PGE2, PTH acts both upstream and downstream of IL-6 signaling. This hormone is expressed in response to hypocalcaemia and, once in circulation, it acts upon osteoblasts to both increase their activity and induce secretion of factors such as RANKL and IL-6 (43), which promote osteoclastic activity. PTH-induced bone loss can be inhibited by blocking IL-6R with neutralizing antibodies (44). Patients who suffer from primary parathyroidism show elevated IL-6 and markers of increased bone resorption (45), further demonstrating how this hormone is involved in IL-6-associated bone diseases.

It would be overly simple to say that IL-6 is solely detrimental to bone, as recent *in vivo* work has shown a key role for it in bone repair. Transgenic mice overexpressing IL-6 have demonstrated enhanced bone loss, faulty osteoid ossification, and reduced osteoblast activity (46), but, other studies have also shown that IL-6 knock-out (KO) mice display abnormalities in bone architecture and delayed fracture healing (47). These IL-6 KO mice demonstrated delayed mineralization and remodeling of bone, with enhanced levels of collagen and cartilage at early stages of healing and reduced osteoclast number (47). This demonstrates a necessity for IL-6-induced bone loss in the correct repair of fractures. This is further evidenced in a diabetes-associated fracture model where reduced osteoclastic activity resulted in delayed repair (48).

IL-6 and Rheumatoid Arthritis (RA)

RA is a chronic, inflammatory condition prevalent in middle-aged people and is the leading cause of work-associated disability in the United States (49). Characterized by inflammation of the synovium of multiple joints of the body, sufferers exhibit pain and stiffness in hands, knees, wrists and feet. Progression of the disease results in detrimental damage to the joint and erosion of both the cartilage and bone. RA patients commonly express high levels of IL-6 intracellularly, which has been shown to have negative correlations clinically with bone mass density (BMD) (50). Increased vascular permeability and extravasation of fluid into synovial regions are key characteristics of RA and induce joint pain. Excess vascularization, a direct effect of IL-6-induced VEGF overexpression, enhances fluid build-up in joints (51). Antibodies against IL-6R can reduce VEGF expression in RA clinically (51).

In IL-6 KO mouse models, mice are protected against arthritis and have decreased osteoclast activity and bone loss (52). In addition, IL-6 neutralizing antibodies have been administered to mice in collagen-induced *in vivo* arthritis models, where they protected the mice from bone lesions and disease progression (53). Similarly, IL-6R antagonists have been shown to reduce osteoclastogenesis and reduce bone resorption in arthritic mouse models (54). The sIL-6R has also been shown to be of importance *in vivo,* as co-administration of mice with IL-6 and sIL-6R resulted in restoration of arthritic diseased states in IL-6 KO models (55). Having said this, some studies using male IL-6 KO mice demonstrated phenotypes of advanced osteoarthritis upon aging, complicating the story (56). Overall, most findings suggest that IL-6 induces RA initiation and progression (57).

Currently, targeting IL-6 therapeutically for RA has been successful in the clinic, although the negative side effects from this therapeutic approach should not be minimized. The U.S. Food and Drug Administration declined to approve Johnson & Johnson's RA anti-IL-6 drug called sirukumab, saying additional clinical data is needed to further evaluate its safety in September of 2017 (58). Sirukumab is a chimeric (murine-human) IgG1κ monoclonal antibody (mAb) that binds and neutralizes human IL-6. FDA panelists were concerned about an imbalance in the number of deaths in patients taking sirukumab compared with those taking a placebo, but Johnson & Johnson are continuing further development and testing of the drug (58). The most common causes of death were major heart problems, infection and cancers (58). However, Genentech's drug known as Actemra® (tocilizumab), the first humanized anti-IL-6 receptor agonist, has been FDA approved since 2010 for RA and was recently approved (November, 2018) as a prefilled autoinjector known as ACTPen™ for RA and patients with giant cell arthritis (GCA), active polyarticular juvenile idiopathic arthritis (PJIA) or active systemic juvenile idiopathic arthritis (SJIA) (59). Tocilizumab can cause serious side effects however (59), and hence safer options are still desired for patients with RA and other inflammatory diseases. Still, effects of tocilizumab on bone look promising, as different clinical trials have reported that patients experienced a decrease in osteogenic inhibitor DKK1, CTX-1 bone turnover markers, and in erosions of bone in the hands, and an increase in OPG with bone histology and P1NP bone formation markers in blood (60–65).

In May of 2017, Regeneron Pharmaceutical's Kevzara® (sarilumab), another IL-6 receptor antagonist, received FDA approval for treatment of adult patients with moderately to severely active RA (66). Similarly to the IL-6/IL-6R-targeting drugs, sarilumab also increases the risk of serious side effects that may lead to hospitalization or death, largely due to the suppression of the immune system (66). These therapies would be greatly improved if IL-6 could be targeted in the tissues or areas of interest rather than systemically. Moreover, better success may be achievable using bi-specific antibodies, such as one termed MT-6194 currently in preclinical studies targeting IL-17A and IL-6R (67). Research into these and other innovative ways to target IL-6 are crucial new directions for the next generation of anti-IL-6/IL-6R therapies, but as many of the current therapies already have FDA approval, despite toxicities, it is unlikely that pharmaceutical industries feel the financial incentive to move in this direction in their Research and Development departments. It may depend on academic scientists, doctors, patients, or

companies that do not have a currently FDA approved anti-IL-6 therapeutic, to push for improvements and innovations in targeting IL-6.

IL-6 and Osteoporosis

Aging increases the production of inflammatory molecules, including IL-6, which explains some of the increased prevalence of inflammatory disorders (diabetes, lupus, and RA) in aging (68–70). Osteoporosis is also common in older and post-menopausal women due to a natural reduction in estrogen levels and high IL-6 levels, adding to increased osteoclastogenesis and pronounced osteopenia (71, 72). Ordinarily, the estrogen isomer 17-β-oestradiol (E2) reduces monocyte secretion of IL-6 and IL-8 (73), and thus after menopause, these pro-osteoclastic cytokines become more abundant (74). E2 interacts with the NFκB pathway, which is ordinarily held in an inactive state by coupling with an inhibitor known as IκBα (**Figure 2**). Certain signals result in IκBα becoming phosphorylated, uncoupling from NFκB and releasing it from its inactive state, allowing for signaling of downstream genes (75). Studies show that E2 promotes IκBα coupling to NFκB, keeping it in an inactivate state (76). This indicates how a loss in estrogen can result in increased IL-6 and NFκB signaling, and a consequential increase in bone loss and osteoporosis.

Bone loss induced by the E2-NFκB-IL-6 pathways can be reversed by administration of E2 (72) or anti-IL-6 neutralizing antibody (77–79). Clinically, post-menopausal women demonstrate an increased risk of osteoporosis, and patients with certain polymorphisms in the IL-6 promoter show further increases in bone resorption rates and reduction in BMD (80). This highlights how variation in IL-6 expression within patients can alter the risk of osteoporosis in estrogen-depleted environments. Currently, targeting IL-6 therapeutically for osteoporosis has not been explored, but this is an interesting clinical future direction that holds great potential if the side effects from these types of therapies can be minimized.

IL-6 AND BONE-METASTATIC CANCER

Paul Ehrlich first proposed the function of the immune system as the first line of defense against cancer in the early twentieth

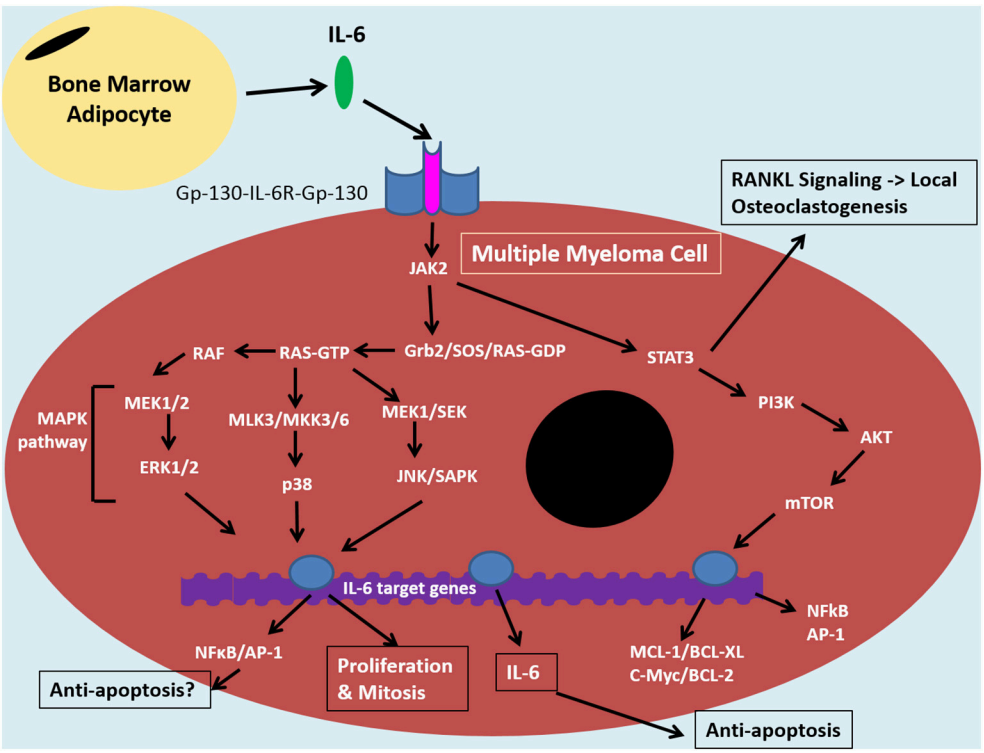

FIGURE 2 | IL-6 signaling in the tumor cell of the bone marrow niche. The bone marrow microenvironment augments MAPK and PI3K/Akt pathways resulting in anti-apoptotic and NF-κB signaling in multiple myeloma cells. Binding of IL-6 to IL-6R and Gp-130 co-receptors induces JAK-2 signaling. This signaling cascade diverges down STAT3/PI3K/Akt pathways and various MAPK pathways, including MEK/ERK. The former is associated with promoting anti-apoptotic proteins: MCL1, BCL-XL, BCL-2 and c-Myc, which contribute to drug resistance. STAT3/PI3K/Akt can also promote NF-κB signaling which results in release of angiogenic and inflammatory molecules, such as IL-6. This can feed into an autocrine positive feedback loop. IκBα can inhibit NFκB through coupling and this interaction can be maintained by certan signals, such as by estradiol. The IL-6 signaling pathway in MM cells is similar to that of bone marrow stromal cells and overall it promotes an inflammatory microenvironment in the bone which results in bone loss, increased tumor burden and disease progression. Bone marrow adipocytes (BMAs) are one cell within the bone microoenvironment thought to feed into this system through secretion of IL-6. This can promote anti-apoptosis and disease progrssion through NF-κB signaling. BMAs, thus, represent an ideal target for MM therapies in order to reduce drug resistance and relapse, instead of targeting the complicated, clonally expanding plasma cell.

century. Since then, the roles of immune and stromal cells, and their secretory repertoires, have been extensively studied in conjunction with cancer. Inflammation in particular has been shown to be key in promoting cancer proliferation and differentiation, with IL-6 being an integral player (81). Many cancer types have been shown to be associated with high serum IL-6 including colorectal (82), breast (83), and prostate cancer (84), as well as MM (85). These cancers induce phenotypic changes in the surrounding stromal cells, creating unique cell types, such as tumor-associated macrophage (TAMs) (86, 87) and cancer-associated fibroblasts (CAFs) (76, 77). These activated stromal cells, along with $CD4^+$ T cells and myeloid-derived suppressor cells (MDSCs), are the main sources of IL-6 in most tumor microenvironments, alongside the tumor cells themselves (88–92). Consequently, these BMM cells are associated with creating pro-oncogenic environments that promote proliferation and progression of cancer cells by favoring angiogenesis and metastasis, and inhibiting apoptosis (93–96). For example, knockdown of IL-6 by RNAi within CAFs can attenuate metastatic phenotypes *in vivo* (94). It is not surprising that level of IL-6 within the serum of various cancer patients is a key prognostic indicator for their response or disease progression (88). As a rule, high levels of IL-6 are associated with aggressive forms of cancers (97–100) and IL-6 levels can act as independent markers of prognosis in certain cancers (101). Furthermore, high expression of IL-6 has been more closely linked to recurrent tumors than primary tumors (102). IL-6 promotes proliferation (103), facilitates an epithelial-to-mesenchymal transition (94, 104) and enhances angiogenesis via VEGF stimulation (105, 106). Increased IL-6 in cancer cells results in subsequent release of IL-6 by stromal cells, thus feeding into the pro-tumoural feed-forward loop and exacerbating the diseased state (107–109).

Chemotherapeutics and ionizing radiation are the most common cancer therapies that work by disrupting DNA and protein synthesis pathways resulting in apoptosis. Yet both are known to stimulate oxidative stress, the NFκB pathway, and consequently IL-6 (110–114). IL-6 stimulation then counteracts the chemotherapy effect by promoting anti-apoptotic pathways to cause drug resistance (115). The anti-myeloma drug bortezomib targets the 26S subunit of the proteasome, the cellular machinery involved in degrading misfolded proteins, and causes an accumulation of unwanted/toxic proteins within cells (116). This accumulation of proteins is particularly seen in cells that already have dysfunctional or excessive protein-folding processes, such as myeloma cells (117). Misfolded protein accumulation results in increased ER stress, cell cycle arrest and programmed cell death (118). However, IL-6 has been shown to induce anti-apoptotic pathways in certain cancers, thus attenuating the efficacy of this drug (119–121). Gemcitabine is a nucleoside analog that causes termination of DNA synthesis (122). Gemcitabine targets fast-replicating cancer cells and resistance to this drug may also be tied to IL-6, as evidenced by a study where gemcitabine-resistance was observed in cancer cells treated with IL-6 and sIL-6R. This was thought to be due to a IL-6-prompted barricade of cells in G0/G1 cell cycle stages, preventing cells from entering the necessary cell-division stages necessary drug-induced replicative-associated destruction (26, 123–125).

As stated above, IL-6 also plays roles in apoptosis, which occurs due to cellular mishaps as a means to reduce malignancy. Interestingly, IL-6 has been shown to play both pro- and anti-apoptotic roles depending on tumor type. In some p53-wild-type breast and colon cancers, when tumors were treated with kinase inhibitors and general chemotherapies, IL-6 was shown to induce apoptosis via p53 and STAT5, with downstream signaling occurring through BAX [the reciprocal protein to anti-apoptotic BCL-2 (126)]. On the other hand, certain cancers, including many bone-metastases, have an opposing phenotype and demonstrate a sustained and even enhanced proliferation due to IL-6 and similar cytokines (113–115).

Bone metastatic cancer cells can become dormant within the BMM, thus protecting them from elimination by the immune system (127). It is common for these tumors to eventually become proliferative and induce osteoclastogenesis through secretion of soluble factors (128, 129). Melanoma cell lines have been shown to home to the bone and induce bone destruction through a TGF-β-IL-11 axis via osteoblasts (130), whilst other cancers facilitate similar pathways, such as TGF-β-RANKL-mediated destruction (131). IL-6 and its bone destructive roles can be tied to certain cancers, including MM and neuroblastoma, both of which induce IL-6 secretion from the surrounding stroma resulting in bone loss through the NFκB pathway (132, 133). MM cells derive from the immune plasma cell, home to the BM, cause bone destruction, and eventually become drug resistant. IL-6 plays a role in all these steps, so this review will focus on the effects of IL-6 in MM, as a disease model, from here on.

IL-6 and MM Homing to the Bone Marrow Niche

Chemokine gradients, such as that created by SDF-1, play a large role homing of cells to the BMM. The CXCR4-SDF1 axis is known to promote IL-6 expression in numerous cancers, including MM (3, 134), and other studies have also indicated that IL-6 may promote CXCR7-SDF1 or CXCR4-SDF1 signaling (135), and as such promote bone-homing (136). SDF1 is constitutively expressed by stromal cells and osteoblasts in the BMM (127, 128), and attracts $CXCR4^+$/$CXCR7^+$ haemopoietic or tumor cells to the BMM (137). B-cells, MM cells, and other bone metastatic cancer cells, including breast and melanoma cells, express CXCR4, which functions in the proliferation and migration of these cells to the SDF1-rich BMM (138–141). The CXCR4-SDF1 axis can also promote the expression of molecules that enable adhesion of cells to the endothelial lining of the BM sinus, such as VCAM1 (142). Additionally, α4β1 integrins can overexpressed due to CXCR4-SDF1 signaling, which can further enhance homing to the BM (142, 143). In contrast, TGF-β1 supresses expression of SDF-1 and has been shown to reduce BM stromal cell (BMSC) migration and adhesion, which could translate to a reduced ability to traffic to the BM (144). It is unsurprising that a CXCR4 inhibitor has been shown to disrupt

MM cell interaction with the BMM and thus increase sensitivity to treatment (145).

Myeloma cells also commonly express cell surface receptors CCR1 and CCR2 (146, 147), which bind MIP-1α and MCP1-3, respectively. Similar to SDF1, these ligands are expressed highly by BMSCs and promote myeloma cell migration and homing to the BMM (148, 149). All three receptor axes (CCR1, CCR2, CXCR4) are interesting from a clinical point of view as patients with low expression of these receptors have shown poor disease prognosis, associated with high serum β2 microglobulin and C-reactive protein (146). The former is a molecule secreted by B cells and acts as a marker of disease progression and poor prognosis in MM. The latter is a marker of systemic inflammation and again marks poor clinical prognosis in MM. It is possible that the downregulation of the receptors results in a reduced ability to home to and reside within the BMM and an increase in circulating myeloma cells, which may cause more systemic spreading of the disease. However, this contradicts our typical understanding that tumor cells are more protected in the BM, and thus cause worse outcomes when lodged in the bone rather than when in circulation. Interestingly, IL-6 KO mice are known to express lower levels of IL-2 (150), a cytokine known to promote CCR1 and CCR2 (151). Taken together, these data indicate that IL-6 may increase MM bone homing through promoting not only CXCR4 and possibly CCR2/3 signaling.

IL-6–Driven Bone Destruction in MM

MM also increases osteoclast number and activity, which leads to hypercalcaemia, renal-insufficiency, anemia, osteopenia and lesions in the bone. The breakdown of bone promotes chronic pain, increases fracture risk, and results in the release of bone-derived growth factors, which feed the tumor cells and promote disease progression (17, 18). This vicious cycle leads to a poor quality of life and ultimately results in prolific dissemination of these plasma cells throughout the body, a fatal condition known as plasma cell leukemia.

A number of factors feed into MM-bone loss including osteocyte-derived sclerostin. In MM patients, elevated circulating sclerostin levels are commonly seen, particularly in advanced stages (152). Sclerostin has anti-osteoblastic properties and anti-sclerostin therapeutics are currently being investigated for use in MM-associated bone loss pre-clinically (153, 154). Anti-sclerostin antibodies also reduce BM adiposity, and hence more research into their effects on bone or cancer though modulating BM adiposity is warranted (155, 156). DKK1 is another Wnt inhibitor involved in MM-mediated bone loss and disease progression that has been investigated as a target in MM bone loss (157). DKK1, like sclerostin, inhibits osteoblastogenesis by blocking differentiation of pre-osteoblasts by impeding the WNT signaling cascade (158). Recently, a bispecific antibody targeting sclerostin and DKK-1 has been shown to promote bone mass accrual and fracture repair in rodents and non-human primates (159). In addition to simply reducing numbers of mature osteoblasts, DKK1 also increases the number of undifferentiated BMSCs, and these undifferentiated BMSCs secrete higher IL-6 amounts than their differentiated counterparts (160), thus feeding into the IL-6-bone-destruction pathway described previously. Inhibition of IL-6 from MSC-conditioned media slowed the proliferation of DKK-1 secreting myeloma cells treated with this media, suggesting that in the myeloma environment, IL-6 increases bone resorption through promoting proliferation of DKK-1-secreting myeloma cells (160). However, in other contexts, a negative correlation between IL-6 and DKK1 was observed, implying that more research into the role of IL-6 in bone destruction would be useful (161).

IL-6 mediated bone loss also occurs due to direct expression and release of IL-6 by MM cells and consequential osteoclast activation. The mechanism of IL-6-mediated osteoclastogenesis is thought to be due primarily to activation of the JAK2/STAT3 axis, resulting in upregulation of RANKL molecules (24) (see **Figure 2**). This overthrows the fine balance between osteoclastic RANKL molecules and their inhibitor, OPG, pushing the equilibrium in favor of bone loss (162). In addition, IL-6 may also make preosteoclasts more sensitive to RANKL stimulation, presumably due to an IL-6-mediated upregulation of RANK receptors, which was previously shown in Paget's disease (163). *In vivo* studies have confirmed IL-6 mediated bone loss in both trabecular and endochondral bone; this bone loss is enhanced in the presence of MM cells and is associated with increased osteoclast differentiation (164). This finding is being investigated in phase II clinical trials, which are currently examining the efficacy of anti-IL-6 treatment on MM bone-loss (NCT01484275).

IL-6 and MM Disease Progression

MM begins as a condition known as monoclonal gammopathy of undetermined significance (MGUS), an asymptomatic stage where levels of immunoglobulin in the blood and risk of fracture are elevated. Blood serum IL-6 levels correspond to prognosis and progression of MM (85); prognosis is worse when levels exceed 7 pg/ml, with an average survival of 2.7 months compared to 53.7 months in those with lower levels (85). IL-6/STAT3 pathways are known to promote angiogenesis via enhancement of VEGF in MM (165). Osteoclasts are also known to secrete pro-angiogenic molecules, and thus in MM when both osteoclast number and activity is enhanced, there is also an increase in the development of vasculature (166). IL-6/STAT3 signaling not only promotes the creation of these new endothelial cells, but also stimulates Ras, Akt and MAPK pathways which promotes the survival of said MM cells (167).

IL-6–Mediated Drug Resistance and Survival in MM

Apoptosis involves the activation of caspase enzymes in a cascade ending in the activation and release of apoptosis-inducing factors from mitochondria. Apoptosis-inducing agents cause DNA fragmentation and chromatin condensation, which ultimately induces cell death (168). IL-6 promotes JAK/STAT3 and consequently PI3K/Akt and MEK/MAPK (169), (**Figure 2**); these pathways are known to upregulate anti-apoptosis proteins Mcl-1, Bcl-XL and c-Myc in primary MM cells, resulting in cell survival and chemotherapy resistance (170, 171). A number of antibodies have been investigated to neutralize IL-6, but little success has occurred clinically (172, 173). However, evidence

indicates that indirect targeting of downstream anti-apoptosis mediators can help reverse the effects of IL-6. *In vitro,* anti-sense targeting strategies against MCL-1 sensitized MM cells to drugs by inhibiting these anti-apoptotic pathways (174). In addition, IL-6-mediated resistance was reversed by targeting CDC34 (175). This molecule is an ubiquitin-conjugating enzyme involved in proteasomal degradation. It has been tied to ubiquitination and degradation of IκBα, the NFκB inhibitor, and thus promotes drug resistance via NFκB signaling. Targeting other molecules within the JAK/STAT3 cascade also appears to aid in resistance reversal, as the Akt inhibitor, MK2206, has also been found to help overcome bortezomib resistance in MM cells induced by co-culturing with IL-6 or MSCs (176). [For more on IL-6 and proteasome inhibitor resistance in MM, we refer the reader to our recent review may (121)].

IL-6 may also cause drug resistance through epigenetic modulation proteins. IL-6 signals via STAT3 and enhances DNA methyltransferase 1, which promotes the methylation and thus deactivation of P53, facilitating cells to avoid cell cycle checkpoint destruction (177). This could clearly have unfavorable effects in MM, promoting drug resistance and/or disease progression. Initial studies into use of general histone deacetylase (HDAC) inhibitors were unfavorable (178), however, subsequent use of selective HDAC inhibitors, such as chidamide, have shown anti-MM and bone protective effect, showing synergistic effects with other therapies such as dexamethasone, carfilzomib and pomalidomide (179).

Bone Marrow Adipocyte (BMA)-Derived IL-6 Contribution to MM Progression

Obesity is an increasingly common condition which increases one's risk of cardiovascular disease, diabetes, certain cancers, and many other diseases. One link to cancer is due to the increased inflammatory state which occurs due to obesity (180). The excess storage of lipids within adipose tissues causes the release of inflammatory molecules, TNF-α and IL-6, and suppression of anti-inflammatory adiponectin (181). This creates a microenvironment of high oxidative stress and inflammation and results in damage to tissues, increasing one's risk of oncogenic mutations and tumorigenesis (180). Increased fat intake, which typically causes obesity over time, is linked to higher levels of BM adiposity in rodents (182); recently, BM adipocytes (BMAs) have been shown to play significant roles in MM drug resistance and disease progression (183), possibly through the secretion of soluble factors, such as IL-6 (184). BMAs have such a profound effect on MM that, clinically, obese and older populations, both of whom suffer from enhanced systemic inflammation and increased BMA levels, demonstrate higher risk of developing MM than other groups of people (185).

BMAs may contribute to disease progression in numerous ways. *In vitro* work has demonstrated an ability of BMAs extracted from MM patient femurs to support MM cell growth and protect them from chemotherapy-induced apoptosis (186). In addition, MM is known to increase expression of PGC-1α within BMAs, resulting in VEGF and GLUT-4 expression (187), which increases proliferation, angiogenesis and metastasis, as described above. BMAs have also been shown to increase expression of autophagic proteins within MM cells, which can promote drug resistance (186). Finally, BMA-derived IL-6 has been hypothesized to contribute to chemotherapy resistance due to an upregulation of anti-apoptotic proteins and inhibition of cell checkpoint proteins (**Figure 2**). The latter pathway remains unverified, yet studies are currently underway to investigate this further. If true, IL-6 and its downstream signaling molecules represent good targets for possible re-sensitization of MM cells in refractory disease. Investigation into the blockade of anti-apoptotic pathways downstream of IL-6 and other IL-6 mediated pathways is warranted. In addition, efforts should be made to find therapeutics that target BMAs specifically, and to investigate the secretory repertoires of all BMM cells to better understand and manipulate the microenvironment into one of anti-tumorigenicity.

Clinically Targeting IL-6 in Cancer

Despite extensive pre-clinical *in vitro* and *in vivo* support for the role of IL-6 in MM and other osteolytic bone cancers, clinical translation results have been dismal. Clinical trial results with siltuximab, formerly CNTO 328, in MM have been disheartening and no clinical trials in other bone cancers have been initiated as of yet. As presented in an abstract for the American Society of Hematology (ASH), 2017 annual meeting, IL-6 blockade did not add benefit to chemotherapy in a phase 2, randomized, double-blind, placebo-controlled multicenter study in patients with high-risk smoldering MM (188). A clinical trial examining if siltuximab can decrease symptom burden after autologous stem cell transplantation for patients with MM or AL amyloidosis is currently underway (ClinicalTrials.gov Identifier: NCT03315026). No data on MM and siltuximab was presented at the ASH 2018 conference. A study of CNTO 328 in Japan from 2011 to 2014 in relapsed or refractory MM patients was halted due to safety concerns (ClinicalTrials.gov Identifier: NCT01309412). An open-label, phase I trial of CTNO with lenalidomide, bortezomib and dexamethasone (RVD) was performed (ClinicalTrials.gov Identifier: NCT01531998), however the efficacy data were limited by the small number of patients since the trial was halted and did not proceed to phase II. The abandonment of the phase II trial was due to the negative outcomes from another phase II study of bortezomib-melphalan-prednisone (VMP) vs. VMP+siltuximab, which demonstrated no significant improvement in progression-free survival. After this finding, further development of siltuximab in symptomatic myeloma was halted by the sponsor (189). Trials have also been initiated examining the safety and efficacy of siltuximab in metastatic, hormone-refractory prostate cancer, renal and other solid tumors (ovarian, pancreatic, colorectal, and others), and the field is currently waiting to see the results of these trials (190).

Tocilizumab, a humanized anti-IL-6R mAb, is currently the subject of investigation in many clinical cancer trials. Current clinical trials are ongoing for metastatic HER2-positive breast cancer resistant to trastuzumab, lymphoblastic leukemia, pancreatic cancer, myeloid leukemia, B-cell chronic lymphocytic leukemia, non-Hodgkin's lymphoma, B-cell lymphoma, and diffuse large B-cell lymphoma (ClinicalTrials.gov). Trials in myeloma were terminated due to low accrual rate (ClinicalTrials.gov Identifier: NCT02057770). In Europe, there

is currently a clinical trial to explore the use of tocilizumab in fibrous dysplasia of bone (FD), a rare, painful bone disease affecting one or several bones (191). If promising data result, more clinical trials may arise studying the drug for bone pain relief applications.

Unfortunately, overall, large randomized trials show no efficacy of IL-6 inhibitors in various cancers, particularly myeloma. These results are despite a full inhibition of C-reactive protein (CRP) production in treated patients, the numerous preclinical studies showing an involvement of IL-6 in these diseases, and initial short-term treatments demonstrating a dramatic inhibition of cancer cell proliferation *in vivo* (192). Similarly, a meta-review of 48 clinical studies concluded that inhibition of IL-6 has unknown and unproven effects on decreasing GI (gastric, pancreatic, colorectal, bile duct and gall bladder) cancer syndromes or improving quality of life (191). A likely explanation for this lack of efficacy is the plasticity of cancer cells and their ability to clonally evolve and develop subclones that are less dependent on IL-6. Moreover, many therapies targeting tumor cells already decrease IL-6 or pathways downstream of IL-6, so that no additive or synergistic effect is derived from the anti-IL-6 therapy. Still, anti-IL-6 therapeutics are able to neutralize IL-6 production *in vivo* and are safe and useful in inflammatory diseases and Castleman disease. Their application may hold promise in treatment of bone-resident cancers if more developed (e.g., bi-specific mAbs against tumor cells and IL-6, or against RANKL and IL-6) therapies can be developed to increase their efficacy. Moreover, getting the treatment more specifically to the cancer may allow for higher doses and less off-target effects, and thus better outcomes, and more research into tumor-homing drug delivery or targeted nanomedicine technology may accelerate this process. As we understand more about the role of the immune system in cancer and the ability for tumor cells to block the immune response, researchers and clinicians may be able to design anti-IL-6 clinical trials using patient populations that are identified to have a positive response to these therapies. Similarly, a better understanding of the full activity of anti-IL-6 therapy will mean that different combination regimens or dosing strategies may be designed to be optimal for different patient subgroups.

CONCLUSIONS

IL-6 is a pro-inflammatory cytokine that promotes NF-κB, MAPK and PI3K/Akt signaling. Together these pathways promote anti-apoptosis signaling and drug resistance in cancer cells, in addition to further inflammatory signaling. The latter contributes to bone destruction and osteopenia, which promotes homing of metastatic cancers to the bone marrow niche. Bone marrow adipocytes, as well as mesenchymal stromal cells, may be a source of IL-6 or other factors, that contribute to chemotherapy resistance in the bone microenvironment. Targeting of these adipocytes, or their secreted factors, may help alleviate refractory disease.

Because MM demonstrates such genetic heterogeneity and high levels of refractory disease, targeting the BMM, along with the tumor cell directly, is an ideal plan of attack. The IL-6 signaling pathway in MM cells promotes an inflammatory bone microenvironment that results in osteopenia, increased tumor burden and disease progression. Bone marrow adipocytes are one cell type within the bone microoenvironment thought to feed into this system through secretion of IL-6 and other adipokines. Thus, bone marrow adipocytes represent an ideal target for MM therapies in order to reduce drug resistance and relapse, instead of targeting the complicated, clonally expanding plasma cells. The more we learn, the better we can target such pathways and not only improve quality of life for patients, but hopefully extend that lifetime as well. As this dream has not become realized in the clinic yet, more efforts on understanding and maximizing the targeting of IL-6 and its downstream pathways may be necessary. Moreover, building mouse cancer models, or tissue-engineered 3D models, that more accurately model the effects of targeting IL-6 in the human should lead to better clinical results in the future and avoid the pitfalls of wasted time and money on unsuccessful clinical trials.

AUTHOR CONTRIBUTIONS

DH wrote the manuscript. CF and MR edited the manuscript. All authors approved the manuscript. MR takes responsibility for the manuscript.

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Physical Activity and Bone Health: What Is the Role of Immune System? A Narrative Review of the Third Way

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Bone tissue can be seen as a physiological hub of several stimuli of different origin (e.g., dietary, endocrine, nervous, immune, skeletal muscle traction, biomechanical load). Their integration, at the bone level, results in: (i) changes in mineral and protein composition and microarchitecture and, consequently, in shape and strength; (ii) modulation of calcium and phosphorous release into the bloodstream, (iii) expression and release of hormones and mediators able to communicate the current bone status to the rest of the body. Different stimuli are able to act on either one or, as usual, more levels. Physical activity is the key stimulus for bone metabolism acting in two ways: through the biomechanical load which resolves into a direct stimulation of the segment(s) involved and through an indirect load mediated by muscle traction onto the bone, which is the main physiological stimulus for bone formation, and the endocrine stimulation which causes homeostatic adaptation. The third way, in which physical activity is able to modify bone functions, passes through the immune system. It is known that immune function is modulated by physical activity; however, two recent insights have shed new light on this modulation. The first relies on the discovery of inflammasomes, receptors/sensors of the innate immunity that regulate caspase-1 activation and are, hence, the tissue triggers of inflammation in response to infections and/or stressors. The second relies on the ability of certain tissues, and particularly skeletal muscle and adipose tissue, to synthesize and secrete mediators (namely, myokines and adipokines) able to affect, profoundly, the immune function. Physical activity is known to act on both these mechanisms and, hence, its effects on bone are also mediated by the immune system activation. Indeed, that immune system and bone are tightly connected and inflammation is pivotal in determining the bone metabolic status is well-known. The aim of this narrative review is to give a complete view of the exercise-dependent immune system-mediated effects on bone metabolism and function.

Keywords: exercise, training, biomechanical loading, inflammatory mediators, inflammasomes

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INTRODUCTION

Exercise, particularly when energetically demanding and sustained, affects all human tissues and organs. Different kinds of exercise (e.g., endurance, high intensity, resistance) have different effects on tissues and organs homeostasis and, consequently, different kinds of training require different adaptive changes that might take place (1). Bone is importantly affected by exercise and bone cells metabolism forcefully adapts to training. This metabolic adaptation reflects, on long-term basis, in a micro-architectural, and possibly macro-architectural, redefinition of bone shape and structure. The biological meaning of this phenomena resides in the physical adaptation of bones (in terms of shape, mass, and strength) to the changed environmental conditions (applied forces) (2). However, the bony response to exercise does not only depend on the biomechanical stimulus but equally relies on other systemic mechanisms that make the bone a center for the integration of the signals generated during and following the exercise (3). A main signal is the metabolic one: bone metabolism is highly-demanding in terms of energy; parallel, during exercise the energy needs increase with the increasing duration and intensity. Being not a lifesaving function, bone metabolism is partially blocked in response to acute exercise, regardless the degree of biomechanical load. This block mainly pertains the anabolic function (osteoblastic function, i.e., bone formation) while leaves almost unaltered the catabolic side (osteoclastic function, i.e., bone resorption). The acute unbalance toward bone resorption makes the calcium, stored in the bone matrix, available to skeletal muscles (SKM) and cardiac muscle for contraction and to the brain to sustain the neuromuscular function (4). On the other hand, the chronically established bone metabolic response to training is driven by the degree of load: weight-bearing/high impact activities (e.g., plyometric) shift the balance toward bone formation and on average increase bone mass and bone mineral density (BMD). Equally demanding activities featured by a low/absent biomechanical load (e.g., swimming, cycling) shift the balance toward resorption and causes a decrease in the average bone mass and BMD (3). Another key signal, generated during and following exercise and affecting bone metabolism, is the immune/inflammatory one. Inflammation, acute and chronic, is a main determinant for bone metabolism and the plethora of inflammatory mediators, produced under either physiologic or pathologic conditions, affects bone cells. Noteworthy, in last 15 years an interdisciplinary branch of study embracing (but not limited to) endocrinology, immunology, orthopedics, and rheumatology, namely osteoimmunology, has developed quickly thus becoming a central subject in metabolic diseases of bone (5). A key role of the osteoimmune networking has been highlighted by the clinical success and safety, over the classical anti-resorptive drugs (6, 7), of the fully human monoclonal antibody denosumab used in the treatment of primary and secondary osteoporosis, in both males and females (8), which is an inhibitor of the prototypic osteoimmunological signaling pathway, the RANK/RANKL system, as described elsewhere (9). Acute exercise represents a powerful inflammatory stimulus itself while the inflammatory response to training is a handbook example of adaptation to a continuous stimulus. Exercise, indeed, initiates a series of inflammatory events, which ultimately, if chronically continued, positively affects health. The inflammatory response to exercise, however, takes place in the immune system (IS), involving mainly the innate compartment with fallouts on the humoral immunity, as well as in tissues other than the IS, e.g., SKM, white and brown adipose tissues (WAT, BAT), brain, liver (3, 10, 11). Notably, the acute response elicited by exercise in terms of inflammatory mediators is often very similar to that observed during pathological acute and chronic inflammatory conditions (e.g., sepsis, obesity, autoimmune diseases, tumors) but what determines the net result of this response (pro- vs. anti-inflammatory) is the milieu in which this response is generated, together with its temporal length and the site of production. As an example, interleukin (IL)-6 expression is strongly induced by time, intensity and type of muscle contractions-dependently as well as in sepsis (various origin) but, in the latter case, it is driven by the previous increase in another cytokine, the tumor necrosis factor (TNF)-α (12). Based on the interdependence between bone and IS, it is implicit that the acute inflammatory response induced by exercise- and the modulation of the inflammatory status induced by chronic training both affect bone cells differentiation and function and, in turn, the bone metabolism. It should be kept in mind that, other than a target of these endocrine and inflammatory stimuli, the bone is itself a source of these mediators and, hence, it actively enters in the regulation/modulation network of the homeostasis (3).

This review aims at summarizing the experimental evidences about the exercise- and training-dependent effects on bone mediated by the IS and the other inflammatory sources in order to depict additional, and less explored, link between bone and inflammation as further mechanisms by which the physically active status is a main determinant for health.

In order to go deep into the description, it is necessary to introduce some key concepts, relative to the exercise physiology, that will help the reader in fully understand the biological meaning underlying the homeostatic response. About the types of muscle actions, one can distinguish between static (isometric) and dynamic (isotonic) actions. During the static (isometric) action, muscles generate force without changing length due to an external resistance (weight of an object) greater than the force produced by the muscle; despite the energy expenditure, no work is done due to lack of movement. The isotonic action, instead, can be either (i) concentric, when muscles produce enough force to overcome the external resistance and the muscle contraction results in a work, or (ii) eccentric, when the muscles lengthens while generate a force due to the opposite movement of the external resistance and the sarcomere shortening (13). Another series of concepts regard the terms physical activity (PA), exercise, and training. PA is defined as any bodily movement produced by SKM resulting in an energy consumption. Everyone performs PA in order to sustain life but the amount is subject to personal. The term "exercise," instead, although often erroneously used interchangeably with PA, is defined as a PA featured by planning, structure, and repetitiveness, which is aimed at maintaining or improving the

TABLE 1 | Common and distinctive features of physical activity and exercise.

Physical Activity	Exercise
Body movement generated by skeletal muscles	Body movement generated by skeletal muscles
Variable energy expenditure	Variable energy expenditure
Positive correlation with physical fitness	Positive correlation with physical fitness
	Planned, structured, and repetitive
	Aimed at maintain/improve physical fitness

physical fitness (14). The common and distinctive features of PA and exercise are summarized in **Table 1**.

When a single bout of exercise (acute exercise) is continued over the time, in the same fashion, it is defined training (exercise training). Finally, the different types of exercise and training can be categorized as follows: (i) endurance, mainly based on the aerobic metabolism (e.g., distance running, road cycling, swimming, triathlon), (ii) resistance (also known as strength), mainly based on the anaerobic metabolism (e.g., weight lifting, discus, hammer, and javelin throw) (15).

HOW DO EXERCISE AND TRAINING AFFECT BONE METABOLISM?

The responsiveness of bone to mechanical stimulation was first theorized by Frost who postulated, with the "mechanostat" hypothesis, bone mass and structure remain constant around a certain threshold of mechanical strains. Bone formation takes place when the strain increases above this threshold, and it results in an increased bone stiffness. When the strain experienced by the bone segment is lower than this threshold bone loss can take place (16). Later, it was shown that the threshold itself is modifiable by several factors, mainly endocrine [parathyroid hormone (PTH), sex hormones, etc.] (17). However, despite its importance, the mechanical strains induced by strenuous PA is very small degree attesting to up to 0.3% (3,000 microstrain) (18). Based on that, it is likely that bone cells are exposed to and integrate different PA-generated mechanical stimuli that altogether imply an amplification of the environmental stimulation. A further level of complexity is due to the fact that different types of bone cells are anatomically exposed to different combinations of stimuli. Bone marrow and endosteal osteoblasts experience the pressure forces generated within the marrow cavity. Osteocytes buried into the matrix with their interconnecting long cellular processes running within the fluid-filled canalicular network experience dynamic fluid flow pressure, shear stress forces, and dynamic electric fields (due to the transit of charged ions in the interstitial fluid). Mature osteoclasts and their precursors, residing in the bone marrow, may be exposed to mechanical stimulation due to dynamic pressure (19). Bone mechanosensitivity is mediated by several cellular components (e.g., membrane, membrane proteins, cytoskeleton, cilia, ion channels). Shear stress and pressure deform the plasma membrane and, consequently, to the cytoskeleton and, in turn, through integrins to the protein machinery mediating the cell-to-matrix adhesion and to the nucleus where it induces the expression of downstream genes (20). In osteoblast, the deformation of the plasma membrane is associated with the activation of ion channels (21), as in osteocytes, whose cilia, protruding out of the dendritic extensions, sense fluid flow and activate channel-mediated ion fluxes that modulate the Wnt signaling pathway (22).

The different nature of the mechanical stimuli together with the number of cell structures involved in mechanosensitivity imply the integration of the different signals generated (19). Indeed, the physical stimulus is translated into several chemical signals including calcium, mitogen-activated protein kinase (MAPK), Wnt, and RhoA/ROCK pathways. For instance, the Frizzled-LRP5/6-mediated activation of Wnt induces the expression of osteoblastic factors, as RUNX2 that promotes the commitment of mesenchymal stem cells (MSCs) toward the osteoblast lineage, induces proliferation and differentiation of pre-osteoblast, and stimulates mineralization. Hence, exercise shifts the adipogenic-to-osteogenic equilibrium, governing the MSC fate, toward the osteoblastic commitment (23, 24). By modulating the OPG and RANKL expression in osteoblasts, Wnt signaling also downregulates osteoclastogenesis and osteoclast activity (25). A key mechanism regulating the Wnt activity that underlies the exercise-associated effects on bone is mediated by sclerostin (Sost) (26). Osteocytes constitutively produce Sost that inhibits the Wnt pathway, thereby osteoblastogenesis and bone formation. Loading activates a molecular response that inhibits Sost expression and, then, allow the activation of Wnt signaling. Noteworthy, also prostaglandin E2 (PGE2), which is induced by strain sensing, by activating its receptors EP2 and EP4, facilitates the nuclear translocation of β-catenin and its transcriptional action (27). Finally, the intracellular signaling generated following mechanical stimulation can be propagated to neighbor cells as calcium (e.g., between osteoblasts and osteocytes) or adenosine triphosphate (ATP; e.g., osteoblast-to-osteoclast) transients through the gap junction network (28, 29).

Another way by which exercise and recovery beneficially affects bone metabolism, mineral content, and structure is by increasing the blood flow to the bone and the consequent improved supply of nutrients supporting its metabolic needs (30, 31). Indeed, also from this point of view, bone is a really active tissue as demonstrated by the fact that blood flow to the bone and glucose uptake within the bone tissue are increased in response to exercise (32). However, as exercise load increases blood flow offs due to the sympathetic response that shifts the flow to the active SKM. Moreover, perfusion in bone, which ranges over a window wider than that expected during exercise, is mainly ruled by chemoreceptors, and hence nutrients, rather than by hypoxia (33). The exercise-induced enhanced bone perfusion also causes an increased efflux of stem cells from bone marrow (e.g., endothelial precursors) (34), consequently to the endothelial production of nitric oxide (35) and sympathetic nervous system activation (36). Finally, exercise-induced muscular-derived adenosine could solve another key role in bone blood flow determination (37).

The Effect of Acute Exercise

Despite the key role of loading, the bone tissue response of the to a single bout of exercise is mainly driven by the

exercise-dependent metabolic requests of noble organs and of those organs that are directly involved in the activity. In other terms, during PA, and mostly dependent upon its intensity and duration, the metabolic needs of non-life saving organs (e.g., the bone, skin, gut) are somehow "sacrificed" in order to have all the fuels available for being used by brain, SKM, and liver (3). After the conclusion of the PA, during recovery, and with the chronic and/or long-term repetition of the act, the loading/impact-induced anabolic response takes place. This means that during PA the bone metabolism is mostly unbalanced toward resorption. This is also a consequence of the key role of bone in calcium homeostasis: PA implies the usage of calcium in terms of functioning of the contractile machinery of the SKM, release, and recycling of neurotransmitters and hormones. Being resorbed, bone make the calcium promptly available. Indeed, two activities both characterized by high energy expenditures but differentiated by kind of biomechanical loading, one featured by high load and impact (mountain ultra-trail) while the other non-impact/loading (cycling), both display an anti-anabolic/pro-catabolic acute response of the bone to the activity (38, 39).

By considering the biomechanical aspects, only dynamic stimuli can generate an osteogenic response that, instead, is not induced by static loading (40) and strain amplitude, number of cycles and interval between the cycles are important, as well (41, 42). Indeed, the anabolic response can be desensitized by long-lasting activities while interval rest between the load cycles may have an osteogenic effect (43, 44). On the contrary, inter-cycles resting activates some mechanotransduction-sensitizing mechanisms that sustain the osteogenic response (28).However, too high magnitudes or number of cycles of load may fatigue the bone tissue and consequently can cause microdamage that, in turns, result in a series of catabolic events including local bone resorption (45).

The Effect of Chronic Exercise and Training

Life-long PA is associated with a better bone quality, thus potentially resulting in a stronger bone, e.g., improved cross-sectional area, BMD, and moments of inertia. These features, for example, and have been observed in gymnasts vs. non-gymnasts (46, 47), and dominant vs. non-dominant limbs of racquet sports players (48) or triple jumpers (49). By applying the same external force, the deformation experienced by "weak bones" is greater than that of "strong bones" and, consequently, it elicits in larger tissue strains. This results in a greater anabolic response in the weaker bone that attempts to become stronger (50). In experimental models, loads causing high strains induce bone formation in loaded areas, while areas with lower peak strains featured by reduced bone formation or even increased bone catabolism (51). Site-specific adaptation to loads takes also place in human: in women, after skeletal maturity, the adaptation to load is related to the energy equivalent strain, which means that regions undergone to high-level strain experience bone apposition than regions undergone to low-level strain (50).

However, the bone adaptation to chronic PA and training mainly depends upon the kind of activity. This was clearly demonstrated by Nikander et al. (52), who evaluate the bone quality of the narrowest section of the femoral neck [areal BMD (aBMD), hip structure analysis (HSA), cross-sectional area (CSA), subperiosteal width (W), and section modulus of strength (Z)] in 225 premenopausal women performing different sports. Women performing high-impact (volleyball, hurdling) and odd-impact (squash, soccer, speed skating, step-aerobic) loading sports displayed the highest aBMD (+23 and +29% vs. non-athletic women), CSA (+22 and +27%), and Z (+22 and +26%) even following adjustment for age-, weight-, and height. Contrarily, low-impact (orienteering, cross-country skiing) and non-impact (cycling, swimming) repetitive loading activities were associated with no gains in bone quality, compared with the inactive controls (52). A Cochrane review evaluating the preventive and therapeutic effects of training on postmenopausal osteoporosis established that exercise has a small (about 3%) but significant effect on bone mass and BMD. According to the meta-analysis, femoral bone mass mostly benefits from high-force non- weight-bearing exercises (e.g., resistance strength training of the lower limbs). Vertebral bone mass, instead, mostly benefits from the combination of exercises featured by different types of dynamic loading. Consequently, the risk of fracture across all exercise groups was not significantly different compared to the control groups (53). Exercise-based, either preventing or therapeutic, strategies aimed at affecting bone health might account for this finding. Indeed, based on our recent overview of systematic reviews and meta-analyses, lifelong age-specific exercise is effective in sustaining bone health in women. School-based short bouts of high-impact plyometric exercises positively affect peak bone mass in young girls, while combined-impact exercises represent the best exercise modality to preserve/improve BMD in both pre- and post-menopausal women (54).

Beside the quite well-depicted effects of chronic exercising on the physical feature of the bone, the effects on blood flow or metabolism of human bone are scarce (33). Although neglected, these studies are of key importance since they highlight a role for bone in regulating the whole-body metabolism (55–57) and also because the bone is central in the release of vascular precursor and immune cells. Thus, it emerges that by influencing bone status, exercise training could potentially affect vascular impairments in pathological conditions such as diabetes (33) and it may regulate the release of immune cells into the circulation and, hence, to directly control the inflammatory status.

As important as loading, or even more important, is detraining. Constant load (use of the skeleton) is essential for osteocyte survival and in case of bone immobilization osteocyte apoptosis occurs (58). Parallel, detraining causes bone loss and, hence, training might be continued to maintain bone mass (59) since unloading and disuse increase bone resorption rate. This situation is encountered, for instance, in astronauts under weightlessness conditions, spinal cord injured patients, and elderly people forced to either partial or total immobilization (19). Unloading affects both the cortical and trabecular portions of the bone: spinal cord injured subjects experience a 20–40% decrease in cortical thickness (60) and an average 14% (range 2–80%) in trabecular density (61); for astronauts the mean BMD loss of the trabecular compartment was 4% at the lumbar spine and 12% at the proximal femur (62).

HOW DO EXERCISE AND TRAINING AFFECT THE INFLAMMATORY RESPONSE AND THE IMMUNE FUNCTION?

Exercise profoundly affects the normal functioning of the IS with immune responses to single bouts being transient while an immune adaptation is likely to take place with training. Exercise dose is important in determining the entity of the immune response: prolonged intense training can have depressive effects (e.g., increased infection risk), while regular moderate-intensity exercise has more balanced effects that mainly results in the improvement of the baseline immune reactivity (63). Several evidences support this effect, chronic exercise has been demonstrated to improve immune, and hence health and behavioral outcomes, in several conditions of deregulated immune response, such as aging, obesity, cancer, and chronic viral infections (e.g., HIV) as well as in preventing their onset (63–66). Interestingly, acute exercise is effective in improving vaccines response (63). These effects are mediated, from one hand, by the cells of the IS belonging to both the innate and adaptive branches and, from the other hand, by all the cells of the body that are induced to express either a pro-inflammatory or anti-inflammatory phenotype. Exercise activates inflammatory cascades involving cells belonging to both the innate and adaptive immunity branches, cytokines, and mediators with active roles in inflammation (myokines, adipokines) that are in turn responsible for the generation of an environment in which recovery, regeneration, and adaptation take place. Exercise duration, mode, and intensity are the determinants for the exercise-induced inflammatory response. At the same time, training exerts anti-inflammatory actions through several distinct mechanisms involving metabolic, endocrine, and immune mediators of various tissues and organs (67). However, exercise exerts its anti-inflammatory effect only after the activation of pro-inflammatory cascades (67).

A special mention might be spent about cortisol. Although this adrenal hormone has anti-inflammatory immunosuppressant actions, and its occasionally increased circulating concentration (e.g., following PA) are beneficial in reducing inflammation, its chronically high levels (i.e., hypercortisolism) and deregulated rhythms are associated with aging and age-associated low-grade inflammation (68). Cortisol and the other endogenous glucocorticoids (GCs) are the final products of the neuroendocrine hypothalamus-pituitary-adrenal cortex (HPA) axis that is responsible for the regulation of both the energy balance and stress response. The physiological circadian fluctuations of GC levels allow the correct functioning of the intermediate metabolism and the development and the maintenance of the homeostasis of a wide all the body tissues, including the bone. Indeed, GCs are essential for bone modeling and remodeling as they promote osteoblastogenesis to maintain the bone architecture (69). Excessive energy intake (unbalanced energy intake-to-energy expenditure ratio) and adiposity are associated with chronic inflammation and stress which are in turn responsible for a deregulated of the HPA axis. Moreover, hypercortisolism is associated with a deregulated energy metabolism that in turn is responsible for the maintenance of the chronic inflammatory state (70). PA, by acting as a chronoenhancer, also impacting on the HPA axis, is able to improve the GC response in healthy subjects as well as to restore the circadian rhythm in age-associated low-grade inflammation dependent hypercortisolism and, hence, to improve the related comorbidities (68).

Effects of Exercise and Training on Inflammation

Effect of Acute Exercise

A single exercise bout starts a series of timely-defined inflammatory events, which mainly depend upon mode, intensity, duration, and training status (i.e., familiarity with the exercise). This cascade starts with a pro-inflammatory phase (1.5–24 h post-exercise) which is then followed by an anti-inflammatory phase that sustains SKM regeneration (24–72 h post-exercise). The exercise-induced inflammatory response is evidenced by the rise of the circulating levels of myokines (i.e., IL-6) and anti-inflammatory mediators (IL-10, IL-1ra). Moreover, exercise downregulates the expression, on the surface of antigen-presenting cells (APCs, e.g., monocytes) of those receptors involved in the recognition of danger signals, i.e., toll-like receptors (TLRs) (67). TLRs are highly evolutionarily conserved transmembrane proteins involved in the recognition of classes of molecules non-specifically associated with pathogens (pathogen-associated molecular patterns, PAMPs) and "danger signals" non-specifically induced/released following tissue damage due to physical, chemical, or biological agents (danger-associated molecular patterns, DAMPs) (71). TLRs activation leads to the expression of inflammatory cytokines. Acute exercise affects the expression of TLRs on monocytes and, hence, by desensitizing these cells to pro-inflammatory stimulation, this results in a push toward the anti-inflammatory phenotype. 2.5 h of cycling at 60% of VO_2max induces a significant decrease in the expression of TLRs on CD14+ monocytes, compared to rest, immediately (TLR2, about −25%) and 1 h post-exercise (TLR1, −60%, TLR2 and TLR4, −50%, TLR3, −30%) (72).

Effect of Chronic Exercise and Training

There are several potential tissue-specific anti-inflammatory mechanisms associated with regular PA and these include reductions in body fat (particularly, visceral fat), enhanced expression and release of contracting muscle-derived anti-inflammatory mediators, downregulated expression of TLRs in monocytes and macrophages, increased expression of anti-oxidant species counteracting the exercise-associated rise in reactive oxygen species (ROS) generation (73).

Exercise causes transient elevations in IL-6 coming from exercising SKM (74). Contrarily, during inflammation, and especially chronic low-grade inflammation, IL-6 is produced in a slightly chronically elevated manner and, in this case, the source is represented by immune cells and hepatocytes (3). Muscle-derived IL-6 has anti-inflammatory effects by inducing other anti-inflammatory cytokines (e.g., IL-1ra and IL-10) that antagonize the pro-inflammatory IL-1β and TNF-α (12). IL-6, but also the exercise-related increased energy needs, stimulate

the release of cortisol that has an immunosuppressant activity (74). The logic around the IL-6 release by the contracting muscle resides in its activity aimed at increasing the usage and delivery of energetic substrates to the myocytes, in concert with the stress hormones (e.g., cortisol, epinephrine). By acting in autocrine and paracrine fashions, indeed, IL-6 stimulates the cellular uptake and the oxidation of glucose and fatty acids by the SKM itself; contemporary, by acting in an endocrine fashion, it induces lipolysis at the AT level and glycogenolysis and gluconeogenesis. The insulin-mimetic effect of IL-6 on glucose uptake is of particular interest. The binding of IL-6 with the IL-6Rα/gp130Rβ receptor complex leads to the activation of AMPK that triggers the plasma membrane translocation of intracellular vesicles bearing the insulin-dependent glucose transporter (GLUT4) allowing glucose uptake regardless the insulin status. This accounts for the beneficial effects of PA on the metabolic function also in impaired glucose tolerance and insulin resistance (75). Moreover, other than stimulating the release of IL-6, exercise training generates an appropriate environment making the IL-6 effect distinctly anti-inflammatory: for instance, aerobic training reduces the expression of TNF-α and IL-1α by mononuclear cells and induces IL-4, IL-10, and TGF-β1 in subjects at high-risks of heart disease (76).

Training exerts its effects by also reducing the activation potential of the innate immune response activation in terms of TLRs in an age-independent manner. Young (18–35 years of age) and elderly (65–80 years of age) active subjects have a one third reduced expression of TLR4 on CD14+ monocytes' surface, compared to their inactive counterparts. Moreover, 12-week of either endurance or resistance training halved TLR4 expression in monocytes from these inactive (old and young) subjects to a level comparable to those found in active age-matched controls, while the intervention was ineffective in the already active subjects (72).

The anti-inflammatory effect of training might is also the result of the modulation of nitric oxide (NO) and ROS production and the consequent activation of their downstream pathways. Exercise induces the synthesis of NO and ROS which are important in inducing anti-inflammatory defensive mechanisms especially by targeting muscle gene expression (77). In the case of ROS, with training, the cyclic exercise-induced spiked production (contrarily to what happens in chronic inflammation) causes the activation of an adaptive response that, in turn, protects SKM from exposure to the exercise-dependent increase of ROS itself. This phenomenon accounts also for the decreased expression of TNFα that may further inflammation (77).

The AT is determinant in defining the inflammatory status. The association of physical inactivity and high caloric intake results in adipocyte and AT hypertrophy. As adipocytes grow, the oxygen supply becomes limiting and the consequent hypoxic stress leads to cell death and necrosis. Necrosis recalls macrophages and potently induces an inflammatory response (78). Notably, this process seems to involve mainly the visceral AT that has a higher inflammatory potential than the subcutaneous one. Exercise-induced caloric imbalance causes lipolysis, aimed at mobilizing fats to be used as fuel by the exercising muscle, with a reduction of the adipocytes' size and, thereby, hypoxic stress and inflammation (79).

Effects of Exercise and Training on Immune Functions

Exercise profoundly affects the IS functioning. An exercise bout causes an important redistribution of leukocytes as a consequence of the hemodynamic response and the increased blood levels of catecholamine and glucocorticoids, but the effects depend upon exercise intensity and duration. Prolonged periods of intensive training can impair immune functions, and particularly those of T-cells, natural killer (NK)-cells, and neutrophils; in elite athletes, during periods of heavy training and competition, mucosal immunity is also affected determining an increased risk of infections of the upper respiratory tract. Contrarily, regular moderate-intensity activities are beneficial having immune-enhancing effects and, as stated above, throughout the reductions of inflammation, increased immune cells turnover, enhanced immune surveillance, and improvement of psychological stress status (63).

Effect of Acute Exercise

An exercise bout increases both the absolute and relative leukocyte counts. Transient leucocytosis takes place already after brief (minutes) dynamic exercise and is more sustained in the case of prolonged endurance exercise (80) and returns to pre-exercise levels within 6–24 h (63). Neutrophils and lymphocytes are mainly involved in this response while a smaller contribute is given by monocytes. The response, however, differs for these cells: during the early phase of recovery (30–60 min after exercise), the neutrophilia is associated with lymphocytopenia that can be particularly pronounced until clinically relevant low levels ($<1.0{\cdot}10^9$/L, which means −30 to −50% compared to pre-exercise values) and can last up to 6 h (80). Other features also characterize this response. Exercise tends to mobilize cytotoxic cells (e.g., NK-cells, and CD8+ and γδ T-cells) (81) and non-lymphocyte effector cells (e.g., CD16+ monocytes and CD16- neutrophils) (82, 83). The exercise-dependent mobilization mainly involves those cells with a higher migration potential, e.g., leukocytes expressing high levels of integrins and intracellular adhesion molecules (84) and a wide range of chemokine receptors (85). Finally, in these leukocytes the expression of adrenoreceptors (β2-ARs) and glucocorticoid receptors is upregulated, and are therefore they are highly responsive to catecholamines and cortisol (81, 86, 87), this indicates that leukocyte trafficking between the blood and tissues is strongly influenced by both the sympathetic branch of the nervous system and the HPA axis activation.

Other than being dislodged from liver, lung, and spleen endothelia due to the exercise-induced increased blood pressure- and cardiac output-mediated shear stress (80), leucocytes come from lymph nodes, intestines, bone marrow, thymus, and SKM that contain large numbers of white cells. The contribution of primary (i.e., bone marrow, thymus) and some secondary (i.e., lymph nodes), lymphoid organs to the initial exercise-induced leucocytosis is limited, since their content in mature/differentiated cells. The bone marrow

likely sustains neutrophilia during recovery from prolonged exercise, while lymph nodes and thymus mainly sustain the restoration of the blood lymphocyte count following the transient lymphocytopenia (63).

About innate immunity, submaximal exercises enhance neutrophil chemotaxis (88), phagocytosis (89), and spontaneous degranulation (90). Also the neutrophil oxidative burst is affected by the exercise although in an intensity-and duration-dependent fashion: cycling at 50 and 80% of VO_2max have enhancing and impairing effects, respectively; moreover, during recovery from moderate-intensity exercise the oxidative burst is enhanced while it is impaired following exhaustive and prolonged activities (91, 92). Also NK-cell cytotoxicity is quickly induced by exercise but this response is followed by a delayed suppression during recovery but this likely mirroring changes in their number (93).

Adaptive immunity appears to be both augmented and inhibited depending on intensity, duration, and modality of exercise (63). In trained triathletes, following a half-Ironman race, an intradermal inoculation containing several recall antigens caused a reduced 48 h-delayed-type compared to both resting triathletes and moderately trained healthy men (94).

Effect of Chronic Exercise and Training

In general, high-intensity and high-volume training is thought to cause short- or long-term immune depressive states that can increase infection risk. Repeated bouts of strenuous exercise, performed without adequate recovery, result in a chronic state of impaired immunity (80). The decline in the count of circulating immune cells is associated with the increased susceptibility to infections: although athletes and healthy age-matched controls have comparable absolute and relative leukocyte counts, endurance athletes may experience reduced resting lymphocyte (runners) and NK-cell (swimmers, cyclists) counts (95–97). Functional declines in adaptive immunity associated with prolonged intensive training are related to unbalanced expression of pro- and anti-inflammatory cytokines and increased plasma levels of stress hormone (e.g., cortisol) (98).

Contrary to the strenuous, exhaustive exercise typically practiced by athletes, moderate-intensity training has beneficial effects on immune function (99). Moreover, exercise mode (i.e., aerobic, resistance, or combined) as well as the condition on which the intervention is addressed are main determinants of the immune effects. Indeed, moderate-intensity exercise training associates with a life-long improvement/maintenance of several aspects of the immune function (99, 100), such as increased response to vaccine (101, 102), viral infections (103, 104), and tumors (105–107), enhanced neutrophil phagocytic activity (108), T-cell proliferation (93, 109), NK-cell cytotoxic activity (93, 110), basal level of cytokines (111) and IL-2 production (112) and decreased number of senescent T-cells (113) and inflammatory response to bacterial challenge (114).

The enhanced adaptive immune response is also sustained by the improvement in systemic low-grade inflammation, driven by the improved inflammatory status of the AT, and hence in the associated adipokine profile (described above). Indeed, the downregulation of TLRs on the surface of monocytes (115) together with the direct exercise-induced M1 (pro-inflammatory)-to-M2 (anti-inflammatory) shift in macrophage phenotype, reduces the infiltration of the AT and, hence, its inflammatory status (116). The stress-related hormones released during exercise that have anti-inflammatory properties are responsible of a further stimulus: cortisol acting as an immunomodulatory and immunosuppressant compound and adrenaline that downregulates the expression of the inflammatory mediators IL-1β and TNF (73, 117). Exercise also decreases the content of cholesterol of the cell membranes that may improve T-cell receptor signaling and the translocation of MHC molecules for antigen presentation (118).

IMMUNO-MEDIATED EFFECTS OF EXERCISE AND TRAINING ON BONE

Inflammasome Activation and Bone Metabolism

The innate immune function depends upon the recognition, by germline-encoded pattern-recognition receptors (PRRs), of PAMPs, derived from invading pathogens, and DAMPs, induced by endogenous stresses. PAMPs-/DAMPs-dependent activation of PRRs triggers the downstream signaling cascades and induces the expression of type I interferons (IFN-α, IFN-β) and pro-inflammatory cytokines (119). Inflammasomes are multimeric protein complexes assembling within the cytosol after sensing PAMPs or DAMPs (120, 121). They serve as scaffolds to recruit the inactive zymogen pro-caspase-1 that oligomerizes allowing the auto-proteolytic cleavage into active caspase-1. Active caspase-1 cleaves the precursor cytokines pro-IL-1β and pro-IL-18 generating the biologically active forms (122–124). Furthermore, when activated, caspase-1 can activate a series of intracellular events that lead to a form of cell-death mediated by inflammation which is known with the term of pyroptosis (125, 126). Several PRRs families are involved in inflammasomes activation, in both mice and humans, including the nucleotide-binding domain, leucine-rich repeat containing proteins (NLRs, NOD-like receptors), and absent in melanoma 2-like receptors (ALRs, AIM2-like receptors) (127). Following stimulation, the relevant NLR or ALR oligomerizes and becomes a caspase-1-activating scaffold. Inflammasomes have been linked to several auto-inflammatory and autoimmune diseases, neurodegenerative diseases (e.g., multiple sclerosis, Alzheimer's disease, Parkinson's disease), and metabolic disorders [atherosclerosis, type-2 diabetes (T2DM), obesity] (126). Inflammasomes play either causative or contributing roles in inflammatory diseases onset, and also increase the severity of the condition in response to host-derived factors (119). Many PRRs can sense metabolic signals, such as free fatty acids (FFAs) and ceramides (CERs), whose blood concentrations increase during aging. These signals activate critical inflammatory signaling cascade pathways, such as IκBα kinase/nuclear factor-κB (IKK/NF-κB), endoplasmic reticulum (ER) stress-induced unfolded protein response (UPR), and NLRP3 inflammasome. Notably, other than in

immune cells, PRRs are expressed in several metabolically active tissues (liver, SKM, AT) where they prime the inflammatory cascades (128).

Besides the established role of inflammation (and age-associated low-grade inflammation) in the pathogenesis of osteoporosis (129–131), very recent findings have linked it to inflammasomes activation. PA counteracts all the molecular mechanisms involved in inflammatory signaling cascades and inflammasome complexes activation (128). In post-menopausal osteoporosis, IL-18 blood levels are increased while those of its antagonist, IL-18BP, are decreased. According to Mansoori et al., IL-18BP enhanced murine osteoblast differentiation and inhibits the activation of NLRP3 inflammasome and caspase-1, *in vitro*, and improved the metabolic and bone statuses in ovariectomized rats (a rodent model of post-menopausal OP) (132). Further evidences derived from the association of NLRP3 mutations with arthropathy and OP (133) and the SIRT1-dependent inhibition of osteogenic differentiation and enhancement adipogenic differentiation, in mesenchymal stem cells (MSC), following lipopolysaccharide (LPS)-induced NLRP3 inflammasome activation (134). It is known that bone matrix organic and inorganic components, released during high-rate bone turnover (e.g., chronic low-grade inflammation, estrogen deficiency, primary hyperparathyroidism), promote osteoclastogenesis. This process, however, was importantly reduced in $Nlrp3^{-/-}$ cells and mice and pharmacologic inhibition of bone resorption (with bisphosphonates, e.g., zoledronic acid) attenuated inflammasome activation *in vivo*. These evidences suggest that the DAMPs-NLRP3 inflammasome axis may represent a novel mechanism supporting bone resorption (135).

PA effectively counteracts all the molecular mechanisms involved in the inflammatory signaling cascades (e.g., IKK/NF-κB, ER-UPR, inflammasomes) although the evidences about the effects of exercise on inflammasome are currently limited to NLRP3 activation and only in mouse models and in obesity. According to Ringseis, in obese mice both endurance (treadmill, 80% VO_2max, 10 weeks) and resistance exercise (intermittent vertical holding, 10 weeks) decrease NLRP3 mRNA in AT and IL-18 in plasma (128). A number of human studies demonstrated that PA reduces plasma IL-18 levels providing the evidence for the exercise-dependent NLRP3 pathway inhibition: 12-week aerobic interval training in males and females with metabolic syndrome; 6-month aerobic training (50–85% VO_2max) in overweight T2DM individuals; 8-week high-intensity training on a rowing ergometer (≥70% VO_2max) in obese. In diet-induced obese rats, exercise strongly reverses TLR4 signaling and IKKβ phosphorylation in AT, SKM, and liver, suggesting a priming role for the exercise-induced inhibition of NLRP3 inflammasome. Key primers of NLRP3 activation are saturated FFA and CERs, whose circulating levels are increased in aging and metabolic dysfunctions while are decreased in response to exercise in obese animals and humans. Exercise may also reduce ER stress that primes NLRP3 activation via ROS production and NF-κB activation (128).

Extra-Immune Systemic Inflammation and Bone Metabolism

Beside the above described prototypic adipo-myokine IL-6 with its anti-inflammatory actions (induction of IL-10 and IL-1ra and inhibition of IL-1β and TNFα), SKM and AT secrete a plethora of active molecules that act in autocrine, paracrine, and hormone-like fashion the blood concentrations of many of which have been associated with several metabolic, immune-related, and age-related pathological conditions (3). It is well-known that the post-exercise rise of circulating IL-6 is supported by SKM (136) but contrarily, chronically slightly elevated blood IL-6 are found in metabolic conditions such as metabolic syndrome and insulin resistance, obesity, and T2DM (137–139). In these cases, the main source of IL-6 is represented by the visceral AT, the liver, and the activated immune cells upon NF-κB signaling (140). Indeed, in both overweight and lean males the contribution of the AT to the circulating amount of IL-6 is mainly in the post-exercise phase (141).

In bone, IL-6 stimulates bone resorption by enhancing osteoclastogenesis/osteoclast differentiation throughout the induction of RANKL expression (142, 143) and by inducing prostaglandin E2 (PGE2) expression in osteoblasts (144–146). Bones from IL-6 transgenic mice developed osteoporosis in association with an increased number of osteoclasts and decreased osteoblasts while, on the contrary, IL-6 knock out (KO) improved the arthritis phenotype, associated with a reduced osteoclast recruitment at the erosion sites, in a murine model of arthritis (147, 148). Ovariectomy in rats, model of postmenopausal osteoporosis, decreased trabecular bone volume (TBV) and impaired hormone and inflammatory profile (decreased estradiol and calcitonin and increased bone-derived IL-1β, IL-6, and cyclooxygenase-2). Contrarily, the treadmill-exercised counterparts displayed an overall improved phenotype (149). Parallel, postmenopausal women have BMD and muscle strength correlated with soluble IL-6 (150).

Low-impact high-intensity interval training (HIIT) acutely increased bone alkaline phosphatase activity (BAP) and the expression of OPG, RANKL, and pro-inflammatory cytokines (IL-1α, IL-1β, IL-6, TNFα) while decreased bone resorption [N-terminal cross-linked telopeptide of type I collagen (NTx)] (151). In obese subjects (n= 173), the degree of obesity and BMD were related to IL-6 levels (in males), osteocalcin (in females), C-reactive protein (CRP), and leptin indicating that adiposity and systemic inflammation are associated with low BMD (152). In obese, a 32-week combined loading training improved muscle strength and BMD at various sites along with an improved metabolic/inflammatory status (decreased CRP, interferon (IFN)-γ, IL-6) (153).

The leukemia inhibitory factor (LIF), a myokine belonging to the IL-6 superfamily, stimulates the proliferation of satellite cell which is essential in post-injury muscle regeneration (e.g., exercise-induced muscle damage, EIMD) and SKM hypertrophy (154). It was induced, at least in term of mRNA, by acute aerobic and resistance exercises (155). In bone, LIF stimulates bone turnover, osteoblast proliferation and bone matrix deposition, and prostaglandin-induced bone resorption depending on

the differentiation stage of the target cells: enhancement of differentiation in progenitors, inhibition of function (e.g., mineralization) in mature osteoblasts (156). Exercise-induced LIF acts on periosteal osteoblast in order to modulate their activity (157).

IL-7 is essential for T-cell and B-cell development (136) but it is also expressed by the contracting muscles where it acts paracrinally to induce migration in satellite cells (158). As such, IL-7 mediates the oestrogens deficiency-induced bone loss: it induces RANKL and TNFα expression in T-cells (159) and, hence, it activates mature osteoclast and stimulates the progenitors differentiation (160) as also observed *in vivo* in mice following systemic administration (161). It also promotes survival and differentiation of dendritic cells, B220+ subset, into osteoclasts (162). However, despite their beneficial effects on bone, exercise and training seem to increase the SKM expression of IL-7. Indeed, plasma IL-7 has been found to be induced, in elite female soccer players, after a 90-min soccer games (163), 30-min post resistance exercise but not after 12 weeks of resistance training (164), while the mRNA expression level was induced in SKM by 11-week long strength training (158). Therefore, as IL-6, the biological significance of SKM- and IS-derived IL-7 resides into basal-to-peak ratio (3).

In addition, IL-15 is considered an exercise-related myokine although the effect of exercise on its expression and secretion by the SKM are not well-understood. Current evidences account for a role of IL-15 in the first phase of adaptation to exercise since it has been found upregulated (in muscle and in blood) in inactive/normally active subjects following an acute bout of exercise but not following the completion of a training program (165). Indeed, IL-15 mRNA expression in SKM was not affected by a 3-h treadmill run in trained subjects (marathon runners) (166) as well as in healthy physically active men after 3-h cycling (167). However, despite no changes in plasma levels after 12-week endurance training, the protein content in SKM was increased (167). Increased blood IL-15 was found after a 30-min run on a treadmill at 70% of maximum heart rate in untrained healthy young men (168), acute resistance exercise in young healthy inactive subjects but not after 10-week chronic training (169) as well as SKM mRNA, in healthy normally active men, after an heavy bout resistance exercise (170). IL-15 is a powerful inducer of TNF-α expression, and hence of RANKL, in osteoblasts and stromal cells, resulting in enhanced osteoclastogenesis. Furthermore, in rat bone marrow cultures, it stimulates pre-osteoclasts differentiation independently from TNF-α (171). IL-15 acts synergistically with RANKL in osteoclastogenesis by activating ERK (172). These data suggest that IL-15 positively regulate osteoclastogenesis (173).

Myostatin, is a member of the transforming growth factor beta (TGF-β) superfamily [also known as growth-differentiation factor (GDF)-8], negatively regulates SKM hypertrophy and hyperplasia (174) and it may cause SKM mass loss during aging (sarcopenia) and with metabolic and inflammatory conditions (175, 176). As such it is negatively regulated by PA (acute endurance (177), acute resistance (178–180), chronic (6-month) aerobic training in overweight and obese men (181). The recovery strategy seems to have in determining the net effect of exercise (182). Different factors act as antagonist of myostatin and among them follistatin (FST) (183), follistatin-like 1 (FSTL1) (184), and decorin (185). Acute resistance exercise did not affect follistatin mRNA expression in SKM from lean young and old men (180) while it had enhancing effects in postmenopausal women (179). Decorin mRNA expression in SKM was induced by chronic combined strength and endurance training (186) but also by acute endurance exercise (187) and, in terms of plasma levels, by acute resistance exercise (186). FSTL1 plasma levels were increased in young healthy men after acute endurance exercise (188) while chronic strength training induced mRNA expression in SKM (189). Myostatin has direct effects on osteoclastogenesis (190). Indeed, osteoclasts number on trabecular bone surfaces is increased in unloading conditions in both wild-type and myostatin KO mice (191), however myostatin deficiency suppresses subperiosteal resorption with unloading, suggesting that at least a part of the effects myostatin on osteoclasts are localized to the muscle-bone interface (192). Wnt-independent bone resorption consequent to strong endurance effort (e.g., ultramarathon) has been associated to increased myostatin and decreased FST (193). FST and its related factors (FSTL1, FSTL3, decorin) are induced by exercise and their importance in bone and muscle development is evidenced in the severe phenotypes consequent to their mutation. Fstl3$^{-/-}$ mice experienced frequent fractures together with the loss of mechanosensitivity which led to the loss of bone gain and Sost response to exercise. Importantly, a decreased FSTL3 expression is associated with aging (176). FST is induced by hyper-gravity and inhibited by microgravity (194).

Although its indisputable role as a myokine (195), brain-derived neurotrophic factor (BDNF) is mostly expressed in the brain, as is its receptor (196, 197), indeed 70–80% of the circulating protein origins from brain (9). BDNF plasma levels are raised by acute endurance and high-intensity, but not low-intensity, exercise in both males and females (195, 198–200) and also in response to chronic endurance training in young adult males (201). On the other hand, 10-week chronic resistance exercise did not affect BDNF serum levels compared to physically inactive (202). Interestingly, BDNF plasma concentrations were found to be lower in young compared to middle-aged women. After high-intensity resistance exercise BDNF follows a biphasic response featured by a decrease 1 h and an increase 24 h post exercise (203). On the contrary, 12-week moderate aerobic training (nordic walking) increased circulating BDNF in middle-aged women in association with improvement in cognitive functions (204). The BDNF receptor, TrkB, is expressed by active trabecular osteoblasts, growth plate hypertrophic chondrocytes during intramembranous ossification, and in osteoblasts and endothelial cells in fracture healing site (205, 206). Mice BDNF conditional KO in brain, beside the metabolic phenotype (hyperphagia, increased abdominal AT, obesity, leptin resistance), displayed increased femur length, high BMD and BMC (207).

The chemokine MCP-1 (also known as CCL2) is the primary ligand for the CCR2 receptor, which is normally expressed on monocyte/macrophages. As such it is a key regulator of osteoclastogenesis and has a pivotal role in inflammation and

tumor-induced osteolysis (208). It is also an adipo-myokine acting as chemoattractant for monocytes and T lymphocytes (209). MCP-1 expression in SKM is strongly induced by acute and chronic resistance exercise, in terms of both mRNA and protein (164, 210), in healthy young and elderly (211). Also acute endurance activities (70% VO_2max for 40 min, high-intensity treadmill running) increase mRNA expression in SKM in lean, obese, and T2DM (164), and circulating protein in well-trained male runners subjects (212). Therefore, expression of MCP-1 seems to be influenced by the intensity of the exercise rather than the kind of activity. Moreover, MCP-1 is affected by acute exercise while its response is not affected by training in both young and elderly healthy subjects, males and females, regardless the type of activity (211, 213).

The pro-inflammatory TNFα, the prototypic early mediator of local inflammation and initiator of the acute phase response, is expressed, other than from the immune cells, also by AT and SKM. In the AT, its expression is related to the fat mass (140) while in the SKM, the mRNA expression is inhibited following training (endurance and resistance) (214, 215). The muscle expression is neither affected by the metabolic status, being comparable between lean, overweight, and T2DM, nor by acute exercise (141, 216, 217). Circulating TNFα concentrations, however, are inversely related with the amount of PA (218) but, contrarily to moderate-intensity exercise, high-intensive training causes a temporary rise in systemic inflammation (e.g., TNFα) during recovery in response to muscle damage (10) as a propaedeutic step needed for the following regeneration (219). TNF-α is a powerful stimulus for bone resorption and is strikingly implicated in inflammatory bone diseases (220). By activating NF-κB signaling, it induces osteoclast differentiation form progenitors in the presence of M-CSF and in the absence of RANKL (221) and it also enhance RANKL sensitivity in osteoclast progenitors by inducing the expression of RANK (222). TNF-α can accelerate RANKL-dependent osteoclastogenesis by activating NF-κB and AP-1 throughout TRAF2/5 and MAPKs cascades (223) and RANKL enhances TNFα-induced osteoclastogenesis via TRAF6-independent signaling (224). TNF-α stimulates osteoclastogenesis also indirectly by inducing the expression of M-CSF and RANKL stromal cells, osteoblasts, and activated T cells (161, 225).

Visfatin/NAMP (alternatively known as pre-B cell colony-enhancing factor, PEBF) (226) is synthesized as both an intracellular form, acting as nicotinamide phosphoribosyltransferase (eNAMPT) in NAD biosynthesis, and extracellular one, visfatin, mainly secreted by the visceral AT, acting as an insulin-mimetic, pro-inflammatory/immuno-modulating adipokine (227). Its circulating levels are associated with obesity/fat mass, insulin resistance (228), and the energy-bone crosstalk (57). NAMPT expression was two-fold higher in SKM of athletes compared to that found in SKM from sedentary obese, non-obese, and T2DM subjects (229). However, in obese (>30 kg/m^2) men no difference circulating visfatin concentrations were found between subjects with high and low cardiorespiratory fitness (230). NAMPT mRNA expression and protein content in the SKM of the exercising leg was doubled compared to the non-exercising limb, of non-obese sedentary individuals after 3 weeks of one-legged endurance exercise training endurance training (231). Acute exercise, instead, affects eNAMPT/visfatin in an intensity-dependent manner: 3-h cycling at 60% VO_2max had no effect (232) while an acute bout of high-intensity running-based anaerobic sprint exercise had an inductive effect (233). *In vitro* visfatin stimulates osteoblast proliferation through the activation of insulin-receptor (234), induces osteoblastic differentiation in association with inhibition of OC expression (235), inhibits osteoclastogenesis throughout the suppression of RANK and NF-AT pathways (236), stimulates adipogenesis in mesenchymal stem cells (MSC) throughout the induction of PPAR-γ (237). Plasma visfatin did not differ between less than moderately trained subjects and experienced ultramarathon runners but, in this latter group it was two-fold induced after a mountain ultramarathon (39). Similarly, despite the worse metabolic profile, sedentary subjects had comparable serum visfatin concentration than professional rowers (238). Patients with metabolic syndrome had higher plasma visfatin than their age-matched counterparts which was correlated with lumbar spine BMD in men (239).Furthermore, in different women population (Chinese, Iranian) visfatin independently predicted BMD (240, 241) although it was not correlated with either BMD or BMC in a cohort of adolescent female athletes, participating in different sports (242).

Adiponectin is a prototypic adipokines that increases fatty acid oxidation and glucose uptake in SKM while inhibiting hepatic gluconeogenesis (243) and its circulating levels are inversely related to BMI and adiposity (244). It has anti-inflammatory effects since it inhibits expression and secretion of TNFα in macrophages and induces the expression of IL-10 (245, 246). Although it is considered a classical adipokine, adiponectin is also expressed by the SKM (247, 248). Plasma adiponectin levels are decreased in obesity and insulin resistance but the SKM expression of its receptors (AdipoR1 and R2) is increased (249). Acute exercise has no clear effects on circulating adiponectin with researches depicting inductive (250, 251), depressing (141) or no effects (252–256), regardless the metabolic state of the subjects. Contrarily, in healthy lean and overweight and obese subjects with impaired glucose tolerance, endurance training increased plasma adiponectin levels and induced the expression of AdipoR1/R2 in SKM (257). In highly-trained professional cyclists, plasma adiponectin was increased during a 3-week stage race (38). Fatouros and co-workers, instead, reported that high-intensity, but not moderate-intensity, either acute or chronic resistance exercise increased plasma adiponectin levels (258, 259), suggest that exercise intensity is a key determinant of the regulation of adiponectin release in blood. Among the adipokines, adiponectin is the most closely associated with BMD (negative) and fracture risk (positively) regardless gender and menopausal status (260). Hence, it exerts negative effects on bone mass although it is inversely associated with fat mass, promotes insulin sensitivity, and fat oxidation. However, bone osteocalcin induces adiponectin expression in adipocytes that, in turn, improves glucose tolerance (261, 262). AdipoRs and adiponectin are expressed by osteoblasts and osteoclasts (263, 264). Current evidences suggest that adiponectin acts autocrinally/paracrinally

to simulate osteoblast function, while systemic adiponectin has inhibitory effects on osteoblasts activity while enhances osteoclastogenesis (265, 266).

Leptin is another adipokine involved in the regulation of energy homeostasis (267) it is an adiposity signal that suppresses appetite. As for adiponectin, also leptin is expressed in SKM (268) but the relative contribution of AT and SKM to circulating leptin has been not fully understood (165). Leptin and leptin receptor KO mice ($ob^{-/-}$ and $db^{-/-}$) which are obese have also higher bone mass and intracerebroventricular infusions improved the metabolic status and reverted the high bone mass phenotype (269). Leptin effects on bone formation are mediated by the sympathetic nervous system (SNS), independently from AT, indeed by high bone mass phenotype obtained following the inhibition adrenergic signaling cannot corrected by intracerebroventricular infusion of leptin (270, 271). In strenuous exercise-induced hypogonadal women, leptin induced oestrogens that partially improved the bone phenotype (272). Circulating leptin was decreased in highly trained professional cyclists during a 3-week stage race (38) and in experienced ultramarathon runners after a mountain ultramarathon (39). However, in these runners resting levels of leptin were significantly lower than their less than moderately trained counterparts (39). Interestingly, in competing professional cyclists the decrease in leptin was associated with increased bone resorption and GluOC-to-GlaOC ratio (38). Similarly, 8-week aerobic training decreased fat mass and leptin, improved insulin sensitivity, and increased both total OC and GluOC in obese young males experienced (273). On the contrary, in competing ultramarathon runners, a comparable trend in leptin was associated with a reduced GluOC-to-GlaOC ratio (39). Finally, soluble leptin, insulin, and OC were increased by bed rest independently from resistive vibration exercises (274). These data indicate that load may regulate leptin release. Leptin mRNA expression in AT after acute endurance exercise were found either unaffected (275) or decreased in lean and overweight subjects (141). Several studies have shown a delayed (24–48 h post exercise) reduction of circulating leptin levels in healthy active men (276–279). Taken together, the current evidences suggest that exercise training decreases plasma leptin levels, while is ineffective on mRNA expression in AT (165). Weight loss in elderly obese accelerated bone turnover but PA can attenuates BMD decrease and stimulated a greater decrease in circulating leptin (280). The detrimental bony effects of leptin also depends upon its pro-inflammatory action: stimulation of neutrophil chemotaxis and phagocytic function, induction of pro-inflammatory cytokines in monocytes, and induction of T helper (Th)-1 cytokines (246). These data suggest that the exercise-dependent beneficial effects on bone may be also mediated by the exercise-dependent reduction in circulating leptin (3).

Resistin, an inflammatory marker, is positively associated with fat mass, waist circumference, and obesity-related diseases, and it causes oxidative stress and nitric oxide production downregulating, thus, determining endothelial dysfunction (281, 282). Both circulating levels and AT mRNA expression are not affected by acute endurance training in overweight and lean males (141, 253). However, the baseline training status of the subjects seems to affect the resistance exercise-dependent response to exercise with regularly training subjects experiencing a decrease over 6 months (281). Also osteoclasts, osteoblasts, and bone marrow-derived MSC express resistin, and *in vitro* it may stimulate both osteoclastogenesis and osteoblastogenesis (234, 235). Similarly to visfatin, resistin induces PPAR-γ expression in MSC and, thus, the adipogenic differentiation (237). It has been negatively associated with BMD (240, 283), although not definitively (284–286), and in postmenopause its circulating levels are doubled compared to premenopause (287). Resistin correlated positively with previous osteoporotic fractures and much more in the presence of diabetes (288).

Irisin is a newly discovered myokine released into the circulation following the cleavage, mediated by unknown proteases, of the transmembrane glycoprotein fibronectin type III domain containing 5 (FNDC5). In target cells, mainly white adipocytes, it induces the expression of the mitochondrial uncoupling protein 1 (Ucp1) that uncouples the respiratory chain from the oxidative phosphorylation: the energy derived from the oxidation of energy substrates (e.g., carbohydrates, fatty acids), and generated by the passage along electrochemical gradient of the electrons, is released as heat. This process normally occurs in the thermogenic BAT rather than in fat-storing WAT. Irisin induces a metabolic shift in the white adipocytes, namely browning, making them expressing an intermediate beige phenotype (289). Irisin is induced by exercise and its circulating levels are higher in trained, males and young subjects than in sedentary, females, and elderly, as a function of the muscle mass and the muscle activation level (290). Along with animal studies (291), researches in human have highlighted that high-intensity acute exercise (292), endurance training (293, 294), cold exposure (295), lifestyle changes as in obese children (296) and pregnant women (297) all increase blood irisin in association with an improved metabolic status. However, these results have not been always replicated and there are still doubts about its physiology (182, 291). Such discrepancies could be, at least partially, imputed to the methodological issues emerged about some commercially available immunoassays when compared to the gold standard mass spectrometry-based method (298). Irisin is also expressed by the AT and respond to PA as for its muscle counterpart (299, 300). Other than being directly induced by exercise, irisin expression is regulated by several exercise-modulated factors such as BDNF, myostatin, follistatin, TGFβ, FFAs, cytokines, betatrophin (291). Irisin could be a link between exercise and BDNF expression (289). Indeed, 30-day voluntary free running-wheel induced FNDC5 expression in mice hippocampus, which in turn into and increased expression of BDNF (301). This correlation might support the neuroprotective effects of exercise. Still, data directly evaluating the impact of exercise (especially in human), its time duration and intensity on irisin, BDNF, and cognitive function are unclear. By affecting adiposity, irisin can improve the inflammatory status and, recently, a direct relationship between irisin concentrations and inflammatory markers in metabolic syndrome has been described (302). Irisin is involved in the SKM-bone endocrine connection and it its involvement in bone mass gain in muscle

disease-associated osteopenia has been proposed (303). Irisin levels have been, indeed, associated with osteoporotic fractures in postmenopausal women (304) with an inverse correlation with fat mass and PA status (305). *In vitro*, irisin promotes osteoblast differentiation while *in vivo* it induces osteoblast proliferation and differentiation, inhibits osteoclast activity, increases cortical BMD (306), and prevents muscle atrophy-induced bone loss (307).

Adaptive Immunity Activation and Bone Metabolism

Besides the role of innate immunity, several examples exist about the interplay between bone and IS. First of all, as already stated, osteoclasts are monocytic/macrophagic origin and M-CSF, a key cytokine for this lineage is also important in osteoclast differentiation (308) and antigen-presenting cells, such as dendritic cells, retain the capability to transdifferentiate into bone-resorbing osteoclasts (309, 310). Moreover, several soluble mediators regulate osteoblasts and osteoclasts differentiation and activity (311). Interestingly, a subset of osteogenic cells, called N-cadherin-positive spindle-shaped osteoblasts are an integral part of the hematopoietic stem cell (HSC) niche and solve a key role in the maintenance of the HSC pool that gives rise to all blood and immune cells (312).

When activate, under inflammatory conditions (e.g., autoimmune diseases, inflammatory bowel diseases, periodontal infections), T- and B-lymphocytes secrete RANKL and TNFα that stimulate osteoclast differentiation and function and, therefore, bone resorption (313). However, under physiological conditions, B-cells represent an important source of the osteoclast inhibitor OPG. For instance, human tonsil B-cells secrete OPG and the activation of the CD40 costimulatory pathway on these cells, *in vitro,* further induced OPG expression (314); parallel, bone marrow B-cells contribute up to 64% of the total OPG, in mice (315). Consequently, B-cells KO mice experienced increased bone resorption rate, reduced BMD and bone mass, in association with low circulating OPG; the restoration of the B-cells pool into young B-cell prevented the bone phenotype. The ligand of CD40, CD40L, is mainly expressed by activated T-cells and the deletion of either CD40 or CD40L on T-cells caused a powerful inhibition of OPG expression in B-cells and bone loss (315). It is, thus, suggested that under physiological conditions B-cells, regulated by the T-cells co-stimulatory action, protect the skeleton by secreting OPG, while under inflammatory conditions B- and T-cells negatively affect bone metabolism by secreting RANKL and inflammatory cytokines (316).

Postmenopause-related estrogen deficiency gives an explicative example. Indeed, oestrogens mediate powerful anti-inflammatory effects and loss of estrogen causes significant proliferation of T- and B-lymphocytes (129, 317). This was demonstrated by the fact that while ovariectomized wild-type rats experienced bone loss, T-cell-deficient null mice were protected by osteoclastic bone resorption (318). RANKL is secreted by activated T but not in conditions of estrogen deficiency, in mice. Contrary, in this condition, circulating and tissue TNFα is raised (318, 319) and TNFα and TNFRI (p55) KO in mice prevented ovariectomy-induced bone loss (318). In agreement to this model, estrogen loss causes the expansion of TNFα-secreting T-cells and TNFα sustains and amplifies the RANKL-induced osteoclast-mediated bone resorption (320). IL-7 expression in several tissues anticipates T-cells expansion (321); this cytokine increases the sensitivity of T-cells to otherwise tolerogenic antigens and, hence, decreases the antigen-dependent T-cell activation threshold (320). Consequently, the differentiation of T-cells into the different T helper subsets (i.e., Th1) leads to TNFα secretion along with IFNγ, that upregulates the expression of CIITA in macrophage, a transcription factor that in turn upregulates MHCII expression and, hence, antigen presentation to T-cells, further amplifying T-cells activation (317). Also the Th17 subset is induced in this process; these cells secrete IL-17A a pro-osteoclastogenic cytokine that induces expression RANKL in osteoblasts. IL-17 expression is raised by ovariectomy (322): treatment with anti-IL-17 antibodies (323) or the IL-17 gene deletion (324) improves bone loss in ovariectomized mice. The downregulation of TGFβ is another step in this process. TGFβ is expressed in response to oestrogens and has immunosuppressive effects by inducing regulatory T-cells (Tregs) that down-regulate T-cells activation (316).

Importantly, T-cell activation-dependent ovariectomy-induced bone loss depends upon antigen stimulation. Indeed, ovariectomy in mice with silenced antigen presentation due to a mutated T-cell receptor, only responsive to chicken ovalbumin: when no antigen is presented, mice were fully protected from ovariectomy-induced bone loss while, after exogenous administration of ovalbumin (i.e., the antigen) the bone response was retained (317). Evidence suggests that, in human, these antigens are derived from the gut microbiota (325, 326) since the gut permeability is regulated by oestrogens (326).

T-cells are critical in the mechanisms of action of parathyroid Hormone (PTH) in bone (327). Chronic elevated production of PTH (hyperparathyroidism, HPT) causes skeletal and extra-skeletal diseases: primary HPT (PHPT) is associated with increased bone turnover and osteopenia (328), while secondary HPT (SHPT) is involved in the pathogenesis of age-associated osteoporosis (329). Continuous PTH infusion mimics PHPT and SHPT, while intermittent administration has pro-anabolic effects on bone (330). T-cells express PTH-1R, the functional G protein coupled PTH receptor and they may contribute to the catabolic effect of PTH, *in vivo* (327, 331). Continuous PTH treatment at doses that mimic HPT failed to induce osteoclast formation, bone resorption, and cortical bone depletion in mice deficient for T-cells (331). On the contrary, intermittent PTH stimulates Wnt10b expression in bone marrow CD8+ T-cells and activate the canonical Wnt signaling in pre-osteoblasts (332).

Currently, there are no available study depicting the effects of the exercise on the relationship between adaptive IS and bone cell function. Hence, in order to improve the use of PA as a therapy for bone loss it is necessary to increase the knowledge in this field.

PRACTICAL IMPLICATIONS, CONCLUSIONS, AND PERSPECTIVES

The complex net of physiological connections linking bone metabolism and IS branches in relationship with physical exercise is, now, only a little depicted. The great majority of the current knowledge concern the inflammation-mediated effects of PA while, only a few is known about the adaptive immunity-mediated effects.

Chronic PA is a powerful stimulus for bone and lifelong exercising is the most effective strategy to improve bone mass (in childhood and adolescence) and to keep bone health (in adulthood and older ages). However, there is no consensus on the best kind of PA to be prescribed at this purpose. There are evidences that sustain the effectiveness of load and impact and this is further improved when the activity is carried on in an intermittent way (54). Therefore, the role of loading is central in this discussion but, importantly, the direct effect of the applied forces onto the skeleton on the immune function are not known and this point must be developed in the next years.

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GL conception, drafting, and reviewing the article. EZ drafting and reviewing the article. GB drafting and reviewing.

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One Disease, Many Genes: Implications for the Treatment of Osteopetroses

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Osteopetrosis is a condition characterized by increased bone mass due to defects in osteoclast function or formation. In the last decades, the molecular dissection of osteopetrosis has unveiled a plethora of molecular players responsible for different forms of the disease, some of which present also primary neurodegeneration that severely limits the therapy. Hematopoietic stem cell transplantation can cure the majority of them when performed in the first months of life, highlighting the relevance of an early molecular diagnosis. However, clinical management of these patients is constrained by the severity of the disease and lack of a bone marrow niche that may delay immune reconstitution. Based on osteopetrosis genetic heterogeneity and disease severity, personalized therapies are required for patients that are not candidate to bone marrow transplantation. This review briefly describes the genetics of osteopetrosis, its clinical heterogeneity, current therapy and innovative approaches undergoing preclinical evaluation.

Keywords: bone disease, osteopetrosis, osteoclasts, hematopoietic stem cell transplantation, gene therapy

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INTRODUCTION

The term osteopetrosis derives from the Greek "osteo," bone, and "petros," stone, to define a genetically heterogenous group of diseases affecting the skeletal tissue, ranging in severity from benign to fatal in early childhood (1). Osteopetrosis is characterized by increased bone mass due to defective resorption activity or differentiation of osteoclasts (2), causing a disequilibrium of bone turnover, deformities, dental abnormalities and impaired mineral homeostasis, and giving rise to structural fragility that causes frequent fractures. Moreover, osteopetrotic patients are characterized by reduction of marrow cavity, affecting hematologic function; related phenotypes are severe anemia, pancytopenia, frequent infections and hepatosplenomegaly (1, 2) and increased frequency of circulating $CD34^{+}$ cells in the peripheral blood (3). The overly dense cranial nerve foramina lead to impairment of neurologic functions with progressive deafness, blindness and nerve palsies (1, 2). Three different forms of osteopetrosis have been described, based on the pattern of inheritance: autosomal recessive osteopetrosis (ARO), autosomal dominant osteopetrosis (ADO) and X-linked osteopetrosis (2, 4). The only cure for osteopetrosis is allogeneic hematopoietic stem cell transplantation (HSCT), that has greatly improved its outcome overtime (5–7). In this review, we describe the different forms of the disease and therapeutic options, highlighting advances in the setting of safer conditioning regimens and alternative therapies to overcome the limited donor availability.

AUTOSOMAL RECESSIVE OSTEOPETROSIS (ARO)

The autosomal recessive form of osteopetrosis (ARO), also known as infantile malignant osteopetrosis (IMO), has an incidence of 1:250000 live births, with higher rates in specific geographic areas because of geographic isolation, high frequency of parental consanguinity or the presence of a founder effect (8). Unless treated with HSCT, ARO is usually fatal within the first 10 years of life (8). Children present with failure to thrive, skull abnormalities (macrocephaly, frontal bossing, choanal stenosis), hydrocephalus, hypocalcemia due to defective calcium mobilization activity of osteoclasts (1) and abnormal tooth eruption with frequent development of dental caries (9). ARO is caused by mutations in different genes that are implicated in osteoclast function (osteoclast-rich osteopetrosis) or differentiation (osteoclast-poor osteopetrosis) (**Figure 1**).

Osteoclast-Rich Osteopetrosis

The most frequent form is caused by mutations in the *TCIRG1* (T cell immune regulator 1) gene, accounting for more than 50% of ARO cases. *TCIRG1* encodes for the a3 subunit of V0 complex of the V-ATPase proton pump, mainly expressed by osteoclasts and gastric parietal cells on apical membrane. The V-ATPase pump acidifies the resorption lacuna in the bone for the dissolution of the hydroxyapatite crystals, that form the bone mineral fraction, and the degradation of the matrix (10). The a3 subunit has also been implicated in the interaction between actin cytoskeleton and microtubules, fundamental for the osteoclast ruffled border formation (8, 11). Accordingly, *TCIRG1*-mutated osteoclasts show defective ruffled border and markedly reduced resorptive activity (11, 12). Moreover, the V-ATPase maintains the low pH in the stomach for the dietary Ca^{2+} absorption (13), and, since gastric acidification is also relevant for calcium uptake, this form of osteopetrosis is characterized by rickets or osteomalacia. The second most frequent form of ARO (17% of the cases) is caused by loss of function mutations in the *CLCN7* (chloride voltage-gated channel 7) gene (2, 14). This gene codes a $2Cl^-/H^+$ antiporter regulated by voltage-gating mechanism, expressed on the osteoclast ruffled border and on the membrane of late endosomes and lysosomes (15). This channel cooperates with the V-ATPase in the acid pH maintenance of the resorption lacuna. CLCN7 is involved in vesicle trafficking in early and recycling endosomes by regulating the luminal Cl^- concentration (16). Mutations in the *CLCN7* gene are responsible for a wide spectrum of clinical manifestations. Biallelic mutations cause a very severe form in which bone defects and hematological failure are associated in some patients with primary neurodegeneration, resembling lysosomal storage disease, cerebral atrophy, spasticity, axial hypotonia and peripheral hypertonia (8, 14, 17). Carrier individuals do not show any overt bone phenotype. CLCN7-deficient osteoclasts have been reported to display impaired endolysosomal trafficking (8). In rare intermediate forms of *TCIRG1*- and *CLCN7*-deficient ARO, milder presentation or later onset and slower progression have been recently reported (18–21).

OSTM1 (osteopetrosis-associated transmembrane protein 1) mutations are reported in 5% of ARO cases (4, 22, 23) and invariably cause osteopetrosis and severe primary neurodegeneration, with a life expectancy lower than 2 years (22, 24–26). OSTM1 has a highly glycosylated N-terminus that has been reported to stabilize CLCN7 protein and to be required, together with its transmembrane region, for CLCN7 Cl^-/H^+ transport activity (15). OSTM1 acts also as an E3 ubiquitin ligase for the heterotrimeric G-protein Gαi3 and potentiates WNT canonical signaling by modulating β-catenin/Lef1 interaction (27, 28).

Less than 5% of ARO cases are caused by mutations in the *SNX10* gene, encoding for the sortin nexin 10 protein, one of the major interactors of the V-ATPase. It is involved in the vesicular sorting of the V-ATPase complex from the Golgi network and in its targeting to the ruffled border (8, 29). In the original work, *SNX10*-dependent osteopetrosis was reported to show few and small osteoclasts (30), while in a more recent paper SNX10-deficient osteoclasts were larger and pale at tartrate-resistant acid phosphatase (TRAP) staining (31). Overall, the severity of clinical manifestations is variable (29, 31, 32).

Rare cases of osteoclast-rich osteopetrosis caused by mutations in other genes have also been reported. For example, osteopetrosis caused by carbonic anhydrase II (*CA-II*) deficiency appears in less than one in a million live births and is associated with cerebral calcification and renal tubular acidosis (2, 33). Carbonic anhydrase II enzyme provides protons to the vacuolar proton pump. Since renal defects are more severe than bone abnormalities, *CA-II* deficiency generally is not considered a classic form of ARO (34).

Loss-of-function mutations in the *PLEKHM1* (pleckstrin homology domain–containing family M member 1) gene cause mild osteopetrosis in the *ia* (incisors absent) rat, as well as an intermediate form of human osteopetrosis (35). PLEKHM1 is a cytosolic protein involved in lysosomal trafficking likely acting as an effector of Rab7 (36, 37). Patient-derived *PLEKHM1*-deficient osteoclasts displayed altered morphology and abnormal podosome distribution (35).

Mutations in *FERMT3* (fermitin family member 3) gene have been reported to cause osteopetrosis in association with leukocyte adhesion deficiency type III (LAD III). *FERMT3* gene is expressed in hematopoietic cells and encodes kindlin-3 protein, necessary for integrin signaling and platelet aggregation (38). Patients affected with *FERMT3*-deficiency are characterized by frequent bleeding and recurrent infections (39, 40).

LRRK1 (leucine-rich repeat kinase 1) gene mutation was found in a single patient affected by osteosclerotic metaphyseal dysplasia, that specifically compromises the methaphyses of long bones, vertebral endplates, costal ends and margin of flat bones (41).

Another mutated gene associated with osteopetrosis is *MITF* (microphtalmia-associated growth factor) that

Abbreviations: ADO, autosomal dominant osteopetrosis; ARO, autosomal recessive osteopetrosis; DOS, dysosteosclerosis; DSF, disease-free survival; GT, gene therapy; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; HSPC, hematopoietic stem progenitor cells; MSC, mesenchymal stem cell; RIC, reduced intensity conditioning; TRAP, tartrate-resistant acid phosphatase.

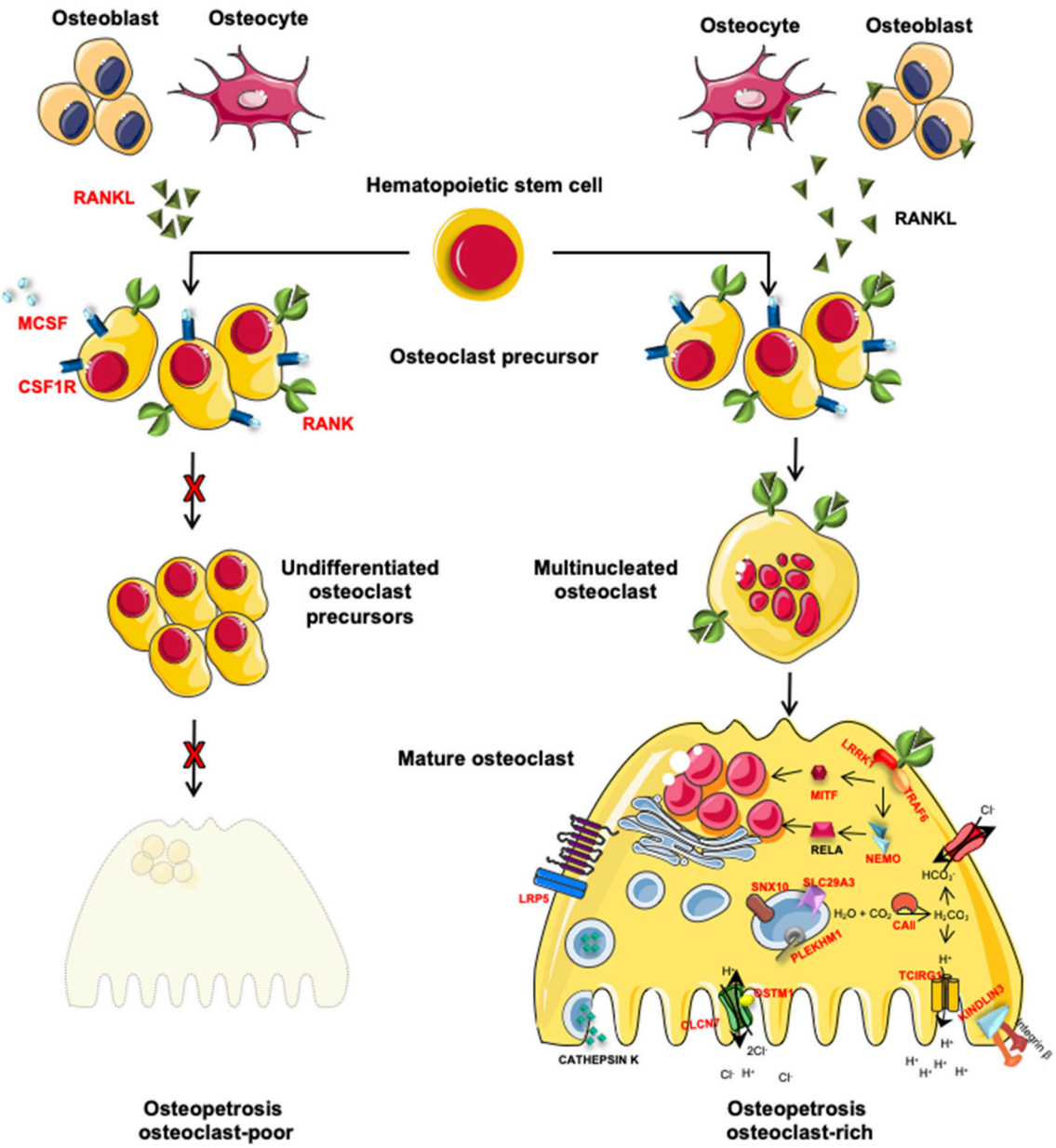

FIGURE 1 | Schematic representation of genes involved in osteoclast-poor and osteoclast-rich osteopetrosis. In red are indicated genes involved in the pathogenesis of ARO. MCSF and RANKL, cytokines secreted by osteoblasts and osteocytes, are necessary for the differentiation of osteoclast precursors into mature and resorbing osteoclasts. When these signals are absent (*TNFSF11* gene mutations) or the pathway is interrupted by the lack of cytokine receptors (*TNFRSF11A* and *CSF1R* gene mutations), osteoclast precursors are not able to differentiate into mature osteoclast causing osteoclast-poor forms of osteopetrosis. Alternatively, if osteopetrosis is caused by mutations in genes encoding for protein necessary for bone resorption, the disease is defined as osteoclast-rich osteopetrosis. On the right of the figure, are indicated genes involved in bone resorption activity with different roles: i.e., acidification of resorption lacunae and pH regulation (*TCIRG1, CLCN7, OSTM1,* and *CAII*), vesicular trafficking and sorting of protein complex to the membrane (*SNX10* and *PLEKHM1*), cytoskeletal rearrangement for ruffle border formation (*FERMT3* and *LRRK1).* Other molecules involved in different signal transductions, essential for osteoclast functions (*MITF, LRP5,* and *IKBKG*) are reported.

encodes for a transcription factor acting downstream RANK/RANKL pathway (42). *MITF* deficiency is responsible for COMMAD (coloboma, osteopetrosis, microphthalmia, macrocephaly, albinism, and deafness) syndrome in two unrelated patients, suggesting a role for *MITF* in regulating various processes beside bone development and homeostasis (43).

Finally, a homozygous mutation in *C16orf57* has been described in poikiloderma and neutropenia associated with osteopetrosis (44). This gene encodes for a phosphodiesterase responsible for modification and stabilization of the U6 small nuclear RNA, fundamental element of the spliceosome machinery (45), however, its pathophysiologic function in osteoclast still has to be elucidated.

Osteoclast-Poor Osteopetrosis

The complete absence of osteoclasts is the key feature of the osteoclast-poor form of osteopetrosis (46). Patients are characterized by absence of TRAP-positive osteoclasts in bone biopsies. The defective osteoclastogenesis is caused by either the lack of RANKL (receptor activator of nuclear kappa B ligand) cytokine (2% of all ARO cases) or of its receptor RANK (4.5% of ARO forms) (47–50). RANKL is encoded by the *TNFSF11* gene and the binding to its receptor RANK, encoded by the *TNFRSF11A* gene, determines the activation of the downstream pathway that drives osteoclast differentiation and activation (51). In bone, RANKL is produced mainly by the stromal compartment in physiological condition, while other cell sources are more important in pathological context (52). Recent evidence suggests that RANKL has also an osteogenic role through an autocrine loop in mesenchymal stem cells (53) and through reverse signaling from the osteoclasts to the osteoblasts (54). In addition, in patients RANKL absence leads to a partial defect in T cell proliferation and cytokine production (50), while RANK-deficiency perturbs B cell memory subset and immunoglobulin production (48, 49).

A rare osteoclast-poor form of osteopetrosis, called dysosteosclerosis (DOS), accompanied by red violet macular atrophy, platyspondyly and metaphyseal osteosclerosis, is caused by mutations of the *SLC29A3* (solute carrier family 29 member 3) gene encoding for a lysosomal nucleoside transporter highly expressed in myeloid cells (21, 55). More recently a novel splice-site mutation in the intron 6 of *TNFRSF11A* has been described in one patient indicating *TNFRSF11A* as additional gene responsible for DOS (56).

A recent report described two affected siblings presenting osteopetrosis associated with severe combined immunodeficiency (SCID) caused by a large deletion on chromosome 11 encompassing *RAG1* and *RAG2* genes and the 5′ region of *TRAF6* (TNF receptor-associated factor 6 gene), the most important adaptor for the RANK/RANKL signaling pathway (57).

Lastly, a heterozygous truncating mutation in the *CSF1R* gene, which encodes for MCSF (macrophage colony-stimulating factor) receptor, was reported in the consanguineous parents of two deceased siblings, showing osteopetrosis and brain malformations (58). This mutation could not be assessed in the probands, however based on this report, this genetic variant could be responsible for the disease in this family (59).

AUTOSOMAL DOMINANT OSTEOPETROSIS

Autosomal Dominant Osteopetrosis (ADO) has an incidence of 1:20,000 live births with clinical onset typically in adolescence or adulthood (4) and cases diagnosed in pediatric age are reported too (18, 60). It is characterized by diffuse osteosclerosis, primarily involving the axial skeleton, and symmetrical defects of the long bones, with no or little modeling defects. ADO form, also known as benign form, is caused by heterozygous missense mutations of the *CLCN7* gene with dominant negative characteristic, in which the mutant subunit is able to dimerize, functionally impairing the protein (12, 17). Patients affected with ADO present a wide range of symptoms: radiographic alterations, frequent atraumatic fractures, osteonecrosis or osteomyelitis, vision and hearing impairment due to cranial nerve compression and occasional bone marrow failure (4, 8, 61). Although *CLCN7* is widely expressed in the body and the biallelic loss of function causes neurodegeneration in some CLCN7-deficient ARO patients, only sporadic cases of cognitive failure have been reported in ADO patients (4, 12).

X-LINKED OSTEOPETROSIS

Osteopetrosis caused by mutations of the *IKBKG* (inhibitor of nuclear factor kappa B kinase subunit gamma) gene, located on the X chromosome, occurs as a moderate complication of the OL-EDA-ID syndrome, lymphedema, anhidrotic ectodermal dysplasia and immunodeficiency (hence, the acronym) (62–65). The *IKBKG* gene encodes NEMO, the regulatory subunit of IKK complex, fundamental for the activation of NF-kB transcription factor to induce osteoclastogenesis (62). Consistently, inhibition of NF-kappaB signaling in mouse models of inflammation showed amelioration of osteolysis and inflammation (66). Bone biopsy evaluation in a patient revealed that osteoclasts were present in normal numbers and showed no morphological abnormalities (63). Thus, OL-EDA-ID is classified as an osteoclast-rich osteopetrosis (67).

CURRENT THERAPIES AND MANAGEMENT OF OSTEOPETROSIS

The majority of osteopetrotic forms are caused by osteoclast dysfunction, while a lower proportion of cases are caused by impaired osteoclastogenesis (8). **Table 1** summarizes the main clinical features in various forms of osteopetrosis. Since osteoclasts derive from the myeloid lineage, HSCT is the recommended treatment. A successful HSCT allows the engraftment of donor-derived osteoclast precursors, which further differentiate and give rise to functional mature osteoclasts, resulting in bone remodeling and haematopoiesis (9). However, HSCT is contraindicated in patients with primary neurodegenerative disease (**Table 1**).

Since secondary neurological defects are not rescued by transplant, genetic diagnosis and HSCT need to be performed as soon as possible (7, 68, 69). To this end, *in utero* HSC transplantation might represent in the future a therapeutic option as demonstrated by successful preclinical studies performed in the *oc/oc* mouse model (70, 71). Multicentre studies reported that patients undergoing HLA-haploidentical HSCT before the age of 10 months, survived with a full donor engraftment. On the contrary, almost all patients receiving HSCT after the age of 10 months underwent graft rejection or autologous reconstitution, even when an haploidentical donor source was used (7). Taken together, these evidences suggest that the fast diagnosis and timing of treatment, play a fundamental role in the long-term efficacy of HSCT (8). The degree of donor

TABLE 1 | Main clinical features and indications for treatment in osteopetrosis.

Gene	Autosomal recessive osteopetrosis	Overall disease severity	Hematological defects	Visual defects	Hypocalcemia	Growth retardation	Primary neurodegeneration	Indication to HSCT
TCIRG1	Osteoclast-rich form	Most often severe	Severe	Mild to severe	Severe	Mild to severe	No	Yes
CICN7	Osteoclast-rich form	Severe to mild	Mild to severe	Mild to severe	Severe	Mild to severe	Yes	To be evaluated based on the severity of CNS involvement
OSTM1	Osteoclast-rich form	Severe	Mild to severe	Mild to severe	Moderate	Mild to severe	Yes	No severe CNS involvement
SNX10	Osteoclast-rich form	Variable	Severe	Severe	Mild	Mild	No	Yes
CAII	Osteoclast-rich form	Moderate	None	Mild	Mild	Moderate	Cerebral calcification	To be evaluated based on cerebral calcification
PLEKHM1	Osteoclast-rich form	Mild	None	None	None	None to moderate	No	No mild presentation
FERMT3	Osteoclast-rich form	Severe	Severe	Mild	Mild	Mild	No	Yes
NEMO	Osteoclast-rich form	Severe	Severe	None	Mild	Moderate	No	Yes
TNFRSF11A/RANK	Osteoclast-poor form	Most often severe	Mild	Mild	Mild	Moderate	No	Yes
TNFSF11/RANKL	Osteoclast-poor form	Intermediate	Mild	Mild	Mild	Severe	No	No

compatibility is another key point to obtain a high rate of 5-years disease-free survival (DFS) after allogenic transplant. Data collected during the years on transplant outcomes, proved that the early diagnosis, the constant monitoring and prompt intervention for the associate comorbidities, the optimization of the donor source in term of HLA-matching and the choice of reduced intensity conditioning regimens allowed higher efficacy and safety of HSCT (9, 69, 72). The most recent report of transplants performed by Ulm and Paris Transplant Units highlighted the improved outcomes of HSCT with 93% of survival using T cell replete matched donor and 80% of survival using T cell depleted haploidentical donor (7). Unrelated cord blood is not recommended because its use is associated to high risk of primary engraftment failure (73). Fludarabine-based conditioning, performed better than the conventional cyclophosphamide-based one, in terms of higher engraftment and reduced toxicity with a higher 5-years DFS. In a selected cohort of 31 patients transplanted from related or unrelated fully matched donors, reduced intensity conditioning (RIC), based on fludarabine, treosulfan and thiotepa with proximal serotherapy dosing using anti-thymocyte globulin, allowed 100% overall survival (69).

The most frequent post-transplant complication is the engraftment failure caused by a delayed hematological reconstitution, due to limited or nearly absent bone marrow space (7) and graft vs. host disease (GvHD) (69). T-cell replete haploidentical graft with the administration of cyclophosphamide after HSCT has been proposed in patients older than 10 months (74). Frequently, transplanted ARO patients showed liver and pulmonary venous-occlusive disease (VOD) (75). Respiratory problems, such as choanal stenosis with upper airway obstructions, capillary leak syndrome, primary pulmonary infections and primary pulmonary hypertension were also frequent. When feasible, the use of a RIC regimen may reduces significantly the incidence of pulmonary hypertension (9, 69).

In addition, central nervous system complications may occur in terms of hydrocephalus, hypocalcaemic convulsions or deterioration of preexisting symptoms. Lastly a recurrent post-transplant risk was the onset of hypercalcemia, that can be treated by the use of Denosumab (76).

ALTERNATIVE TREATMENTS AND FUTURE THERAPIES

Despite recent improvement in the HSCT outcome, the availability of HLA-matched donors remains an open issue. For individuals lacking compatible donor, a strategy based on gene therapy (GT) has been proposed. The protocol would exploit the use of genetically modified $CD34^+$ cells, isolated from peripheral blood without the need of pharmacological HSC mobilization (3). The efficacy and the feasibility of GT have been studied in the *oc/oc* murine model, to evaluate neonatal transplantation of genetically corrected HSC in the context of *TCIRG1*-dependent osteopetrosis. Retroviral vectors were able to improve bone resorption and survival of *oc/oc* mice (77). Unfortunately, clinical trials in which immunodeficient patients were treated with this type of vector showed the risk of leukemia (78). In recent years, lentiviral vector GT has proven to provide clinical benefit in

patients affected by a number of diseases, avoiding the leukemic side effects (79, 80). Moreover, transduction of $CD34^+$ cells from the blood of *TCIRG1*-deficient patients with a lentiviral vector achieved the correction of the osteoclast dysfunction *in vitro* (81).

ARO caused by osteoclast extrinsic deficiency, such as *TNFSF11* mutations, requires a different approach. In particular, a replacement therapy has been evaluated at the preclinical level: the product of the *TNFSF11* gene, RANKL cytokine, has been administered pharmacologically to *Tnfsf11* knockout mice, rescuing bone defects and hematopoietic organ architecture (82). Additional strategies could be considered, for example, mesenchymal stem cell (MSC) transplantation to replace the osteoblast precursor population (83); however clinical application still raises doubts and challenges, thus this is far from a mature therapeutic option. The second method exploited the use of biotechnological devices, implanted subcutaneously, to release soluble RANKL and allowing osteoclastogenesis in *Tnfsf11* knockout mice (84). More recently, a promising biomimetic scaffold, seeded with *Tnfsf11* knockout MSC, overexpressing human soluble RANKL after transduction with lentiviral vector has been developed. When implanted subcutaneously, the 3D system was well tolerated and was able to drive the differentiation of TRAP positive cells (85).

Regarding new approaches for the treatment of ADO2, small interfering RNA has been demonstrated to silence specifically the mutated *CLCN7* allele, and to be effective and safe *in vitro* on human cells and *in vivo*, in an ADO2 mouse model (86). Therefore, efforts have been undertaken to move into the clinic (87). Alternatively, the administration of different doses of IFN-γ partially reduced whole-body bone mineral density of ADO2 mice, although further studies for clinical applications are needed (88).

CONCLUSIONS

Genetic dissection of osteopetrosis has unveiled the complex scenario of molecules involved in the pathogenesis of this disease. Early genetic diagnosis is important to establish treatment and thus prevent worsening of the clinical signs. However, despite new molecular techniques have defined ARO molecular complexity, there is the need to further understand their clinical heterogeneity and design novel and suitable cure to these patients. To this end, significant progress has been made in the treatment of ARO thanks to the improvement of novel conditioning regimens and source of donor HSPC, however additional work remains to be done to overcome the limited availability of donors or lack of a therapy for patients carrying RANKL defects or presenting with neurodegenerative osteopetrosis. On this basis, efforts are currently ongoing to further extend the number of molecular players causative of the disease in parallel with the design of novel clinical strategies to be offered as curative treatment for different forms of osteopetrosis.

AUTHOR CONTRIBUTIONS

SP, VC, and AV wrote the manuscript. CS and EP critically revised the manuscript and contributed to design the figure.

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frontiers in Endocrinology

MINI REVIEW
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The Uncovered Role of Immune Cells and NK Cells in the Regulation of Bone Metastasis

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Bone is one of the main metastatic sites of solid tumors like breast, lung, and prostate cancer. Disseminated tumor cells (DTCs) and cancer stem cells (CSCs) represent the main target to counteract bone metastatization. These cells often localize in bone marrow (BM) at level of pre-metastatic niche: they can remain dormant for years or directly grow and create bone lesion, according to the different stimulations received in BM. The immune system in bone marrow is dampened and represents an appealing site for DTCs/CSCs. NK cells have an important role in controlling tumor progression, but their involvement in bone metastasis formation is an interesting and not fully investigated issue. Indeed, whether NK cells can interfere with CSC formation, kill them at the site of primary tumor, during circulation or in the pre-metastic niche needs to be elucidated. This review focuses on different aspects that regulate DTC/CSC life in bone and how NK cells potentially control bone metastasis formation.

Keywords: bone metastases, NK cells, cancer stem cells, dormancy, immune response

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INTRODUCTION

Although many cancer patients benefit from more efficient treatments of primary tumors and become long survivors, the overall probability to develop metastases is increasing, making this aspect of the disease a key target for researchers and clinicians (1). Bone is one of the main metastatic sites for different solid tumors including breast, lung, and prostate cancer (2). Bone metastasis formation and evolution is strongly influenced by a complex cross talk occurring among tumor, immune, and bone cells (3, 4). BM, besides cell precursors, contains different types of resident or recirculating mature immune cells, including Dendritic cells (DC), macrophages, granulocytes, myeloid derived suppressor cells (MDSCs), NK cells, and different T and B lymphocyte subsets. Although some of these cells (i.e., macrophages, granulocytes, lymphocytes, and NK cells) are endowed with effector functions and directly involved in pathogen elimination, virtually all BM immune cells can produce a variety of cytokines, chemokines, or other factors possibly influencing the local tissue homeostasis. Moreover, subtypes of bone cells, such as osteoclasts (OCs), originate from immune progenitors and use "immune" receptor/ligand pairs to rule their maturation and also to govern their bone degradation activity, a process significantly involved in metastasis formation (5).

Immune cells are poorly effective in the control of metastasis formation and growth, and this is true also for bone metastases, in spite of the consistency of the immune system in the bone niches. The definition of immunotherapeutic approaches in the metastatic disease is nevertheless attractive, especially considering NK cells, a subset of powerful effectors of the innate immunity endowed with

anti-tumor activity. These cells have been shown to kill pro-metastatic tumor initiating cells and, recently, also to control metastases in animal models. On the other hand, NK cell heterogeneity and the complexity of their functional interactions with the local tumor microenvironment indicate that specific studies need to be addressed to define their role in bone metastases.

DTCS COLONIZE BONE MARROW AND ACTIVATE THE BONE METASTATIC VICIOUS CYCLE

In primary tumors, genetic, and epigenetic changes favor the switch of malignant cells to less differentiated forms through a process called epithelial-to-mesenchymal transition (EMT) (6). Cells rising from this switch can leave the primary tumor site becoming disseminated tumor cells (DTCs). DTCs can express cancer stem cell (CSC) profiles and properties such as resistance to chemotherapy and ability to home in BM for long time. Indeed, DTCs can migrate to distant organs and establish in BM at level of the premetastatic niches, which are induced by soluble factors or extra-cellular vescicles released in circulation by the primary tumor (7). The presence of DTCs in BM has clinical relevance, since it is associated to an increased risk to develop bone metastases (8–11).

In the BM, DTCs can also compete with hematopoietic stem cells (HSC) (12, 13) and establish in the niche by interacting with different elements including osteoblasts (OBs), endothelial cells, and Extracellular Matrix (ECM). OBs constitutively express CXCL12 and attract CXCR4-expressing tumor cells (14). Using mouse models, it has been shown that breast, lung, and prostate cancer cells overexpressing CXCR4 and CXCR7 increased their ability to extravasate and colonize bone (15, 16), and CXCR4 inhibition decreased bone and lung metastases (17, 18). Integrins and cadherins are other crucial factors for the interactions between DTCs and niches (16, 19). In breast cancer, the vascular-endothelial molecule-1 (VCAM-1) binds with high affinity $\alpha4\beta7$ and $\alpha4\beta1$ on OC precursors, leading to osteoclastogenesis, and $\alpha4$ or VCAM-1 blocking antibodies effectively inhibit bone metastasis (20). Integrins can also interact with osteopontin (OPN), an ECM protein overexpressed in tumors and associated to tumor cell migration, metastases, and poor prognosis (21, 22).

Breast and prostate cancer DTCs can live in BM in a dormant state in pre-metastatic niche for years before starting to grow and to form metastases (5, 23). Indeed, the outgrowth of DTCs from dormant state, depends both on factors released by bone microenvironment, such as fibronectin, collagen I, and periostin (24), and by physical factors such as acid pH, hypoxia, high extracellular calcium concentration (25), which also cause disruption of the balanced physiological bone remodeling due to OC and OB activity (26). Remarkably, an increased OC activity generates the physical space for tumor expansion and induces the release from the bone matrix of molecules that further stimulate tumor cell proliferation, creating the vicious cycle (27, 28). Tumor cells in turn secrete PTHrP, activated vitamin D, tumor necrosis factor (TNF), matrix metalloproteinases (MMPs), interleukin-6 (IL-6), and other factors, which stimulate the expression of the receptor activator of nuclear factor NF-kB ligand (RANKL) on OBs, leading to the final stimulation of osteoclastogenesis from local OC precursors (3, 20).

INTERACTION BETWEEN IMMUNE SYSTEM AND BONE FAVOR TUMOR CELL SURVIVAL AND PROLIFERATION

A fundamental molecular link between immune system and bone is represented by the axis comprising RANKL, its receptor RANK, and the natural decoy receptor osteoprotegerin (OPG) (29, 30). RANK/RANKL interaction activates osteoclastogenesis, while OPG counteracts this effect by competing with RANK to bind RANKL (31). OBs and BM stromal cells are the main producers of both RANKL and OPG in physiological conditions (32), however, B or activated T cells can influence the RANKL/OPG ratio, end eventually osteoclastogenesis by producing OPG or RANKL, respectively (33). Literature data report that T cells could directly carry on a modulatory action on OCs through production of different factors such as IL-7, RANKL, TNFα (34–38). Circulating OC precursors from bone metastatic patients have been shown to differentiate into mature OCs in a T cell dependent way, in the absence of the classical OC inducers M-CSF, and RANKL (39). On the other hand, in mouse models, it has been shown that T cells exert a fundamental anti-tumor effect, regardless of OC status. Indeed, PLCγ2-KO mice, with dysfunctional OCs and impaired T-cell activation, showed increased bone tumor growth despite protection from bone loss, whereas Lyn-KO mice with numerous OCs and increased T-cell responses, showed impaired tumor growth in bone despite enhanced OC activity and osteolysis. The injection of antigen-specific wild-type cytotoxic CD8(+) T cells in both these mouse models normalized tumor growth in bone, suggesting their important role in the regulation of tumor bone metastases (40). T cells can limit tumor cell diffusion by releasing IFNγ, which also affects osteoclastogenesis, indeed lack of IFNγ has been related to the increase of bone metastases (41).

Tumor cells modify the surrounding microenvironment, indeed it has been shown that BM from breast cancer patients differed from that of healthy subjects in its cellular composition as well as the activation status of cells from the innate immune system (macrophages, NK cells) and from the adaptive immune system (T cell subsets) (42). Many immature and suppressor immune cell types are present in bone, such as T regulatory cells, which must maintain a balanced immune-reactivity (43), and MDSCs, which stimulate osteoclastogenesis (44). In breast cancer, infiltrating T regulatory cells produce RANKL, promoting OC differentiation, activity, and subsequent bone lesions (45).

MDSCs are increased in cancer patients from 2 up to 25% (46) suppressing innate and adaptive immune response, thus sustaining tumor growth and metastatization (47). In breast cancer, MDSCs, derived from bone metastatic microenvironment, can differentiate into mature and functional OCs *in vitro* (48).

NK CELLS ARE ENDOWED WITH POWERFUL ANTI-TUMOR FUNCTIONS

NK cells can kill a variety of tumor cells of different origin and types (49–52). This wide range of reactivity is ensured by the expression at the cell surface of several receptors capable of activating or inhibiting the main functions of NK cells, including the release of cytolytic granules (49, 53). Thus, thanks to their HLA-I-specific inhibitory receptors and a complex and heterogeneous group of activating receptors, NK cells can sense the HLA-I expression decrease that often characterizes tumor cells and recognize different ligands that can be variably induced on cells undergoing tumor transformation (**Table 1**). Different patterns of NK receptors are engaged during contact with pathological or non-pathological cells, regulating the activation, and the intensity of the cytolytic response (49, 50, 53, 54). Most NK cells express the FcγIII-receptor (CD16), which is a strong activator of cytotoxicity and enables NK cells to mediate the Antibody-Dependent Cellular Cytotoxicity (ADCC).

NK cells can attack tumor cells by releasing pro-apoptotic factors, including TNF-α and Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (69, 70), or cytokines capable of inhibiting tumor cell proliferation and promoting the inflammatory response, such as IFN-γ. In addition, NK cells can release chemokines (CCL3, CCL4, CCL5, and XCL1) capable of attracting T cells, DC, and monocytes (71, 72) and give rise to specific cross-talks promoting and regulating the adaptive anti-tumor response (73–75). Finally, NK cells can also amplify their recruitment at the tumor site by releasing a chemotactic form of HMGB1 molecule upon interaction with tumor cells (76).

In order to appropriately evaluate the role of NK cells in the control of tumors it should be also considered that the NK cell population is rather heterogeneous as it includes different cell subsets, each characterized by peculiar functional capabilities (77). In humans, the CD56brightCD16dim/neg ($CD56^{bright}$) and the CD56dim/CD16bright ($CD56^{dim}$) cells represent the two most studied NK cell types. The $CD56^{bright}$ NK cells largely produce IFN-γ in response to monokines but are poorly cytotoxic. These cells constitute 5–10% of circulating NK cells, and, in line with their pattern of chemokine and homing receptors (i.e., CD62L, CCR7, CXCR3, and CXCR4), represent most LN-NK cells and an important fraction of tissue NK cells in different organs. The $CD56^{dim}$ cells release IFN-γ upon triggering of major activating receptors (NKp46, NKp30, NKp44, and CD16) and are highly cytotoxic. They represent 90–95% of PB NK cells and predominate in spleen, lungs, and kidney although in different percentages. Moreover, CD56dim NK cells express chemokine receptors (CXCR1, CX_3CR1, and CXCR4)

TABLE 1 | Overview of the major NK cell receptors and Ligands involved in tumor cell recognition.

	NK Receptor	Ligand(s)	Ligand expression on tumor cells	References
Inhibitory receptors	KIRs*	HLA-I (HLA-A,B,C)	Down-regulated in certain tumor cells	(50, 54)
	CD94:NKG2A	HLA-E (non-classical HLA-I)	Down-regulated in certain tumor cells	(50, 54, 55)
	LILRB1	HLA-I (HLA-A,B,C)	Down-regulated in certain tumor cells	(50, 54)
		HLA-G (non-classical HLA-I)	Up-regulated in certain tumors	(55–57)
Activating receptors	NKp46	HSPG	Up-regulated/modified in different tumor cells	(58, 59)
		Complement Factor P (properdin)	?	(60)
		Additional still unknown ligands**		(50, 61)
	NKp44	HSPG	Up-regulated/modified in different tumor cells	(58, 59)
		MLL5 isoform	Ectopically expressed at the cell surface of tumor cells of hematologic and solid tumors	(62)
		PDGF-DD	Soluble factor released by several tumors (induces NKp44-dependent cytokine release)	(63)
		Nidogen-1	Decoy extracellular ligand expressed by different tumor cell lines (inhibits NKp44-dependent cytokine release)	(64)
	NKp30	HSPG	Up-regulated/modified in different tumor cells	(58, 59)
		BAT3	Up-regulated in different tumor cells (released in exosomes)	(65)
		B7-H6	Highly expressed in different tumor cells	(66)
	NKG2D	MICA/B, ULBP1-6	Up-regulated in tumors of epithelial and non-epithelial origins	(67)
	DNAM-1	CD155, CD112	Up-regulated in many tumor cell types	(68)

**KIRs, Killer-cell immunoglobulin-like receptor; NKG2A, Natural Killer Group 2A; LILRB1, Leukocyte Immunoglobulin Like Receptor B1; NKG2D, Natural Killer Group 2 D; DNAM-1, DNAX Accessory Molecule-1; HLA, Human Leukocyte Antigen; HSPG, Heparan Sulfate Proteoglycans; MLL5, mixed-lineage leukemia protein-5; PDGF-DD, platelet-derived growth factor—isoform dimer DD; BAT3, human leukocyte antigen (HLA)-B-associated transcript 3; MIC, MHC class I chain-related protein; ULBP, UL16 binding proteins.*

***Different tumor cell lines bind recombinant soluble NKp46 receptors and/or are killed by NK cells in a NKp46-dependent way but the putative ligand on these cells has not yet been identified.*

that allow their possible recruitment to inflamed peripheral tissues (77, 78). The assessment of NK cells in tissues and the definition of their anti-tumor potential are rather complicated. Indeed, tissues comprise both potentially cytotoxic NK cells that recirculate from PB, but also stably resident cells expressing specific markers of tissue retention (CD69, CD49a, and CD103) and chemokine receptors (CCR5, CXCR6) (79–82). These latter cells may display unique functions, possibly organ-specific, not necessarily oriented to tumor cell killing.

ROLE OF NK CELLS IN THE CONTROL OF SOLID TUMORS AND METASTATIC SPREAD

Several studies using different mice models have documented that NK cells can control tumor insurgence, growth, and metastasis dissemination (83–86). Remarkably, the role of NK cells in the control of tumors has also been suggested in different human studies. In a 11-year follow-up study on more than 8,000 healthy individuals, Imai et al. initially showed that insurgence of tumors of different types inversely correlated with the levels of natural cytotoxic activity of peripheral blood lymphocytes (87). More recently, different groups have found correlations between the quantity and the quality of tumor infiltrating or PB-NK cells and a more favorable prognosis or the lower number of metastases at diagnosis (88). In this last decade, it has also become evident that a plethora of mechanisms of tumor escape can strongly reduce the efficacy of NK cells. Within the tumor microenvironment, different immune suppressor cells (including Tregs and MDSCs), tumor-associated fibroblasts (TAF), and tumor cells can produce soluble factors (TGF-β, PGE2, IDO-derived kynurenine) which inhibit expression and function of the major activating receptors (89, 90). Similar effects on activating receptors are induced also by soluble decoy ligands shed by tumor cells or released as extracellular molecules (64, 89, 91, 92). Finally, exposure to hypoxia, which often characterizes tumor tissues, can also cause activating receptor down-regulation (93). Remarkably, some of these suppressive mechanisms, such as those induced by hypoxia and TAFs, appear to minimally affect the ADCC function (94). On the other hand, hypoxia and tumor cells can modulate the repertoire of chemokine receptors on NK cells and favor the preferential recruitment of CD56bright cells (poorly cytotoxic and unable to mediate ADCC) (95). The NK-cell recruitment into neoplastic tissues may also be influenced by the chemokine profile induced in the tumor microenvironment. Human lung and breast tumors have been shown to express higher CCL19 (a CD56bright cell attracting chemokine) and lower CXCL12 compared to their normal tissue counterpart (96), while in mice, BM with MM showed increased CXCL9 and CXCL10, decreased CXCL12, down-modulation of CXCR3 on NK cells, and selective reduction of KLRG1^{-} cytotoxic NK cells (97). Collectively, the above-described mechanisms can account for the observation that in different tumor tissues the NK cell infiltrate is often limited or constituted by CD56bright or altered (poorly functional) CD56^{dim} cells (50, 89).

Another important issue regards the so-called immune-checkpoints. Different pairs of receptor-ligands are available to the immune system to regulate or terminate excessive (dangerous) responses. Some of these receptors, such as PD-1, TIM-3, TIGIT, and SIGIRR, can be also expressed by NK cells, especially by those associated to tumors, and control different NK cell functions including cytotoxicity. Blocking or overcoming these checkpoints, by specific monoclonal antibodies or activating cytokines can improve the NK-mediated control of carcinogenesis or metastasis formation (98–101).

THE AMBIGUOUS ROLE OF THE NK:CSC CROSS-TALK IN THE CONTROL OF METASTASIS FORMATION

Whether NK cells can interfere with CSC formation, or kill CSC at the site of primary tumor, during circulation, or in the pre-metastatic niches represents an interesting and still incompletely investigated issue. Several reports have indicated that NK cells can kill tumor cells with features of CSC derived from different tumors (glioma, melanoma, colon, prostate, and breast) (102). Consistent with these findings, CSCs of different origins have been shown to express or even up-regulate the ligands for NKG2D, DNAM1, and NKp30 NK-activating receptors and cells undergoing EMT showed up-regulated NKG2D-Ls (88, 102). In addition, EMT induction in lung cancer cells could promote increased NK cell-mediated metastasis-specific immunosurveillance in RAG1$^{-/-}$ mice (103). On the other hand, it has also been shown that NK cells could induce melanoma cells to undergo EMT, upregulate the expression of stemness markers, reduce proliferative capability, thus acquiring characteristics reminiscent of the CSC phenotype. Moreover, EMT increased ability of melanoma cells to suppress NK cell cytotoxicity against tumor cells (104).

IS THERE ANY ROLE FOR NK CELLS IN BONE METASTASES?

Although the role of NK cells in contrasting bone metastases has been recently suggested in breast cancer preclinical models (105), an established knowledge on this issue is still lacking. BM is where NK cells mature and differentiate from CD34+ progenitors; therefore, it contains precursors at different stages. Once NK cells have matured, changes in the expression of the key receptors CXCR4 and S1P5 (down- and up-regulated, respectively) drive their egress from the CXCL12-containing BM and their recruitment to blood where the S1P5-ligand S1P is abundant (106). Besides the immature NK cell precursors, BM also contains a reservoir of mature NK cells, recirculating from the blood, which can be mobilized upon inflammatory stimuli (107). BM also includes a substantial population of resident CXCR6+CD69+ NK (BMrNK) cells, which may be poorly effective against tumor cells. Indeed, compared to classical NK cells, BMrNK cells display lower proliferative capacity, cytolytic granule content, DNAM1, and higher TIGIT expression (108).

The heterogeneity of the NK cells in the BM and their still poorly defined interaction with the metastatic niche, together with the possible cross-talk between PB-NK and CSC/EMT cells

add a layer of complexity to the issue of how NK cells can influence bone metastasis formation and progression (**Figure 1**). In the bone, the RANK-RANK-L axis, whose deregulation is important in metastasis formation, also influences NK cells. The signaling of RANK-L in leukemia cells can induce the release of NK-suppressing factors (109), whereas, under inflammatory conditions (knee arthritis) NK cells can stimulate OCs through activation of RANKL pathway (110). NK cells can either favor or inhibit generation of OCs depending on the release of TNF-α or IFN-γ, respectively (111, 112). On the other hand, OCs have been shown to contribute to the induction of efficient NK cells, capable of inhibiting growth of poorly differentiated tumors in humanized BLT mice (113). This effect is in line with the ability of OCs to produce NK-stimulating cytokines such as IL-12, IL-15, and IL-18. Finally, OCs are targets of NK cells, as they express MHC class I molecules at low levels and are killed by IL-2 treated NK cells (114).

CONCLUDING REMARKS

Understanding the reason why and how in many patients' metastases can overcome the surveillance of NK cells is still poorly understood. Studies are rapidly progressing to define how to properly activate NK cells by cytokine combinations and unleash their potential by blocking their checkpoint receptors. The crucial mechanisms that govern entrance and egress of NK cells in the bone metastatic niche and modulate the NK cell killing capability within the bone lesions are lacking. Addressing these questions will significantly increase

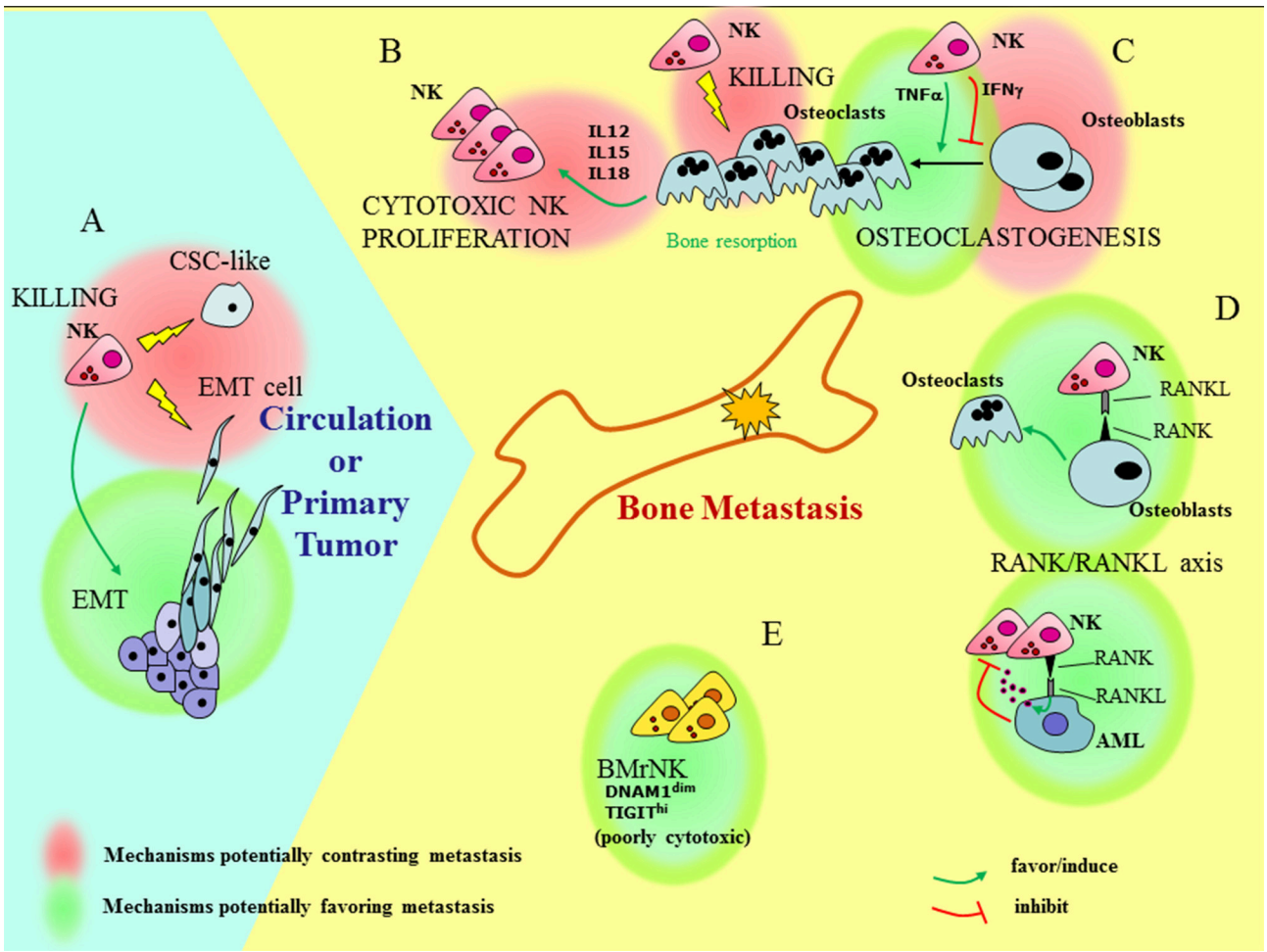

FIGURE 1 | Understanding how NK cells can influence bone metastasis. Outside and inside the bone (light blue and yellow fields, respectively), different functional interactions involving BM resident, recirculating, or PB NK cells can have contrasting effects on bone metastasis formation and tumor progression. **(A)** At the site of primary tumors NK cells may favor EMT. On the other hand, NK cells can kill pro-metastatic tumor cells, such as cells that have undergone EMT (EMT cells), and cells with features of CSC (CSC-like). **(B)** OCs can induce proliferation of highly cytotoxic NK cells via the release of IL12, IL15, and IL18. **(C)** The interaction of NK cells with OCs gives rise to contrasting effects: NK cells can either favor or inhibit generation of OCs depending on the release of TNF-α or IFN-γ, respectively and kill OCs. **(D)** The RANK/RANKL axis may play a role in the cross-talk between NK cells and bone microenvironment: the signaling of RANK-L in leukemia cells (AML) can induce the release of NK-suppressing factors; on the other hand, RANKL-expressing NK cells provide signals for OC generation. **(E)** Besides recirculating PB-NK cells, BM also contains resident poorly cytotoxic NK cells (BMrNK) which may hardly eliminate tumor cells.

the therapeutic options for NK cells in the treatment of bone metastatic disease.

AUTHOR CONTRIBUTIONS

IR revised the literature and wrote the paragraphs concerning bone metastasis. MV revised the literature and wrote the paragraphs concerning NK cells.

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Docosahexaenoic Acid Inhibits Inflammation-Induced Osteoclast Formation and Bone Resorption *in vivo* Through GPR120 by Inhibiting TNF-α Production in Macrophages and Directly Inhibiting Osteoclast Formation

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Docosahexaenoic acid (DHA) is an n-3 fatty acid that is an important structural component of the cell membrane. DHA exerts potent anti-inflammatory effects through G protein-coupled receptor 120 (GPR120), which is a functional receptor for n-3 fatty acids. DHA also regulates osteoclast formation and function. However, no studies have investigated the effect of DHA on inflammation-induced osteoclast formation *in vivo*. In the present study, we investigated whether DHA influences osteoclast formation, bone resorption and the expression of osteoclast-associated cytokines during lipopolysaccharide (LPS)-induced inflammation *in vivo*, and then we elucidated the underlying mechanisms by using *in vitro* experiments. *In vitro* experiments revealed both receptor activator of NF-kB ligand (RANKL)- and tumor necrosis factor-α (TNF-α)-induced osteoclast formation was inhibited by DHA. Supracalvarial administration of LPS with or without DHA was carried out for 5 days and then the number of osteoclasts, ratio of bone resorption pits and the level of type I collagen C-terminal cross-linked telopeptide were measured. All measurements were significantly lower in LPS+DHA-co-administered mice than LPS-administered mice. However, this DHA-induced inhibition was not observed in LPS-, DHA-, and selective GPR120 antagonist AH7614-co-administered mice. Furthermore, the expression of RANKL and TNF-α mRNAs was lower in the LPS+DHA-co-administered group than in the LPS-administered group *in vivo*. TNF-α mRNA levels were decreased in macrophages co-treated with LPS+DHA compared with cells treated with LPS *in vitro*. In contrast, RANKL mRNA expression levels from osteoblasts co-treated with DHA and LPS *in vitro* were equal to that in cells

treated with LPS alone. Finally, the inhibitory effects of DHA on osteoclast formation *in vitro* were not observed by using osteoclast precursors from GPR120-deficient mice, and inhibition of LPS-induced osteoclast formation and bone resorption by DHA *in vivo* was not observed in GPR120-deficient mice. These results suggest that DHA inhibits LPS-induced osteoclast formation and bone resorption *in vivo* via GPR120 by inhibiting LPS-induced TNF-α production in macrophages along with direct inhibition of osteoclast formation.

Keywords: osteoclast, DHA, LPS, mouse, GPR120

INTRODUCTION

Osteoclasts, which are specialized bone resorbing cells derived from hematopoietic stem cells, play important roles in bone remodeling and in bone destruction in diseases such as rheumatoid arthritis, osteoporosis, and periodontal disease (1). Two important cytokines, receptor activator of NF-kB ligand (RANKL) and macrophage colony stimulating factor (M-CSF), are required for osteoclast formation and osteoclast function (2). M-CSF is also essential for proliferation of osteoclast precursors. Tumor necrosis factor-α (TNF-α) has also been reported to induce osteoclast formation both *in vitro* (3–5) and *in vivo* (6, 7). TNF-α is central to pathological bone disorders including inflammation (8).

Lipopolysaccharide (LPS), which is a major constituent of the cell wall of Gram-negative bacteria, induces inflammation and pathological bone destruction (9, 10). LPS also induces the production of pro-inflammatory cytokines, such as TNF-α from macrophages and other cells at sites of inflammation (11, 12). Furthermore, LPS stimulates osteoblasts to produce and express osteoclast-related cytokine RANKL (13). These cytokines have been linked to LPS-induced osteoclast formation and bone resorption in both *in vivo* and *in vitro* studies (9, 14).

It has been reported that polyunsaturated fatty acids confer some beneficial effects on cardiovascular diseases (15), autoimmune disorders and inflammatory disorders such as rheumatoid arthritis, inflammatory bowel disease, and dysmenorrhea (16, 17) and diabetes (18). Docosahexaenoic acid (DHA), a well-known dietary n-3 polyunsaturated fatty acid, is a long-chain polyunsaturated fatty acid that has 22 carbon atoms and 6 double bonds. DHA is used as a food supplement and has favorable effects against certain cancers, diabetes and cardiovascular diseases (19, 20).

G protein-coupled receptors (GPRs) play a pivotal role as signaling molecules for many cellular functions. G protein-coupled receptors are seven transmembrane domain receptors that regulate many physiological and pathological responses (21–24). It has been reported that free fatty acids can activate receptors GPR40 (free fatty acid receptor: FFAR1), GPR41 (FFAR3), GPR43 (FFAR2), GPR84, and GPR120 (FFAR4) and long-chain fatty acids can activate GPR40 and GPR120 (25, 26). GPR120, also known as free fatty acid receptor 4 (FFAR4), has also been implicated in homeostatic metabolic regulation in immune processes and inflammatory *in vivo* (27). Therefore, GPRs are leading targets for drug development for many human diseases. Especially, GPR120 has gathered attention owing to its potential role in the regulation of many inflammation-related diseases such as diabetes and obesity (21, 26–31).

It has been reported that dietary n-3 fatty acids inhibit bone loss in ovariectomized mice owing to their inhibitory effects on osteoclast formation (32). Some studies showed the inhibitory effects of DHA on osteoclast formation and activity *in vitro*. It has been reported that DHA inhibits osteoclast formation and function in human CD14+ monocytes (33). It has also been shown the differential effects of DHA on osteoclast formation in the murine monocytic cell line RAW 264.7 (34). These *in vitro* studies give some insight into the effects of DHA on osteoclast formation, however, the effects of DHA on osteoclast formation *in vivo* remain unclear. Furthermore, the effects of DHA through GPR120 on osteoclast formation *in vivo* have not been investigated.

In the present study, we showed the effects of DHA on LPS-induced osteoclast formation and bone resorption via GPR120 in a murine experimental model and elucidated the underlying mechanisms by using *in vitro* experiments.

MATERIALS AND METHODS

Animals and Reagents

Eight- to ten-week-old male C57BL6/J mice were obtained from CLEA Japan (Tokyo, Japan). C57BL6 mice bearing the *Ffar4*-floxed gene were generated by an outsourced research company (Transgenic Inc., Fukuoka, Japan) as schematically illustrated in **Supplementary Figure 1**. In the conditional *Ffar4*-targeting vector designed according to the recombineering-based construct, two loxP sites were inserted into the introns immediately upstream and downstream of the exon 1 such that an FRT-containing neomycin resistance cassette was included in the loxP-flanked region. The targeting vector was introduced into ES cells, and homologous recombination-positive clones were isolated and used for generating germline-chimeric mice by the embryo aggregation method. Heterozygous mutant mice bearing the homologous mutation were produced and bred with the Flp mice (Jackson Laboratory, Bar Harbor, USA) to remove the neomycin resistance cassette. To generate systemic *Ffar*4 knock out mice [$Ffar4^{(dE1/dE1)}$ mice], the floxed mice were crossed with transgenic mice expressing Cre -recombinase under the control of the chicken actin promoter (CAG). All animal care and experiments were conducted according to Tohoku University rules and regulations. Four mice were randomly distributed

in each experimental group. DHA (Sigma-Aldrich, St Louis, MO, USA), *Escherichia coli* LPS (Sigma-Aldrich) and GPR120 antagonist AH7614 (Tocris Bioscience, Bristol, United Kingdom) were used in this study. For *in vitro* study, recombinant mouse RANKL (35) and TNF-α (6) were obtained as previously described, and recombinant mouse M-CSF was prepared from an M-CSF-expressing cell line (CMG14-12) (36).

Histological Analysis

In a previous study, daily subcutaneous supracalvarial administration of 100 μg LPS to mouse calvariae for 5 days significantly induced osteoclast formation (37, 38). Therefore, we followed the same protocol, dose and LPS administration period in this study. Four mice were randomly distributed in each group and received daily subcutaneous injections of phosphate-buffered saline (PBS; negative control group), LPS (100 μg/day, positive control group), LPS (100 μg/day), and DHA (100 μg/day) with or without AH7614 (100 μg/day) and DHA (100 μg/day). *Ffar4*$^{(dE1/dE1)}$ mice were subjected to daily subcutaneous injections of LPS (100 μg/day, positive control group) and LPS (100 μg/day) and DHA (100 μg/day). Calvariae were resected immediately after sacrifice on the 6th day. After fixation in 4% PBS-buffered formaldehyde, all calvariae were demineralized in 14% ethylenediaminetetraacetic acid (EDTA) for 3 days. After dehydration, calvariae were embedded in paraffin and sectioned perpendicular to the sagittal suture (5-μm thickness) using a microtome (Leica, Wetzlar, Germany). Sections were stained for tartrate-resistant acid phosphatase (TRAP) and hematoxylin counterstain as previously described (37, 38). Osteoclasts were identified as TRAP-positive cells containing three or more nuclei. The number of osteoclasts located at the mesenchyme of the sagittal suture was counted in all slides as described previously (37, 38). In addition, the percentage of interface of bone marrow space covered by osteoclasts and the number of osteoclast of interface of bone marrow space were histomorphometrically determined in specimens derived from each sample.

Preparation of Osteoclast Precursors for Osteoclast Formation

Osteoclast formation was evaluated as previously described (37, 38). Briefly, femoral and tibial epiphyses from 8 to 10-week-old male C57BL/6J mice were cut, and then the bone marrow was flushed using a 25-gauge needle and syringe prefilled with alpha-modified minimal essential medium (α-MEM; Sigma-Aldrich). Harvested cells were then cultured in α-MEM containing 10% fetal bovine serum, 100 IU/mL penicillin G (Meiji Seika, Tokyo, Japan) and 100 μg/mL streptomycin (Meiji Seika) with M-CSF. Adherent cells were harvested using trypsin-EDTA solution (Sigma-Aldrich). Harvested cells were then cultured in the presence of M-CSF until they reached confluency. Cells were recognized as osteoclast precursors. Osteoclast precursors were seeded at 5×10^4 cells per 200 μL of culture medium in a 96-well plate and cultured for 5 days in medium containing M-CSF (100 ng/mL); M-CSF (100 ng/mL) and RANKL (100 ng/mL) or TNF-α (100 ng/mL); M-CSF (100 ng/mL), RANKL (100 ng/mL), or TNF-α (100 ng/mL), and DHA (100 ng/mL) with or without AH7614 (100 ng/mL); or M-CSF (100 ng/mL) and DHA (100 ng/mL). After fixation with 4% PBS-buffered formaldehyde, cells were permeabilized with 0.2% Triton X-100 and TRAP staining was performed to visualize active osteoclasts. TRAP-positive cells containing three or more nuclei were counted under a light microscope (37, 38).

Isolation of Murine Macrophages

Eight- to ten-week old C57BL/6J mice were sacrificed to obtain peritoneal macrophages; 5 mL sterile ice-cold PBS (pH 7.4) was injected into the peritoneal cavity and then the fluid was aspirated out to collect cells. Collected cells were incubated for 1 h and then non-adherent cells were removed. Adherent cells were cultured an additional 24 h and used as peritoneal macrophages (37, 38).

RNA Preparation and Real-Time RT-PCR Analysis

Calvariae from *in vivo* experiments were frozen in liquid nitrogen and crushed by Micro Smash MS-100R (Tomy Seiko, Tokyo, Japan) in 800 μL TRIzol reagent (Invitrogen, Carlsbad, CA) for each sample. Total RNA was extracted with an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Osteoblasts (Cosmo Bio, Tokyo, Japan) or macrophages were cultured in culture medium supplemented with PBS; LPS (100 ng/mL); LPS (100 ng/mL); and DHA (100 ng/mL) with or without AH7614 (100 ng/mL); or DHA (100 ng/mL). After 24 h culture, total RNA was extracted from osteoblasts or peritoneal macrophages. cDNA was synthesized for each sample from 2 μg total RNA with oligo-dT primers (Invitrogen) and reverse transcriptase in a total volume reaction of 20 μL. To assess the gene expression value, the Thermal Cycler Dice Real Time System (Takara, Shiga, Japan) was used for real-time RT-PCR. Each reaction comprised a total volume of 25 μL containing 2 μL cDNA and 23 μL of a mixture of SYBR Premix Ex Taq (Takara) and 50 pmol/μL primers. PCR cycling conditions were as follows: 95°C for 10 s for initial denaturation followed by 45–60 amplification cycles, with each cycle comprising a denaturation step of 95°C for 5 s and then an annealing step of 60°C for 30 s. Relative expression levels of RANKL and TNF-α mRNAs were calculated by normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. Primer sequences used for cDNA amplification were as follows: 5′-GGTGGAGCCAAAAGGGTCA-3′ and 5′-GGGG GCTAAGCAGTTGGT-3′ for GAPDH; 5′-AGGCGGTGC TTGTTCCTCA-3′ and 5′-AGGCGAGAAGATGATCTGA CTGCC-3′ for TNF-α; and 5′-CCTGAGGCCAGCCATTT- 3′ and 5′-CTTGGCCCAGCCTCGAT-3′ for RANKL as already reported (37, 38).

Analysis of Bone Resorption

To evaluate the bone resorption area, calvariae were fixed in 4% PBS-buffered formaldehyde and then scanned with microfocus computed tomography (CT) (ScanXmate-E090; Comscan, Kanagawa, Japan). Three-dimensional images of the calvariae were prepared using TRI/3D-BON64 software (RATOC System Engineering, Tokyo, Japan), and the ratio of the bone

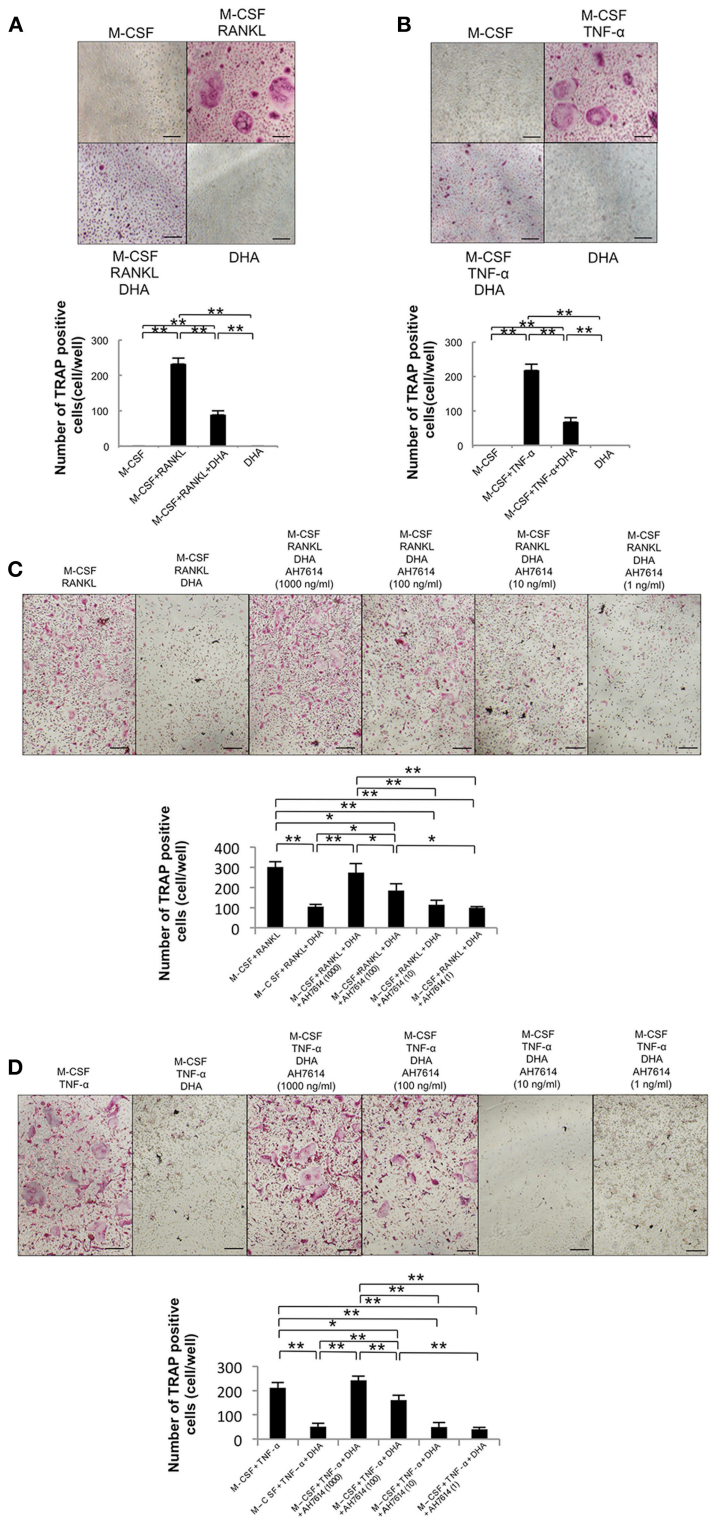

FIGURE 1 | DHA inhibits RANKL- and TNF-α-induced osteoclast formation via GPR120 *in vitro*. **(A)** Microscopic images and numbers of TRAP-positive cells following RANKL-induced osteoclast formation. Osteoclast precursors were cultured with M-CSF, M-CSF+RANKL, M-CSF+RANKL+DHA, or M-CSF+DHA for 5 days, followed by staining with TRAP solution. **(B)** Microscopic images and numbers of TRAP-positive cells following TNF-α-induced osteoclast formation. Osteoclast precursors were treated with M-CSF, M-CSF+TNF-α, M-CSF+TNF-α+DHA, or M-CSF+DHA for 5 days, followed by staining with TRAP solution. **(C)** Microscopic images and numbers of TRAP-positive cells following RANKL-induced osteoclast formation under AH7614 treatment. Osteoclast precursors were cultured with

(Continued)

FIGURE 1 | M-CSF+RANKL, M-CSF+RANKL+DHA, or M-CSF+RANKL+DHA and various concentration of AH7614 (1,000, 100, 10, and 1 ng/mL). **(D)** Microscopic photos and numbers of TRAP-positive cells following TNF-α-induced osteoclast formation under AH7614 treatment. Osteoclast precursors were cultured with M-CSF+TNF-α, M-CSF+TNF-α+DHA, or M-CSF+TNF-α+DHA and various concentration of AH7614 (1,000, 100, 10, and 1 ng/mL). Data are expressed as means ± SD ($n = 4$; $^{**}p < 0.01$, $^{*}p < 0.05$). Statistical significance was determined by Scheffe's test. Scale bars = 100 μm.

resorption area to the total area was measured using ImageJ (NIH, Bethesda, MD) (37, 38).

Measurement of Serum C-Terminal Telopeptide (CTX) of Type I Collagen

Serum was obtained from mice after 5 days of daily supracalvarial administration of PBS, LPS, LPS+DHA with or without AH7614 or DHA alone. Serum CTX levels were evaluated using a mouse CTX assay kit (IDS, Tyne and Wear, UK) by measuring absorbance at 450 nm using a microplate reader (Remote Sunrise; Tecan, Kawasaki, Japan) with a reference wavelength of 620 nm (37, 38).

Statistical Analyses

All data are expressed as means ± standard deviation (SD). Differences between groups were assessed using Scheffe's test, one-way ANOVA and the Turkey-Kramer test. Statistical significance was assumed at a threshold of $p < 0.05$.

RESULTS

DHA Inhibits RANKL- and TNF-α-Induced Osteoclast Formation via GPR120 Activation *in vitro*

First, we examined the influence of DHA on RANKL- and TNF-α-induced osteoclast formation to clarify whether DHA directly influences osteoclast precursor cells (**Figures 1A,B**). A large number of TRAP-positive cells were noted among osteoclast precursor cells cultured with M-CSF and RANKL or TNF-α. In contrast, the number of TRAP-positive cells was markedly decreased when cells were cultured with M-CSF and RANKL or TNF-α with DHA. Then, we investigated whether the inhibitory effect of DHA is diminished by GPR120 antagonist AH7614 (**Figures 1C,D**). AH7614 dose-dependently increased the number of TRAP-positive cells compared with cells cultured with M-CSF and RANKL or TNF-α with DHA.

DHA Inhibits LPS-Induced Osteoclast Formation via GPR120 Activation *in vivo*

To assess whether LPS-induced osteoclast formation is inhibited by DHA via GPR120 *in vivo*, LPS and DHA with or without AH7614 were injected into mouse calvariae. When LPS was administered for 5 consecutive days, many large multinucleated TRAP-positive cells were noted within the suture mesenchyme of histological sections (**Figure 2A**), the higher percentage of interface of bone marrow space covered by osteoclasts and the higher number of osteoclast of interface of bone marrow space were observed (**Figure 2B**). In contrast, the mean number of TRAP-positive cells and the percentage of osteoclast area of interface of bone marrow space were markedly decreased in the LPS and DHA co-administered group compared with the LPS group, while the inhibitory effect of DHA was attenuated by co-administration with AH7614 (**Figures 2A,B**). Moreover, TRAP mRNA levels were remarkably lower in the LPS and DHA group than in LPS or LPS and DHA with AH7614 co-administered groups (**Figure 2C**).

DHA Inhibits LPS-Induced Bone Resorption via GPR120 Activation *in vivo*

The ratio of bone resorption area to total area in three-dimensional images of mouse calvariae was evaluated and compared in each group. Many bone destruction defects were observed in the LPS group compared with the PBS or DHA group. However, the bone destruction defects were significantly reduced in the LPS and DHA co-administered group, which was increased again in the LPS and DHA with AH7614 co-administered group (**Figures 3A,B**). We next evaluated serum CTX levels by using a mouse CTX assay kit. Serum CTX levels in the LPS group were significantly increased compared with those in the PBS or DHA group, although the LPS with DHA group had significantly lower CTX levels than the LPS group (**Figure 3C**). Additionally, serum CTX levels were significantly increased in the LPS and DHA with AH7614 co-administered group compared with the LPS and DHA group.

DHA Inhibits Expression of LPS-Induced TNF-α and RANKL mRNAs *in vivo*

The expression levels of osteoclast-related cytokines in calvariae were determined by using real-time RT-PCR (**Figures 4A,B**). RANKL and TNF-α mRNA levels were significantly elevated in the LPS group compared with the PBS group. In contrast, RANKL and TNF-α mRNA expression levels were significantly lower in the LPS and DHA group than those of the LPS group. Furthermore, treatment with GPR120 antagonist AH7614 elevated RANKL and TNF-α mRNA expression levels compared with the LPS and DHA group.

DHA Suppresses LPS-Induced TNF-α Expression in Macrophages but Does Not Inhibit RANKL Expression in Osteoblasts

To precisely determine how DHA inhibits osteoclast formation via GPR120, we investigated RANKL mRNA expression levels in osteoblasts *in vitro*. RANKL mRNA expression levels were upregulated by LPS treatment compared with PBS or DHA alone. However, osteoblasts treated with both LPS and DHA demonstrated similar RANKL mRNA expression levels compared with cells treated with LPS alone. Moreover, RANKL mRNA expression levels were similar between the LPS and DHA

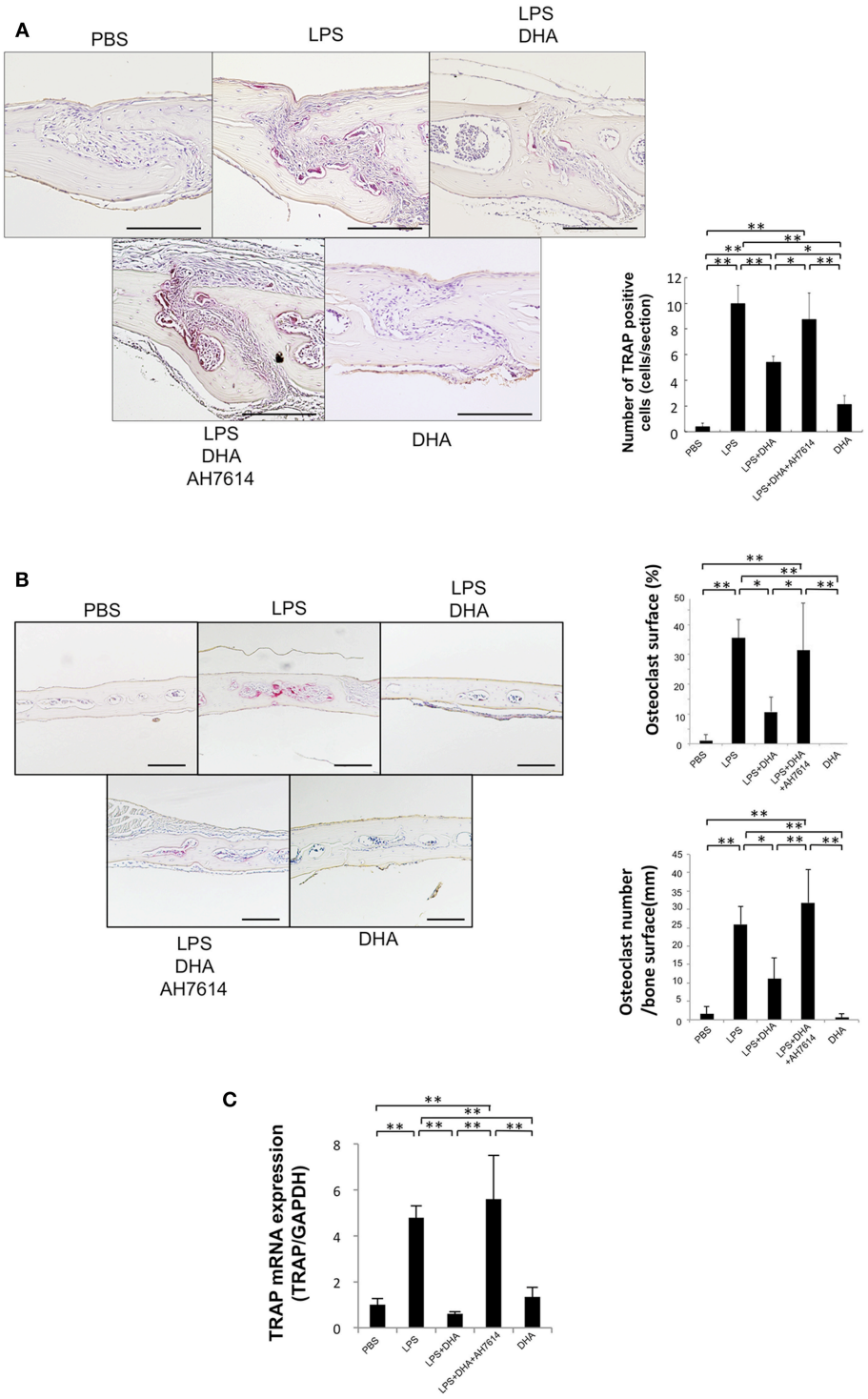

FIGURE 2 | DHA inhibits LPS-induced osteoclast formation via GPR120 activation *in vivo*. **(A)** Histological sections of calvariae were prepared from C57/BL6 mice following 5 days of supracalvarial administration of PBS, LPS (100 μg/day), LPS (100 μg/day)+DHA (100 μg/day), LPS (100 μg/day)+DHA (100 μg/day)+AH7614 (100 μg/day), or DHA (100 μg/day). Sections were stained with TRAP solution and hematoxylin for counterstaining was performed. TRAP-positive cells appeared dark red. Scale bars = 100 μm. Numbers of TRAP-positive cells in the suture mesenchyme of calvariae from mouse groups administered PBS, LPS, LPS+DHA;

(Continued)

FIGURE 2 | LPS+DHA+AH7614 or DHA. Data are shown as means ± SD. Statistical significance was determined by Scheffe's test ($n = 4$; $^{**}p < 0.01$, $^{*}p < 0.05$). **(B)** Histological sections of calvariae after 5 days of daily supracalvarial administration of PBS, LPS (100 μg/day), LPS (100 μg/day)+DHA (100 μg/day), LPS (100 μg/day)+DHA (100 μg/day)+AH7614 (100 μg/day), or DHA (100 μg/day). Sections were stained with TRAP solution and hematoxylin for counterstaining was performed. The percentage of interface of bone marrow space covered by osteoclast and the number of TRAP-positive cells per millimeter of interface of bone marrow space were evaluated. Data is expressed as means ± standard deviation (SD). Statistical significance was determined by Scheffe's test ($n = 4$; $^{**}p < 0.01$ $^{*}p < 0.05$). **(C)** Expression levels of TRAP mRNA in calvariae of the mouse groups determined by real-time RT-PCR analysis. Data are expressed as means ± SD. Statistical significance was determined by one-way ANOVA and Turkey-Kramer tests ($n = 4$; $^{**}p < 0.01$, $^{*}p < 0.05$).

with AH7614 co-administered group and the LPS and DHA group (**Figure 5A**).

We next investigated whether DHA inhibits LPS-induced TNF-α expression in macrophages *in vitro*. TNF-α mRNA expression levels were upregulated by LPS treatment, while they were decreased by LPS and DHA treatment. Moreover, treatment with GPR120 antagonist AH7614 significantly increased TNF-α mRNA expression levels compared with LPS and DHA (**Figure 5B**).

DHA Does Not Suppress LPS-Induced Osteoclast Formation, Bone Resorption and Production of Osteoclast-Related Cytokines (TNF-α and RANKL) in *Ffar4*$^{(dE1/dE1)}$ Mice

To clarify the role of GPR120 in osteoclast formation, we generated and used *Ffar4* knockout mice [*Ffar4*$^{(dE1/dE1)}$]. Osteoclast precursor cells from *Ffar4*$^{(dE1/dE1)}$ mice were treated with M-CSF, M-CSF, and RANKL with or without DHA or DHA alone, and then the number of TRAP-positive cells was counted. The number of TRAP-positive cells was increased in the M-CSF and RANKL group. However, there was no significant difference in the number of TRAP-positive cells between the M-CSF and RANKL with DHA group and the RANKL group (**Figure 6A**). Similar results were observed in osteoclast formation following TNF-α and DHA treatment (**Figure 6B**).

We also evaluated osteoclast formation *in vivo* using *Ffar4*$^{(dE1/dE1)}$ mice. Supracalvarial administration of LPS with or without DHA was carried out for 5 days, and then the number of TRAP-positive cells at the mesenchyme of the sagittal suture was counted. Furthermore, the percentage of interface of bone marrow space covered by osteoclasts and the number of osteoclast of interface of bone marrow space were evaluated. In the LPS group, a great number of TRAP-positive cells and the higher percentage of osteoclast area of interface of bone marrow space was observed in both wild type and *Ffar4*$^{(dE1/dE1)}$ mice. There was no difference in the number of TRAP-positive cells and the percentage of osteoclast area of interface of bone marrow space between the LPS and DHA co-administrated group and the LPS group. However, the number of TRAP-positive cells and percentage of osteoclast area of interface of bone marrow space were decreased in the LPS and DHA co-administrated group compared with that of wild type mice (**Figures 6C,D**). There is no significant difference of osteoclast number and percentage of osteoclast area in both PBS injected wild type and *Ffar4*$^{(dE1/dE1)}$ mice as control (**Supplementary Figure 2**).

Finally, we investigated bone resorption in *Ffar4*$^{(dE1/dE1)}$ mice by using microfocus CT. There was no significant difference between the ratio of bone resorption area in wild type and *Ffar4*$^{(dE1/dE1)}$ mice administered LPS alone. In contrast, a marked decrease was observed in the ratio of bone resorption area in wild type mice co-administered LPS and DHA, while no significant change was observed in *Ffar4*$^{(dE1/dE1)}$ mice co-administered LPS and DHA compared with respective mice that received LPS alone (**Figure 6E**).

DISCUSSION

Favorable effect of food intake and supplement of n-3 fatty acids such as DHA were provided against skeletal disorders such as osteoporosis and rheumatoid arthritis (19, 20). There are some *in vitro* studies of effect of DHA on osteoclast formation by using RAW264.7 cell (34, 39) and human CD14+ monocytes (33). GPR120, which is one of the cell surface receptor, has an important role in the regulation of inflammation. Therefore, GPR120 is being watched with keep interest as one of the potential therapeutic target in several inflammatory disorders. In this study, we showed that DHA inhibits LPS-induced osteoclast formation and bone resorption *in vivo* via GPR120 by inhibiting LPS-induced TNF-α production in macrophages and directly inhibiting osteoclast formation.

In the present study, we focused on GPR120, because several studies showed GPR120 is highly expressed in RAW264.7 cells, intraperitoneal macrophages, osteoclast precursors and osteoclast (27, 40, 41). It has been reported that DHA can activate not only GPR120 but also GPR40 (42). GPR40 activation by using its selective agonist GW9508 by blocked osteoclast formation (43). However, Kim et al. reported that GPR120 was highly expressed but GPR40 is very low in osteoclast precursors and osteoclast (41). Therefore, we thought that GPR120 might play important role in osteoclast formation and function.

Several studies showed that DHA inhibits osteoclast formation *in vitro*. Kim et al. reported that DHA inhibits RANKL-induced osteoclast formation in primary murine macrophages by suppressing the activation of NF-kB and MAPKs (44). Sun et al. also showed that DHA inhibits RANKL-induced osteoclast formation in bone marrow macrophages (32). Furthermore, DHA reduces RANKL-induced osteoclast formation in RAW 264.7 cells (34, 39) by inhibition of c-Fos expression (34). It has also been reported that DHA inhibits osteoclast formation and function in human CD14+ monocytes (33). In the present study, we initially investigated whether DHA can inhibit RANKL- and TNF-α-induced osteoclast formation by directly affecting osteoclast precursors *in vitro*. Our results

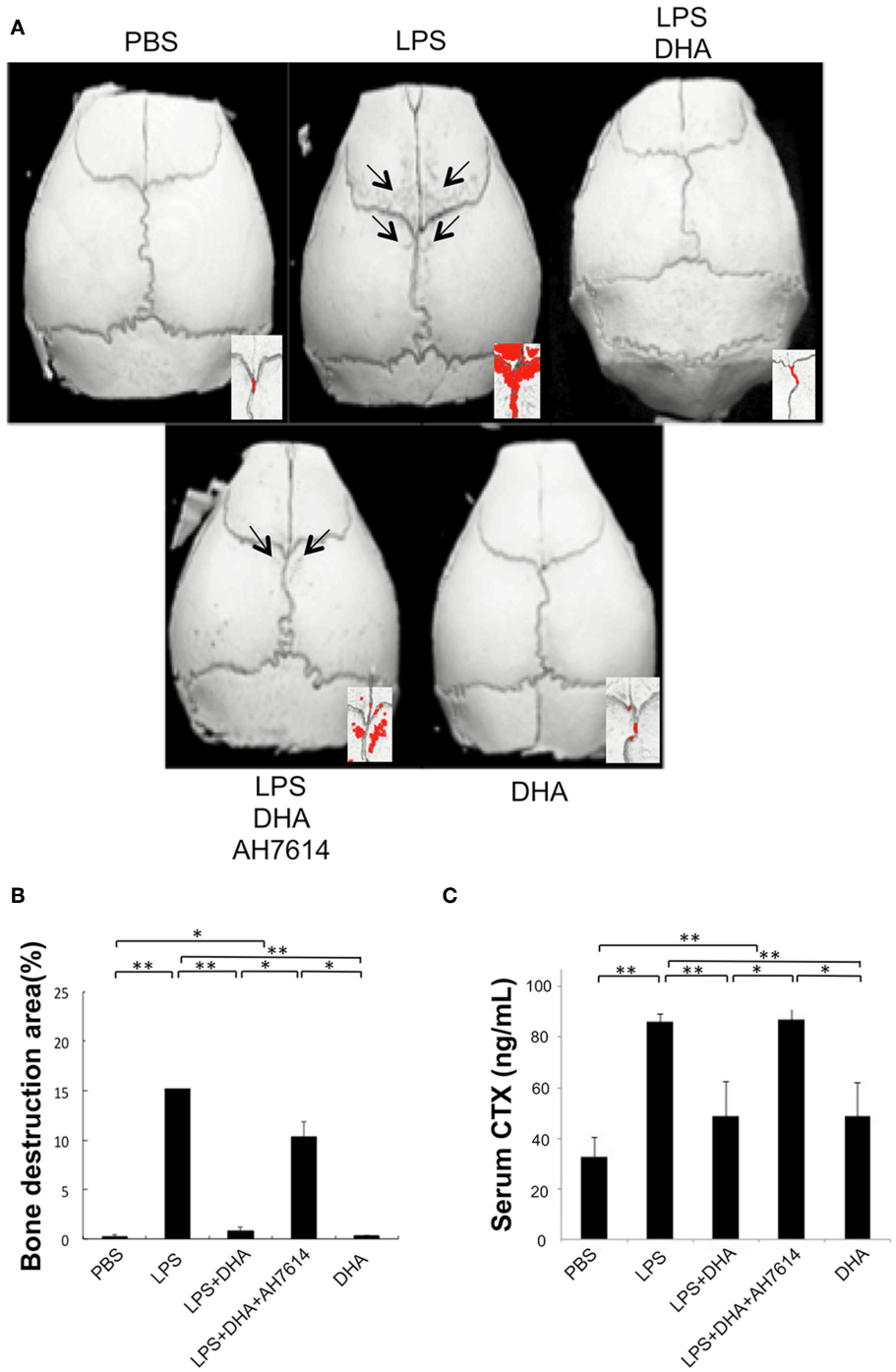

FIGURE 3 | DHA inhibits LPS-induced bone resorption via GPR120 activation *in vivo.* **(A)** Micro-CT reconstruction images of calvariae. Images of calvariae resected from mice after 5 days of daily supracalvarial administration of PBS, LPS (100 μg/day), LPS (100 μg/day)+DHA (100 μg/day), LPS (100 μg/day)+DHA (100 μg/day)+AH7614 (100 μg/day), or DHA (100 μg/day). Arrows indicate bone resorption areas and red areas indicate extensive bone resorption. **(B)** Ratio of bone resorption area to total bone area. Results are expressed as means ± SD ($n = 4$; $^{**}p < 0.01$, $^{*}p < 0.05$). Differences were determined by Scheffe's test. **(C)** Serum CTX levels *in vivo*. Serum was prepared from mice following 5 days of daily supracalvarial administration of PBS, LPS (100 μg/day), LPS (100 μg/day)+DHA (100 μg/day), LPS (100 μg/day)+DHA (100 μg/day)+AH7614 (100 μg/day), or DHA (100 μg/day). Enzyme-linked immunosorbent assays was performed to determine concentrations of circulating CTX. Data are shown as means ± SD ($n = 4$; $^{**}p < 0.01$, $^{*}p < 0.05$). Differences were determined by one-way ANOVA and Turkey-Kramer tests.

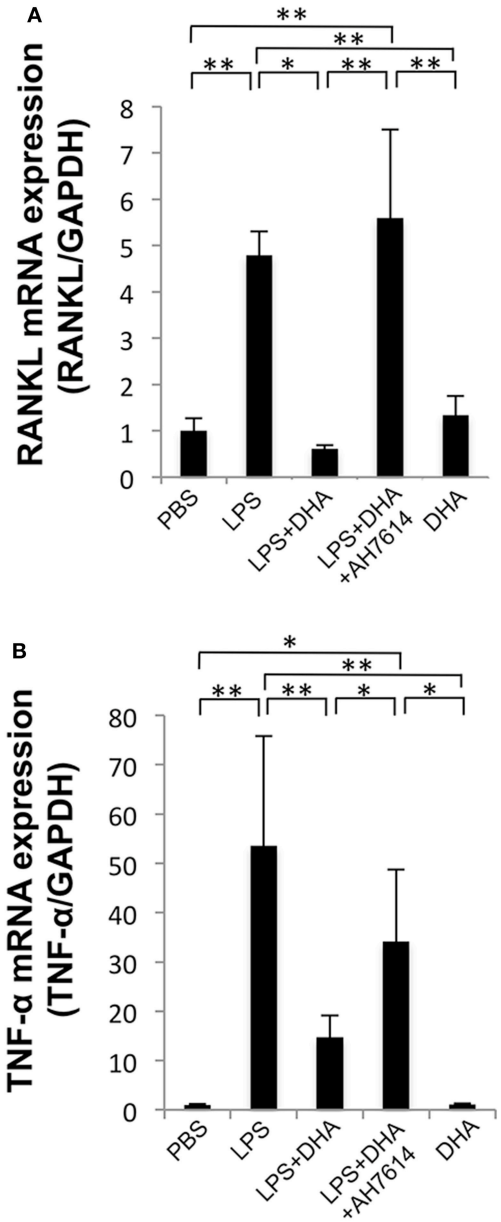

FIGURE 4 | DHA inhibits expression of LPS-induced TNF-α and RANKL via GPR120 *in vivo*. **(A)** Expression levels of RANKL mRNA in mouse calvariae *in vivo*. **(B)** Expression levels of TNF-α mRNA in mouse calvariae *in vivo*. TNF-α and RANKL mRNA levels were determined using real-time RT-PCR. Total RNA from mouse calvariae was isolated after 5 days of daily supracalvarial injections of PBS, LPS (100 μg/day), LPS (100 μg/day)+DHA (100 μg/day) with or without AH7614 (100 μg/day) and DHA (100 μg/day). Expression levels of RANKL and TNF-α mRNA were normalized to that of GAPDH. Data are shown as means ± SD ($n = 4$; $^{**}p < 0.01$, $^{*}p < 0.05$). Differences were determined by one-way ANOVA and Tukey-Kramer tests.

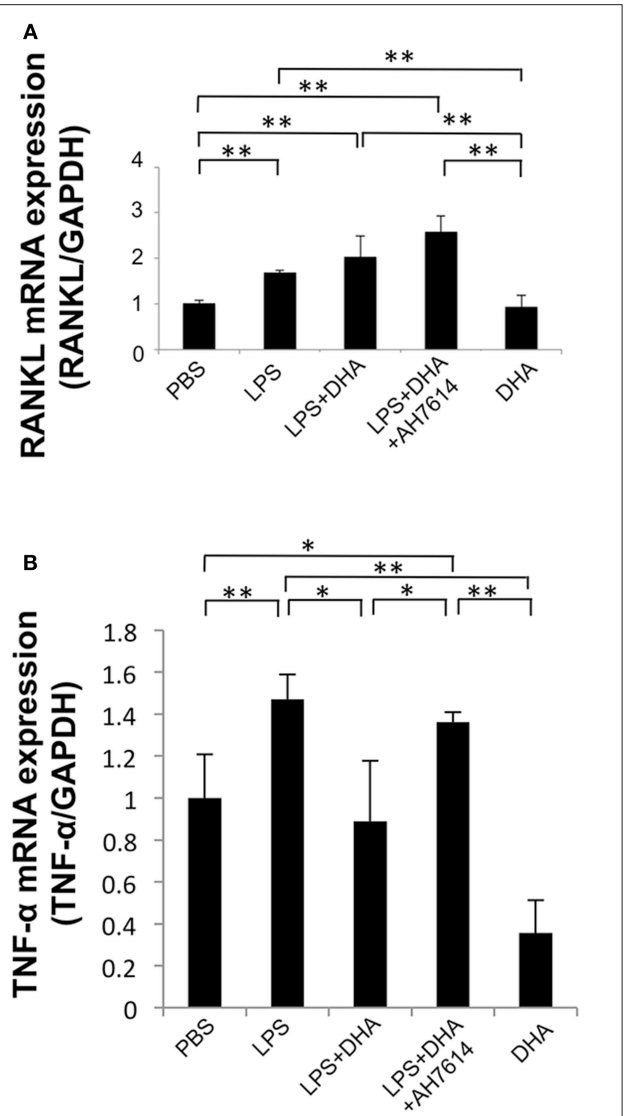

FIGURE 5 | DHA suppresses LPS-induced TNF-α mRNA expression in macrophages but does not affect LPS-induced RANKL mRNA expression in osteoblasts. **(A)** Osteoblasts from cranial bone were incubated with PBS, LPS, LPS+DHA with or without AH7614, or DHA alone. After 24-h culture, total RNA was isolated and RANKL mRNA levels were determined using real-time RT-PCR. **(B)** Peritoneal macrophages were cultured with PBS, LPS, LPS+DHA with or without AH7614 or DHA alone. After 24-h culture, total RNA was isolated and TNF-α mRNA levels were determined using real-time RT-PCR. RANKL and TNF-α mRNA levels were normalized to that of GAPDH. Data are expressed as means ± SD ($n = 4$; $^{**}p < 0.01$, $^{*}p < 0.05$). Differences were determined by one-way ANOVA and Turkey-Kramer tests.

indicated that DHA directly inhibited RANKL- and TNF-α-induced differentiation of osteoclast precursors. In addition, we investigated whether DHA inhibits RANKL- and TNF-α-induced osteoclast formation via GPR120 activation by using GPR120 antagonist AH7614 *in vitro*. AH7614 dose-dependently increased the number of TRAP-positive cells in both RANKL- and TNF-α-induced osteoclast formation in cells cultured with DHA. These results suggested that DHA inhibits osteoclast formation via GPR120 *in vitro*.

It has been reported that DHA inhibits bone loss in ovariectomized mice owing to its inhibition of osteoclast generation and activation *in vivo* (32). In addition, perinatal DHA supplementation is associated with decreased bone

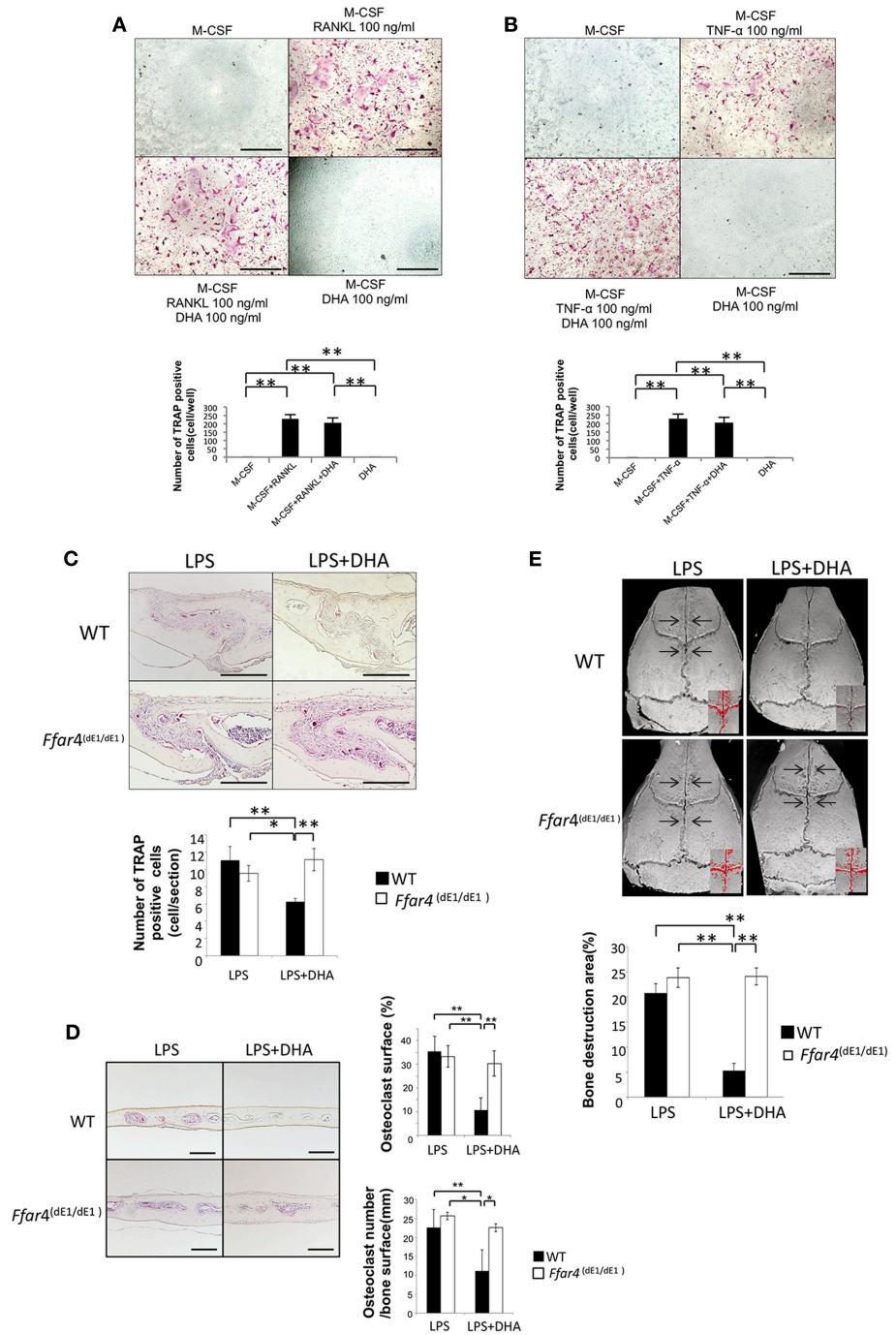

FIGURE 6 | DHA does not suppress LPS-induced osteoclast formation, bone resorption and production of osteoclast-related cytokines (TNF-α and RANKL) in *Ffar4*$^{(dE1/dE1)}$ mice. **(A)** Microscopic images and numbers of TRAP-positive cells. Osteoclast precursor cells from *Ffar4*$^{(dE1/dE1)}$ mice were treated with M-CSF, M-CSF+RANKL with or without DHA or DHA alone. TRAP staining was performed and TRAP-positive cells were counted. **(B)** Microscopic images and numbers of TRAP-positive cells. Osteoclast precursor cells from *Ffar4*$^{(dE1/dE1)}$ mice were treated with M-CSF, M-CSF+TNF-α with or without DHA or DHA alone, and then TRAP staining was performed. TRAP-positive cells were counted. Scale bars =100 μm. **(C)** Histological sections of calvariae were prepared from *Ffar4*$^{(dE1/dE1)}$ mice after 5 days of daily supracalvarial administration of LPS (100 μg/day) with or without DHA (100 μg/day). Sections were stained with TRAP solution and hematoxylin counterstaining was performed. TRAP-positive cells were counted. Scale bars =100 μm. **(D)** Histological sections of calvariae were prepared from *Ffar4*$^{dE1/dE1}$ mice after 5 days of daily supracalvarial administration of LPS (100 μg/day) with or without DHA (100 μg/day). The percentage of interface of bone marrow space covered by osteoclast and the number of TRAP-positive cell per millimeter of interface of bone marrow space were evaluated. **(E)** Micro-CT reconstruction images of calvariae. Images of calvariae excised from *Ffar4*$^{(dE1/dE1)}$ mice after 5 days of daily supracalvarial administration of LPS (100 μg/day) with or without DHA (100 μg/day). Arrows indicate bone resorption areas and red areas indicate extensive bone resorption. Data are shown as means ± SD ($n = 4$; $^{**}p < 0.01$, $^{*}p < 0.05$). Differences were determined by Scheffe's test.

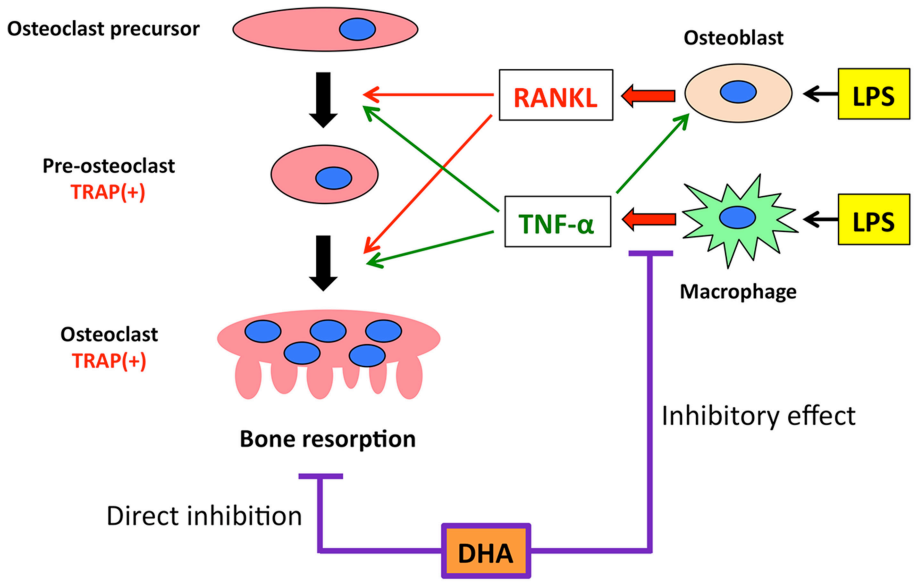

FIGURE 7 | Putative model of the inhibitory effect of GPR120 activation by DHA in LPS-induced osteoclast formation and production of osteoclast-related cytokines (RANKL and TNF-α) *in vivo*. Both RANKL and TNF-α have essential roles in inducing osteoclast differentiation. When osteoclast precursors are treated with RANKL, TRAP-positive pre-osteoclasts are induced. These cells fuse and differentiate into osteoclasts under the influence of RANKL, subsequently inducing bone resorption. TNF-α acts in a similar manner as RANKL to induce osteoclast differentiation. These cytokines also affect LPS-induced osteoclast formation and bone resorption *in vivo*. LPS administration induces RANKL expression in osteoblasts and TNF-α expression in macrophages. Furthermore, LPS-induced TNF-α production increases RANKL expression in osteoblasts. GPR120 activation directly inhibits osteoclast precursor differentiation and LPS-induced TNF-α expression in macrophages. However, the inhibitory effect of GPR120 may be unrelated to RANKL expression in osteoblasts.

resorption, decreased osteoclast density and a higher bone mass in rats (45). Periodontitis rats, which are generated by oral infection of *Porphyromonas gingivalis*, treated with fish oil containing DHA had signified less alveolar bone resorption (46). In this study, DHA inhibited osteoclast formation induced by LPS. Daily supracalvarial administration of 100 μg of DHA for 5 days inhibited LPS-induced osteoclast formation *in vivo*. The inhibitory effect of DHA on bone resorption induced by LPS was also evaluated. The increase in bone resorption was evaluated using the ratio of the bone resorption area to total area in microfocus CT images. We also determined bone destruction by evaluating the serum CTX level. The increase in bone resorption was significantly lower in the LPS and DHA group than in the LPS group. These results indicated that DHA inhibits LPS-induced osteoclast formation and bone destruction *in vivo*. The results of the effect of DHA on osteoclast formation are similar to those reported in previous studies (33, 34, 39, 44). Furthermore, AH7614 increased osteoclast formation and bone resorption in the LPS and DHA group. Taken together, these results indicated that DHA inhibits LPS-induced osteoclast formation via GPR120 *in vivo*.

Several reports showed that DHA inhibits pro-inflammatory cytokine production from several cell types. DHA can inhibit TNF-α and interleukin (IL)-6 production in primary mouse macrophages and RAW 247.6 cells by binding to GPR120 (27). DHA also reduces LPS-induced production of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, in THP-1 macrophages (47). Furthermore, DHA reduces IL-1β expression in bone marrow-derived macrophages (48). We suspect that the mechanisms of inhibitory effect of LPS-induced osteoclast formation and bone resorption *in vivo* by DHA are conceivable via either of two possible mechanisms. One possible mechanism is DHA blocks expression of LPS-induced cytokines associated with osteoclast formation. It is well-known that RANKL and TNF-α are important factors in osteoclast formation (2, 6, 7). Several groups showed that LPS is able to induce TNF-α and RANKL expression *in vivo* (13, 49, 50). In the present study, TNF-α and RANKL mRNA expression levels were increased in LPS-treated mice. On the other hand, TNF-α and RANKL mRNA expression was suppressed in DHA and LPS co-administered mice in comparison with LPS-treated mice. Our results were consistent with the hypothesis that DHA inhibits osteoclast formation by suppressing the expression of LPS-induced production of osteoclast-associated cytokines. Another possible mechanism of DHA inhibition is that DHA directly suppresses osteoclast formation by influencing cell differentiation. Our results indicated that DHA directly inhibited both RANKL- and TNF-α-induced differentiation of osteoclast precursor cells into osteoclasts. Moreover, AH7614 increased the concentration of osteoclast-related cytokines in LPS and DHA-treated mice. The results suggested that DHA inhibited LPS-induced production of osteoclast-related cytokines via GPR120 *in vivo*. These results indicated that the inhibitory effects of DHA on osteoclast formation by LPS *in vivo* are due to both decreased production of osteoclast-associated

cytokines and direct actions of DHA on osteoclast precursors through GPR120.

Next, we evaluated whether DHA inhibits LPS-induced TNF-α mRNA expression in macrophages. The results indicated that downregulation of TNF-α mRNA by DHA might result from a direct action of DHA on macrophages. AH7614 increased LPS-induced TNF-α mRNA expression in macrophages with DHA treatment. Therefore, the results suggested that DHA inhibited LPS-induced TNF-α in macrophages via GPR120. In addition, we investigated the influence of DHA on LPS-induced RANKL expression in osteoblasts, but no effect of DHA on LPS-induced RANKL expression was found. Our results suggested that DHA likely suppresses LPS-induced osteoclast formation *in vivo* by directly inhibiting LPS-induced TNF-α expression in macrophages. This inhibition subsequently blocks RANKL expression in osteoblasts, although there is no effect of DHA on LPS-induced RANKL expression in osteoblasts, in addition to direct inhibition of osteoclast formation.

Finally, we evaluated whether both RANKL- and TNF-α-induced osteoclast formation is inhibited by DHA through GPR120 and whether DHA inhibits LPS-induced osteoclast formation and bone resorption *in vivo* via GPR120 by using GPR120-deficient mice. The effects of DHA on osteoclast formation *in vitro* disappeared by using osteoclast precursors from GPR120-deficient mice and inhibition of LPS-induced osteoclast formation and bone resorption by DHA *in vivo* disappeared in GPR120-deficient mice. The results strongly suggested that the effect of DHA on osteoclast formation *in vivo* and *in vitro* was maintained via GPR120.

In conclusion, we demonstrated that DHA inhibits LPS-induced osteoclast formation and bone destruction *in vivo* and directly inhibits RANKL- and TNF-α-induced osteoclast formation *in vitro*. The underlying mechanisms by which DHA inhibits LPS-induced osteoclast formation and bone destruction *in vivo* were associated with its inhibition of LPS-induced TNF-α production in macrophages and direct inhibition of RANKL- and TNF-α-induced osteoclast formation via GPR120 (**Figure 7**).

AUTHOR CONTRIBUTIONS

AK and HK contributed to conception, design, data acquisition, data analysis, data interpretation, and drafting of the manuscript. HK and AI contributed to critical revision of the manuscript. KK, SO, JQ, W-RS, FO, TN, AM, and YN collected the samples and performed data analyses. HK and IM supervised the project. All authors provided final approval and agree to be accountable for all aspects of the work.

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SUPPLEMENTARY MATERIAL

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Effects of Vitamin E-Stabilized Ultra High Molecular Weight Polyethylene on Oxidative Stress Response and Osteoimmunological Response in Human Osteoblast

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High Crosslink process was introduced in the development of joint prosthetic devices, in order to decrease the wear rate of ultrahigh molecular weight polyethylene (UHMWPE), but it also triggers the formation of free radicals and oxidative stress, which affects the physiological bone remodeling, leading to osteolysis. Vitamin E stabilization of UHMWPE was proposed to provide oxidation resistance without affecting mechanical properties and fatigue strength. The aim of this study is to evaluate the antioxidant effect of vitamin E added to UHMWPE on oxidative stress induced osteolysis, focusing in particular on the oxidative stress response in correlation with the production of osteoimmunological markers, Sclerostin and DKK-1, and the RANKL/OPG ratio compared to conventional UHMWPE wear debris. Human osteoblastic cell line SaOS2 were incubated for 96 h with wear particles derived from crosslinked and not crosslinked Vitamin E-stabilized, UHMWPE without Vitamin E, and growth medium as control. Cellular response to oxidative stress, compared to not treat cells, was evaluated in terms of proteins O-GlcNAcylation, cellular levels of OGA, and OGT proteins by immunoblotting. O-GlcNAcylation and its positive regulator OGT levels are increased in the presence of Vitamin E blended UHMWPE, in particular with not crosslinked Vit E stabilized UHMWPE. Conversely, the negative regulator OGA increased in the presence of UHMWPE not blended with Vitamin E. Vitamin E-stabilized UHMWPE induced a decrease of RANKL/OPG ratio compared to UHMWPE without Vitamin E, and the same effect was observed for Sclerostin, while DKK-1 was not significantly affected. In conclusion, Vitamin E stabilization of UHMWPE increased osteoblast response to oxidative stress, inducing a cellular mechanism aimed at cell survival. Vitamin E antioxidant effect influences the

secretion of osteoimmunological factors, shifting the bone turnover balance toward bone protection stimuli. This suggests that Vitamin E-Stabilization of UHMWPE could contribute to reduction of oxidation-induced osteolysis and the consequent loosening of the prosthetic devices, therefore improving the longevity of total joint replacements.

Keywords: vitamin E, high-molecular-weight polyethylene (HMWPE), oxidative stress, osteoblasts, proteins O-GlcNAcylation, osteoimmunological markers

INTRODUCTION

One of the main problems in total hip arthroplasty is osteolysis triggered by ultrahigh molecular weight polyethylene (UHMWPE) wear particles (1) and different strategies have been developed to improve the oxidation and wear resistance.

High crosslink process was developed in order to decrease the wear rate of UHMWPE (2), but it also triggers the formation of free radicals (3), leading to oxidative degradation of the material through a cascade reaction with oxygen (4). In order to reduce oxidation, Vitamin E was introduced in UHMWPE stabilization, to provide oxidation resistance without affecting mechanical and fatigue strength of the material (5, 6). Vitamin E in the most abundant and effective antioxidant in the body, able to react with free radicals in cell membrane and protect polyunsaturated fatty acids from degradation due to oxidation (7). Polyethylene has a lipid—like molecular structure and its oxidation follows a similar mechanism of oxidation of lipids *in vivo* (8).

The physiological intracellular redox state is maintained in equilibrium by the balance of antioxidants and reactive oxygen species (ROS)- producing enzyme. Oxidative stress occurs when the overproduction of ROS is not balanced by an adequate level of antioxidants (9). The redox state affects the physiological process of bone remodeling (9). Indeed, changes in the ROS/antioxidant balance are involved in the pathogenesis of bone loss. In particular, oxidative stress activates the differentiation of osteoclasts from their precursors, while inducing osteoblasts apoptosis, thus shifting the balance toward osteoclastogenesis and bone resorption (9). High levels of ROS reduce osteoblast differentiation and activity, therefore reducing mineralization and bone mass (10, 11). On the contrary, antioxidant may contribute to osteoblasts differentiation and activity, promoting bone formation (9, 11).

Moreover, oxidative stress promotes the inflammatory response and directly interferes with the osteoimmunological regulation of bone remodeling, based on the action of the RANKL/RANK/OPG system. The receptor activator of NF-κB (nuclear factor-κB) ligand (RANKL) is a key factor stimulating the differentiation and activation of osteoclasts, and therefore, is essential for bone remodeling. The binding of RANKL to its receptor RANK leads to osteoclasts differentiation, while the decoy receptor Osteoprotegerin (OPG) counteracts this effect by binding and blocking RANKL. ROS act as specific secondary messengers in signaling pathways involved in RANKL-induced osteoclast differentiation (12). The expression of RANKL and OPG is sensitive to oxidative status that reduces OPG expression and induces RANKL expression, thus shifting the balance toward bone loss. An excess of oxidative stress also induces apoptosis of osteocytes, resulting in the reduction OPG production and increase of Sclerostin and DKK-1, two inhibitors of the WNT pathway involved in the osteoimmunological regulation of bone remodeling (13, 14). The expression of WNT pathway inhibitors seems to be induced by inflammatory mediators and aging (15), both conditions characterized by an increase in oxidative stress.

In this context, knowing that oxidative stress plays an important role in bone remodeling disorders, it's extremely important to study the effect of antioxidant agent (Vitamin E) added to UHMWPE that may reduce oxidative stress, thus modulating the inflammatory process and the regulation of bone remodeling by osteoimmunological mediators, eventually protecting from periprosthetic bone loss. Several studies in the recent years evaluated the clinical advantages of Vitamin E added to UHMWPE (16–20), but there is little if no evidence focusing in particular on the protective role of Vitamin E from oxidative stress in correlation with inflammation and the consequent periprosthetic bone loss.

One of the main mechanisms of cellular oxidative stress response is the modification of intracellular proteins by monosaccharides of O-linked β-N- acetylglucosammine, known as O-GlcNAcylation (21). This is a post-translational modification of nuclear and cytoplasmic proteins, which consists in the attachment of a single N-acetylglucosamine (O-GlcNAc) to serine and threonine residues of a protein (22).Two enzymes regulate this process: O-GlcNAc transferase (OGT) that catalyzes the addition of O-GlcNAc to the hydroxyl group of serine or threonine residues of a protein, and O-GlcNAcase (OGA) that removes O-GlcNAc from proteins (23, 24). Levels of O-GlcNAc are induced in response to stress, in order to prevent apoptosis and promote cell survival mechanism, and are considered a target of mammalian stress response (25). The protein O-GlcNAcylation, OGA and OGT levels are therefore considered markers of cellular response to oxidative stress.

The aim of this study is to evaluate the antioxidant effect of vitamin E added to UHMWPE and the association with the main osteoimmunological biomarkers (RANKL/RANK/OPG), and WNT pathway inhibitors, in order to better understand how the antioxidant effect of Vitamin E can prevent periprosthetic inflammation and the consequent o and prosthetic loosening.

METHODS

UHMWPE Particles Generation

UHMWPE wear particles were generated as previously described in Galliera et al.(26) by four different UHMWPE articular inserts (raw material GUR 1020): Material (1) moderately cross-linked vitamin E-blended UHMWPE (60 kGy electron-beam irradiated) (vitamin E concentration 0.1 wt%), EtO sterilized (*Vital-XE®, Permedica S.p.A.*); Material (2) standard UHMWPE (without vitamin E and not cross-linked), EtO sterilized *(Permedica S.p.A);* Material (3) vitamin E-blended UHMWPE (vitamin E concentration 0.1 wt%) not cross-linked, EtO sterilized (*Vital-E®, Permedica S.p.A.*).

All UHMWPE wear particles were generated as described previously (26). Briefly, the articular inserts were rubbed for 10 days at 230 rpm against ceramic ball heads using a combined drilling and tapping machine (IM company, Italy), applying a load of 1,000 N. The resulting UHMWPE particles were released directly into a closed sterile recipient containing 500 mL of ultrapure water with 0.2% sodium azide (antibacterial additive).

Cell Culture and Treatment With Wear Particles

SAoS2 cells, a permanent line of human osteoblast-like cells, were obtained from a partner institute and grown in RPMI 1,640 (Invitrogen, Germany) with L-glutamine, 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (GIBCO, USA). Cells were cultured in 5% CO_2 at 37°C in 12-well culture plates (Corning, USA).

Treatment with HUMWPE wear particles was performed as previously described (26). Briefly, after sterilization by UV irradiation overnight, the three types of wear particle (described above) diluted in growth media at a concentration of 1:1 or pure media (as control) were added to the cell culture. After 96 h of incubation, the supernatants of each well were collected and stocked at −20°C for ELISA assays, while cells were washed with PBS twice and then lysed for 15 min at 4°C in lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 20 mM NaF, 1 mM Na3VO4, 0.5% v/v NP40, 10 mg/ml leupeptin, 10 mg/ml aprotinin, 1 mg/ml pepstatin A). Insoluble material was removed by centrifugation at 13,000 g for 10 min, supernatants were collected and assayed for protein concentration with Coomassie Protein Assay (Pierce). Then samples were analyzed by immunoblotting.

Evaluation of Oxidative Stress Parameters: Immunoblotting and Densitometry Analysis

Forty micrograms of cell proteins were separated by SDS electrophoresis under denaturating conditions using 6–10% polyacrylamide gels. SDS-PAGE gels were electrophoretically transferred on PVDF membrane in Tris-glicine buffer, using the Mini Transblot System (Bio-Rad Laboratories, Richmond, VA). O-GlcNAc levels were measured by anti-b-O-linked N-Acetylglucosamine (OGlcNAc) CTD 110.6, an antibody that specifically recognizes endogenous levels of O-GlcNAc, linked to both serine and threonine residues of proteins, 1:1000 dilution (Cell Signaling). Other primary antibodies were used as follows: anti-OGA 1:3000 dilution (Sigma-Aldrich), anti-OGT 1:500 dilution (Sigma–Aldrich), and anti-Histone H3 1:2000 dilution (Cell Signaling).

Each membrane was washed three times for 10 min and then incubated with the appropriate secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) for 1 h. For the immunological detection of proteins, MINI HD 9 System (Uvitec Limited, Cambridge UK) was used. Band density was quantified Quantity One Software (Bio-Rad Laboratories).

Evaluation of Oxidative Stress Parameters: ROS Generation Assay

ROS production was tested by OxiSelect™ *In vitro* ROS/RNS Assay kit [Green Fluorescence (Cell Biolabs)], according to manufacturer's protocol. Briefly, the OxiSelect™ *In vitro* ROS/RNS Assay Kit is an *in vitro* assay for measuring total ROS/RNS free radical activity. Unknown ROS or RNS samples or standards are added to the wells with a catalyst that helps accelerate the oxidative reaction. After a brief incubation, the prepared DCFH probe is added to all wells and the oxidation reaction is allowed to proceed. Samples are measured fluorometrically against a hydrogen peroxide or DCF standard. The assay is performed in a 96-well fluorescence plate format that can be read on a standard fluorescence plate reader. The free radical content in unknown samples is determined by comparison with the predetermined DCF or hydrogen peroxide standard curve.

Evaluation of Osteoimmunological Biomarkers: ELISA Assay

The osteoimmunological biomarkers were evaluated by ELISA assay in SaOS2 supernatant. In particular, RANKL was measured using an ELISA Duo Set assay (R&D System, Minneapolis, MN, USA), while DKK-1, OPG, and Sclerostin were measured by ELISA Quantikine colorimetric sandwich assays (R&D System, Minneapolis, MN, USA), according to the manufacturer's protocols.

RANKL: CV intra assay 8.01% and inter assay 6.2%; OPG: CV intra assay 7.3% and inter assay 6.9%; DKK-1: CV intra assay 2.7 % and inter assay 5.4 %, SOST: CV intra assay 2.1 %, and inter assay 8.2%.

Evaluation of Cell Vitality

Cell viability was assessed quantitatively using the resulting Alamar Blue® test, a non-toxic test for cells as it exploits the reducing power of living cells by measuring their metabolic activity quantitatively, which makes it possible to analyze, in different timing, the same proliferating cell population. This test was also used as an indirect index of cell proliferation. The assay exploits the mitochondrial activity of viable cells capable of maintaining an environment of reducing inside the cell. Resazurin, the active component of Alamar Blu, is a compound able to cross the membranes, and once it enters the cell, it gets reduced in Resorufin and its color turns from blue to red. The reduction was measured by a spectrophotometer at 560 and 600 nm (Glo Max, Promega). The results obtained by both the readings were analyzed following the indications provided by the

assay protocol by calculating the percentage of cell viability as the difference in the reduction between the treated samples and the samples control (medium + Alamar Blue®).

Evaluation of Mineralization

The cells were seeded in 6-well plates at a density of 0.8 × 10^5 cells/well and stabilized for 24 h. To induce osteoblast differentiation, Osteogenic medium (Promocell), containing 50 μg/ml L-ascorbic acid and 10 mM β-glycerophosphate, was added to culture for 11 days. The culture medium was changed every 3–4 days. Then, the cells were fixed in 10% formalin for 10 min and stained with the 40 mM Alizarin Red-S (pH 4.2; Sigma-Aldrich; Merck KGaA) for 15 min, all at RT. For quantification of Alizarin red S, 500 μl citrate solution containing 20% methanol and 10% acetic acid was added for 20 min at RT, and the absorbance of supernatants was measured at 570 nm using a GloMAx Fluorescence Reader (Promega). The plates were observed under the Leica Microscope DML B2/11888111 equipped with Leica camera DFC450 at ×100 magnification.

Statistical Analysis

Statistical analysis was performed with dedicated statistical software (GraphPad Prism 7); normality of distribution of the groups was verified by KS normality for all the parameters evaluated. Statistical analysis was performed with one-way ANOVA, considering $p < 0.05$ as significant and $p < 0.001$ as highly significant. Data are expressed as the mean ± standard deviation (SD).

RESULTS

Oxidative Stress Response

To investigate the effect of the of Vitamin E blended UHMWPE on O-GlcNAcylation process, we measured cellular O-GlcNAC levels by western blot using CTD110.6 antibody. As shown in **Figure 1,** Panel A, O-GlcNAcylation levels increased in the presence of Vitamin E blended UHMWPE (in particular with not crosslinked Vit E blended UHMWPE) while, conversely, they fall in the absence of Vitamin E.

Densitometric analysis showed a significative ($p < 0.005$) increase in O-GlcNAC levels in the presence of not-crosslinked Vit E blended HMWPE and a significative ($p < 0.005$) decrease in Vitamin E absence (**Figure 1B**).

In order to determine whether the observed variation in O-GlcNAC levels was caused by an alteration of the ratio between the two O-GlcNAc cycling enzymes, we examined protein expression of OGA and OGT by western blot analysis (**Figures 2A,B**). As shown in Panel A, a significant increase ($p < 0.01$) of OGT protein level was found in the presence of not-crosslinked Vitamin E blended UHMWPE, whereas a significant increase ($p < 0.05$) of OGA enzyme was observed in Vitamin E absence. The OGT/OGA expression ratio shows a behavior consistent with the observed O-GlcNAC levels (**Figure 2B**).

ROS production was evaluated during the treatment, at 24, 48, 72, and 96 h (**Figure 4C**).The results show that at early time points (24 h), there is a significative increase of ROS induced by M1 (crosslinked HUMWPE vitamin E added), while M3 (not crosslinked V, Vitamin E added UHMWPE) display a significative decrease compared to M2 (not crosslinked V, UHMWPE without Vitamin E). This effect attenuates at 48 h, resulting in an insignificant difference between M1 and M2, maintaining a low but weakly significant level in response to M3. At 72 h, there is a little and insignificant increase in ROS in response to M1 and M3, which then turns into comparable levels, with no significative difference between the three materials at 96 h.

Osteoimmunological Biomarkers

The secretion of osteoimmunological markers in the supernatant was measured after 96 h of incubation with material 1, 2, and 3, as described in the Method Session, and growth medium as control. The two main osteoimmunological pathways were evaluated: the RANK/RANKL/OPG, by measuring the secretion of RANKL and OPG from the osteoblast cell line SaOs2 pathway, and the Wnt pathway, by measuring the secretion of the two main Wnt inhibitors, Sclerostin (SOST), and DKK-1.

RANKL production (**Figure 3A**) displayed a insignificant increase in response to M1 material, compared to control, and it resulted comparable to control in response to M3 material. On the contrary, RANKL displayed a strong and significant increase in response to material M2. Conversely, OPG (**Figure 3B**) displayed a significant reduction in response to material M2, while it showed a significant increase in response to M1 and an even higher increase in response to material M3.

In order to evaluate the trend of bone remodeling regulators *in vitro*, the RANKL/OPG ratio was calculated (**Figure 3E**). In all the conditions, the RANKL/OPG ratio resulted largely <1.However, while in response to M1 and M3 the RANKL/OPG ratio was very low and comparable to control, in response to M2 the RANKL/OPG ratio displayed a very significant increase.

Sclerostin, a marker of bone resorption, displayed a significant increase in response to material M2, while it showed a significant decrease in response to material 1 and a little, even significant decrease in response to material M3 (**Figure 3C**). The secretion of the other Wnt inhibitor DKK-1 displayed no significant changes in response to material M1 compared to control (**Figure 3D**).

Cell Vitality and Mineralization After Exposure to M1, M2 and M3 Wear Debris

Cell were plated at the concentration of 10^3cells/mL, and 24 h after, when they resulted completely adherent, the incubation with wear particles started and lasted for 96 h, during the exponential growth phase. Doubling time of Saos cell is 37 h and they can be cultured in logaritimic growth for 7 days before splitting, when they reach the concentration of 3 × 10^5cells/mL.

Cells were equally plated for treatment with wear particles and control medium. The vitality and the growth rate of the Sa0S2 cell were evaluated by Alamar blue, and treated as well as not treated cells displayed exactly the same viability and growth rate, as shown in **Figure 4A**.

Mineralization assays were performed in the presence of material 1, 2, and 3, and only mineralization medium as control.

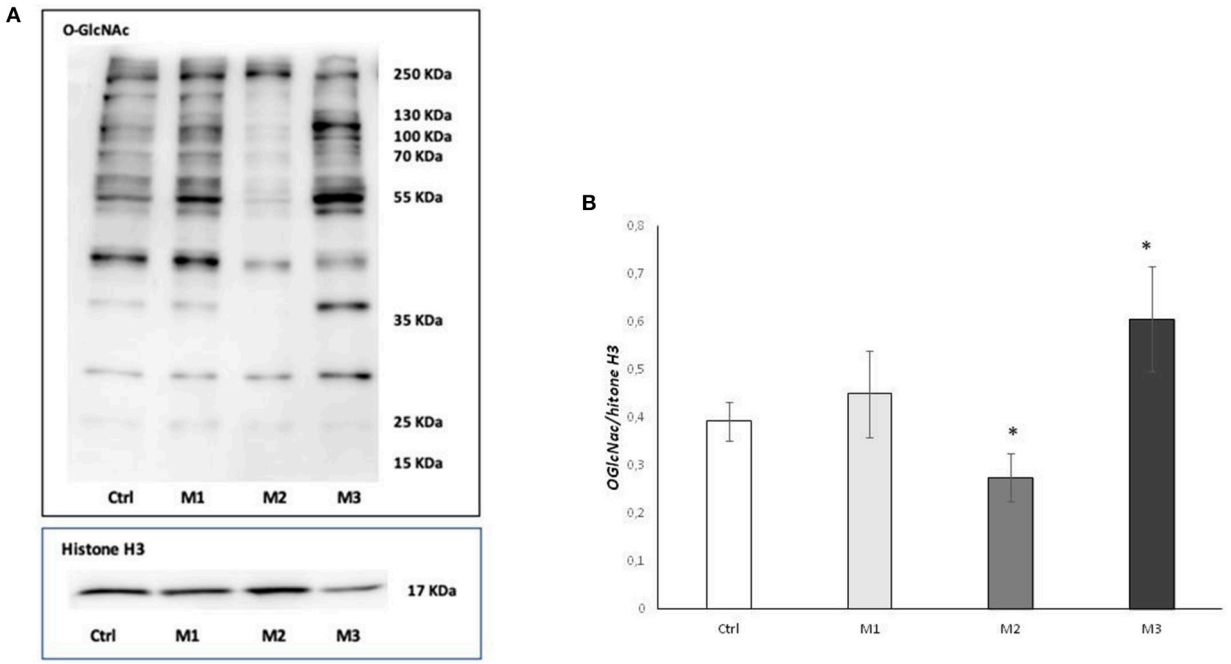

FIGURE 1 | Total O-GlcNAcylations after exposure to M1, M2, and M3 wear debris. **(A)** O-GlcNAcylations of cell proteins examined by western blotting. The western blot image is representative of three experiments. **(B)** Densitometric analysis of proteins expression was performed using Histone H3 as loading control. *$P < 0.05$ vs. Ctrl. Materials M1, M2, M3 are described in the Methods section, as follows: Material M1 a moderately cross-linked vitamin E-blended UHMWPE, EtO sterilized; Material M2 standard UHMWPE (without vitamin E and not cross-linked), EtO sterilized; Material M3 vitamin E-blended UHMWPE not cross-linked, EtO sterilized. Cnotrol (white bar) Material M1 (light gray bar), material M2 (medium gray bar), material M3 (dark gray bar).

As shown in **Figures 4B,D**. The three different kinds of material did not affect the mineralization of SaoS2.

DISCUSSION

Implant materials can release wear particles which may elicit adverse reactions in patients, such as local inflammatory response leading to tissue damage, which eventually results in loosening of the implant. In the case of ultra-high molecular weight polyethylene (UHMWPE), the inflammation is further boosted by the oxidation of the material, which has been recognized as a potential limiting factor for the longevity of total joint replacements (6). In order to reduce UHMWPE oxidation, chemical stabilization with Vitamin E was introduced over the past decade. Vitamin E, when added to UHMWPE, has been shown to suppress the oxidation cascade by reducing both alkyl and peroxy radicals (27). The antioxidant effect of Vitamin E has been extensively studied from the biochemical point of view (17, 28), but the comprehensive effect on cellular response to oxidative stress and the correlation with inflammatory response and bone resorption still needs to be fully elucidated. For this reason, this study aimed to evaluate the effects of Vitamin E addition to UHMWPE on both the aspects of inflammation leading to bone tissue damage: on the one hand the cellular response to oxidative stress and on the other hand the production of osteoimmunological mediators, that combine the regulation of inflammatory response to bone remodeling (23). In order to evaluate the effect of Vitamin E added, different variants of UHMWPE were evaluated. In the Vitamin E- UHMWPE production, the radiation cross linking process is required to reduce wear, but it also increases the oxidation of UHMWPE (24). For this reason, we evaluated two different types of Vitamin E-stabilized UHMWPE, one crosslinked, and one not crosslinked, compared to not crosslinked UHMWPE, without Vitamin E.

Cells and tissue respond to oxidative stress, environmental and injury by reprogramming gene expression, transcription, transduction, and post trasductional protein modification in order to stat pathways of repair and survival. In particular oxidative stress induces a dynamic O-GlcNAcylation (25, 29), promoting the glycosilation of some proteins and the deglycosilation of others, in order to prevent apoptosis and promote the cell survival mechanism (29). The dynamic O-GlcNAcylation is cycled by two enzymes, the O-GlcNAc transferase (OGT), which catalyzes the addition of O-GlcNAc residues, and the O-GlcNAcase (OGA), which removes O-GlcNAc residues. The level of O-GlcNAcylation, OGA, and OGT levels are therefore markers of cellular response to oxidative stress and were measured in the present study to evaluate the response of the osteoblastic cell line SaOs2 to wear debris from crosslinked Vitamin E—stabilized UHMWPE, not-crosslinked Vitamin E—stabilized UHMWPE and not-crosslinked UHMWPE without vitamin E, compared to not-treated cells. As shown in

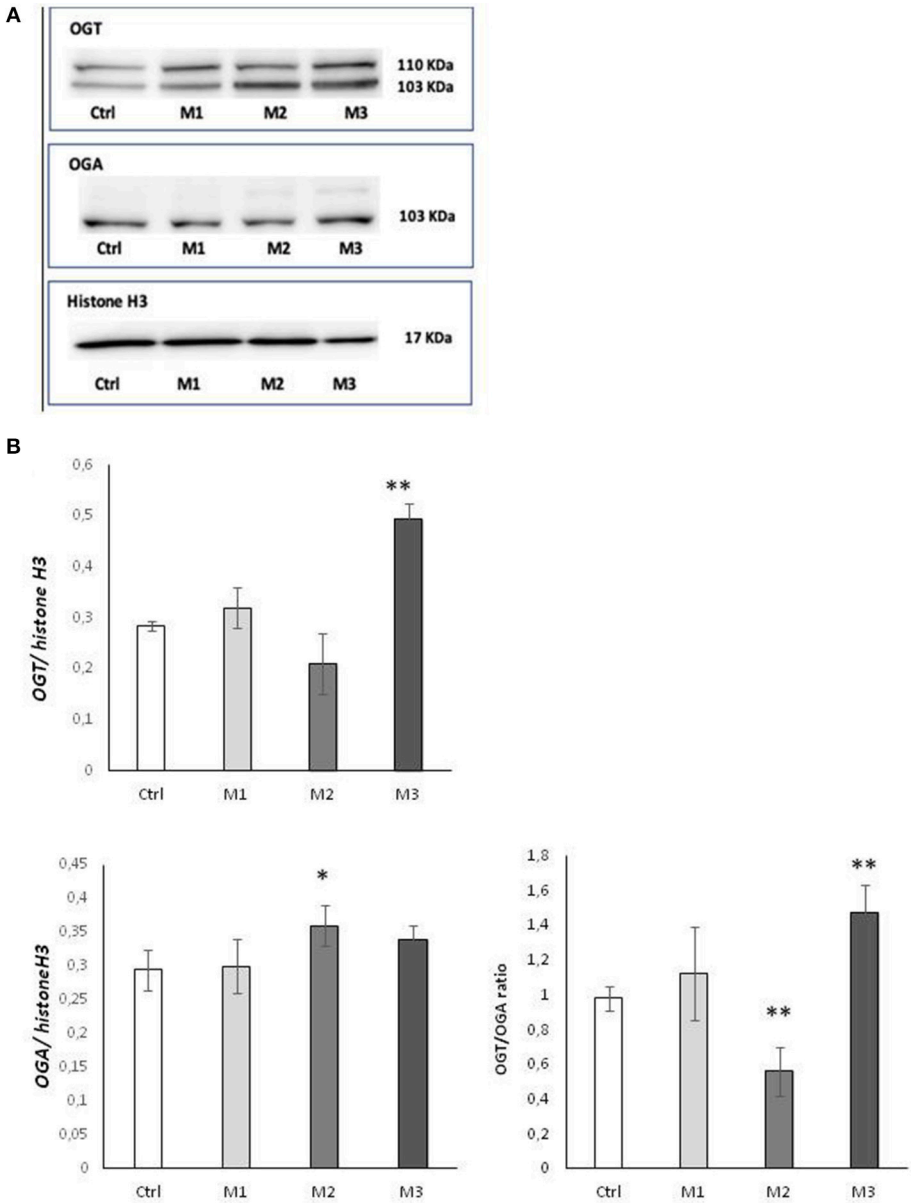

FIGURE 2 | OGA and OGT levels after exposure to M1, M2, and M3 wear debris. **(A)** Cellular levels of OGA and OGT proteins examined by western blotting. The western blot image is representative of three experiments. Densitometric analysis of proteins expression was performed using Histone H3 as loading control. **(B)** OGT/OGA expression ratio. *$P < 0.05$ Vs. Ctrl; **$P < 0.01$ Vs. Ctrl. Materials M1, M2, M3 are described in the Methods section, as follows: Material M1 a moderately cross-linked vitamin E-blended UHMWPE, EtO sterilized; Material M2 standard UHMWPE (without vitamin E and not cross-linked), EtO sterilized; Material M3 vitamin E-blended UHMWPE not cross-linked, EtO sterilized. Control (white bar) Material M1 (light gray bar), material M2 (medium gray bar), material M3 (dark gray bar).

Figure 1, O-GlcNAcylation is significantly reduced in response to notVitamin E-stabilized UHMWPE, while they are increased in response to Vitamin E—stabilized UHMWPE, compared to controls, with a significant increase in the case of not-crosslinked Vitamin E—stabilized UHMWPE (material 3), indicating that the presence of Vitamin E increase the ability of Saos2 Cells to respond to oxidative stress due to UHMWPE. This result is also confirmed by the specific evaluation of OGA and OGT: OGT production showed the same changes observed for O-GlcNAcylation, with a significant increase in response to not-crosslinked Vitamin E—stabilized UHMWPE, a little but not significant increase in response to crosslinked Vitamin E—stabilized UHMWPE and a decrease in response to not-crosslinked UHMWPE without Vitamin E. These results are in accordance with previous reports indicating that stress induced O-GlcNAcylation is coincident with increased protein expression of OGT of OGT (29–31). OGA exert the opposite role of OGT (21), and even though less is known about the regulation of OGA levels in stresses cells, recent studies reported that increased O-GlcNAcylation levels are associated with a decrease in OGA (29,

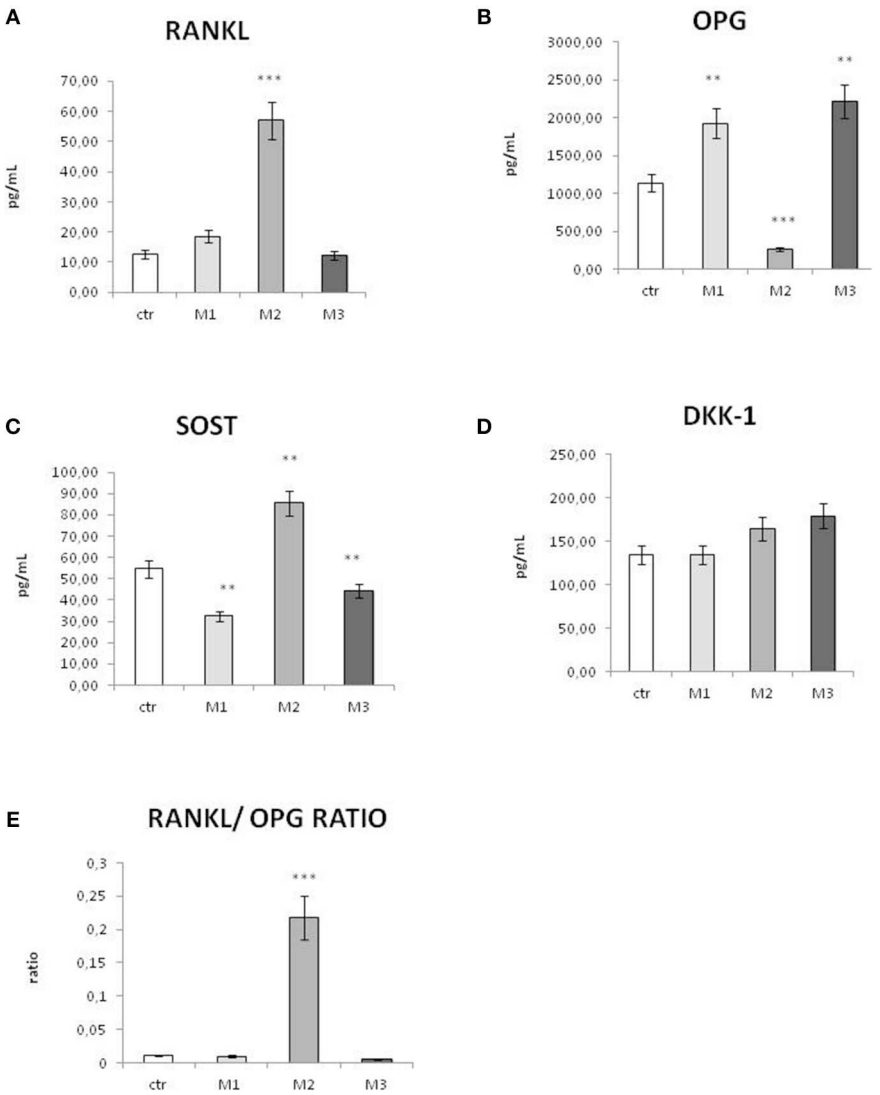

FIGURE 3 | Secretion of osteoimmunological biomarkers in the cell culture supernatant after exposure to M1, M2, and M3 wear debris. Concentrations (picograms per milliliter) of RANKL **(A)**, OPG **(B)**, Sclerostin (SOST, **C),** and DKK-1 **(D)**, RANKL/OPG ratio **(E)** in the cell culture supernatant of control (white bar), material M1 (light gray bars), material M2 (medium gray bars), material M3 (dark gray bars). Materials M1, M2, M3 are described in the Methods section, as follows: Material M1 a moderately cross-linked vitamin E-blended UHMWPE, EtO sterilized; Material M2 standard UHMWPE (without vitamin E and not cross-linked), EtO sterilized; Material M3 vitamin E-blended UHMWPE not cross-linked, EtO sterilized. Control (white bar) Material M1 (light gray bar), material M2 (medium gray bar), material M3 (dark gray bar). $^{*}p < 0.05$, statistically significant; $^{**}p < 0.01$; $^{***}p < 0.005$.

31). Consistently with these evidences, we observed a significant increase of OGA in response to material 2 (UHMWPE without Vitamin E), in correspondence to a decrease of OGT, and no significant change in response to material 1 and 3 (UHMWPE with Vitamin E). The overall effect is more evident by evaluating OGT/OGA ratio, showing that UHMWPE without Vitamin E induced a very significant decrease in OGT/OGA ratio while not-crosslinked Vitamin E—stabilized UHMWPE induced a very significant increase in OGT/OGA ratio, shifting the balance toward increased cell survival. These results suggest that the addition of Vitamin E to UHMWPE increases the ability of SaOs2 cells to respond to oxidative stress induced by UHMWPE, while the absence of Vitamin E reduces the cell response to oxidative stress. Material 1 (crosslinked Vitamin E—stabilized UHMWPE) stimulated an increase in OGT/OGA ratio but in a minor extent of its not-crosslinked counterpart (material 3), indicating that the cross linking process contributes to generate oxidative stress and weakly reduces the beneficial effect of Vitamin E-stabilization. This is in accordance with the literature reporting that the cross linking process is a strong source of UHMWPE oxidation (6, 32, 33). Taken together, these results suggest that the Vitamin E-stabilization, in particular in absence of cross linking, stimulates cellular response to oxidative stress, in order to promote cell survival.

The results of ROS production are in line with recent literature evidences indicating that UHMWPE induce oxidative stress,

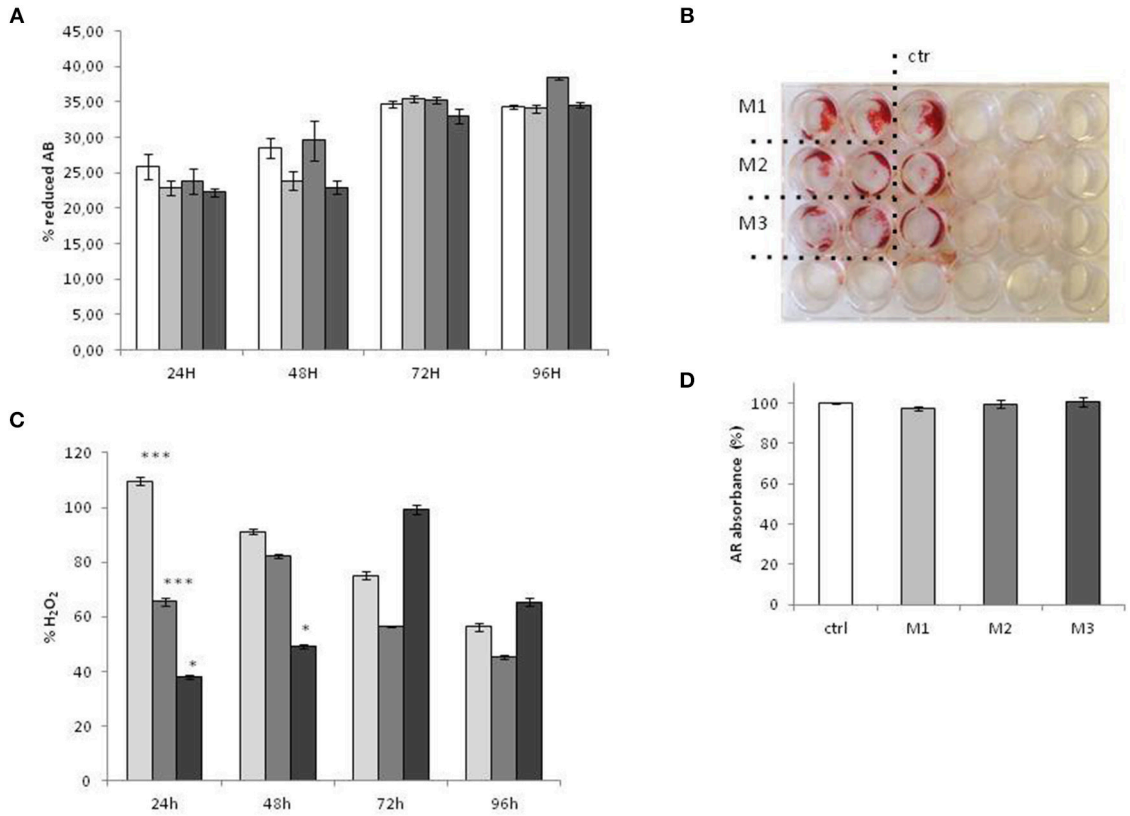

FIGURE 4 | Cell Vitality, mineralization, and ROS production after exposure to M1, M2, and M3 wear debris. **(A)** vitality and the growth rate of Sa0S2 cell evaluated by Alamar blue **(A,B)**. The vitality of the cells is expressed as percentage of reduced Alamar Blue (%AB), as indicated in Alamar Blue protocol. Mineralization Assay was evaluated by alizarin Red (AR) staining. The plates were observed under the Leica Microscope DML B2/11888111 equipped with Leica camera DFC450 at ×100 magnification **(B)**. For quantification of Alizarin red S, 500 μl citrate solution containing 20% methanol and 10% acetic acid was added for 20 min at RT, and the absorbance of supernatants was measured at 570 nm using an Fluorescence Reader (GloMAx). The mineralization is expressed as percentage of Alzarin Red staining (% ARS) of treated cells vs. untreated control **(D)**. **(C)** ROS production was tested by OxiSelect^{TM} *In vitro* ROS/RNS Assay kit. ROS production is expressed as percentage of H_2O_2 production vs. untreated control. Materials M1, M2, M3 are described in the Methods section, as follows: Material M1 a moderately cross-linked vitamin E-blended UHMWPE, EtO sterilized; Material M2 standard UHMWPE (without vitamin E and not cross-linked), EtO sterilized; Material M3 vitamin E-blended UHMWPE not cross-linked, EtO sterilized. Control (white bar) Material M1 (light gray bar), material M2 (medium gray bar), material M3 (dark gray bar). *$p < 0.05$; **$p <$ 0.01, statistically very significant; ***$p < 0.005$.

even when modified (34) with Vitamin E. Indeed, Vitamin E exerts an antioxidant effect mainly *in vivo* as a scavenger of oxidant molecules, in particular lipid-soluble peroxyl radical, when they reach a high concentration, in order to prevent cell damage (35). On the other hand, is should be noted that low levels of oxidative stimuli, such as ROS generation (36), induce a cellular adaptive response to upregulate the defense capacity against subsequent oxidative stress (35, 37–39). Consistently, O-GlvNAcylation response proceeds as a two-step process. An initial ROS stimulus stimulates O-GlvNAcylation which, in turn, activates an intracellular signaling cascade leading to a long-term oxidative defense process. Indeed, a significative reduction in ROS generation is reported to be reduced by a sustained O-GlvNAcylation (40). In our results, the early and transient ROS production in response to M1 and M3 could be considered a priming factor that induces, at later time points, the O-GlvNAcylation as a mechanism of long-term antioxidant defense. Indeed, at a time point of 96 h, O-GlvNAcylation was observed in concordance with a decrease in ROS and a stabilization, with no significative difference between the three materials. These results indicate that Vitamin E added to WHMWPE does not have an immediate antioxidant effect (24 h) on the reduction ROS generation, but a more long term effect, stimulating, by an initial and transient ROS increase, the O-GlvNAcylation, which leads in turn to a antioxidant defense process.

These results are consistent with the final goal of Vitamin E adding to UHMWPE, to prevent the long-term effect of UHMWPE oxidation.

Oxidative stress is related to the inflammatory response, which in turn, affects bone turnover and remodeling by means of osteoimmunological mediators (9).

Vitamin E has also been shown to influence inflammatory cytokine production (17, 41). We recently showed the effect of Vitamin E—stabilized UHMWPE wear particles on osteoimmunological molecule's gene expression and secretion at early time points (26), so in this study, we aimed to analyze

in parallel the effects of Vitamin E added to UHMWPE on oxidative stress response and osteoimmunological response. In particular, O-GlcNAcylation has been reported to affect the production of osteoimmunological factors (42). In our study, the osteoimmunological factors RANKL and its negative regulators confirmed our previous report, showing an increase in the bone-resorptive marker RANKL and a correspondent reduction of the osteo-protective marker OPG in response to UHMWPE without Vitamin E, while when vitamin E is added to stabilize UHMWPE, there is an strong reduction in RANKL, and an increase in OPG. In order to give a comprehensive result, we measured the RANKL/OPG ratio, which is considered a better parameter than the single RANKL and OPG values to evaluate the trend toward bone resorption or bone formation stimuli (43). A number of studies have shown that bone remodeling is dependent on the ratio of RANKL to OPG: if RANKL is higher bone resorption dominates, while when OPG is higher, the balance is shifted toward bone formation (44, 45). Thus, OPG acquired its name from its ability to protect bone from excessive resorption by counteracting the osteoclastic effects of RANKL (46, 47). Over the last decade, innovative, efficacious treatments for osteolysis have been developed specifically targeting the RANKL/OPG ratio, in order to reduce the incidence of related implant failures (43). In our study, RANKL/OPG ratio showed a trend toward an increase in this ratio when UHMWPE is not stabilized with Vitamin E, while the addition of Vitamin E restores the bone turnover stimuli to levels comparable to controls.

These results are also confirmed by the evaluation of two other main osteoimmunological factors, Sclerostin (SOST), and DKK-1, two inhibitors of the Wnt pathway involved in bone remodeling regulation. Wnt Signaling stimulates OPG expression, while the Wnt inhibitors Sclerostin and DKK-1 prevent this effect (13), thus promoting bone resorption. Our results confirmed an increase in Sclerostin expression in correspondence to a decrease in OPG expression, in response to material 2 (UHMWPE without Vitamin E), and a decrease of Sclerostin in correspondence to the treatment with Vitamin E-stabilized UHMWPE. In this case, no significant difference was observed among crosslinked and not-crosslinked Vitamin E-stabilized UHMWPE, suggesting that the cross linking process does not affect the expression of these osteoimmunological biomarkers. A different effect was observed for DKK-1 secretion, showing no significant decrease in response to material 1 and a little increase in response to material 3, while as expected it displayed an increase, even though not significant, in response to material 2. The exact mechanism of DKK-1 regulation of OPG production still needs to be elucidated, and these results suggest that it could be different from the Sclerostin mechanism. It is known that Sclerostin and DKK-1 can act separately and even alternatively in the regulation of bone turnover (48). We have already shown that Vitamin E-stabilized UHMWPE wear debris had different effects on Sclerostin and DKK-1 (26), and recent evidence indicated that these two Wnt inhibitors can be differently influenced by treatments (49) or pathological conditions (50). Consistently with these results, recent evidence showed that redox regulating mechanisms are able to affect cytokine and osteoimmunological factors produced by bone tissue and that the expression of RANKL, OPG and Sclerostin are redox regulated processes (51, 52). In particular, among Wnt pathway inhibitors, Sclerostin can be affected by oxidative stress, showing the same reduction in response to antioxidant as RANKL expression and RANKL/OPG ratio (52).

The limitation of this study is that the effect of Vitamin E stabilization was not tested on primary human osteoblasts, which could better reproduce the *in vivo* conditions, but on an immortalized human osteoblast cell line. Nevertheless, this choice was made in order to introduce as few variables as possible, since the effect of Vitamin E stabilization of UHMWPE on osteoblasts was unknown. Osteoblastic–like SaOS-2 cells are considered a valuable system for studying osteoblast functions and response to oxidative stress (10). Moreover, the choice for this immortalized cell line provides some advantages, such as a more stable and standardized growth condition than the primary cell culture.

In conclusion, taken together, these results suggest that the Vitamin E stabilization of UHMWPE produces two synergic effects on osteoblasts: on the one hand, it improves the ability of osteoblasts to respond to oxidative stress, inducing the cellular mechanism of defense, such as dynamic O-GlcNAcylation; on the other hand, the antioxidant effect influences the secretion on osteoimmunological factors, stimulating bone protective osteoimmunological factors such as OPG and reducing the RANKL/OPG ratio. This effect observed *in vitro*, could reflect *In vivo* through inducing a shift of the bone turnover balance toward bone protection. This suggests that the Vitamin E-Stabilization of UHMWPE could contribute to reduce oxidation- induced osteolysis (6, 32, 53) and the consequent loosening of the prosthetic device, therefore, improving the longevity of total joint replacements.

DATA AVAILABILITY

The raw/processed data required to reproduce these findings cannot be shared at this time due to legal or ethical reasons

AUTHOR CONTRIBUTIONS

LM, EG, and VR contributed conception and design of the study. NP, LM, and GG performed the oxidative stress evaluation. MMCR and GG revised critically data interpretation. EG wrote the draft of the manuscript and performed osteoimmunological assays. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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Updates on Osteoimmunology: What's New on the Cross-Talk Between Bone and Immune System

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The term osteoimmunology was coined many years ago to describe the research field that deals with the cross-regulation between bone cells and the immune system. As a matter of fact, many factors that are classically considered immune-related, such as InterLeukins (i.e., IL-6, -11, -17, and -23), Tumor Necrosis Factor (TNF)-α, Receptor-Activator of Nuclear factor Kappa B (RANK), and its Ligand (RANKL), Nuclear Factor of Activated T-cell, cytoplasmatic-1 (NFATc1), and others have all been found to be crucial in osteoclast and osteoblast biology. Conversely, bone cells, which we used to think would only regulate each other and take care of remodeling bone, actually regulate immune cells, by creating the so-called "endosteal niche." Both osteoblasts and osteoclasts participate to this niche, either by favoring engraftment, or mobilization of Hematopoietic Stem Cells (HSCs). In this review, we will describe the main milestones at the base of the osteoimmunology and present the key cellular players of the bone-immune system cross-talk, including HSCs, osteoblasts, osteoclasts, bone marrow macrophages, osteomacs, T- and B-lymphocytes, dendritic cells, and neutrophils. We will also briefly describe some pathological conditions in which the bone-immune system cross-talk plays a crucial role, with the final aim to portray the state of the art in the mechanisms regulating the bone-immune system interplay, and some of the latest molecular players in the field. This is important to encourage investigation in this field, to identify new targets in the treatment of bone and immune diseases.

Keywords: osteoimmunology, RANKL, osteoclasts, osteoblasts, immune cells, inflammation, rheumatoid arthritis, osteoporosis

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INTRODUCTION

Evidence collected over the years draw bone researchers to the conclusion that bone accomplishes several unexpected functions besides its classical role in locomotion, protection of vital organs and in the regulation of calcium and phosphate homeostasis. As a matter of fact, it is now well-accepted that bone has a role in the regulation of glucose metabolism, energy expenditure (1–3), male fertility and cognitive functions, through osteoblasts secretion of osteocalcin (4). Therefore, we can assume that bone is a central organ, capable of regulating several other tissues and to be in turn influenced by them.

An intriguing aspect testifying the versatility of bone and its cells, is its deep cross-talk with the immune system. This led to the establishment of a new interdisciplinary field, named

osteoimmunology, thanks to the great contribution of studies by Takayanagi and many others. These pointed out the pivotal role of some classical immunoregulatory factors in osteoclast differentiation (5) as well as the cross-talk between autoimmune diseases, such as Rheumatoid Arthritis (RA), and bone destruction (6). Intriguingly, the cross-talk between the immune system and bone is bidirectional, meaning that bone cells also influence immune cells.

In this review, we will describe the main milestones in the history of osteoimmunology, as well as the latest findings enriching this discipline, with the final aim to have a state-of-the-art reference of the mechanisms regulating the bone-immune system interplay. This is important to encourage investigation in this field, in order to identify new targets in the treatment of bone and immune diseases.

BONE BIOLOGY

In the past, bone was seen as a static tissue, a simple "scaffolding" for all the other organs. Now we know bone is actually extremely dynamic, undergoing continuous cycles of modeling during growth and remodeling during adulthood, which guarantee adequate mechanical properties and proper bone shape (7). Bone modeling and remodeling are guaranteed by the action of three types of bone cells: osteoclasts, which resorb bone, osteoblasts, which depose bone, and osteocytes, which are former osteoblasts buried in bone matrix, controlling bone mechano-physiology, and able to resorb and depose bone. The cycle of bone remodeling happens following these 4 phases: (1) latent phase: bone-lining cells are activated by osteocytes following a stimulus, starting osteoclast differentiation (**Figure 1**) and exposing the bone surface; (2) activation phase: osteoclasts resorb the portion of bone left exposed by the bone-lining cells. When they are done resorbing, they detach from bone and undergo apoptosis; (3) reverse phase: macrophage-like reverse cells migrate to the resorbed lacuna and clean it of the debris left by osteoclasts. Reverse cells also secrete factors that summon osteoblasts in the resorption lacuna; (4) formation phase: this is the longest phase in bone remodeling, lasting up to 6 months. Osteoblasts occupy the resorption lacuna and fill it up with organic osteoid matrix, which they then mineralize (7). In this last phase, osteoblasts may undergo apoptosis, or embed themselves in the bone matrix they produce, eventually becoming osteocytes (8). Bone modeling and remodeling are very similar as far as the mechanisms and the cellular players go. The key difference is that modeling happens during growth and fracture repair, and guarantees bone mass accrual, while remodeling happens in adulthood, does not change bone mass, but keeps mechanical properties at physiological levels, by continuously renewing the bone matrix. Although this is quite an accurate depiction of "normal" bone modeling/remodeling, it has become clear in the last years, that the molecular and cellular players involved in the maintenance and accrual of bone mass are many more than originally expected. A key player in the maintenance of bone mass in bone pathophysiology is undoubtedly the immune system, giving rise to an extremely important field of research: osteoimmunology. This will be discussed in the following paragraphs and chapters.

OSTEOIMMUNOLOGY

The term osteoimmunology was likely adopted for the first time by Arron and Choi (9), to describe the phenomenon of T-cell-mediated regulation of osteoclasts. Most of the research projects related to bone-immune system cross-talk are quite recent and mainly focused on the influence of the immune system on osteoclast physiology. As a matter of fact, these cells share a common origin with immune cells, since they both arise from bone marrow hematopoietic stem cells (10). Moreover, like other hematopoietic cells, osteoclast precursors can be detected as circulating cells in blood and their number increases under inflammatory conditions, characterized by high levels of the potent inflammatory cytokine Tumor Necrosis Factor (TNF)-α (11, 12).

Bone Cells and the Immune System

In the following paragraphs, the many ways through which bone cells are linked to the immune system, and how they regulate immunity, will be discussed.

Osteoblasts

A pivotal work demonstrating a close connection between bone and the hematopoietic compartment came in 2003, when Calvi and colleagues demonstrated that mice genetically engineered to express a constitutively active PTH/PTHrP receptor in osteoblasts, had more Hematopoietic Stem Cells (HSCs). This was due to an increase in osteoblastic Jagged1, which in turn mediated said effect through activation of Notch1 (**Figure 2**). Intriguingly, when Calvi and colleagues myeloablated WT mice, and subsequently performed bone marrow transplantation, treatment with intermitting doses of PTH improved the engraftment rate to 100%. This could be important for human conditions that require similar procedures (13). Likewise, Zhang et al. found a direct correlation between the number of a subset of osteoblasts, called Spindle-shaped-N-cadherin^{+}CD45^{-} Osteoblasts (SNOs) and the number of HSCs. Moreover, long-term HSCs were found bound to SNOs by an N-cadherin/βcatenin-dependent mechanism (14). These data clearly demonstrate that a subpopulation of osteoblasts plays a crucial role in HSCs regulation, thus identifying in the bone marrow the so called "endosteal niche" besides the well-known vascular niche (15).

Later on, Zhu and colleagues definitely demonstrated that osteoblasts contribute to the commitment and differentiation of B lymphocytes from hematopoietic stem cells (16). In particular, in mice subjected to osteoblast conditional ablation, B-lymphocyte differentiation was compromised because of the lack of transition from Rag2^{-} to Rag2^{+} committed lymphoid progenitors. This effect was likely due to osteoblast secretion of InterLeukin (IL)-7 and C-X-C motif chemokine Ligand (CXCL)12 *alias* Stromal cell-Derived Factor (SDF) 1, two cytokines pivotal for B cells differentiation (17, 18). A collection

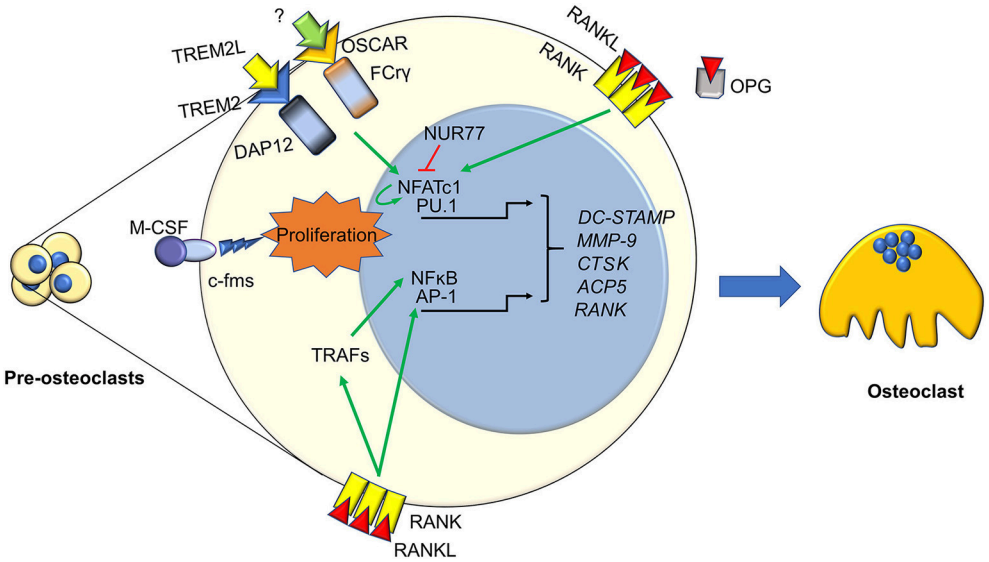

FIGURE 1 | Immune factors in osteoclastogenesis. Osteoclasts differentiation from pre-osteoclasts involves several factors, most of which are derived from the immune system. Signaling from TREM2, OSCAR, c-FMS, and RANK cause the nuclear translocation of several transcription factors activating pre-osteoclasst proliferation and differentiation. These include the master osteoclastogenesis controllers NFATc1, which also self-amplifies, and NFkB, along with the early commitment factor PU.1 and AP1. This is enacted both directly by RANK, and indirectly through TRAFs and PLCγ. Osteoclastogenesis can be hindered by several factors, two key ones are the decoy receptor for RANKL, OPG, and the immune factor NUR77, which can inhibit NFATc1 stopping its self-amplifcation loop. The final outcome of these molecular pathways is the transcription of key osteoclast genes, such as DC-STAMP, MMP9. *CTSK*, ACP5, and RANK, which eventually results in the generation of a mature osteoclast.

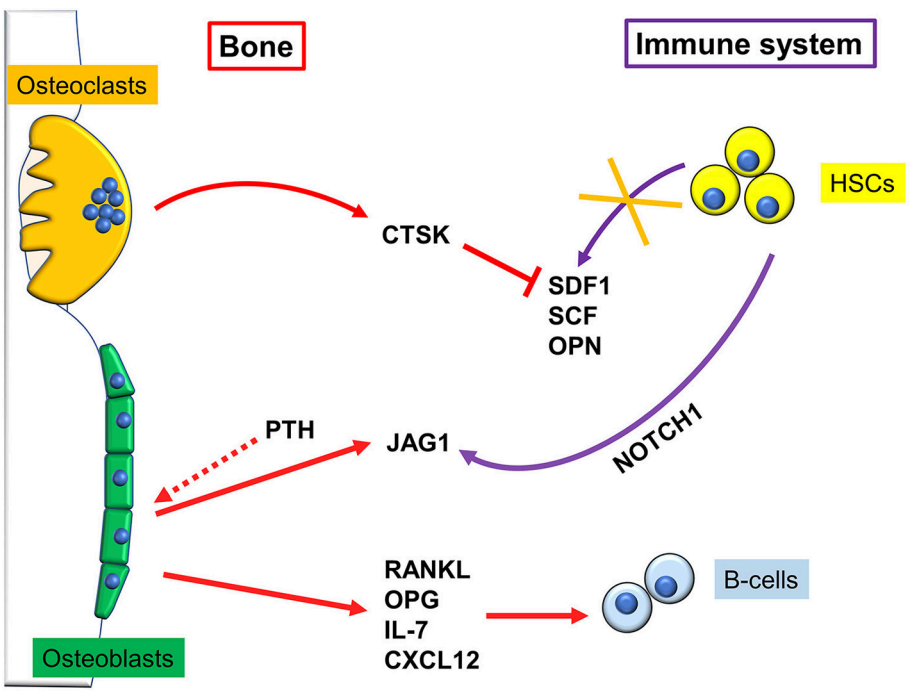

FIGURE 2 | Regulation of immune cells by bone cells. Osteoclasts reduce hematopoietic stem cells (HSCs) homing by secreting cathepsin K (CTSK), which in turn degrades stromal cell-derived factor (SDF)1, stem cell factor (SCF), and osteopontin (OPN) depriving the bone niche of HSC-binding sites, which causes their mobilization. Osteoblasts, after stimulation with pro-osteoblastogenic factors such as intermittent parathyroid hormone (PTH), express Jagged1 (Jag1), which binds NOTCH1 on HSCs, and allows them to engraft and survive into the endosteal niche. B cells and bone cells communicate in multiple ways. For example, osteoblasts produce IL-7 and the chemokine CXCL12, that are fundamental for B-cells survival and activity.

TABLE 1 | Factors produced by immune cells influencing osteoblast activity.

Factor	Source	Action	References
IL-11	Bone marrow stromal cells	Increase osteoblast activity	(19)
IL-6	Bone marrow stromal cells, osteoblasts, macrophages, muscle tissue, fibroblasts	Reduce osteoblast differentiation and activity	(20, 21)
IFN-γ	T-cells, NK-cells	Increase osteoblast activity	(22, 23)
IL-17F	Th17 cells	Increase osteoblast activity	(24, 25)
IL-15	PG-stimulated stromal cells, NK-cells	Reduce viability, increase apoptosis	(26)
OSM	B-cells	Increase osteoblast activity	(27)

of the main immune-derived factors promoting or hindering osteoblast differentiation and activity is present in **Table 1**.

Osteocytes

Osteocytes are the main producers of Receptor Activator of Nuclear factor Kappa B ligand (RANKL) in the bone, therefore since this cytokine is crucial not only for osteoclasts but also for lymphocyte development (see next paragraph) it is conceivable that this cell type could influence the immune system. Indeed, it has been demonstrated that RANKL arising from osteocytes contributes to the increased osteoclastogenesis and bone loss observed in estrogen deficient conditions. Moreover, specific deletion of the Rankl gene in osteocytes also prevented the increase in B cell formation caused by estrogen deficiency (28). In support of the relationship between osteocytes and the immune system, Sato and colleagues found that *in vivo* ablation of osteocytes leads to severe lymphopenia, caused by the loss of lymphoid- supporting stroma in the thymus and in the bone marrow, which is reverted by re-establishing the osteocyte population (29).

Osteoclasts

Osteoclasts have been shown to regulate the HSC niche both directly and indirectly through osteoblasts (**Figure 2**). Firstly, osteoclasts can increase HSC mobilization by secreting cathepsin K, a crucial protein for osteoclast function, which cleaves SDF1, OsteoPontiN (OPN), and Stem Cell Factor (SCF), depriving the bone niche of HSC-binding sites. Consequently, HSCs mobilize to the circulation and are no longer kept quiescent (30). Furthermore, it has been shown that *oc/oc* mice, which have an inactivating mutation in the *T Cell, Immune ReGulator 1, ATPase, H^+ transporting, lysosomal V0 protein A3* (*Tcirg1)* gene and therefore almost entirely lack osteoclasts activity (31), have an overrepresented Mesenchymal Stem Cell (MSC) fraction. However, despite the higher number of precursors, MSCs differentiate less into osteoblasts, which impairs osteoblast-mediated HSCs homing to bone (32). Therefore, osteoclasts number and activity need to be tightly regulated, because any alteration could lead to excessive HSCs mobilization. Furthermore, *oc/oc* mice present with improper B lymphopoiesis, which is blocked at the pro-B stage, leading to fewer mature B-cells. T-cell activation is also affected, leading to a form of B-T-cells immunodeficiency (33).

As it is well-known, osteoclast differentiation strictly relies on the RANKL/RANK pathway (34, 35). RANKL interacts with its receptor RANK expressed by osteoclast precursors, thus recruiting TNFR-Associated Factors (TRAFs), which in turn trigger osteoclast differentiation by stimulating nuclear translocation of Nuclear Factor k-light-chain-enhancer of activated B cells (NFkB), Activator Protein 1 (AP1) complex and Nuclear Factor of Activated T-cells, cytoplasmic, calcineurin-dependent 1 (NFATc1) (36). All these factors stimulate transcription of several osteoclast-specific genes, such as Tartrate Resistant Acid Phosphatase (TRAcP), calcitonin receptor, cathepsin K, OSteoClast Associated Receptor (OSCAR), alpha V β3 integrin, Matrix Metalloproteinase (MMP) 9, and Dendritic Cell-Specific Transmembrane Protein (DC-STAMP) the latter involved in osteoclast fusion [**Figure 1**, (7)].

Of note, RANKL is also produced by activated T-lymphocytes as soluble form and is expressed in lymph nodes and thymus. Its importance in the immunological context was demonstrated by the fact that mice lacking RANKL showed not only a bone phenotype, resulting in osteopetrosis due to the lack of osteoclasts, but also presented with immunological defects, with impaired lymphocytes development and lack of lymph node organogenesis (37). Consistently, Dougall et al. demonstrated that RANK is essential for osteoclast and lymph node development, since RANK knockout mice showed an osteopetrotic phenotype along with a lack of peripheral lymph nodes and a marked deficiency in B and T lymphocytes (38). In contrast, OsteoProteGerin (OPG) is a decoy receptor for RANKL, belonging to the TNF Receptor (TNFR) superfamily, which prevents RANKL interaction with its receptor RANK, eventually leading to inhibition of osteoclast formation (35). Not only osteoblasts but also B lymphocytes produce OPG, thus concurring to regulate osteoclastogenesis (39).

The awareness of a close interconnection between bone and immune system was increased by Takayanagi et al. (40), who demonstrated that mice lacking Immunoreceptor Tyrosine-based Activation Motif (ITAM)-harboring adaptors, Fc Receptor common Gamma subunit (FcRγ) and DNAX-Activating Protein (DAP)12, presented with an osteopetrotic phenotype caused by a reduction of osteoclast differentiation. Indeed, they showed that RANKL/RANK pathway requires the ITAM-dependent costimulatory signals to activate osteoclast differentiation (5). Later, they found that FcRgamma and DAP12 associated to OSCAR and receptor expressed on myeloid cells

2 (TREM2), respectively, eventually leading to PhosphoLipase C (PLC)γ activation, which in turn activates calcium signaling, necessary for NFATc1 auto-amplification (41, 42). Osteoclasts also express Bruton's Tyrosine Kinase (Btk) and Tec, which have a physiological role in B cells (43), and mice double knock out for both these kinases manifest osteoclast-poor osteopetrosis, likely due to a suppression of the RANKL induced phosphorylation of PLCγ (44).

Regulation of osteoclast formation is a complex mechanism, calling into questions more and more pathways. One of the last is NR4A1 (alias Nur77), which belongs to the orphan nuclear receptor family, already known to be a key regulator of myeloid and lymphoid differentiation and function (45, 46). Recent findings demonstrated a role for this nuclear orphan receptor in the suppression of osteoclast differentiation (47). Moreover, Scholtysek and colleagues clarified a role for NR4A1 in controlling pre-osteoclast recruitment and migration, with an effect linked to the myeloid lineage. In fact, myeloid-specific but not osteoblast-specific deletion of NR4A1 resulted in osteopenia due to an increase of osteoclast number (48). The immune-related factors influencing osteoclast formation and biology are many more; a list of the most important ones is presented in **Table 2**.

TABLE 2 | Secreted and membrane-bound immune factors promoting osteoclastogenesis.

Factor	Source	References
RANKL	Osteoblasts, osteocytes, neutrophils, sinoviocytes, T-cells	(28, 37, 49)
OPG	Osteoblasts, B-cells	(35, 50)
M-CSF	Osteoblasts, activated T-cells	(6, 51, 52)
TNFα	Activated leucocytes	(11, 12)
IL-1α,−1β	Activated leucocytes, osteoblasts, synoviocytes, endothelial cells	(53)
IL-7	Osteoblasts, bone marrow stromal cells, leucocytes	(53)
IL-8	Activated leucocytes	(53)
IL-11	Bone marrow stromal cells	(19)
IL-23	Dendritic cells, Th17 T-cells	(24, 54)
IL-34	Dendritic cells, Th17 T-cells, synoviocytes, osteoblasts	(24, 53)
Prostaglandins	Bone marrow and bone cells	(55)
SOFAT	T-cells	(56)
IL-6	Bone marrow stromal cells, osteoblasts, macrophages, muscle tissue, fibroblasts	(19–21, 57, 58)
IFN-γ	T-cells, NK-cells	(22, 23)
IL-17A	Th17 cells	(24, 59, 60)
IL-15	PG-stimulated stromal cells, NK-cells	(26)

Immune Cells in Bone Physiology

As described, bone cells can influence the immune system, and employ several immune factors for their physiologic function. The opposite is also true: immune cells can influence bone health in many ways, as will be described in the following paragraphs.

T-Cells

T-cells are a key component of adaptive immunity. These small and relatively rare cells have a key role not only in immunity, but also in osteoimmunology. T-cells are not all created equal, and in this group, we can find cytotoxic $CD8^+$ T-cells, $CD4^+$ T-helpers, further subcategorized in Th1, Th2, Th17, and T-reg cells, the latter having an important role in preventing excessive or improper (e.g. self-directed) immune response (61). The links between T-cells and bone biology are numerous: essentially all the subtypes of T-cells are able to influence bone cells (mostly osteoclasts). However, a particularly important role for Th17 and T-reg cells is emerging. Th17 cells have been proposed to be the most osteoclastogenesis-inducing T-cells. They are characterized by the expression of a cytokine signature: IL-17A, IL-17F (hence the name), IL-22, IL-26, and IFN-γ (62). These cells can induce Macrophage Colony-Stimulating Factor (*M-CSF*) and *RANKL* expression in osteoblasts and stromal cells (6), produce RANKL and TNF-α, while parallelly increasing *RANK* expression in osteoclast precursors (63). These features make them potent osteoclastogenesis inducers, which have been already described as players in human bone diseases, such as RA (64) and multiple myeloma (65). With regards to T-reg cells, their role is clearly anti-osteoclastogenic, and it is enacted through a soluble factors-mediated mechanism, as well as a contact-mediated mechanism (24). In fact, co-culture experiments of whole Peripheral Blood Mononuclear Cells (PBMCs) or T-reg-depleted PBMCs resulted in higher osteoclast formation in the former (66), which seems to be dependent on TGF-β and IL-4, while the latter was found to be Cytotoxic T-Lymphocyte Antigen (CTLA)4-dependent (67). In the aforementioned reports, *in vitro* bone resorption was reduced up to 80% by the action of T-reg cells, which makes them potentially very important in autoimmune osteolysis-inducing disease such as RA. In fact, this has been proposed and demonstrated in mice (68), although human studies are still lacking to date.

Dendritic Cells

Dendritic Cells (DCs) are antigen-presenting cells entrusted with the important role of directing cell-mediated immunity toward the right targets, as quickly as possible and avoiding self-immunity (69). Their role in bone biology has in fact been historically thought as mostly indirect, through T-cells (70). Dendritic cells are in fact not only able to present antigens to T-cells, but also to regulate their activity and subtype balance through cytokine signaling (69, 71). However, another interesting concept that could be important for RA is that DCs can transdifferentiate into osteoclasts through M-CSF and RANKL stimulation, as if they were osteoclast precursors. Since DCs are numerous in and around the inflamed synovium in RA, they could well-contribute to the osteolytic disease in RA (72). However, DCs have not been further investigated in the last years in osteoimmunology in human studies.

Neutrophils

Neutrophils also play a role in bone biology, and particularly in inflammation-induced bone loss (73). In fact, neutrophils are usually the first cell type migrating to damage sites,

including bone (74), and they can secrete many chemokines, cytokines and small molecules, which are able to act as immunomodulatory factors. For example, by CCL2 and CCL20 secretion, neutrophils are able to summon Th17 cells (73) which in turn cause bone loss, as discussed in the above paragraphs. However, absence of neutrophils can be even more damaging to bone tissue, as it eventually results in local IL-17-driven inflammatory bone loss (75). Remarkably, activated neutrophils express RANKL in the inflammatory site, and if that site is the synovium, they can actively participate in osteoclastogenesis, which increases RA-related osteolysis (76). In summary, although the role of neutrophils in osteoimmunology is not cut-and-dried, the general consensus is that activated neutrophils are osteoclastogenesis inducers, both directly and indirectly.

B-Cells

B-cell development relies on the production of several factors, including RANKL, OPG, IL-7, and CXCL12, which are produced by bone marrow stromal cells and osteoblasts [**Figure 2**, (50)]. As already mentioned, RANK knockout mice presented with a reduction in the number of mature $B220^+IgM^+$ and $B220^+IgG^+$ B-cells in lymph nodes (38, 77). Further studies demonstrated that not only RANKL arising from the bone marrow/bone compartment is crucial for B-cell development, but also B-cells themselves produce RANKL, which then acts as an autocrine factor (78). However, when RANK was conditionally deleted in the pro-B cells, B cell development was not affected (79) thus suggesting that RANKL could interact with an alternative receptor.

The evidence that B cells produce RANKL suggests that they could influence osteoclasts, and this is the case. In fact, Onal and colleagues demonstrated that mice lacking RANKL in B lymphocytes were partially protected from ovariectomy-mediated bone loss, through a mechanism counteracting the increase in osteoclasts number that is a hallmark of this mouse model. Conditional knock out of RANKL in T-lymphocytes had no effect on ovariectomy-induced bone loss (78). Interestingly, IL-7 transgenic mice showed focal osteolysis, besides the expected increase of pro-B and pre-B cells, while mice lacking the IL-7 receptor showed suppression in B-lymphocyte development associated to an increased bone mass (80).

In vitro studies have shown that purified B cells can be driven to differentiate into osteoclasts when treated with RANKL, thus acting as a source of osteoclast progenitors in *vitro* (81, 82). In contrast, *in vivo* lineage-tracing studies to investigate whether cells committed to the B cell lineage can act as osteoclast progenitors found that this was not the case, therefore the authors conclude that the role of B cells is not to act as osteoclast progenitors but as cells supporting osteoclasts formation (28).

Natural Killer (NK) Cells

Natural Killer (NK) cells, as all other lymphocytes, play a role in the regulation of bone homeostasis. In fact, several reports emerged stating that they are particularly important in bone destruction induced by RA, and they are also able to induce osteoblast cell death (26, 83, 84). This makes NK cells a potential therapeutic target to reduce RA-induced bone destruction. However, NK cells have also been described as helpful and necessary to slow down RA by recent reports (85), casting doubts over the efficacy of a hypothetical anti-NK-cells treatment in RA.

Osteomacs and Bone Marrow Macrophages

As other organs in the body, bone and bone marrow present with resident macrophages, which include bone marrow macrophages and osteal macrophages (86). The latter, also known as osteomacs, are F4/80 positive and TRAcP negative, and are located close to the bone surface. It has been recently demonstrated that in 2-day-old mouse calvarial osteoblasts a small population of $CD45^+F4/80^+$ osteomacs can be detected (87). This subpopulation cooperates with osteoblasts and megakaryocytes to promote hematopoietic progenitor and HSC function (87, 88). The same authors also demonstrated that highly purified $CD45^+F4/80^+$ osteomacs from neonatal calvarial osteoblasts can differentiate into TRAcP positive osteoclasts able to resorb bone (88).

Notably, *in vitro* and *in vivo* studies demonstrated a role for these bone macrophages in osteoblast differentiation by producing Bone Morphogenetic Proteins (BMPs) (89) and Oncostatin M (27). Moreover, Chang and colleagues found that depletion of osteal macrophages in primary osteoblasts inhibited their differentiation (90). *In vivo* approaches able to selectively ablate osteal macrophages but not osteoclasts showed that their lack determined a decrease in bone formation, a reduction in bone growth in young mice and osteoporosis (90–92). Therefore, osteomacs are versatile cells, able to regulate bone mass, become osteoclasts, and actively participate in the homeostasis of the immune system.

Inflammation and Inflammatory Factors

Several cells of the immune system (T and B cells, NK cells, monocyte/macrophage and dendritic cells) produce InterFeroN (IFN)-γ, which has a pivotal role in innate and adaptive immune responses as well as in the regulation of inflammation (22, 23). In bone, IFN-γ affects both osteoblasts and osteoclasts. The former, which produce low levels of this cytokine, are indeed positively affected, since IFN-γ increases osteoblast differentiating genes, like Runt related transcription factor 2 (Runx2), Osterix, ALkaline Phosphatase (ALP) and Osteocalcin (93, 94) and mice knock out for the IFN-γ receptor showed reduced osteoblast differentiation (95). Conversely, IFN-γ has an inhibitory effect on adipogenesis (96).

Much data has been collected about the inhibitory effect of IFN-γ on osteoclast differentiation [for a review see (23)]. This protein is known to counteract the effect of M-CSF on osteoclast precursors by reducing the expression of its receptor c-fms, eventually leading to a reduced pool of Rank-positive preosteoclasts (97). Furthermore, IFN-γ promotes TRAF6 degradation (53, 98), thus inhibiting the downstream signaling which involves JNK and NFkB, while it induces osteoclast apoptosis by activating Fas-FasL-mediated death signaling (99). However, other studies show a pro-osteoclastogenic effect of IFN-γ in the late state of differentiation. This depends on the

upregulation of NFATc1 and c-fos, which in turn stimulate *DC-STAMP* expression, thus promoting osteoclast fusion (100). Another indirect pro-osteoclastogenic effect of IFN-γ is due to its ability to increase the secretion of CXCL10, also known as Interferon gamma induced Protein 10 (IP-10), by macrophages and this cytokine in turn stimulates the production of RANKL and TNF-α by T cells (101).

Inflammatory diseases, such as RA, periodontal disease (see later), systemic lupus erythematous, inflammatory bowel diseases and cystic fibrosis are all characterized by bone loss (6, 102–106), which is not only secondary to the employ of anti-inflammatory corticosteroid therapies (107), but is also due to a direct effect of inflammatory cytokines on osteoclasts, thus creating a sort of "vicious cycle".

So far, a plethora of inflammatory cytokines have been identified as positive modulators of osteoclasts [for a review see (53)]. Above all the proinflammatory cytokine TNF-α, which directly stimulates osteoclastogenesis by a mechanism independent of RANKL (108) as well as indirectly, by promoting RANK expression on preosteoclasts (109) and increasing RANKL and M-CSF production by osteoblasts and activated T cells (50, 51).

The IL-6 family includes at least three pro-osteoclastogenic cytokines: IL-6, -11 and -23. The first stimulates osteoclastogenesis by a mechanism independent of RANKL, since the presence of OPG does not blunt this effect (57). This is accompanied by a positive stimulation of RANKL production by stromal cells and osteoblasts (19, 58). As for osteoblasts, IL-6 reduces their differentiation *in vivo* (20) and *in vitro* by affecting the MEK2 and Akt2 pathways (21).

Also, IL-11 has a pro-osteoclastogenic effect (19), while on the osteoblast side it seems to be pro-osteogenic (110, 111).

IL-23 is mainly produced by dendritic cells and macrophages, and it indirectly stimulates osteoclastogenesis by increasing RANK and RANKL expression by osteoclast precursors and osteoblasts, respectively (54). Other factors strongly promoting osteoclastogenesis are prostaglandins (55), IL-1α, -1β, -7, -8, and -34 (53).

OSTEOIMMUNOLOGY IN BONE AND IMMUNE DISEASES

As it is easy to imagine, the link between bone and the immune system in physiology is also maintained in pathological conditions: many diseases affecting bone have an immunologic origin, while some immunological disorders, such as acute myeloid leukemia, can originate from bone-derived signals, as will be described in the following paragraphs.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a T helper (Th) type 1, degenerative disease with strong genetic predisposition factors (up to 50%), characterized by synovitis, persistent inflammation and the generation of antibodies against endogenous proteins, especially rheumatoid factor (112). Takayanagi's group demonstrated a close connection between RA and osteoclast deregulation (6). Indeed, despite the high levels of IFN-γ, which is known to inhibit osteoclastogenesis, the T-cells' activation observed in this pathology results in an exacerbated osteoclast activation. This is due to the action of a subset of activated T-cells, called Th-17 because they produce IL-17. Of note, in a mouse model of RA, IL-17 ablation reduced bone destruction (59). This cytokine is of interest, because it stimulates the expression of more pro-osteoclastogenic cytokines, including IL-6, IL-8, and TNF-α also in absence of RANKL (113, 114). Moreover, in the synovium of RA patients, IL-17 induces the production of IL-32, which in turn stimulates IL-17 expression (60) creating a feed-forward loop. Therefore, this cytokine plays a crucial role not only in the onset of RA but also in the bone destruction that this disease presents with (6). Furthermore, IL-6 production increases RANKL expression in synoviocytes, leading to further exacerbated osteoclastic bone destruction (49).

Osteoporosis

Estrogen deficiency is the leading cause of osteoporosis in post-menopausal women. Pacifici and colleagues found that this condition results in increased production of inflammatory cytokines. This was a cornerstone discovery, that elucidated a new facet of post-menopausal osteoporosis, describing it as an inflammatory disease (115). The protective effect of estrogen in bone has been well characterized and is mainly due to a direct action on both osteoclasts and osteoblasts. In the former, estrogens significantly increase apoptosis (116, 117) and reduce RANKL-dependent osteoclast formation (118), while in osteoblasts they exert an anabolic effect by at least increasing osteoblast survival and collagen I production (119). Consistently, it has been demonstrated that estrogens suppress RANKL production not only in osteoblasts but also in T and B cells (120), while the lack of estrogens increases the release of pro-osteoclastogenic cytokines (i.e., TNF-α and RANKL) by activated T cells (121–123) thus indirectly identifying immune cells as additional players in the onset of osteoporosis. Estrogens withdrawal also leads to a significant increase of B-cells' number (124).

Regarding the role of immune cells-derived-IFN-γ in osteoporosis, results are conflicting (23). In particular, while Breuil and colleagues found reduced secreted levels of IFN-γ by $CD4^+$ T-cells in osteoporotic patients (121) previous studies did not find any differences (125, 126).

Periodontal Disease

Another pathological phenomenon in which immune cells deregulation causes bone loss is periodontal disease, where activated B and T cells concur to stimulate osteoclast resorption by producing RANKL (127, 128). Consistently, Weitzmann and colleagues identified a T cell-secreted cytokine, named Secreted Osteoclastogenic Factor of Activated T-cells (SOFAT) that promotes osteoclastogenesis independently of RANKL (129, 130). Moreover, increased mRNA expression of SOFAT has been found in human periodontal samples, while SOFAT injection induced osteoclast formation in a mouse model of periodontal disease (56).

Bone Fracture Repair

This physiological process is orchestrated not only by bone cells, but also by immune cells, whose deregulation can delay fracture repair (131, 132). In fact, in conditions of B and T cells depletion, an impairment of bone regeneration, due to a reduction of osteoblast differentiation and bone mineralization, has been observed (25, 133). Consistently, immunocompromised HIV patients present with a delay of fracture healing repair (131).

One of the earliest phases of bone healing is characterized by an inflammatory state, with the release of IL-1, IL-6, and TNF-α, which recruit B and T lymphocytes, the latter having a pro-osteogenic role by releasing IL-17F (25). In fact, at variance with IL-17A, known for its pro-osteoclastogenic role, the pro-inflammatory cytokine IL-17F is expressed during bone healing, where it increases Col1a1, osteocalcin, and bone sialoproteins in treated osteoblasts (25). Neutrophil granulocytes are also present during the early phase of bone repair, and they clean debris and damaged cells in the site of injury (134). Another crucial step for bone fracture repair is the recruitment of MSCs which again involves immune cells (i.e., NK cells and macrophages) since they produce chemoattractant molecules such as CXCL7 (alias NAP2) and Monocyte Inflammatory Protein (MIP)-1α (135, 136). Once recruited, MSCs are fostered to differentiate toward the osteoblast lineage, through macrophage-derived BMPs (137) while activated monocytes stimulate the expression of Runx2 (138). At the same time, once the bone-repair process activates, the inflammatory reaction should be switched off in order to avoid any inflammation-derived damage. To this aim, MSCs exert an immunosuppressive role, by stimulating the differentiation of T-reg lymphocytes, inducing the apoptosis of the pro-inflammatory Th1 and Th17 lymphocytes and inhibiting migration of B-lymphocytes (139–142).

Myelodysplasia and Acute Myeloid Leukemia

Another strong link between bone and the immune system is the fact that osteoblasts can influence the progression of pre-neoplastic and neoplastic transformations in the myeloid lineage. In fact, osteoblasts are able to slow down leukemia progression in mouse, creating an unfavorable microenvironment for leukemic blast growth (143). Consistently, osteoblast number is reduced by more than half in leukemic patients. Simulating this situation by mouse genetics, causes leukemic blasts to grow faster and engraft better (143). The same authors demonstrated that osteoblasts have another tight link to human leukemia: osteoblasts that have been genetically engineered to express a constitutively active form of β-catenin, are able to induce leukemic transformation in myeloid cells, causing MyeloDysplaSia (MDS) and then Acute Myeloid Leukemia (AML) (144).

The concept of bone cells inducing malignant transformation, however, was not new, since already a few years earlier, Raaijmakers and colleagues found that ablating the miRNA processing protein dicer from osteoblast progenitors induces dysfunctional haematopoiesis, eventually leading to MDS and AML development (145). The field of "niche-induced leukemia" has received much attention, and still many groups are working on this topic to date.

LATEST DEVELOPMENTS IN OSTEOIMMUNOLOGY

In the last few years, despite much of the field has already emerged, several groups are still actively discovering new molecules that can be considered part of the osteoimmunology world. This has been the case for a secreted protein named homologous to Lymphotoxin, exhibits Inducible expression and competes with HSV Glycoprotein D for binding to Herpesvirus entry mediator, a receptor expressed on T lymphocytes (LIGHT, a.k.a. Tumor Necrosis Factor SuperFamily member 14, TNFSF14), which has been linked to increased bone resorption in osteoarthritis more than 10 years ago (146, 147), and has known a *renaissance* in the last few years as target for bone loss (148) and biomarker for bone disease in multiple myeloma (149). This molecule seems to have a dual effect in bone: high levels are linked to bone loss, and so is its absence. The mechanisms involving it are therefore quite complex, although agonists and antagonists of the LIGHT pathway are in development and testing. This behavior is also common to another regulator of bone mass that has recently emerged in the last few years: LipoCaliN-2 (Lcn2). This protein is also called Neutrophil Gelatinase-Associated Lipocalin (NGAL), since it can bind and stabilize MMP9, a crucial factor for neutrophil extravasation. Furthermore, Lcn2 is also readily overexpressed during inflammation, and following treatment with TNFα, IL-17, and IL-1β, and its role in inflammatory diseases is only starting to emerge; what is sure is that this molecule can be considered a player in innate immunity. In 2009, we discovered that Lcn2 is strongly overexpressed in osteoblasts following *in vitro* mechanical unloading (150). Furthermore, we confirmed these findings *in vivo*, which led to the concept that Lcn2 is a mechanoresponsive gene that regulates bone homeostasis (151). Surprisingly though, removing this protein genetically, reduces bone mass instead of increasing it (152). This is likely due to the fact that Lcn2 impairs osteoblasts when overexpressed, and impairs energy metabolism when removed, which causes an indirect osteoblast dysfunction (152). The role of Lcn2 in bone is still under investigation by ours and other groups where it has been found to influence hematopoiesis (153), and even appetite through the melatonin receptor MC4R (154).

CONCLUSIONS

The concept of osteoimmunology is aging well, almost 20 years since the term was coined. This way of interpreting bone and the immune system has been steadily providing new insights about how the two of them operate and cooperate. As an example, the role of pro-inflammatory cytokines in promoting osteoclastogenesis, and the many parallelisms between immune cells and osteoclasts have proved crucial to understand the

biology of these giant bone-eating cells. Intriguingly, the control mechanisms between bone and the immune system are complex, tightly interconnected, and involve many players. The underlying complexity of this field has made it difficult for researchers to find clear-cut results, the kind that leads to the direct clinical application. Nevertheless, thanks to the effort of many scientists, nowadays clinics can use drugs, classically employed to treat osteoporosis, for immunological diseases [e.g., Denosumab for RA; (155)]. In conclusion, although the study of osteoimmunology has provided many answers, it also raised more questions, which we need to answer in order to improve standards of care for patients of both immune and bone disorders, by exploiting the cross-talk between these two remarkable systems, which are actually starting to look like a single one.

AUTHOR CONTRIBUTIONS

MP prepared the cartoons and the tables. NR drafted the manuscript, MP and NR finalized, reviewed and approved the manuscript in its final form.

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Mechanisms Involved in Childhood Obesity-Related Bone Fragility

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Childhood obesity is one of the major health problems in western countries. The excessive accumulation of adipose tissue causes inflammation, oxidative stress, apoptosis, and mitochondrial dysfunctions. Thus, obesity leads to the development of severe co-morbidities including type 2 diabetes mellitus, liver steatosis, cardiovascular, and neurodegenerative diseases which can develop early in life. Furthermore, obese children have low bone mineral density and a greater risk of osteoporosis and fractures. The knowledge about the interplay bone tissue and between adipose is still growing, although recent findings suggest that adipose tissue activity on bone can be fat-depot specific. Obesity is associated to a low-grade inflammation that alters the expression of adiponectin, leptin, IL-6, Monocyte Chemotactic Protein 1 (MCP1), TRAIL, LIGHT/TNFSF14, OPG, and TNFα. These molecules can affect bone metabolism, thus resulting in osteoporosis. The purpose of this review was to deepen the cellular mechanisms by which obesity may facilitate osteoporosis and bone fractures.

Keywords: osteoporosis, low grade inflammation, osteoimmunology, osteoclast, cytokines

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INTRODUCTION

Childhood obesity represents an international public health problem with epidemic proportions (1). The World Obesity Federation showed a strong increase of childhood overweight and obesity in several low-, middle-, and high-income regions over the past three decades (2). In the USA ∼17% of children and adolescents are obese, representing a risk for health status in adulthood and life expectancy (3, 4).

The excess of adipose tissue causes inflammation, oxidative stress, apoptosis and mitochondrial dysfunctions (5, 6). Therefore, obesity can lead to the onset of type-2-diabetes, liver steatosis, cardiovascular and neurodegenerative diseases which can develop early in life (7–12). Different studies have shown a susceptibility to skeletal fractures in obese children (13–23), suggesting that adipose tissue affects bone metabolism (24, 25). Therefore, the excess of fat could reduce the peak of bone mass reached during childhood and adolescence, with a potential osteoporotic risk in adulthood (1, 26). The bone fragility in obese population is due to an increase in fall injury risk, an unbalanced diet and a low physical activity. Despite the knowledge about the interplay between bone and adipose tissue is still growing, recent findings suggest that the influence of adipose tissue on bone can be fat-depot specific. In fact, the visceral fat storage may determine negative consequences on bone, while normal fat depots seem to affect positively the skeleton (27).

Furthermore, obesity can act in a different way on specific skeletal compartments (i.e., trabecular vs. cortical) and sites (i.e., weight-bearing vs. non-weight-bearing) (28). The aim of our review was to overview the cellular mechanisms by which obesity regulates bone remodeling leading to osteoporosis and fracture risk (**Figure 1**).

MESENCHYMAL STEM CELL FATE

The link between obesity and osteoporosis can be explained by the common stem cell precursor shared by osteoblasts and adipocytes (29). Two groups of crucial factors, CEBP-α, -β, - δ, and PPAR-α, -γ2 and -δ, need to be activated to attain a complete adipocytic differentiation of a mesenchymal stem cell. Otherwise, activation of other crucial factors (i.e., RUNX2, BMP2, TGF-β, and Osterix) are required to shift the differentiation of a mesenchymal cell into osteoblast (29). The differentiation "switches" characterizing stem cell fate are strictly linked to the stimuli present in the microenvironment. Furthermore, adipocytes cultured from marrow display the capability to revert to a proliferative status and thus differentiate in osteoblasts (30).

OBESITY AND BONE TURNOVER

The link between obesity and bone turnover has been evaluated both in humans and murine models, and the excess of fat mass is associated with reduced bone mineral density (BMD) (31–34). Obesity influences bone metabolism by different mechanisms. It stimulates pre-osteoblasts to differentiate toward adipocytes rather than osteoblasts, thus filling the cavities of bone marrow with adipocytes rather than trabecular bone with consequent bone fragility increase (35). Consistently, in obese adolescents and young adults, total and trabecular BMD and trabecular number have been inversely related with marrow adipose tissue (MAT) at the distal tibia, but not with lumbar spine MAT (36). Obesity can also enhance bone resorption by the increase of pro-inflammatory cytokine levels [Tumor Necrosis factor alpha (TNFα) and interleukin-6 (IL-6)], which promote osteoclast formation and activity by affecting RANKL/RANK/OPG pathway (37, 38). Bone marrow fat also may regulate osteoclastogenesis by producing RANKL (39). Obese subjects show low serum levels of adiponectin (40), an adipokine that inhibits osteoclast formation and activity (41). High leptin levels associated with reduced adiponectin may stimulate both macrophage accumulation into the adipose tissue (42) and adhesion of macrophages to endothelial cells (43). Several studies have demonstrated the impact of obesity on bone remodeling. Weiler et al. found that body fat percentage is correlated with suboptimal achievement of peak of bone mass in a cross-sectional study involving 60 girls (10–19 years old) (44). Goulding et al. showed that severe obesity is associated with higher risk of distal forearm fractures in boys aged 3–19 years (16).

Furthermore, Hsu et al. reported an increased risk for osteoporosis and non-spine fractures related with high percentage of body fat in a cross-sectional study involving 7,137 men, 2,248 postmenopausal women and 4,585 premenopausal women aged 25–64 years old (33). In leptin-deficient (ob/ob) obese mice, a reduction of femoral BMD, trabecular bone volume, and cortical thickness has been observed (45). Using a mouse model of diet-induced obesity, it has been found that mice fed with a high fat diet (HFD) had cancellous bone loss in the proximal tibia, together with a significant body weight increase (46). In the models, an increase of leptin and TRAP serum levels, a high RANKL/OPG ratio in cultured osteoblasts, and in the number of osteoclasts was observed (46–48). HFD determines an augment of bone marrow adiposity together with a reduction of BMD in several bone segments, and an increase of IL-6, TNFα, peroxisome proliferator-activated receptor γ (PPARγ) (49). Additionally, HFD decreases intestinal absorption of calcium, through the production of unabsorbable calcium soaps by free fatty acids (50–52).

HIGH LEVELS OF PRO-INFLAMMATORY CYTOKINES IN OBESITY

Obesity is characterized by a low-grade chronic inflammation. The discovery of high TNFα levels in the adipose tissue of obese mice offered the initial demonstration of a cross-talk between obesity and inflammation (27). Furthermore, the detection of leptin, hormone produced by adipocytes, further sustained the idea that adipose tissue is not only an energy storage but it is also a dynamic endocrine organ (53, 54). In fact, the chronic inflammatory status associated to obesity is characterized by abnormal cytokine production, and activation of signaling pathways of inflammation, with consequent development of obesity-related diseases (55). Adipose tissue is rich of macrophages, key source of inflammatory cytokines (56, 57). Obese subjects produce higher amounts of TNFα and pro-inflammatory cytokines (IL-6 and C-reactive protein) in adipose tissue than lean controls (58–60). Furthermore, the levels of adiponectin are lower in obese patients respect to controls (40). Obesity has also been related with inflammatory musculoskeletal diseases (i.e., osteoarthritis) (61). The low-grade inflammation which characterizes obesity may also influence endochondral longitudinal bone growth together with change in nutrients, minerals, and hormone metabolism (62). In obesity, the altered levels of numerous molecules inhibit osteoblastogenesis, as TNFα, DKK1, sclerostin, IL-6, serotonin, and advanced glycation end products (AGEs) [revised in Roy et al. (63)]. Interestingly, many pro-inflammatory cytokines involved in obesity are also crucial players of osteoclast formation and activation, and are known to be involved in bone disease (64–66), suggesting a link between obesity and bone turnover. In particular, in this review we focused the attention on MCP1, TRAIL, LIGHT, OPG, and TNFα (**Figure 2**).

MCP1

The chemokine Monocyte Chemotactic Protein-1 (MCP1) interacts with the receptor CCR2, its expression is ubiquitous and it is up-regulated by numerous stimuli. Firstly, MCP1 has

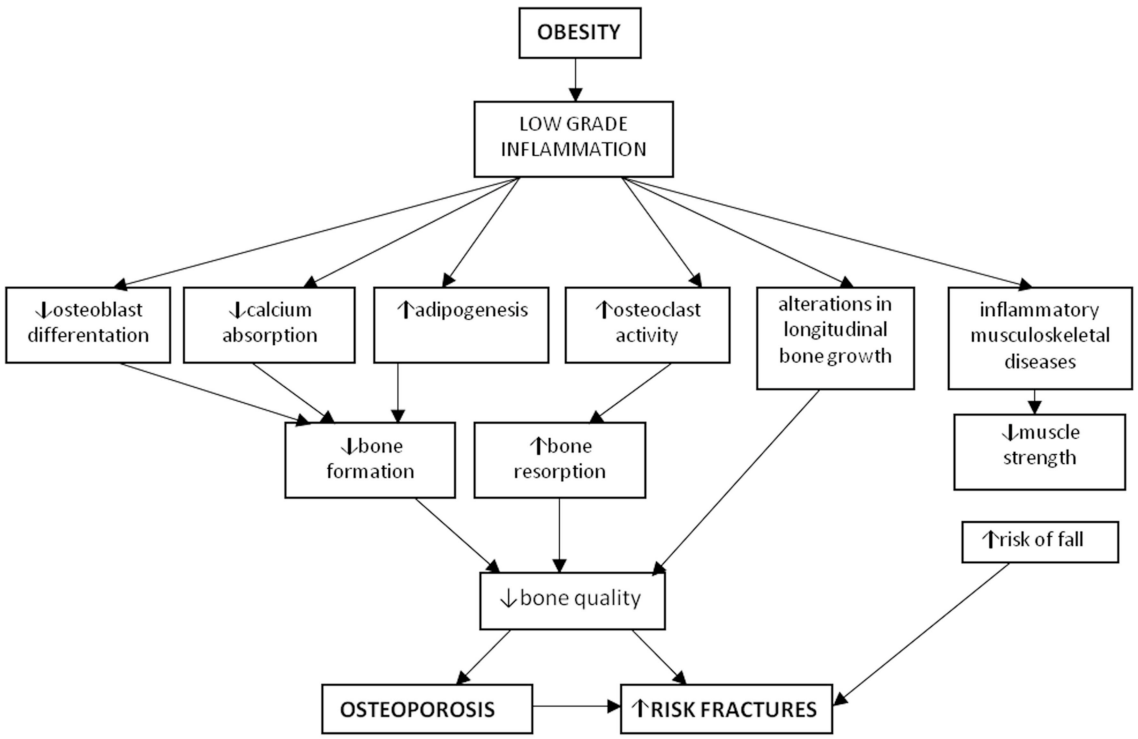

FIGURE 1 | Osteoporosis and obesity. Obesity is characterized by a low-grade chronic inflammation leading to increased osteoclastogenesis and adipogenesis, together with decreased osteoblastogenesis and muscle strength thus determining osteoporosis and increased risk of fractures.

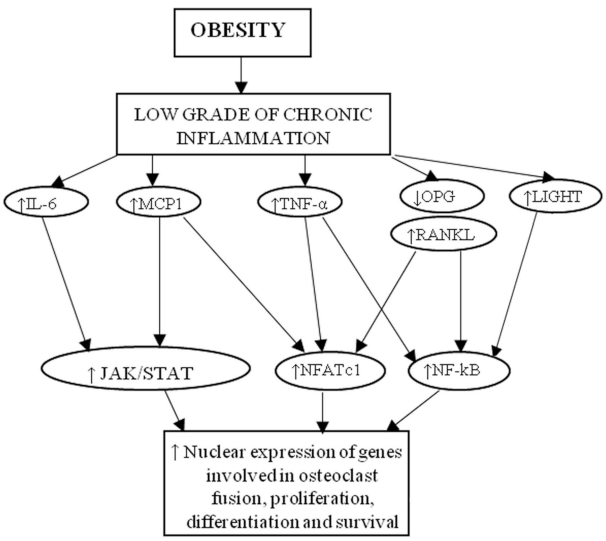

FIGURE 2 | Cytokines linking obesity to osteoporosis. MCP-1, RANKL, IL-6, TNFα, and LIGHT activate intracellular pathway that induce the nuclear expression of genes involved in osteoclast formation, activity and survival.

been purified from myelomonocytic cell line THP-1 (67), but it is expressed by numerous normal cells, such as endothelial cells (68–72), fibroblasts (73, 74), mononuclear cells (73, 75–82), mast cells (83), epithelial cells (84), keratinocytes (85), melanocytes (86), smooth muscle cells (68, 87, 88), mesothelial cells (89), adipocytes (90, 91), mesangial cells (92–96), chondrocytes (97), osteoblasts, astrocytes (98, 99), and microglia (99). In untreated normal cells, MCP1 levels are low, while tumor cell lines produce MCP1 constitutively (67, 100–104). The expression of MCP1 can also be downregulated by glucocorticoids (e.g., dexamethasone), cytokines (e.g., IL-13), and nitric oxide (79, 80, 97, 105–109). The expression of MCP1 and its receptor is higher in subcutaneous and visceral adipose tissues of obese patients than controls (90). Additionally, in omental fat of subjects with severe obesity, an increase of MCP1 expression together with an elevated macrophage infiltration was found (91). MCP1 levels are higher in obese adults (110) and children (111) compared to aged-matched controls. In obese patients MCP1 levels were augmented by fructose expenditure (112), reduced by low-glycemic index diet (113), and modulated by PTH (114). Moreover, 1α-25-dihydroxy-vitamin D decreases MCP1 production by adipocytes (115). CCR2-deficient mice fed with a HFD showed insulin resistance and reduced accumulation of visceral fat (78). Furthermore, MCP1 exerts a pro-angiogenic action (116), thus contributing to the expansion of adipose tissues.

MCP1 interaction with CCR2 on monocytes/macrophages leads to osteoclastogenesis via JAK/STAT and Ras/MAPK signaling pathways. However, RANKL co-treatment is mandatory to generate active bone resorptive osteoclasts (117).

TRAIL

TRAIL is a TNF superfamily member, initially known for its selective pro-apoptotic activity on cancer cell death (118). In humans, TRAIL binds to its death domain (DD)-containing receptors, DR5 and DR4, as well as decoy receptors osteoprotegerin (OPG), DcR1 and DcR2. In contrast to humans, mice express only one death receptor, mDR5, showing about 60% sequence homology to human DR4 and DR5 (118), and as decoy receptors, mDcR1, mDcR2 and OPG. TRAIL also affects non-cancer cell viability and activity, such as thymocytes (119), neural cells (120), hepatocytes (121), osteoclasts (122, 123), stem cells (124), valvular interstitial cells (125, 126), vascular smooth muscle cells (127), and osteoblasts (128–130). TRAIL pro-apoptotic signal in undifferentiated osteoblasts determines the activation of caspases (131). In lymphomonocyte cultures from donors TRAIL directly induces osteoclastogenesis in the absence of RANKL, whereas generates an inhibitory action when used simultaneously to RANKL (132). This last condition is associated with the inhibition of the phosphorylation of P38/MAPK (133). TRAIL controls homeostasis of the immune system in health and disease. Zoller et al. demonstrated that TRAIL determines an inflammatory status in pre-adipocytes and adipocytes (134). Funcke et al. reported that TRAIL induces the proliferation of human pre-adipocyte via ERK1/2 activation (135). Consistently, TRAIL takes part in the pathogenesis of metabolic diseases, i.e., obesity (121, 136). It has been demonstrated a positive association between TRAIL serum levels and body fat, serum lipid concentrations (137), waist-circumference and fat mass in healthy subjects (138). TRAIL serum levels were also positively correlated with higher energy balance (139), LDL and waist circumference, supporting a significant link between visceral adiposity and TRAIL (140). Even if these reports demonstrated high TRAIL levels in obesity, other authors failed to show such correlation (125, 138, 141, 142). Furthermore, a positive correlation between weight gain and TRAIL has been demonstrated in obese animal models. In detail, in adipose tissues of leptin-deficient mice the expression of TRAIL was significantly higher respect to wild-type mice (125). Furthermore, TRAIL levels decreased following an overnight fasting, and then rescued following feeding (125). Otherwise, results derived from TRAIL-treated wild-type and HFD fed, or TRAIL-deficient mice, support a defensive role for TRAIL in obesity. Bernardi et al. reported that in mice fed with a HFD, weekly injections of TRAIL generated a smaller fat mass compared to controls. TRAIL-mediated weight loss was linked to decreased transcript levels of TNFα, caspase-3, MCP1, augmented apoptosis in adipocytes, and decreased IL-6 serum levels (143). Consistently, $TRAIL^{-/-}ApoE^{-/-}$ mice fed with HFD showed high levels of IL-6 and MCP1, together with adipocyte hypertrophy and weight gain respect to $ApoE^{-/-}$ mice (144). Although Di Bartolo et al. (144) and Bernardi et al. (143) suggest that TRAIL may be beneficial to treat obesity, conversely Keuper et al. (125) found that TRAIL stimulated *in vitro* insulin resistance in adipocytes. Thus, considering the effect of TRAIL on adipose tissue together with its pro-osteoclastogenic and osteoblastic pro-apoptotic effects, further studies are needed to elucidate the role of TRAIL in obesity and related bone disease, overall in childhood.

LIGHT/TNFSF14

LIGHT (homologous to Lymphotoxins exhibiting Inducible expression and competing with herpes simplex virus Glycoprotein D for herpes virus entry mediator [HVEM], a receptor expressed by T-lymphocytes) is part of TNF superfamily (TNFSF14) and a crucial cytokine of the TNF-lymphotoxin network (145–148). It is expressed by natural-killer cells, activated T-cells, granulocytes, monocytes, and immature dendritic cells (149–151). LIGHT can bind two receptors, lymphotoxin-beta receptor (LTβR) and Herpes virus entry mediator (HVEM). LTβR is present on stromal and myeloid cells (146), HVEM on hematopoietic, epithelial and endothelial cells (151, 152). LIGHT-HVEM interaction determines a potent T-cell co-stimulatory effect (153–156). LIGHT-deficient mice showed an impaired activity of CD8+ T-cells and reduced trabecular bone (157–159). LIGHT has a pro-osteoclastogenic effect and we demonstrated that its high levels are linked to bone-disease patients (160–163). LIGHT triggers osteoclastogenesis through the phosphorylation of Akt, nuclear factor-κB (NFκB) and JNK pathways, it indirectly also inhibits osteoblastogenesis through immune cells (160). Moreover, LIGHT is involved in adipogenesis (164, 165). In detail, Tiller et al. reported that LIGHT inhibits adipose differentiation without affecting adipocyte metabolism (166). Otherwise, Kim et al. demonstrated that LIGHT has a key role in adipose tissue inflammatory responses through the increase of macrophages/T-cell infiltration and the release of inflammatory cytokines. In this system LIGHT effect is HVEM-mediated (164). HVEM deficiency displays a protective role against adipose tissue inflammation induced by ovariectomy (165). It has been reported that LIGHT signaling attenuates beige fat biogenesis (167). Human studies demonstrated high LIGHT levels in obese adults compared to controls (168). Interestingly, our preliminary results showed high LIGHT levels in obese children (169).

OPG/RANKL

Osteoprotegerin (OPG), soluble receptor for TRAIL and RANKL, is part of the TNF receptor superfamily. OPG, primarily known as bone resorption inhibitor, shows also anti-apoptotic and anti-inflammatory effects (170). OPG role has been evaluated in metabolic diseases (171). Indeed, low levels of OPG have been found in non-alcoholic fatty liver disease (NAFLD), important consequence of obesity (172, 173). Erol et al. found that obese children showed significantly lower OPG levels compared to the controls. A reduction of OPG levels in obese subjects has been described in some studies (174, 175), otherwise no relationship has been found between BMI and OPG in other reports (176, 177). Interestingly, Ugur-Altan et al. (174) found that the lowest OPG levels are associated with the highest HOMA-IR values, and serum OPG levels negatively correlated with fasting insulin, HOMA-IR,

and glucose. Otherwise, Suliburska et al. (178) showed that obese adolescents displayed higher OPG levels compared to controls, that positively correlated with insulin resistance. Studies on adults reported a potential correlation between metabolic syndrome, insulin resistance, NAFLD, and OPG levels (172–181). These studies demonstrated that in NAFLD the levels of OPG in sera could be utilized as a non-invasive liver damage indicator (174).

Obesity is also associated with increased secretion of RANKL by osteoblasts as well as elevated levels of the RANKL/OPG ratio (182). RANKL-RANK interaction leads to the activation of the transcription factors NFκB and AP-1, which in turn activates nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1). The latter translocates into the nucleus, thus inducing the expression of genes involved in osteoclast formation and activity.

TNFα

TNFα is a pro-inflammatory molecule involved in the regulation of inflammatory response, cell differentiation, proliferation, and apoptosis (183). TNFα binds two receptors, type 1 or 2, and activates NFkB and MAPK signaling (184), and is produced mainly by stromal–vascular cells and adipose tissue macrophages (185). TNFα is an inhibitor of osteoblastogenesis (186), adipogenesis and adipocyte differentiation, mainly by binding TNFR1 and activating the NFκB, ERK1/2 and JNK pathways (187). Another mechanism by which TNFα inhibits adipogenesis is the activation of Wnt/β-catenin pathway and inhibition of transcription factors, such as PPARγ and C/EBPs (188, 189). High levels of TNFα have been found in obese and diabetic subjects (58). The TNFα treatment in 3T3-L1 cells and rats induces insulin resistance (190), whereas the suppression of TNFα and receptor genes improves insulin sensitivity in ob/ob rodent model (191). Moreover, TNFα upregulates miR-155 and miR-27 by activating the NFκB pathway, thus inhibiting early adipogenic transcription factors, i.e., C/EBPβ and CREB (192, 193). TNFα also down-regulates miR-103 and miR-143, which accelerate adipogenesis (194).

TNFα shows a pro-osteoclastogenic effect that can be direct or indirect. In detail, for the direct mechanism TNFα binds to TNFR1 through NF-κB, JNK and p38 with consequent activation of NFATc1, which promotes the transcription of genes involved in osteoclast formation and activity. Moreover, TNFα indirectly affects osteoclast formation by promoting RANKL expression in bone marrow stromal cells (195). Otherwise, TNFα promoted osteoclastogenesis only in the presence of RANKL permissive levels (196).

CONCLUSIONS

Although childhood obesity has not been yet identified as a direct cause of osteoporosis, several cellular mechanisms linked to the accumulation of fat in the body can contribute to osteoporosis and bone fractures. Low grade chronic inflammation commonly exists in obese populations and the cytokines negatively affect bone health. Obesity positively regulates osteoclasts functioning by up-regulating the production of RANKL, LIGHT, TRAIL, TNFα, MCP1 and inhibiting osteoblastogenesis, thereby accelerating bone resorption. Future investigations on the relationship between cytokines and adipogenesis are expected to lead to the improvement of management strategies for osteoporosis associated to obesity.

AUTHOR CONTRIBUTIONS

MF and GB write the review. FC, MG, and SC critically revised the paper. GC, GD, and MC performed the bibliographic research and realized the figures. All the authors critically revised the paper.

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The Role of Osteocytes in Inflammatory Bone Loss

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Osteoimmunology investigations to-date have demonstrated the significant interactions between bone surface cells, osteoclasts and osteoblasts, and immune cells. However, there is a paucity of knowledge on osteocytes, cells embedded in the bone matrix, and their role in inflammation and inflammatory bone loss. Osteocytes communicate through various mechanisms; directly via dendritic processes and through secretion of proteins that can influence the formation and activity of osteoblasts and osteoclasts. Some osteocyte proteins (e.g., interleukin-6 and RANKL) also have roles within the immune system. In the context of mechanical loading/unloading, the regulatory role of osteocytes is well understood. More recent data on osteocytes in various inflammatory models suggest they may also aid in orchestrating inflammation-induced changes in bone turnover. In inflammatory conditions, osteocytes express multiple pro-inflammatory cytokines which are associated with increases in bone resorption and declines in bone formation. Cytokines are known to also influence cell population growth, maturation, and responsiveness via various signaling modalities, but how they influence osteocytes has not been greatly explored. Furthermore, osteocytes may play regulatory roles in orchestrating bone's response to immunological changes in inflammatory conditions. This review will address what is known about osteocyte biology in physiological conditions and in response to varying immunological conditions, as well as highlight key areas of interest for future investigations.

Keywords: osteocyte, cytokines, inflammation, tumor necorosis factor (TNF), sclerostin

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Bone continually adapts to its internal and external environment, undergoing formation and resorption to maintain bone mass. Bone loss is an imbalance of bone formation and resorption which occurs in many conditions including disuse, aging, and chronic inflammatory conditions such as inflammatory bowel disease (1), rheumatoid arthritis (2), psoriasis (3), and systemic lupus erythematosus (4). While comparatively less studied to disuse or age-related bone loss, inflammatory conditions are also associated with increased fracture incidence (4–8). Multiple other conditions with known inflammatory components are also associated with bone loss and increased bone fragility including type 2 diabetes (9), chronic kidney disease (10), spinal cord injury (11), and aging-related osteoporosis (12). While the role of immune factors and inflammation on various bone cells (osteoblasts, osteoclasts, stromal cells, marrow immune cells, bone precursor cells) have been extensively researched (13), the osteocyte response to inflammatory stimuli has been comparably less investigated. However, osteocytes have increasingly become appreciated as key regulators of both osteoclasts and osteoblasts, orchestrating changes in bone turnover.

INTERACTIONS OF THE IMMUNE SYSTEM AND BONE

The immune system interacting with bone physiology is being elucidated in the burgeoning field of osteoimmunology. For example, cytokines are signaling proteins released by various cell-types that modulate the direction of an immune response but can also influence the cellular phenotype of immune and parenchymal cells. In general, pro-inflammatory cytokines are grouped as Th_1, while anti-inflammatory cytokines as Th_2 (14). Notably, cytokines can interact with bone cells leading to increased bone resorption and decreased bone formation that, over time, leads to inflammatory bone loss (15). Key Th_1 cytokines include TNF-α and IL-1β, both stimulators of osteoclastogenesis (16–19). Additionally, these cytokines increase the production of RANKL, a key osteoclastogenesis regulator (20–22). TNF-α can also increase production of OPG, the decoy receptor for RANKL (23). IL-6 has equivocal roles in bone physiology, but, with TNF-α, IL-6 synergizes to stimulate osteoclasts and increase production of RANKL and OPG (21). IL-17 is also a potent stimulus for osteoclastogenesis (24) and sensitizes osteoclast pre-cursors to RANKL (25).

In addition to stimulating an increase in osteoclasts and bone resorption, Th_1 cytokines interact with osteoblast development and function. For example, TNF-α inhibits osteoblast genes and differentiation factors (e.g., *RUNX2*), and reduces bone collagen synthesis (20, 26–28). TNF-α also inhibits the anabolic effects of IGF-I on osteoblasts (29) and induces osteoblast apoptosis (20, 30). Interleukin-1β (IL-1β) has been shown to also suppress bone formation (31). In addition to osteoblasts and osteoclasts, other cell types residing in the bone environment such as bone marrow stromal cells and osteoblast/osteoclast-precursor cells can be influenced in their function by cytokines (32, 33). This illustrates the critical role of immunological factors, such as Th_1 cytokines, in bone biology.

Th_2 cytokines, such as IL-4 and IL-10, are less well understood in the context of bone physiology. In cell culture models, both IL-4 and IL-10 have been shown to inhibit osteoclasts and reduce RANKL production (34–37). Another cell culture study found both IL-4 and IL-13 increased OPG mRNA expression; thus, decreasing RANKL mediated osteoclastogenesis *in vitro* (38). Furthermore, IL-10 transgenic knockout mice have low bone mass and increased fragility which alludes to an influential role of IL-10 in regulating bone turnover (39). There exist many other cytokines within the Th_1 and Th_2 classes and other subsets (Th_9, Th_{17}, Th_{22}, T_{fh}) that have roles not yet delineated in bone physiology, highlighting areas of future research. Finally, the interaction of these cytokines with osteocytes has been minimally investigated.

OSTEOCYTE BIOLOGY

Osteocytes are the longest living bone cell, making up 90–95% of cells in bone tissue in contrast to osteoclasts and osteoblasts making up ∼5% (40). Osteocytes form when osteoblasts become buried in the mineral matrix of bone and develop distinct features. Residing within the lacuna of the mineralized bone matrix, osteocytes form dendritic processes that extend out from their cell bodies into spaces known as canaliculi. Through these dendritic processes, osteocytes form networks interfacing with other osteocytes, cells on bone surfaces, and the marrow (40). Through these communication networks, osteocytes sense the local and systemic environment within the bone.

Osteocytes also coordinate the actions of osteoblasts and osteoclasts via several mechanisms. First, osteocytes express and release proteins that signal to osteoblasts, osteoclasts, and other bone-residing cells to respond to environmental changes. Osteocytes express important factors for the maintenance of mineral homeostasis including SOST, Phex, DMP1, and FGF23 (41). Sclerostin, the protein encoded by the SOST gene, is an antagonist of the Wnt/β-catenin system, with increased sclerostin expression leading to a suppression of bone formation (42–44). Osteocytes also produce RANKL and OPG, critical regulators of osteoclastogenesis. While osteoblasts and other bone-residing immune cells also produce RANKL, it is now appreciated that RANKL synthesized by osteocytes is a significant source of RANKL driving osteoclast formation for bone remodeling (45–47). Additionally, osteocyte apoptosis signals to increase osteoclast activity driving targeted bone resorption (41, 48, 49). Elucidating osteocyte function in the context of osteoimmunology may provide further insight to the imbalance of resorption vs. formation seen in inflammation-induced bone loss.

THE ROLE OF OSTEOCYTES IN ADAPTATIONS TO MECHANICAL STRAINS

In the past few decades, the central role of osteocytes in response to mechanical strains has been explored and identified. Osteocytes sense mechanical strains through fluid flow shear stress through the lacuna-canalicular network and changes in interstitial hydrostatic pressure (50–52). Decreased mechanical strains also induce osteocyte apoptosis leading to decreased bone mass and strength (53, 54). Some preliminary evidence suggests that high mobility group box 1 (HMGB1), an alarmin (55), may be released during osteocyte apoptosis thereby triggering RANKL and other immune factors (56). It is unknown what other immune-related factors may be released during apoptosis and the signaling cascades that follow.

Mechanosensory signals also trigger osteocytes to release various proteins that impact bone turnover. RANKL and OPG are also known to be mechanosensitive (57) and mice lacking osteocyte RANKL are protected from disuse-induced bone loss (46). Furthermore, unloading-induced osteocyte apoptosis initiates an increase in osteocyte RANKL (54). Prevention of osteocyte apoptosis in animal models of unloading mitigates increases in osteocyte RANKL (54, 58). Disuse is also characterized by elevated osteocyte sclerostin in conjunction with decreased bone formation rate (59, 60). Other mechanosensitive osteocyte proteins include insulin-like growth factor-I (IGF-I) and IL-6 which both are upregulated with loading (60–63). The role of osteocytes in the mechanosensory capabilities

of bone highlight the important role these cells play in bone adaptations to the environment. Some osteocyte proteins known to have mechanosensory roles such as RANKL and IL-6 are also signaling molecules in the immune system and play key roles in inflammatory processes. This suggests that other cytokines may perhaps have similar dual roles responding under conditions of loading/unloading and under conditions of inflammation.

OSTEOCYTES AND CYTOKINES

Burgeoning research has shown cytokines directly impact osteocyte apoptosis and cause the release of cytokines that influence bone turnover. In cell culture models, osteocyte apoptosis can be induced by both TNF-α and IL-1β (64–66). A mouse infectious osteomyelitis model lead to increases in osteocyte apoptosis, as well as elevations in gene expression of TNF-α, IL-1β, IL-6, and IL-17 in the femur; the same treatment in TNF-α deficient mice resulted in fewer apoptotic osteocytes (67). We previously demonstrated in rats with inflammatory bowel disease decreased osteocyte density and increased apoptosis concurrent with elevated osteocyte TNF-α (68, 69). Therefore, one mechanism of increased bone resorption in inflammatory conditions is through the direct effect of pro-inflammatory cytokines on osteocyte apoptosis which, in turn, increases osteoclastic driven resorption.

Osteocytes also express pro-inflammatory cytokines. TNF-α is expressed in the MLO-Y4 osteocyte-like cell culture line (70, 71), as is IL-6 (61). Cultured human trabecular bone chips expressing osteocyte-specific genes also express TNF-α, IL-6, IL-1β, and IL-8 (72). Other cell culture osteocyte lines have increased expression of pro-inflammatory cytokines with exposure to monosodium urate crystals (73), Brucella abortus infection (74) and orthopedic implant materials (75). Immunohistochemical analysis of rat bones demonstrate elevated osteocyte TNF-α, IL-6, and IL-17 in various inflammatory conditions (68, 69, 76). Therefore, osteocytes express cytokines that can increase osteoclastogenesis and inhibit osteoblast formation or activity.

Osteocytes themselves respond to circulating pro-inflammatory cytokines influencing their cytokine expression. For example, exposing MLO-Y4 osteocytes in culture to IL-17 increases expression of TNF-α (71). In cultured human bone chips with osteocyte-enriched cells, gene expression of TNF-α, IL-1β, and IL-6 is elevated upon treatment combinations of TNF-α, IL-1β, and IL-6 (72). Based on the supporting data from these *in vitro* studies, cytokines influence osteocytes in a positive-feedback mechanism leading to even greater cytokine expression. This would suggest osteocytes may amplify an inflammatory bone state resulting in increased production of factors altering bone turnover and increasing bone loss.

Many cytokines also alter osteocyte signaling proteins. Osteocyte-to-osteoclast signaling is enhanced by multiple pro-inflammatory cytokines largely through RANKL signaling. Culture media from IL-1β-treated MLO-Y4 cells increased osteoclastogenesis *in vitro* (77). Furthermore, blocking IL-17A prevented the increase in osteocyte RANKL due to continuous parathyroid hormone exposure (78).

MLO-Y4 cells treated with IL-6/IL-6R and co-cultured with osteoclast precursors also results in increased osteoclastogenesis due to elevated RANKL (79). Furthermore, RANKL-positive osteocytes are elevated in animal models of inflammatory conditions including periodontitis (80–82), spinal cord injury (76), and inflammatory bowel disease (68, 69). Furthermore, in rat models of inflammatory bowel disease and spinal cord injury, RANKL-positive osteocytes were associated with increases in osteoclast surfaces (68, 76). In contrast to RANKL, OPG is less well understood in conditions of inflammation. Treatment of cultured human osteocytes- with a combination of IL-1β, TNF-α, and IL-6 upregulates OPG (72). In rodent inflammatory bowel disease and spinal cord injury models, OPG-positive osteocytes were elevated (68, 69, 76). Therefore, while the exact role of OPG is not known, it is known that inflammatory cytokines regulate osteoclastogenesis in part through osteocyte-mediated RANKL/OPG signaling.

Inflammatory signals also influence osteocyte proteins controlling bone formation. Wnt proteins are key mediators of osteoblastogenesis and govern the formation of the skeletal development. Both sclerostin and Dickkopf-related-1 (Dkk-1) inhibit the Wnt signaling pathway in bone. Dkk-1 is upregulated by TNF-α and blockade against Dkk-1 in transgenic mice with inflammatory arthritis prevents bone loss (83); however, osteocyte-specific deletion of Dkk1 did not protect against inflammatory arthritis-induced bone loss (84). Interestingly, Dkk1 expression was inhibited in osteoblast cell culture treated with IL-17A; whether this is also true in osteocytes is unknown (85). Sclerostin has also been shown to bind to LRP5/6 and inhibit Wnt signaling *in vitro* and *in vivo* (42, 86), and be transcriptionally activated by TNF-α (87). In human osteocyte-enriched cell cultures, serum from rheumatoid arthritis patients, IL-1β alone, and a combination of IL-1β, TNF-α, and IL-6 all increased SOST expression (72). Furthermore, osteocyte sclerostin is elevated in animal models of high fat diets with elevated serum and osteocyte TNF-α (87), inflammatory arthritis (83), periodontitis alveolar bone (81, 82), spinal cord injury (76), and inflammatory bowel disease (68, 69). Beyond direct effects of pro-inflammatory cytokines on osteoblasts and bone formation, the inflammation-induced elevation of inhibitors of bone formation contributes to a state of low bone formation.

While outside the scope of this review, additional osteocyte proteins involved in mineral homeostasis and metabolism are influenced by inflammatory signals. Fibroblast growth factor 23 (FGF23), a phosphate regulator synthesized by osteocytes, has increased expression in inflammatory conditions (72, 88, 89). IL-17A has been shown to decrease various genes of osteocyte proteins involved in mineral metabolism including Dmp1 and Phex (90). Therefore, it is clear that osteocytes respond to inflammatory signals through various mechanisms including increased expression of cytokines and altered expression of regulatory proteins.

MECHANOSENSING AND INFLAMMATORY SIGNALS

Crucial to osteocyte function is sensing and responding to bone interstitial fluid shear stress and mechanical strains. Furthermore, there is some overlap in signaling proteins between inflammation and mechanosensing (sclerostin, RANKL, OPG, etc.). What is not fully understood is if mechanosensing is tied with osteocyte inflammatory responses and vice versa. Utilizing pulsatile fluid flow in MLO-Y4 cells, TNF-α, and IL-1β treatment inhibits fluid flow-induced increases in calcium uptake and nitric oxide release indicating a potential blunting of the osteocyte response to mechanical strains (65). Another investigation found media from MLO-Y4 cells cultured with IL-1β induced osteoclastogenesis, while IL-1β-cultured cells that also underwent pulsatile fluid flow prevented osteoclastogenesis (77). Pulsatile fluid flow reduced MLO-Y4 expression of TNF-α- and IL-17A-induced increases in TNF-α and RANKL (71), inhibited TNF-α-induced osteocyte apoptosis (66), and increased IL-6 production in osteocyte cultured cells (61).

With aging, osteocytes develop morphological adaptations and changes in the lacunocanalicular system that may impair their mechanosensory function and ability to communicate (91, 92). In addition, osteocytes of aged mice also express a senescence-associated secretory-phenotype, expressing multiple pro-inflammatory cytokines including IL-17A, IL-1A, and IL-6, likely contributing to age-related bone loss (93). Prevention of the pro-inflammatory secretome of senescent cells in aged mice with a JAK inhibitor improved bone mass and strength (94). It has been hypothesized that exercise to increase mechanical strain on bone could improve the senescent phenotype in aging (95). It remains to been seen whether a lack of mechanical loading and a lack of adequate mechanosensory ability or pro-inflammatory senescent markers occurs first during aging in osteocytes.

To our knowledge, there are no investigations directly assessing the influence of mechanical loading on osteocyte-related proteins in animal models of inflammatory conditions to determine the mechanical loading effects on osteocyte inflammatory changes. However, it is possible that in inflammatory pathologies, such as spinal cord injury where both chronic systemic inflammation and disuse are present, the inflammatory status with the lack of mechanical loading on osteocytes could exacerbate bone loss. Further work needs to be done on the interaction of inflammation and mechanical strains in osteocytes.

FUTURE DIRECTIONS

With the accumulating knowledge of the role of osteocytes in inflammatory bone loss, future areas of interest may include therapeutically targeting osteocytes in inflammatory bone loss conditions. In inflammatory conditions, bone-specific treatments like bisphosphonates, anti-RANKL, and anti-sclerostin, all improve bone outcomes, but have no effect on inflammatory measures (96–98). Anti-inflammatory treatments, like anti-TNF, may improve bone mass (99), but potentially have negative side effects (100, 101). Therefore, viable treatments for inflammatory conditions are still needed. By directly impacting osteocytes via senolytic treatment or a JAK inhibitor in aged mice prevented the inflammatory senescent osteocyte phenotype (94). Additionally, in rodent models of inflammatory bowel disease, a soy protein diet and treatment with exogenous irisin decreased the inflammatory status of osteocytes and improved bone turnover (69, 102). Other anti-inflammatory treatments that improve bone in inflammatory conditions, like resolvin E1 (103), need to be examined for their impact on osteocytes. Furthermore, low-grade inflammation may be beneficial in some conditions like fracture healing (104). The role of other cytokines in bone physiology still needs to be elucidated as well as the relative contribution of osteocytic pro-inflammatory cytokines vs. those from other cell types in the marrow. Finally, the magnitude and duration of mechanical forces that could influence osteocyte-immune crosstalk has yet to be examined.

CONCLUSIONS

Osteocytes play a central role in orchestrating changes in bone turnover. In this review, we present literature that supports an overlap between classical osteocyte regulatory proteins with known mechanosensory functions (RANKL, OPG, sclerostin, etc.) and immune factors that directly impact osteocytes and their communication with osteoblasts and osteoclasts. Pro-inflammatory signals stimulate osteocyte apoptosis, increase osteocyte cytokine production, and alter osteocytic proteins controlling bone turnover. Therefore, osteocytes are key players in inflammatory bone loss. This indicates that osteocytes may be targets for preventing/treating inflammatory bone alterations.

AUTHOR CONTRIBUTIONS

CM and SN drafted and edited the manuscript and approved the final version.

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REVIEW
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Modulatory Effects of Plant Polyphenols on Bone Remodeling: A Prospective View From the Bench to Bedside

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During the past, a more comprehensive knowledge of mechanisms implicated in bone resorption processes has driven researchers to develop a compound library of many small molecules that specifically interfere with the genesis of osteoclast precursors cells. Natural compounds that suppress osteoclast commitment may have therapeutic value in treating pathologies associated with bone resorption like osteoporosis, rheumatoid arthritis, bone metastasis, and periodontal disease. The present review is focused on the current knowledge on the polyphenols derived from plants that could be efficacious in suppressing osteoclast differentiation and bone resorption.

Keywords: biomolecules, polyflavonoids, bone remodeling, titanium implant, coating surfaces

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INTRODUCTION

Bone Cells and Cell Guest Outsider

The strength and integrity of the human skeleton depends on a delicate balance between bone resorption and formation. Bone resorption represents a final step due to the interaction between cells that involve resorption of bone (osteoclasts, OC), and that synthetize bone matrix (osteoblasts, OB). Bone remodeling allows to adapt bone tissue to mechanical forces and to maintain phosphocalcic homeostasis through coordinated steps of formation and resorption (1, 2).

This equilibrium is close controlled by physical parameters (i.e., mechanical stimulations) and several polypeptides (hormones, cytokines) (3, 4). In addition, bone remodeling cycle maintains the integrity of the skeleton through the balanced action of its cell types.

Cells responsible for bone resorption include (1) bone-forming osteoblast, a cell that produces the organic bone extracellular matrix; (2) the bone resorbing osteoclast that dissolves bone mineral and enzymatically degrades extracellular matrix and inorganic components (5); (3) osteocyte, the maturative phase of osteoblast that acts as a mechanosensor and like an endocrine cell (6); (4) the bone lining cell, that probably may have a role in coupling bone resorption to bone formation (7).

Basically, osteoblasts and osteoclasts (and their precursors) are the central players of bone remodeling and each factor that affects these cells, in the end, affects all the process.

Numerous signaling pathways carry on the activities of osteoblast and osteoclast cells and these include nuclear factor kappa-light-chain-enhancer of activated B cells (RANkL) and Bone Morphogenetic Protein (BMP) (8). RANkL/RANK signaling axes regulates osteoclast recruitment and sustenance in normal bone modeling and remodeling and is adversely regulated by osteoprotegerin (OPG) (9).

An asymmetry between bone resorption and formation can result in bone diseases for instance in osteoporosis and in other bone resorbing pathologies.

Osteoporosis appears to be related with an impairment of bone mass through the reduction of osteogenesis and an enhancement of osteoclastic bone resorption, which results in bone fractures (10). At the present treatment options are limited, having issues with their efficacy, and long-term use. For example, the antiresorptive drugs are efficacious in reducing fracture risk (11, 12). Anti-resorptive agents, such as bisphosphonates and denosumab decrease bone loss through the inhibition of the differentiation and catabolic activity of osteoclasts in part by promoting osteoclast apoptosis. Since bone formation is coupled to bone resorption, inhibition of bone resorption is followed by a decrease in osteoblast activity that affects to adequately restore bone mass and bone quality due to increased microdamage collagen formation (13).

By considering papers on adverse effects of pharmacotherapy (estrogens, bisphosphonates) in the treatment of osteoporosis, there is an increasing request for complementary and alternative medicine.

Plant based therapies (i.e., Polyphenols) are safe options at the common treatment of osteoporosis thanks to diminished side effects and costs; in this context various plant components have been assessed for their potential role in the management of osteoporosis. Polyphenols are phytochemicals normally found in the plant kingdom, whose several biological effects have been reported to be protective against chronic diseases, including neurodegenerative and cardiovascular pathologies, cancer, and bone resorption pathologies (14). Depending on the number of phenol rings they comprehend and on the radicals bound to them, polyphenols can be split into different groups: phenolic acids, flavonoids, stilbenes, tannins, coumarins, and lignans. Recent papers based on molecular characteristics of dietary polyphenols have outlined the advantage in their prevention and management of bone resorption diseases (15). Polyphenols can safeguard bone integrity through the decreasing of oxidative stress, the reduction of inflammation by proinflammatory signaling and the modulation of osteoblastogenesis/osteoclastogenesis, by an osteoimmunological action.

The aim of this review paper was achieved on the development of osteoclast-targeting plant polyphenols that could be a great value for the prevention or treatment of bone resorption.

METHODS

Studies for this narrative review were screened using the online database PubMed. Keywords used to search for articles included bone, polyphenols, iicarin, green tea polyphenols, anthocyanins, phloridzin, oleuropein, resveratrol, quercitrin, plorydzin, dried plum, citrus flavonoid, osteoblasts, osteoclasts, bone remodeling, bone resorption, periodontal disease, osteoporosis, oral health. Articles were selected based on the two aspects of the review (cellular and molecular behavior—clinical relevant scenarios). To discuss the cellular characteristics of the bone, milieu articles were selected based on how well they delineate the subject matter. Experimental studies dealing with the impact of polyphenols activity on bone cellular signaling were included. For the remaining section, were included studies based on the broadest spectrum of clinical applications presented and on the strength of evidence provided. Since the topic is relative newborn in its clinical utilization, most of the selected studies were *in vitro*.

POLYPHENOL COMPOUNDS AND BONE METABOLISM

Polyphenols are natural molecules derived from plants isolated and characterized in the fruits, and vegetables. Bioactive phenolic compounds were known as health benefit components due to their antioxidant and anti-inflammatory features. Up to now, there are almost 8,000 recognized polyphenols of which ∼500 are biologically active. They can be obtained from plentiful natural sources and even from food/ beverage industrial products with low costs, thus using resources in a feasible way (16). They can preserve bone health by the action of five possible mechanisms: (i) by decreasing of bone loss through the activity of antioxidants, (ii) by diminishing bone loss through anti-inflammatory action, (iii) by improving osteoblastogenesis, (iv) by reducing osteoclastogenesis, and (v) through osteoimmunological activity. Many flavonoids substances have displayed positive impact on bone metabolism. For example, dietary soy isoflavones suppress bone depletion in rodents and post-menopausic women (17). One of the most studied bioactive compounds during recent years is iicarin, a heavy flavonoid component of Chinese tea, which has been reported to have intriguing osteogenic properties both *in vitro* and *in vivo* (18–21).

Recent researches have identified molecular targets in cell signaling pathways that influence bone structure. Some bioactive compounds seem to have bone anabolic action, which has important implications beyond the inhibition of bone resorption through suppressing osteoclast activation. Probably, the positive actions of these compounds are mainly due to their antioxidant characteristics, since they can act as scavengers of reactive oxygen species (ROS). Considering these properties polyphenols could affect bone metabolism through impairment of inflammatory mediators (22), such as cytokines, primarily involved in supporting osteoclast differentiation and resorption (23), consequently enabling to a decrease in bone resorption (**Figure 1**). Curcumin, for example, can enhance several aspects of bone health in subjects with osteoporosis by acting on different steps in the recruitment and activation of osteoclast cells, increasing mineral density and mechanical strength. Mechanisms that have been suggested involve the downregulation of NF-κB, RANKL, NO production of reactive oxygen species and inflammatory cytokine synthesis (24, 25).

Moreover, a lot of research has recently focused on the use of plum extract due to its high polyphenol content. Graef et al. (26) demonstrated that the polyphenolic extract from dried plums is responsible for reducing osteoclast differentiation and activity through a process of downregulation of osteoclast differentiation acting on the primary bone marrow–cells. In according with

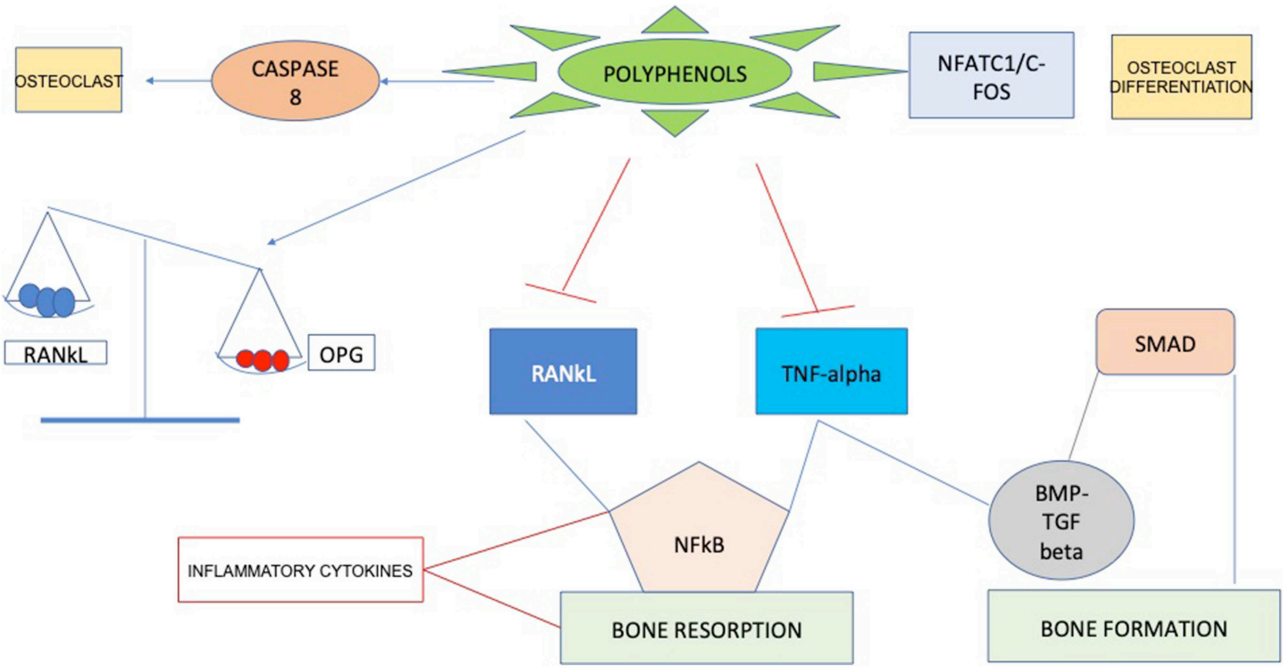


FIGURE 1 | Schematic pathway illustrating the action of polyphenols on bone remodeling.

other studies, this which was mediated through the suppression of Nfatc1, that is the regulator of osteoclast differentiation process (27).

CLINICAL ASPECTS

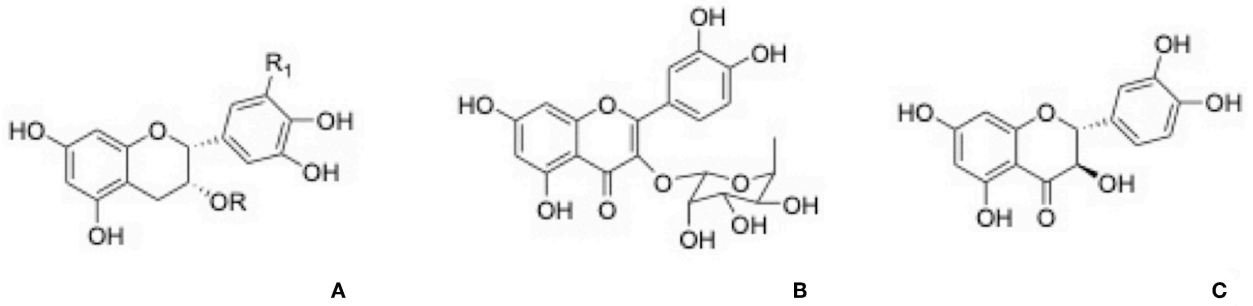
Osteoporosis

Strategies to avoid osteoporosis include decreasing bone loss induced by acute post-menopause estrogen deficiency. As for other chronic diseases, there is increasing indication that inflammation could be part of the etiology of osteoporosis (28). In particular, oxidative stress regulates the promotion of an enhancement in bone resorption, differentiation, and activity of osteoclast cells, so it has a substantial influence on the incidence of osteoporosis. In general, oxidative stress is described as the state of asymmetry between pro-oxidants and antioxidants. It is assumed that there is a correlation between reactive oxygen species (ROS) and its pathogenesis (29). Polyphenols are able of scavenging ROS and downregulating inflammatory cytokine, including osteoclast differentiation factors, including OPG, TNF-α, RANKL (30). For these reasons, antioxidant-rich foods may represent a possible approach for slowing down age-related bone mass reduction and enhancing bone remodeling. Dietary polyphenols have been related with bone health, which may be in part imputable to their antioxidant capability. For this reason, complementary and alternative medicine has generated interest as a natural chance linked to disease prevention. Natural antioxidant supplementation has been investigated to support the reduction of bone loss caused by oxidative stress (31).

Green tea, for instance, is well-known for its content in phenolic compounds including (–)-epigallocatechin 3-gallate (EGCG), (–)-epicatechin gallate (ECG), (–)-epicatechin (EC), and (–)-epigallocatechin (**Figure 2A**). Shen et al. (32, 33) reported that green tea polyphenols inlet reduces degeneration of bone microarchitecture in rats with chronic inflammation by the downregulation of TNF-α modulating cancellous and endocortical bone compartments. Between Green tea active components, epigallocatechin gallate has attracted attention for its potential health benefits and this molecule was largely investigated for its effect on osteoporosis. The bone formation was affected by EGCG through the enhancement of the alkaline phosphatase activity in osteoblastic cells and bone mineralization, related to the suppression of the osteoclast cells differentiation and of the formation of oxidative stress-induced calcium stone formation in rats (34).

Vester et al. (35) demonstrated that stimulation of primary human osteoblasts with low doses of green tea extracts during oxidative stress over 21 days improved mineralization and had beneficial effect on extra-cellular matrix production with higher gene expression of osteocalcin and collagen1α1 during osteoblasts differentiation.

Anthocyanins are water-soluble glycosides including a variety of compounds comprising pelargonidin, cyanidin, delphinidin, peonidin, petunidin, malvidin (**Figure 3**) (36). The health effects of anthocyanins are mainly due to their ability to impair oxidative stress (37). Anthocyanin chalcones and quinoidal bases with a double bond linked to the keto group are antioxidants in scavenging free radicals (38). Therefore, berry food and drinks could diminish the effects of age-related bone loss and decrease the risk of osteoporosis in humans (39).



COMPOUND	R	R1
ECGC	Gallic acid	OH
ECG	Gallic acid	H
EC	H	H
EGC	H	OH

FIGURE 2 | (A) Structures of main green tea polyphenols. **(B)** Quercitrin. **(C)** Taxifolin.

In other studies, Devareddy et al. reported that 5% blueberry treatment (w/w) for 100 days inhibited the loss of whole-body bone mineral density due to the ovariectomy in rats, and suppressed femoral mRNA levels of bone turnover biomarkers that were enhanced by estrogen deficiency (40).

Phloridzin, a dihydrochalcone contained in apples, apples juice, and purees, has been demonstrated to contribute to the antioxidant activity (**Figure 4A**). The activity of phloridzin on glucose uptake and diabetes has been explored and reported in many research works (41). The phlorizdin intake displayed a protection against ovariectomy-induced osteopenia under inflammatory patterns by the downregulation of inflammation markers and bone resorption (42).

In a murine model, Kim et al. found that phloretin (not glucoside form of phloridzin) inhibited receptor activator of NF-κB ligand (RANKL)-induced formation of multinucleated osteoclasts and diminished bone resorption area produced during the osteoclast differentiation process (43).

Oleuropein (**Figure 4B**), a secoiridoids esterified with hydroxytyrosol found in the olive tree and derivates, is well-known for its pharmacological activities including antioxidant, anti-inflammatory, anti-cancer, antimicrobial, and antiviral, cardioprotective against acute adriamycin cardiotoxicity, anti-ischemic, and hypolipidemic (44, 45). Puel et al. displayed the dose-dependent bone-sparing effect of oleuropein showing a reduction of bone loss and at the same time a downregulation of inflammatory biomarkers in ovariectomized rats (46).

Resveratrol (**Figure 4C**) is a stilbene phytoalexin found in many plant species and it is crucial polyphenol found in red wine. It has been considered that dietary resveratrol could affect as an antioxidant, promoting nitric oxide production, platelet aggregation and enhancing high-density lipoprotein cholesterol. In addition, resveratrol was shown to be a chemo preventive agent and exhibits anti-inflammatory, neuroprotective, and antiviral properties (28, 47).

The PGF2α-effect on OPG synthesis was suppresses by resveratrol through the inhibition of the MAP kinase pathways in osteoblasts (48).

Also, resveratrol downregulates bone morphogenic protein-4 (BMP-4) and stimulates VEGF synthesis through the inhibition of p70 S6 kinase in osteoblasts (49) via sirtuin-1 (SIRT1) activation (50).

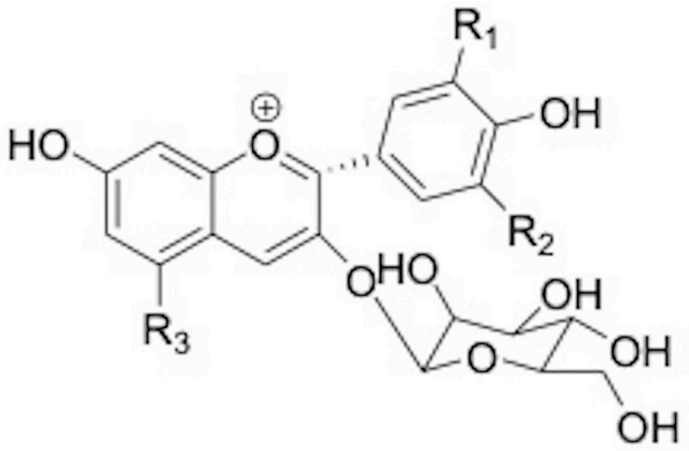
The effect of above described compounds on osteoblast and osteoclast activity, are summarized in **Table 1** (16, 19, 20, 26, 29, 35, 37, 39, 43–45, 50–52).

Periodontal Disease

Periodontitis is a devastating inflammatory disease of tooth-supporting tissues, which are composed by cementum, periodontal ligament, and alveolar bone, due to imbalance between oxidative stress and antioxidant activity.

Of note, inflammatory stimulation by periodontal bacteria increases the production of crevicular fluid and modulate the production of leukocytes, which, in order to deactivate periodontal pathogens, liberate single oxygen, and hypochlorous acid into the crevicular fluid (53).

The subsequent oxidative stress is counteracted by the antioxidant activity of ascorbate, albumin, and urate



ANTHOCYANIN	R_1	R_2	R_3
Delphinidin 3-glucoside	OH	OH	H
Delphinidin 3,5-diglucoside	OH	OH	glucoside
Cyanin 3-glucoside	OH	H	H
Cyanin 3,5-glucoside	OH	H	glucoside
Pelargonin 3-glucoside	H	H	H
Pelargonin 3,5-glucoside	H	H	glucoside

FIGURE 3 | Structures of main anthocyanins.

characteristic of the crevicular fluid and derived from plasma. When there is instability between oxidative stress and antioxidant activity, periodontal tissue demolition may appear. These remarks suggest that antioxidant rich diets might protect periodontal tissue from development and progression of pathologies, particularly in subjects exposed to environmental and dietary sources of oxidative stress (54, 55).

Based on clinical studies about the biochemical properties delivery of polyphenols formulations there is emerging line of natural therapies for periodontitis that may maximize and improved oral health among more populations. Actually, administration of tea polyphenols, by holding green or black tea, in the mouth for 2–5 min enhance the antioxidant capacity of saliva, and daily use of two fresh grapefruits for 2 weeks increases the phagocytic capability of the polymorphonuclear leucocytes inside the gingival crevicular fluid (56).

Following these suggestions polyphenoids may be employed in dentistry as a prophylaxis against bacterial infection and plaque formation, and as adjuvant therapies to aid post-operative healing of dental sockets and other traumatized tissues. Remarkable, in a recent study researchers find out that quercitrin, a glicoside of quercetin, and ramnose (**Figure 2B**) present in tartary buckwheat and in the bark of several oak, was found bioactive *in vitro* on human gingival fibroblasts, downregulating the gene expression of markers linked to inflammation and overexpressing genes that modulate different categories of collagen (57).

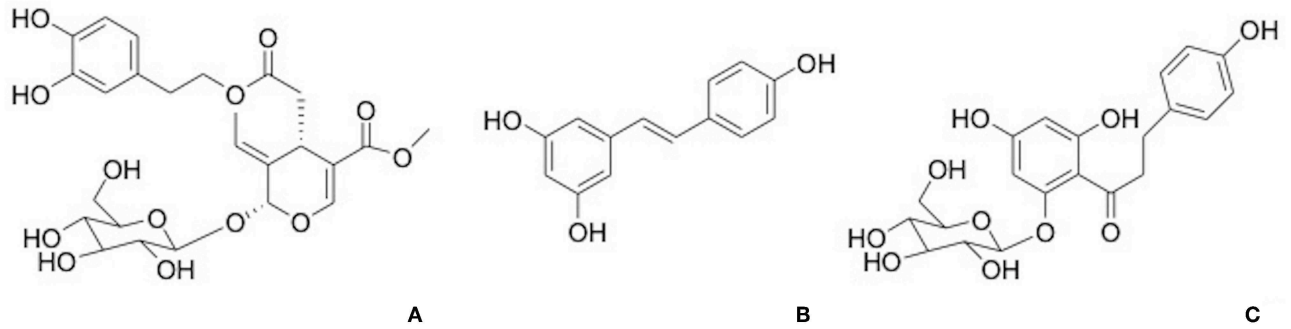


FIGURE 4 | Structures of phloridzin **(A)**, oleuropien **(B)**, and resveratrol **(C)**.

TABLE 1 | Effect of different polyphenols on osteoclasts and osteoblasts activity *in vitro*.

Compound	Effect on osteoclasts	Effect on osteoblasts
Iicarin	– (20)	+ (19)
Green tea polyphenols	– (16)	+ (35)
Anthocyanins	– (29)	+ (37)
Phloridzin	– (43)	+ (39)
Oleuropein	– (45)	+ (44)
Resveratrol	– (51)	+ (50)
Dried plum	– (26)	+ (52)

–, Reduced cellular activity.
+, Enhanced cellular activity.

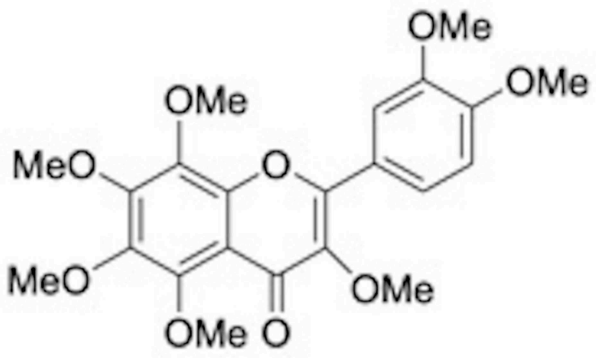


FIGURE 5 | Structure of 3,5,6,7,8,3,4-Heptamethoxyflavone.

Matsumoto et al. have reported that a citrus flavonoid 3,5,6,7,8,3′,4′-Heptamethoxyflavone (HMF) (**Figure 5**) found in Valencia oranges clearly suppressed the osteoclast formation and PGE2 production induced by IL-1. In mouse calvarial organ cultures, HMF attenuated the bone resorption elicited by LPS. HMF inhibits bone resorption induced by inflammation preserving bone mass and contributing to keep away from tooth loss (58).

POTENTIAL APPLICATIONS IN ORAL MEDICAL DEVICES

Due to recent progression in molecular biology and in the bone remodeling pathways many polyphenols are believed promising molecules able to act on osteoblast differentiation and on mineralization without the high financial impact of other osteoinductive factors.

These results are correlated with an exciting application of flavonoids like bioactive surfaces that could be an option to the use of growth factors i.e., in oral medical devices (59). Noteworthy, the use of growth factors, such as BMPs, in biomedical devices has lately gained several negative points regarding its stability, administration, bioactivity, and bioavailability. They are frequently very expensive and very limited in respect on the regulatory approval (60). By another side, flavonoid compounds are cheap, bioavailable, easy to find in daily food and, for this reason, probably easily be translated into clinical applications. For this rationale plant-derived products represent an innovative and interesting candidates for biomaterial applications, including dental research fields. Biomolecules can be believed as promising field relating to the improving of the bioactivity of biomaterial, and a safe substitute to pharmaceuticals, animal-derived compounds or growth factors. With the aim to attempt to obtain a faster osteointegration to speed up the overall treatment process, the use of biomimetic agents represents an interesting research area related with implant dentistry. For example, the functionalization with flavonoids conferred an osteopromotive characteristic to the Titanium surface as discussed by Cordoba et al. (61). In their study, researchers created a bioactive interface based on the covalent immobilization of flavonoids taxifolin (**Figure 2C**) and quercitrin on titanium surfaces.

POLYPHENOLS SAFETY AND TOXICITY

The consumption of food polyphenols such as flavonoids has been associated with a wide range of health benefits both disease preventive and therapeutic agents, including optimizing

cardiometabolic health, cancer prevention, and to a lesser extent positively impacting brain functioning in humans.

Diet-derived polyphenols are considered safe based on their long history of use as food or as traditional medicines, recently it is becoming the idea that these specialized metabolites could have toxic effects at pharmacological concentrations, and in several diseases or polypharmaceutical contexts (62).

The risk of toxic effects is increased using pharmacological doses in prevention/treatment and supplement situations and genetic polymorphisms or molecule–drug interactions that decrease/increase the bioavailability. Few reports on toxicity of these compounds are reported, therefore investigations of the side effects of polyphenols is necessary.

CONCLUSIONS

Bone pathologies majorly including osteoporosis osteoarthritis and oral diseases are becoming frequent with the growth in the aging population, a fact which is frightening if we consider the estimations that by 2030 20% of Europeans and 30% of the US population will be over the age of 65. There are only few standard therapies available for the treatment and prevention of these pathologies so a possible protective effect by natural molecules in multifactorial dysmetabolic disease could be a substitute to rise above side effects of conventional therapies.

Majority of the clinical researches underlined in this review, would attempt to demonstrate the relationship between polyphenols intake and bone turnover regulation. However, in spite of abundant *in vitro* and *in vivo* animal models, up to the present time, there is an absence of consistent human studies relating with polyphenol consumption. Although it was largely described the main role of antioxidative mechanism of polyphenols in prevention and treatment of bone remodeling diseases, it would be required more studies focused on the application of these compounds as therapeutic alternative in bone resorption diseases.

AUTHOR CONTRIBUTIONS

VN selected studies and wrote the manuscript and takes responsibility for the manuscript. ND, SN, FC, FB, and RD analyzed and compared literature data, and edited the manuscript. All authors approved the manuscript.

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The Autophagy in Osteoimmonology: Self-Eating, Maintenance, and Beyond

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It has been long realized that the immune and skeletal systems are closely linked. This crosstalk, also known as osteoimmunology, is a primary process required for bone health. For example, the immune system acts as a key regulator in osteoclasts-osteoblasts coupling to maintain the balanced bone remodeling. Osteoimmunology is achieved through many cellular and molecular processes, among which autophagy has recently been found to play an indispensable role. Autophagy is a highly conserved process in eukaryotic cells, by which the cytoplasm components such as dysfunctional organelles are degraded through lysosomes and then returned to the cytosol for reuse. Autophagy is present in all cells at basal levels to maintain homeostasis and to promote cell survival in response to cellular stress conditions such as nutrition deprivation and hypoxia. Autophagy is a required process in immune cell activation/polarization and osteoclast differentiation, which protecting cells from oxidative stress. The essential of autophagy in osteogenesis is its involvement in osteoblast differentiation and mineralization, especially the role of autophagosome in extracellular calcium transportation. The modulatory feature of autophagy in both immune and skeleton systems suggests its crucial roles in osteoimmunology. Furthermore, autophagy also participates in the maintenance of bone marrow hematopoietic stem cell niche. The focus of this review is to highlight the role of autophagy in the immune-skeleton interactions and the effects on bone physiology, as well as the future application in translational research.

Keywords: osteoimmunology, bone remodeling, autophagy, immunomodulation, stem cell

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INTRODUCTION

The skeletal bone is a dynamic tissue with a life-long continuous renovation termed bone remodeling (1). This remodeling consists of bone resorption and formation and plays a fundamental role in the maintenance of bone homeostasis (2). Bone remodeling is kept in balance under physiological conditions, as the amount of bone resorption equals to that of formation (3). This balance is achieved via sophisticated regulations originated from the immune system (4). The link between the immune and skeleton systems has been identified for almost fifty years and termed as "osteoimmunology" (5). Further research into osteoimmunology has recognized the complex mutual regulations between immune cells and bone cells, that at one level, immune response

determines the balance of bone remodeling, whereas on another level, bone cells mediate the polarization and function of immune cells (3, 4, 6). This interaction consists of multiple factors such as cytokines, receptors, signaling pathways (4); and it has been recently indicated that autophagy plays elementary roles in both immune (7) and skeletal (8) systems. Autophagy is defined as the delivery of cytoplasmic materials to the lysosome in animal cells or the vacuole in plant and yeast cells (9). Especially in eukaryotic cells, autophagy plays a key role in homeostasis maintenance (10, 11). Moreover, autophagy promotes cell survival in response to stress conditions such as nutrition deprivation and hypoxia (12). Autophagy is required in the differentiation of osteoclast and osteoblast (13, 14); meanwhile, it participates in the immune cell polarization/function and therefore regulates immune response (7, 15), suggesting a complex and rather intriguing role in osteoimmunology. This review highlights the effects of autophagy in the immune-skeleton interactions and proposes the regulation of autophagy for future application in bone regeneration.

AUTOPHAGY MECHANISM

There are mainly three types of autophagy, known as macroautophagy, microautophagy, and chaperone-mediated autophagy (9). The current review will focus on macroautophagy (hereafter referred to as autophagy), a highly conserved "self-eating" lysosomal degradation pathway in eukaryotic cells to clear intracellular waste (10, 11). Autophagy initiates with the sequestration of cytoplasmic organelles within double-membrane vesicles known as autophagosomes, which then fuse with lysosomes to form autolysosomes to degrade or recycle the autoghagic contents, such as damaged organelles, intracellular pathogens, glycogens, lipids, and nucleotides proteins (9, 16). Concomitantly, the cytosolic form of microtubule-associated protein 1A/1B-light chain 3 (LC3-I) is converted to form LC3-phosphatidylethanolamine conjugate (LC3-II), which is attached to the autophagosome membrane and then degraded (17, 18). This conversion from LC3-I to LC3-II is identified as one of the autophagy hallmarks. On the other hand, microautophagy is defined as the direct engulfment of small cytoplasmic portions by inward deformation of the lysosomal or late endosomal membrane (9, 19). Chaperone-mediated autophagy does not require membrane reorganization: the substrate proteins containing a KFERQ-like pentapeptide are selectively targeted by cytosolic heat shock cognate 70 (Hsc70) protein, then directly translocated into the lysosomal lumen (9, 20).

Autophagy is maintained at basal levels in all cell types, which plays a "quality control" role to maintain cellular homeostasis (11). On the other hand, autophagy is induced in response to stress conditions such as nutrient deprivation, oxidative stress, hypoxia and infection, which is one of the main strategies to promote cell survival (12, 21). Autophagy facilitates the "recycle" of cellular components and therefore provides energy for cells under starvation (22). Another important function of autophagy is to scavenge malfunctioning/damaged proteins and organelles (22). For example, autophagy-mediated clearance of damaged mitochondria, also termed as "mitophagy," inhibits reactive oxygen species (ROS) accumulation and thereby protecting cell from oxidative stress and apoptosis (15, 23). This has been identified as a required process during bone cell differentiation and immune cell polarization, making a central role in osteoimmunology.

AUTOPHAGY IN THE SKELETAL SYSTEM

General Bone Biology

As the main constituent of the vertebrate skeletal system, bone serves as supporters and protectors of organs in the body (24). Bone consists of cellular components such as osteoclast, osteoblast, and osteocyte, as well as collagen, osteoid and inorganic mineral deposits (25). Osteoclast and osteoblast are the major players in bone remodeling (26). Originated from the hematopoietic stem cells (HSCs), osteoclast is considered as the major cell type responsible for bone resorption (25). The macrophage–monocyte lineage-derived osteoclast precursors fuse with each other to form a giant, multinucleated cell—the osteoclast (27). On the other hand, the mesenchymal stem cells (MSCs)-derived osteoblast is the major bone formation cell (25), which builds the osteoid matrix and eventually differentiate into osteocyte, the most abundant cell type in bone (28). The fact that osteoclastogenesis is dependent on osteoblast-derived receptor activator of nuclear factor factor-kappa B ligand (RANKL) (29, 30), suggests "coupling" between osteoclast and osteoblast, therefore linking osteoclastogenesis to osteogenesis (31). RANKL binds with receptor activator of nuclear factor factor-kappa B (RANK) on osteoclast-precursors, therefore initiating the differentiation of osteoclast. On the other hand, osteoblast produces osteoprotegerin (OPG), a decoy receptor of RANKL, to interrupt osteoclastogenesis (32). Hence, the balance between RANKL and OPG determines the outcome of bone remodeling (33). Furthermore, osteoblast secrets other factors to regulate osteoclastogenesis and osteogenesis in a paracrine or endocrine manner (34). For example, osteoblast is one of the major source of macrophage colony-stimulating factor (M-CSF) —a major factor for osteoclast differentiation— in the bone microenvironment (31, 35–37). Osteoblast-derived semaphorin 3A (Sema3A) and Wnt16 have been found to reduce osteoclastogenesis via interrupting the RANKL-RANK signaling (38, 39), while osteoblast-originated Wnt5 induces osteoclast differentiation by enhancing RANK expression in osteoclast-precursors (40, 41). Osteoblast also produces factors such as vascular endothelial growth factor A (VEGF-A) to induce osteogenesis (34, 42). Beside osteoblast, osteocyte is considered as another critical producer of RANKL, which also produces sclerostin (SOST) to reduce osteogenesis, therefore acting as the orchestrator of bone remodeling (43–46).

Autophagy in the Differentiation/Function of Osteoclast, Osteoblast, and Osteocyte

Recent studies have identified the importance of autophagy in osteoclast differentiation and function. Autophagy activation has been reported during the osteoclastogenic process. During the RANKL-induced osteoclast differentiation, the autophagic

protein levels (such as autophagy related (ATG) 5/7/12) and the LC3-II/LC3-I ratio have been reported to increase in accompany with degradation of p62 (also known as SQSTM1/sequestome1) (47). This degradation plays an essential role in the generation of filamentous actin (F-actin) ring, a key feature of osteoclatogenesis (47, 48). Mutant p62 results in abnormal osteoclasts with increased size, number, multinuclearity, and activity (49). The autophagic proteins Atg5/7/4B and LC3 have also been reported to play decisive roles in regulating the osteoclast-ruffled border (RB) generation and the lysosomal secretion (**Figure 1**), thereby determining osteoclast function *in vitro* and *in vivo* (50). Especially, in rheumatoid arthritis (RA) patients, autophagy is found activated by the pro-inflammatory cytokine tumor necrosis factor α (TNF- α) in osteoclasts, which results in induced osteoclastogenesis and bone resorption *in vitro* and *in vivo*, suggesting a central role of autophagy in the pathogenesis of inflammatory bone loss (51).

Autophagy has long been considered as a necessary part in cell differentiation (22). In a recent study, induced autophagy has been found during osteoblast differentiation and mineralization *in vitro*; autophagosomes act as cargos to transport the intracellular mineral crystal-like structures to facilitate the extracellular mineralization (52). Autophagy inhibition can result in impaired mineralization *in vitro* and decreased bone mass/volume *in vivo* (**Table 1**), which is usually followed by induced oxidative stress and RANKL production (52). These results suggest the fundamental roles of autophagy during osteoblast differentiation and mineralization, which serves as mineralization vehicles, protects osteoblast from increased oxidative stress and moreover, reduces osteoblast-derived RANKL production and thereby inhibits osteoclastogenesis during bone formation (52). This is in accordance with the phenomenon that autophagy deficiency/inhibition in osteoblast leads to an osteoporotic-like phenotype with induced osteoclast differentiation (62). Another study found out that bone morphogenetic protein 2 (BMP2), an osteoinductive agent for clinical-use, leaded to increased protein levels of beclin-1 and lysosome-associated membrane protein 2 (LAMP2) (63), both of which are known as autophagy markers (64, 65). On the other hand, autophagy inhibition has been found to affect the differentiation and immunoregulatory capacities of MSCs in ovariectomy-induced osteoporosis model mice (66). In the field of bone tissue-engineering and biomaterials development, the autophagy-inductive bioactive silica nanoparticles have been found to promote osteogenesis (67), suggesting that autophagy could be a potential therapeutic target for bone repair (**Figure 1**).

Autophagy is particularly critical for terminally-differentiated cells such as neurons and osteocytes, which provides these cells with "intracellular refreshment" to enable the cellular homeostasis and function during their long-life periods (13, 22). Osteocyte is terminally-differentiated osteoblast embedded in bone matrix. The long dendritic processes of osteocyte facilitate the communication within osteocytes as well as the connection with bone surface, making osteocyte capable of sensing mechanical or bio-chemical stimulus from the microenvironment (14). Osteocyte in response to the stimulation therefore acts as director in bone remodeling such as producing RANKL and sclerostin (43, 44, 46, 68). Living in a hypoxic and potentially nutrient poor environment, osteocyte has been reported to keep higher levels of autophagy than the bone surface osteoblast *in vivo* (13, 69). Accordingly, it is found that the terminally-differentiated osteocytes show induced autophagy, as compared with the pre-osteocytes *in vitro* (69). Genetic autophagy suppression (selective *Atg7*-deletion) in murine osteocytes results in skeletal changes in young adult mice (**Table 1**), such as decreased bone mass and volume, reduced osteoclastogenesis and impaired bone formation, as well as induced ROS in the bone marrow; a phenotype similar to the aging bone (70). Another study has also found that autophagy-activation/inhibition is involved in glucocorticoids-related bone loss (71). All these studies suggest that autophagy at a certain level maintains the homeostasis and function osteocyte to facilitate the physiological balance of bone remodeling (**Figure 1**).

AUTOPHAGY IN OSTEOIMMUNOLOGY

Regulations of the Immune System on Bone Remodeling

The crosstalk between skeletal and immune systems was initially identified by the finding that immune cells-originated interleukin-1 (IL-1) could induce osteoclastogenesis (5). Since then, more evidence has revealed the regulatory role of immune system on bone remodeling (4). T-helper cells, derived from the adaptive immune system, have been found to play a critical role in inflammatory bone loss in arthritis via producing RANKL (72). Besides RANKL, the type 17 helper T (Th17) cell-originated IL-17 has long been identified as a key pre-inflammatory cytokine that promotes osteoclastogenesis (73, 74); while the Th2 cell-originated IL-4 and IL-10 are considered as inhibitors for osteoclasts (75–78). On the other hand, the immune-suppressive regulatory T (Treg) cells (79), inhibit osteoclastogenesis either in a direct cell-to-cell contact-dependent manner (cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on Treg cells binding with CD80 and CD86 on osteoclast precursors), or via production of IL-4, IL-10, and TGF-β (80, 81). Cells from the innate immune system, such as macrophages, not only serve as osteoclast precursors (82), but also participate in the osteoclastogenesis regulation. Macrophages are a population of cells with three subsets: (1) non-activated M0 macrophage; (2) pro-inflammatory M1 macrophage, which is classically activated by microbe-derived lipopolysaccharide (LPS) or Th1 cells-derived IFNγ; and (3) anti-inflammatory M2 macrophage, which is alternatively activated by Th2 cells-derived IL-4 or IL-13 (83–86). M1 macrophage induces osteoclastogenesis by producing cytokines such as IL-1α/β (87, 88), IL-6 (89–91), TNF-α (92–95); while M2 macrophage reduces osteoclast differentiation via secretion of IL-10 and TGF-β (85, 96, 97).

The regulation of immune system on osteogenesis is not so clear-cut. There are conflicting results regarding the positive/negative effects of inflammatory/anti-inflammatory cytokines on osteoblast differentiation, known as IL-1 (98–101), IL-17 (102–104), TNF-α (100, 101, 105, 106) and IFNγ (107, 108).

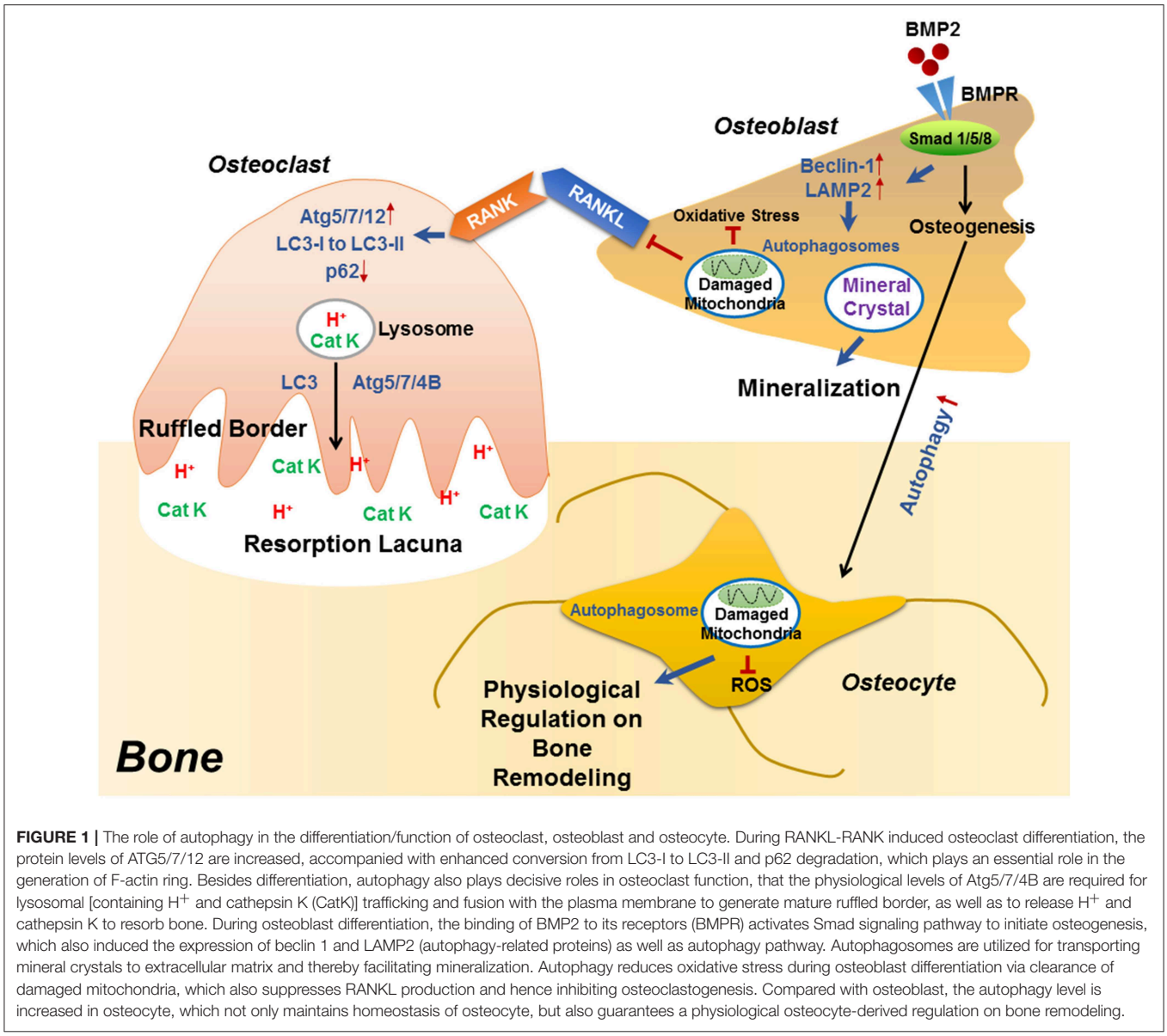

FIGURE 1 | The role of autophagy in the differentiation/function of osteoclast, osteoblast and osteocyte. During RANKL-RANK induced osteoclast differentiation, the protein levels of ATG5/7/12 are increased, accompanied with enhanced conversion from LC3-I to LC3-II and p62 degradation, which plays an essential role in the generation of F-actin ring. Besides differentiation, autophagy also plays decisive roles in osteoclast function, that the physiological levels of Atg5/7/4B are required for lysosomal [containing H^+ and cathepsin K (CatK)] trafficking and fusion with the plasma membrane to generate mature ruffled border, as well as to release H^+ and cathepsin K to resorb bone. During osteoblast differentiation, the binding of BMP2 to its receptors (BMPR) activates Smad signaling pathway to initiate osteogenesis, which also induced the expression of beclin 1 and LAMP2 (autophagy-related proteins) as well as autophagy pathway. Autophagosomes are utilized for transporting mineral crystals to extracellular matrix and thereby facilitating mineralization. Autophagy reduces oxidative stress during osteoblast differentiation via clearance of damaged mitochondria, which also suppresses RANKL production and hence inhibiting osteoclastogenesis. Compared with osteoblast, the autophagy level is increased in osteocyte, which not only maintains homeostasis of osteocyte, but also guarantees a physiological osteocyte-derived regulation on bone remodeling.

An interesting finding is that the pro-inflammatory cytokine IL-6 could induce osteogenesis through the oncostatin M (OSM)-STAT3 signaling pathway, suggests the inflammatory response, at a certain level, could initiate osteoblast differentiation (109–115). This is in accordance with the phenomenon that early stage inflammation with macrophage infiltration is regarded as indispensable in bone fracture healing (116). However, this inflammation will be gradually quenched, as the M1 to M2 macrophages conversion happens along with bone repair. This conversion has been found to improve bone formation (116, 117). The M2 macrophage-derived factors, such as BMP2 and TGF-β, are identified to promote osteoblast differentiation and functions, as well as to enhance mineralization (118, 119). These findings suggest that the transformation from the M1 macrophage-mediated inflammatory microenvironment, to the M2 macrophage-mediated regenerative one, should be a required part in bone formation; modulation of M2 polarization should be considered as a potential therapeutic approach for bone regeneration.

The Role of Autophagy-Mediated Immunomodulation in Bone Remodeling

Autophagy is now identified as a multifunctional pathway in immunity such as lymphocyte differentiation (22), pathogen elimination (120), antigen presentation and inflammation regulation (7, 15). T cell-specific ATG genes deletion (such as *Atg7* or *Atg5*) results in decreased T lymphocyte counts, mitochondria accumulation, and induced apoptosis in mature T cells (121, 122). This is due to the critical role of autophagy-mediated mitochondria clearance in the development of thymocytes into circulating mature T cells (123). Beside differentiation, autophagy also provides barriers against invading

TABLE 1 | Effects of *Atg* gene-knockout on osteoimmunology *in vivo*.

Cell type	*Atg 5*-/-	*Atg 7*-/-	Possible effects on bone resorption	Possible effects on bone formation
Osteoclast	Differentiation↓ (50)	Impaired cathepsin K secretion (53)	Downregulation	Unknown
Osteoblast	Differentiation↓ RANKL production↑ (52)	Differentiation↓ (54)	Upregulation	Downregulation
Osteocyte	Unknown	Aging-like phenotype (54)	Downregulation	Downregulation
M1 macrophage	Polarization↑ (55, 56)	Polarization↑ Aging-like phenotype (57)	Upregulation	Downregulation
M2 macrophage	Polarization↓ (55, 56, 58)	Polarization↓ Aging-like phenotype (57)	Upregulation	Downregulation
Th17 cell	Polarization↑ (59)	IL-17 production↑ (60)	Upregulation	Upregulation
Treg cell	Polarization↓ (61)	Polarization↓ (61) Function↓ (60)	Upregulation	Unknown

pathogens, that autophagosome and autophagolysosome are utilized for selective-detection and elimination of intracellular pathogens (120, 124–127). Especially, the immunomodulatory roles of autophagy have been found in both innate and adaptive immune responses, making autophagy a potential key regulator in osteoimmunology.

The importance of autophagy has long been addressed in macrophage polarization and inflammatory response. Although autophagy is induced by toll-like receptor 4 (TLR4) signaling during M1 macrophage polarization (128), further research has suggested that autophagy plays an immunosuppressive role in macrophage inflammatory response (129). *Atg5*- or *Atg16L1*-deficiency on macrophage is found to direct M2 macrophage to polarize toward a M1-like phenotype with induced secretion of pro-inflammatory cytokines (55, 58). Mice with *Atg5*-knockout macrophages showed induced systemic inflammation (56). Primary bone marrow-derived macrophages (BMDMs) obtained from this mice type exhibited abnormal polarization, that the M1 polarization was increased while the M2 polarization was impaired (**Table 1**), which further indicating that autophagy-deficiency would induce inflammatory response in macrophages (56). In mice with *Atg 7* gene deletion in the hematopoietic system (vav-*Atg7*-/- mice), monocytes failed to differentiated into macrophages under M-CSF stimulation (130). Moreover, macrophages obtained from vav-*Atg7*–/– mice were found to have a phenotype similar to aged macrophages, which showed reduced abilities of phagocytosis and nitrite burst, while induced inflammatory response (**Table 1**); suggesting that autophagy maintained at a certain level would correct the abnormities in immune system to prevent aging associated chronical inflammation (57). It has been demonstrated that autophagy inhibition (either by *Atg* gene deletion or pharmacological intervention) results in induced IL-1β secretion of macrophage, suggesting autophagy limits the inflammatory response of macrophage (15, 131, 132). Further studies have found that inflammatory stimulus causes mitochondrial damage, and then consequently results in induced ROS release/apoptosis in macrophage. ROS interact with NF-κB signaling pathway and then activate the NLRP3 inflammasome to trigger the secretion of IL-1β and IL-18, therefore eventually initiating the inflammatory cascade (132–134). During this process, autophagy scavenges the damaged mitochondria through a collaboration between p62 and LC3, that p62 selectively recognizes damaged mitochondria by its UBA domain, which, collectively, combines with LC3 and ensures the lysosomal degradation of damaged mitochondria, thereby interrupts the inflammatory cascade (134–136). As mentioned before, the macrophage inflammation has been demonstrated to induce osteoclastogenesis and bone loss, while the conversion from pro-inflammatory M1 toward anti-inflammatory M2 phenotypes has been suggested to improve bone repair (116, 117). Therefore, this autophagy-mediated regulation on macrophage response should be considered as beneficial for bone regeneration. The nanomaterials-derived autophagy induction has been found to potentially introduce a polarization toward M2 macrophage and thereby improve osteogenesis (137), which further suggests that autophagy could be a potential immunomodulation target in regenerative medicine, especially for therapies against disorders with inflammatory bone loss, such as arthritis (138), periodontitis (139), periapical lesions (140).

Besides its role in the innate immunity, autophagy also acts as a key regulator in the adaptive immune response, such as T cell activation and polarization. Autophagy promotes major histocompatibility complex (MHC) class II-mediated antigen presentation via inducing the fusion of antigens to LC3 in $CD4^+$ T cells (141), which facilitates the elimination of autoreactive $CD4^+$ T cells (123). On the other hand, although autophagy-dependent antigen presentation is required in in antimicrobial response of dendritic cells (DCs) (141, 142), the autophagy-deficient DCs show hyper-stable interactions with T cells and thereby enhance T cell activation, suggesting the modulatory role of autophagy to prevent excessive T cell response (143). Consistent with this idea, a study has found that graphene quantum dots (GQDs) induce the tolerogenic phenotype of DCs in an autophagy-dependent manner, which show reduced capacity in antigen-presenting and thereby reduce T cell inflammatory response via introducing the polarization of Th1 and Th17 cells toward Th2 and Treg cells, respectively (144). Autophagy also directly inhibits nuclear factor-κB (NF-κB) activation in in antigen-activated T cells and thereby suppresses

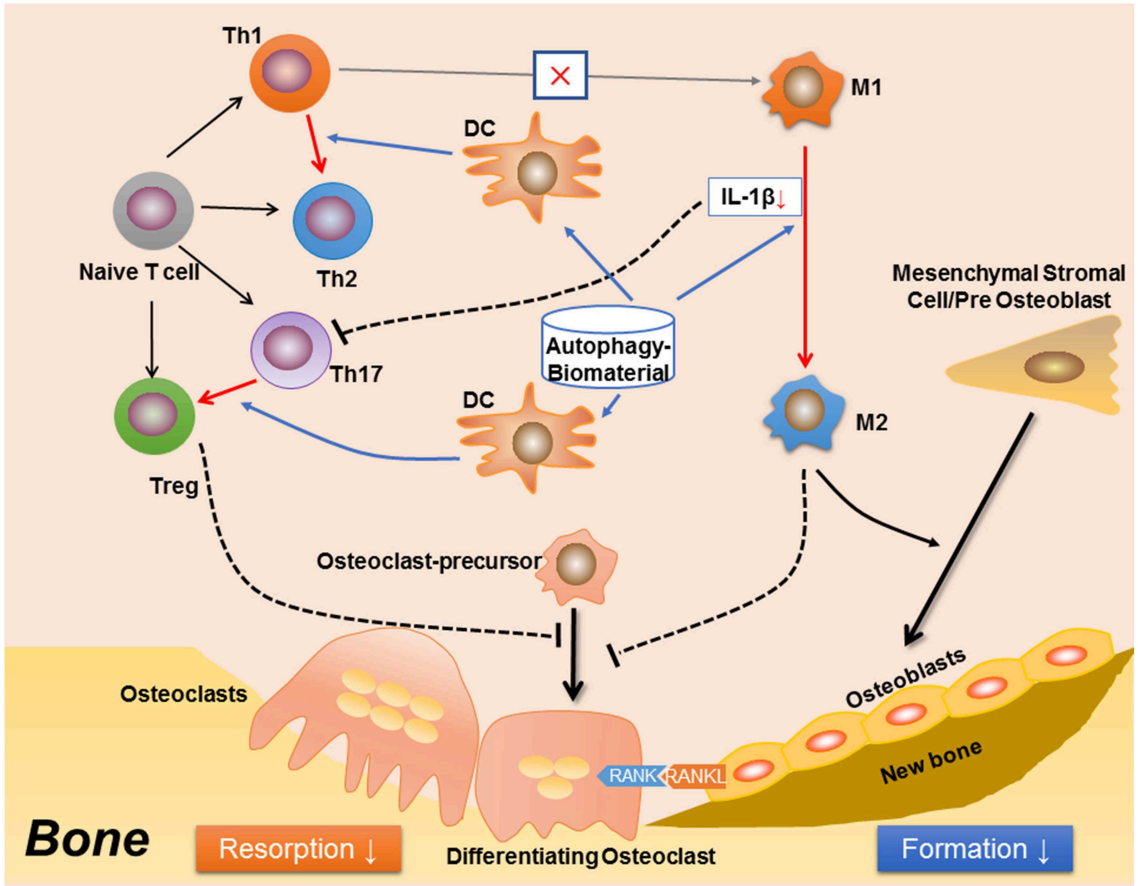

FIGURE 2 | A proposed effect in osteoimmunology regarding biomaterial with the property of inducing autophagy (autophagy-biomaterial). The autophagy-biomaterial should induce bone repair by suppressing the inflammatory response. On one hand, autophagy-induction directly reduces macrophage inflammation and IL-1β secretion. The decreased IL-1β also impedes the polarization and function of Th17 cell (Th17). On the other hand, autophagy-induction in dendritic cell (DC) interrupts its interaction with T cells, thereby inhibiting T cell inflammatory response via introducing the polarization from Th1 toward Th2 cells, as well as that from Th17 to Treg cells. The Th1 to Th2 conversion would in turn reduce M1 polarization while induce M2 polarization. Hence, the autophagy-biomaterial creates an immune microenvironment favoring bone regeneration: the limited inflammatory responses of T cell and M1 macrophage reduce osteoclastogenesis, while the conversion of M1 to M2 macrophages improves osteogenesis.

inflammation (145). On the other hand, autophagy prevents the secretion of macrophage-derived IL-1β (132–134), a cytokine known as promoting Th17 cell response via collaboration with IL-6 and TGF-β (59). In mice with *Atg5*-deficient myeloid cells, $CD4^+$ Th17 cell response is induced (59), further suggesting the role of autophagy in preventing inflammation (**Table 1**). Although inflammatory cytokines such as IL-17 has been reported to induce osteogenesis, excessive IL-17 production results in enhanced RANKL secretion and osteoclastogenesis (73, 74) and therefore is still considered as detrimental for bone regeneration. Especially, autophagy-mediated conversion from Th1 to Th2 cells would in turn induce the polarization from M1 to M2 macrophages (146, 147), a central part in bone regeneration (116, 117). Hence, it could be presumed that autophagy-derived immunomodulation on T cells creates a microenvironment favoring bone repair.

The role of autophagy in osteoimmunology has been further demonstrated in the pathogenesis of autoimmune rheumatic diseases, such as rheumatoid arthritis (RA), a disease with abnormities in organs including joints, heart, vascular system, lungs, and skin (148, 149). Chronical inflammation, as well as bone and cartilage destruction are typical syndromes in RA (150), which mainly due to the interactions within local cells, known as immune cells (such as T and B cells, macrophages), synovial fibroblasts, chondrocytes, as well as osteoclasts and osteoblasts. As autophagy plays decisive roles in osteoclastogenesis, the inhibition of autophagy successfully reduces bone destruction and osteoclast formation in experimental arthritis mouse models (151), suggesting drugs with autophagy inhibition could be used to prevent bone loss in RA patients (148). In addition, autophagy protects cells from apoptosis—a crucial mechanism to extinguish excessive inflammation (132–134, 148), therefore playing essential roles in the pathogenesis and progression of RA, via regulating the balance between immune cell survival and death (148). Induced autophagy/reduced apoptosis have been observed in synovial fibroblasts and synovial tissues obtained

from RA patients (152–154), while autophagy inhibition has been found to reduce synovial inflammation in a collagen induced arthritis (CIA) rat model (148, 155). Autophagy hyper-activation has been found in $CD4^+$ T cells obtained from CIA mouse, and autophagy is considered to regulate T and B lymphocytes homeostasis to maintain the RA chronical inflammatory response (148, 156). Besides, autophagy dysregulation in endothelial cells is considered as responsible for atherosclerosis in RA (157). Especially, autophagy participates in the pathogenesis of RA via inducing the generation of citrullinated peptides, which consequently interrupting immune tolerance (150). The anti-cyclic citrullinated peptide (anti-CCP), an autoantibody (against citrullinated peptides) produced by immune cells upon activation of self-antigens, is a general marker for clinical RA diagnosis (150, 158, 159). The RA patient-derived anti-CCP antibody (Ab) has been found to induce osteoclastogenesis and bone loss (160). Autophagy is recently considered to participate in both the presentation of citrullinated peptides and the generation of anti-CCP Ab (148). Autophagy is required for the antigen presenting cells (APCs) to perform the presentation of citrullinated proteins (161). Furthermore, autophagy is involved in the citrullination processes of Normal Human Bronchial Epithelial (NHBE) cells and human synovial fibroblasts (150, 162). Increased citrullinated peptide production has been observed following autophagy-induction in human synovial fibroblasts from RA patients, and the autophagy level is significantly associated with that of anti-CCP Ab in early-stage RA patients (150), suggesting the fundamental role of autophagy in RA establishment via inducing the generation of citrullinated peptides (150, 163). All these studies indicate that autophagy-derived modulation on osteoimmunology plays a central part not only in physiological bone homeostasis but also in pathological bone diseases, which needs further study in the future.

FUTURE REMARKS & CONCLUSION

Many questions still remain un-resolved regarding the role of autophagy in osteoimmunology. For example, although autophagy is indispensable in osteoclastogenesis, rapamycin (also named as sirolimus), an autophagy inducer via inhibition of the Ser/Thr protein kinase mTOR (mammalian target of rapamycin) (164, 165), has been found to reduce osteoclastogenesis and bone resorption in a mouse model of arthritis, an effect similar to anti-TNF (by Infliximab) treatment (166). It is also found that rapamycin reduces osteoclastogenesis in young rats (167) and post-transplant bone resorption in renal transplant patients (168). This is quite contrary to the positive effect of autophagy in osteoclast differentiation and function as mentioned before. It is presumed that autophagy plays a more maintenance than regulatory role in the differentiation of osteoclast, which is induced and kept in a certain level in response to energy/metabolism variations or intracellular accumulation of damaged organelles such as mitochondria. Whereas, in immune response, autophagy might act more as a regulator to quench the inflammation fire (7), which in turn reduce bone resorption (**Figure 2**). The rapamycin-mediated inhibition of bone loss might be achieved via immunomodulation, suggests that autophagy is an attractive target for osteoimmunology regulation to improve bone tissue regeneration (**Figure 2**). Autophagy might also participate in the mutual regulations between immune-skeletal systems. Previous studies have suggested that the immunosuppressive role of MSCs is achieved through the programmed death 1/ programmed death-ligand 1 (PD-1/PDL1) (169), an autophagy-related signaling pathway (170). Another study has found that the autophagy regulator p62 plays a central role in maintenance of the "macrophage-osteoblast niche," which is indispensable for the retention of HSCs in bone marrow (171). It is also found that compared with the undifferentiated MSCs, the osteogenically differentiated MSCs not only induce the recruitment of macrophages, but also regulate local macrophage response in a VEGFA–C-X-C motif chemokine 12/C-X-C chemokine receptor type 4 (VEGFA-CXCL12/CXCR4) axis dependent manner (172). As CXCR4 signaling has been reported to regulate autophagy via the cross-talk with mTOR (173–175), hence, autophagy should be involved in MSC-mediated regulation on immune cells. Furthermore, It could be predicted that immune cells, especially macrophage, regulate bone remodeling in an autophagy-dependent manner, as both the M1 and M2 macrophage-derived cytokines have been found to modulate autophagy, such as IL-1 (176), TNF-α (177–179), IL-10 (180–182), TGF-β (183, 184); the fundamental role of M1-M2 conversion in osteogenesis might be partially due to different autophagy levels and metabolism states during the differentiation from osteoblast to osteocyte.

In summary, autophagy, a conserved "self-eating" pathway present in all mammalian cells, plays a complex role in osteoimmunology, that at one level, autophagy maintains the cellular homeostasis during differentiation of osteoclast and osteoblast, facilitates the survival and function of osteocyte, and more importantly, direct the immune response to avoid the damage of excessive inflammation. Therefore, the definition of autophagy as "self-eating" should be more appropriate as "self-editing" in maintaining bone homeostasis. This regulatory role in osteoimmunology suggests autophagy could be a novel therapeutic target (e.g., autophagy-inductive biomaterial) to improve bone regeneration in the future translational medicine (**Figure 2**).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. LX: involved in the concept and design of the article and wrote the manuscript. YX: involved in the conception and design of the article and reviewed the manuscript.

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Investigation of NF-κB-94ins/del ATTG and CARD8 (rs2043211) Gene Polymorphism in Acute Lymphoblastic Leukemia

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NLRP3 inflammasome has been widely implicated in the development and progression of various hematological diseases. However, how NLRP3 inflammasome contributes to the pathogenesis and clinical features of acute lymphoblastic leukemia (ALL) is still unknown. Here, in ALL patients' bone marrow, we investigated the single-nucleotide polymorphisms (SNPs) and expression of NLRP3 inflammasome related genes, NF-κB, NLRP3, IL-1β, IL-18, Caspase-1, and ASC. A total of 308 ALL patients and 300 healthy participants were included in this study. D allele and DD genotype under codominant model of NF-κB-94ins/del ATTG were showed as a protective factor in susceptibility of ALL. As for CARD8 (rs2043211), AT/TT genotype under dominant model and TT genotype under codominant model greatly increased the ALL susceptibility. We further studied the relationship between NLRP3 inflammasome genetic polymorphisms and clinical relevance. The results showed that DD genotype of NF-κB-94 ins/del ATTG and AT/TT genotype of CARD8 (rs2043211) contributed to lower WBC count and T-cell immunophenotype, respectively. Moreover, we also found that AT and TT genotypes of CARD8 (rs2043211), GT and TT genotypes of IL-1β (rs16944), and TT genotype of IL-18 (rs1946518) were associated with higher mRNA expression of NLRP3 inflammasome related genes and secretion of downstream cytokines. In conclusion, NF-κB-94 ins/del ATTG and CARD8 (rs2043211) genotypes might serve as novel biomarkers and potential targets for ALL.

Keywords: NLRP3, polymorphism, NF-κB, CARD8, acute lymphoblastic leukemia

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INTRODUCTION

Acute lymphoblastic leukemia (ALL) is a clonal disorder driven by accumulation of progenitor cells in the peripheral blood and bone marrow (1, 2). Although adult ALL accounts for only 20% of leukemia, it is more common in childhood acute leukemia (80% of cases). Nowadays, through traditional chemotherapy, the cure rate of ALL can only reach about 20–40% in adults compared to 80% in children (3, 4). Adult patients present higher risk features at diagnosis, more likely to come up with chemotherapy resistance and higher relapse rate after complete remission (CR) (5). Multiple studies reported that the genetic variants of some metabolism and inflammation related genes contribute to ALL pathogenesis and prognosis (6, 7).

Inflammasomes are large cytosolic multiprotein complexes that assemble in response to infection or stress-associated stimuli and lead to the activation of caspase-1-mediated innate immune responses (8). The NOD-like receptor family was the first family of sensor proteins discovered to form inflammasomes. Among the inflammasomes, the NLRP3 inflammasome has been the most thoroughly studied. NLRP3 inflammasome is composed of NLRP3, the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD) and caspase-1 (9). NLRP3 inflammasome can be activated by exogenous pathogen invasion or *in vivo* cell damage and death, and then lead to the interaction of the PYD domain in NLRP3 and ASC proteins. Assembled inflammasome triggers the cleavage of dormant procaspase-1 into its active form, which turns the inactive proinflammatory cytokines pro-IL-1β and pro-IL-18 into mature and biologically active IL-1β and IL-18, respectively (10, 11). Caspase recruitment domain-containing protein (CARD) 8 interacts with cleaved caspase-1 and then triggers downstream inflammatory response through nuclear factor (NF)-κB pathway (12). NLRP3 inflammasome could modulate cellular levels of the glucocorticoid receptor and affect glucocorticoid resistance of ALL patients (13). However, it is still unknown whether and how NLRP3 inflammasome contributes to the pathogenesis and clinical features of ALL.

Nowadays, the advanced high-throughput techniques have boosted the discovery of genetic variations including copy number variants (CNVs), indels (deletions or insertions), structural variants and single nucleotide polymorphisms (SNPs) (14). SNPs of NLRP3 greatly influence pathogenic challenges and lead to disease outcome, such as autoimmune diseases (15), cardiovascular diseases (16), and malignant tumors (17). The SNP of CARD8 rs2043211 polymorphism has been confirmed to be associated with cardiovascular diseases according to a meta-analysis (18). Accumulating evidences have revealed that NF-κB-94 ins/del ATTG polymorphism is associated with cancer risk such as gastric cancer, hepatocellular carcinoma, and lung cancer (19–21). The polymorphism rs1946518 of NLRP3 effector molecule IL-18 has been shown to influence periodontitis (22) and hepatocellular carcinoma (23). The SNP rs16944 of the other NLRP3 effector molecule IL-1β has been reported contributing to cervical carcinoma (24) and gastritis risk (25). Thus, polymorphism of NLRP3 inflammasome related genes showed strong relevance with malignant tumors, but the regulatory role of the SNP of NLRP3 inflammasome related genes in the ALL bone marrow microenvironment has not been reported until now.

Therefore, to determine the susceptibility and clinical significance of the NLRP3 inflammasome in ALL, we examined the SNPs of four NLRP3 inflammasome related genes, including NF-κB-94ins/del ATTG, CARD8 (rs2043211), IL-18 (rs1946518), and IL-1β (rs16944). We are looking forward to provide evidences for clinical management of ALL.

MATERIALS AND METHODS

Study Subjects and Sample Collection

A total of 308 ALL patients and 300 sex- and age-matched healthy controls were included in this study. The diagnosis of enrolled ALL was based on NCCN Guidelines for acute lymphoblastic leukemia (ALL) (26). The demographic and clinical characteristics of these subjects were shown in **Table 1**. The protocol was approved by the Medical Ethics Committee of Qilu Hospital of Shandong University. Informed consents were obtained from the participants.

TABLE 1 | The demographic and clinical characteristics of ALL patients and controls.

	ALL patients	Controls
No.	308	300
Age, mean ± SD	38.76 ± 17.02	40.06 ± 12.98
Gender (M/F)	184/124	167/133
WBC count (<30*10^9/L / >30*10^9/L)	240/68	NA
Hb (<60 g/L/ > 60 g/L)	250/58	NA
Cr (<115 g/L/ > 115 g/L)	298/10	NA
Spleen (Splenomegaly/Non-Splenomegaly)	248/60	NA
AST (<125 g/L/>125 g/L)	291/17	NA
Fasting blood-glucose (normal/abnormal)	242/66	NA
Immunophenotype (T/B)	66/242	NA
Ph(Ph+/Ph–)	235/73	NA

For determination of polymorphisms, we collected anti-coagulated bone marrow or peripheral blood from ALL patients or peripheral blood from healthy controls. For testing the association of NLRP3 molecules mRNA expression or plasma levels with the genetic polymorphisms, we used bone marrow samples from newly-diagnosed ALL patients. Bone marrow or peripheral blood samples were centrifuged at 430 g for 5 min, and the plasma supernatants were frozen at −80°C for the determination of cytokines by ELISA. The mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation, and re-suspended in sterile PBS and centrifuged again for 5 min at 430 g to remove remaining Ficoll solution. The mononuclear cells were stored −20°C for DNA and RNA extraction.

DNA Extraction and Genotyping

DNA was extracted using TIANamp Genomic DNA Kit (Tiangen, Beijing, China) according to the supplier's recommendations. The concentration and purity of DNA were accessed at 260/280 absorbance using NanoDrop spectrophotometer. The genotype of CARD8 (rs2043211), IL-18 (rs1946518), or IL-1β (rs16944) was performed using TaqMan® allelic discrimination assay (Cat# 4351379; Thermo Fisher Scientific, USA) in accordance with the manufacturer's instruction. Followed the standard protocol, 3 μl TaqMan Universal PCR Master Mix (2×), 0.15 μl pre-designed primers and probes (40×), 1 μl (100 ng/μl) of DNA and 1.85 μl ddH_2O make up the 6 μl volume reaction system. The NF-κB-94 ins/del ATTG polymorphism was examined by using the forward primer: 5′-CCG TGC TGC CTG CGT T-3′, reverse primer: 5′-GCT GGA GCC GGT AGG GAA-3′, and probe 1: 5′-VIC-ACC ATT GAT TGG GCC -MGB-3′ and probe 2: 5′-FAM-CGA CCA TTG GGC C -MGB-3′. A total 10 μl volume reaction system was composed of 5 μl TaqMan Universal PCR Master Mix (2×), 0.8 μl (10 μM) forward primer, 0.8 μl (10 μM) reverse

TABLE 2 | The primer sequences of real-time quantitative PCR.

Gene	Forward (5′-3′)	Reverse (5′-3′)
NF-κB	TCC AGA CCA ACA ACA ACC CC	GAT CTT GAG CTC GGC AGT GT
NLRP3	CAG ACT TCT GTG TGT GGG ACT GA	TCC TGA CAA CAT GCT GAT GTG A
IL-1β	GCC CTA AAC AGA TGA AGT GCT C	GAA CCA GCA TCT TCC TCA G
IL-18	GCT TGA ATC TAA ATT ATC AGT C	GAA GAT TCA AAT TGC ATC TTA T
Caspase-1	AAA TCT CAC TGC TTC GGA CAT G	GGA ACT TGC TGT CAG AGG TCT T
ASC	TGG ATG CTC TGT ACG GGA AG	CCA GGC TGG TGT GAA ACT GAA
GAPDH	GCT CTC TGC TCC TCC TGT TC	GTT GAC TCC GAC CTT CAC CT

TABLE 3 | The association between selected SNPs and susceptibility of ALL.

SNP	Model/Allele	Genotype/Allele	Controls		ALL patients		*P* value
			Count	%	Count	%	
NF-kB 94ins/delATTG	Allele	W	339	56.5	383	62.2	
		D	261	43.5	233	37.8	0.047
	Codominant	WW	98	32.7	119	38.6	
		WD	143	47.7	145	47.1	
		DD	59	19.6	44	14.3	0.027
CARD 8rs2043211	Dominant	AA	93	31	62	20.1	
		AT+TT	207	69	246	79.9	0.002
	Codominant	AA	93	31	62	20.1	
		AT	134	44.7	172	55.8	
		TT	73	24.3	74	24	0.004

primer, 0.2 μl (10 μM) probe 1, 0.2 μl (10 μM) probe 2, 2.0 μl ddH_2O and 1 μl (100 ng/μl) of DNA. ABI7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) was applied for TaqMan SNP genotyping assay, conditions as followed: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of amplification (92°C denaturation for 15 s, 62°C annealing/extension for 60 s). ABI7500 software v 1.3 and TaqMan Genotyper software were used to analyze the genotyping.

Real-Time Quantitative PCR Detection for NF-κB, NLRP3, IL-1β, IL-18, Caspase-1, and ASC

We determined the mRNA expression of NF-κB, NLRP3, IL-1β, IL-18, Caspase-1, and ASC by real-time quantitative PCR. RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). A total of 1 μg RNA was used for reverse transcription of total 10 μl volume by applying the PrimeScript RT reagent Kit Perfect Real Time (Takara Bio, Japan). The quantitative PCR was performed on the LightCycler 480II real-time PCR system (Roche, Switzerland) according to standard protocol. The primers were shown in **Table 2**. The quantitative PCR is a 10 μl volume, comprised of 5 μl of 2× SYBR Green Real-time PCR Master Mix, 1 μl of cDNA, 0.8 μl of the forward and reverse primers, and 3.2 μl ddH_2O. The results were expressed relative to the number of GAPDH transcripts used as an internal control.

ELISA for IL-18 and IL-1β

The levels of IL-18 and IL-1β in plasma were determined with a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) in accordance with the manufacturer's recommendations (lower detection limits were 62.5 pg/ml for IL-18 and 3.9 pg/ml for IL-1β; IL-18 ELISA kit was bought from Abcam and IL-1β ELISA kit was bought from R&D systems, USA).

Statistical Analysis

The *p* value of the Hardy–Weinberg equilibrium (HWE) was calculated using the calculator available at the Helmholtz Center Munich website. In addition to allelic frequencies, we analyzed genotypic frequencies under three genetic models, specifically, the codominant, dominant, and recessive models. Association between the SNPs and ALL susceptibility was calculated by a chi-squared ($\chi 2$) test or a Fisher's exact test. Univariate logistic regression analysis was used to analyze *p* values and odds ratios (ORs) with a corresponding 95% confidence interval (95% CI). A two-tailed $p < 0.05$ was considered statistically significant. All statistical analysis was performed using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

The Polymorphisms of NF-κB-94 ins/del ATTG and CARD8 (rs2043211) Contribute to the Susceptibility of All

We examined SNPs of NLRP3 inflammasome related genes: NF-κB (-94 ins/del ATTG), CARD8 (rs2043211), IL-18 (rs1946518), and IL-1β (rs16944) in 308 ALL patients and 300 healthy controls. The distribution of AT/TT genotype under dominant and TT genotype under codominant models for rs2043211 in CARD8

were found significantly different between ALL patients and controls. In addition, both the D allele in allelic frequencies and DD genotype under the codominant model of NF-κB−94 ins/del ATTG were significantly associated with the susceptibility to ALL ($p < 0.05$; **Table 3**).

Univariate logistic regression analysis was used for analyzing NF-κB and CARD8. For 94 ins/del ATTG in NF-κB, the frequency of D allele was lower in ALL patients compared to controls (37.8 vs. 43.5%), and it was significantly associated with ALL susceptibility (OR = 0.790, 95% CI 0.628–0.994, $p = 0.044$). Furthermore, the frequency of the DD genotype of NF-κB (14.3 vs. 19.6%) was obviously lower in ALL rather than homozygote insertion or wildtype (WW) genotype under codominant model, which hinted a significant association with ALL susceptibility (OR = 0.641, 95% CI 0.383–0.946, $p = 0.043$). For CARD8 under dominant model, the AT/TT genotype was significantly higher in ALL patients compared to controls (79.9 vs. 69.0%), and was associated with ALL susceptibility (OR = 1.783, 95% CI 1.230–2.583, $p = 0.002$). As for codominant model, both AT and TT genotypes of CARD8 (55.8 vs. 44.7%; 24.0 vs. 24.3%, respectively) were much higher in ALL compared to controls, and it significantly contributed to ALL susceptibility rather than AA genotype (OR = 1.925, 95% CI 1.300–2.583, $p = 0.001$; OR = 1.521, 95% CI 0.964–2.399, $p = 0.072$, respectively; **Table 4**). Interestingly, the distribution of CARD8 rs2043211 and NF-κB-94 ins/del ATTG was found different between ALL patients and controls. NF-κB polymorphism demonstrated a protective effect, while AT/TT genotype of CARD8 (rs2043211) greatly increased the ALL susceptibility.

DD Genotype of NF-κB-94 ins/del ATTG and AT/TT Genotype of CARD8 (rs2043211) Contribute to Lower WBC Count and T-Cell Immunophenotype, Respectively

According to NCCN Guidelines for ALL, several prognostic indicators are listed to indicate the prognosis. Our study analyzed the associations between these prognostic indicators and four SNPs of NLRP3 inflammasome genes, aimed to figure out

TABLE 4 | NF-κB-94 ins/del ATTG and CARD8 (rs2043211) contribute to susceptibility of ALL.

SNP	Model/Allele	Genotype/Allele	Controls		ALL patients		OR (95%CI)	*P* value
			Count	%	Count	%		
NF-kB 94ins/delATTG	Allele	W	339	56.5	383	62.2	1.000	
		D	261	43.5	233	37.8	0.790 (0.628–0.994)	0.044
	Codominant	WW	98	32.7	119	38.6	1.000	
		WD	143	47.7	145	47.1	0.835 (0.568–1.189)	0.317
		DD	59	19.6	44	14.3	0.614 (0.383–0.986)	0.043
CARD8rs2043211	Dominant	AA	93	31	62	20.1	1.000	
		AT+TT	207	69	246	79.9	1.783 (1.230–2.583)	0.002
	Codominant	AA	93	31	62	20.1	1.000	
		AT	134	44.7	172	55.8	1.925 (1.300–2.852)	0.001
		TT	73	24.3	74	24	1.521 (0.964–2.399)	0.072

TABLE 5 | The polymorphisms of NF-κB-94 ins/del ATTG and CARD8 (rs2043211) were associated with prognosis characteristics of ALL.

SNP	Model/Allele	Genotype/Allele	Clinical characteristic				OR (CI95%)	*P* value
			Count	%	Count	%		
NF-kB 94ins/del ATTG	Recessive		WBC Count<30		>30			
		WW+WD	192	80	64	94.1	1.000	
		DD	48	20	4	5.9	0.250 (0.087–0.721)	0.010
	Codominant		WBC Count<30		>30			
		WW	91	37.9	28	41.2	1.000	
		WD	109	45.4	36	52.9	1.073 (0.609–1.892)	0.807
		DD	40	16.7	4	5.9	0.325 (0.107–0.998)	0.048
CARD8 rs2043211	Dominant		IPT (B-cell)		IPT (T-cell)			
		AA	55	22.7	7	10.6	1.000	
		AT+TT	187	77.3	59	89.4	2.479 (1.071–5.738)	0.034
	Codominant		IPT (B-cell)		IPT (T-cell)			
		AA	55	22.7	7	10.6	1.000	
		AT	127	52.5	45	68.2	2.748(1.182–6.559)	0.019
		TT	60	24.8	14	21.2	1.833(0.689–4.876)	0.225

the correlation between NLRP3 genetic polymorphisms and prognosis of ALL.

We found that NF-κB-94 ins/del ATTG was significantly associated with the lower white blood cells (WBC) count, which is supposed to be a poor prognostic indicator as long as WBC count over $30*10^9$ /L. After univariate logistic regression analysis, compared with WD/WW genotype of NF-κB under recessive model, DD genotype significantly associated with a lower WBC count (20.0 vs. 5.9%; OR = 0.250, 95% CI 0.087–0.721, $p = 0.010$). Under codominant model, DD genotype also associated with a lower WBC count compared to WW genotype (16.7 vs. 5.9%; OR = 0.325, 95% CI 0.403–0.998, $p = 0.048$). NF-κB-94 ins/del ATTG under both recessive and codominant model, patients with DD genotype tend to have a lower WBC count (**Table 5**).

Furthermore, CARD8 (rs2043211) was also found to be correlated with immunophenotype of ALL, which primary divided into T-cell immunophenotype and B-cell immunophenotype. According to NCCN Guideline for ALL, immunophenotype is a strong prognostic indicator. Compared with AA genotype under dominant model, AT/TT genotype was associated with T-cell immunophenotype (77.3 vs. 89.4%; OR = 2.479, 95% CI 1.071–5.738, $p = 0.034$). Under codominant model, AT genotype was also associated with T-cell immunophenotype compared to AA genotype (52.5 vs. 68.2%; OR = 2.748, 95% CI 1.182–6.559, $p = 0.019$; **Table 5**). Thus, DD genotype of NF-κB-94 ins/del ATTG was showed as a protective factor, while AT/TT genotype of CARD8 (rs2043211) associated with a poor prognosis.

NF-κB-94 ins/del ATTG, IL-18 (rs1946518) and IL-1β (rs16944) Contribute to Clinical Characteristics of All Patients

The NLRP3 inflammasome genetic polymorphisms are also correlated with other clinical indicators of diagnosis, treatment approaches, and complication management. For NF-κB-94 ins/del ATTG, univariate logistic regression analysis revealed that DD genotype ($p = 0.046$) was

TABLE 6 | The association between selected SNPs and clinical characteristics of ALL patients.

SNP	Model/Allele	Genotype/Allele	Clinical characteristics				OR (CI95%)	*P* value
			Count	%	Count	%		
NF-kB	Recessive		Hb<60		>60			
94ins/delATTG		WW+WD	43	74.1	213	85.2	1.000	
		DD	15	25.9	37	14.8	0.498 (0.251–0.986)	0.046
			Splenomegaly		Non-Splenomegaly			
	Codominant	WW	99	39.9	20	33.3	1.000	
		WD	120	48.4	25	41.7	1.031 (0.541–1.966)	0.926
		DD	29	11.7	15	25	2.560 (1.165–5.625)	0.019
	Allele		Splenomegaly		Non-Splenomegaly			
		W	318	64.1	65	54.2	1.000	
		D	178	35.9	55	45.8	1.512 (1.010–2.262)	0.045
IL-18 rs1946518	Recessive		Splenomegaly		Non-Splenomegaly			
		GG+GT	194	78.2	38	63.3	1.000	
		TT	54	21.8	22	36.7	2.080 (1.135–3.811)	0.018
			AST<125		>125			
		GG+GT	223	76.6	9	52.9	1.000	
		TT	68	23.4	8	47.1	2.915 (1.083–7.848)	0.034
	Codominant		Glu<6.1		>6.1			
		GG	59	24.4	22	33.3	1.000	
		GT	121	50	34	51.5	0.754 (0.405–1.401)	0.371
		TT	62	25.6	10	15.2	0.433 (0.189–0.990)	0.047
	Allele		Glu<6.1		>6.1			
		G	239	49.4	78	59.1	1.000	
		T	245	50.6	54	40.9	0.675 (0.457–0.998)	0.049
IL-1β rs16944	Recessive		Cr<115		>115			
		GG+GT	219	73.5	4	40	1.000	
		TT	79	26.5	6	60	4.158 (1.143–15.122)	0.031
	Allele		Cr<115		>115			
		A	302	50.7	5	25	1.000	
		G	294	49.3	15	75	3.082 (1.106–8.587)	0.031

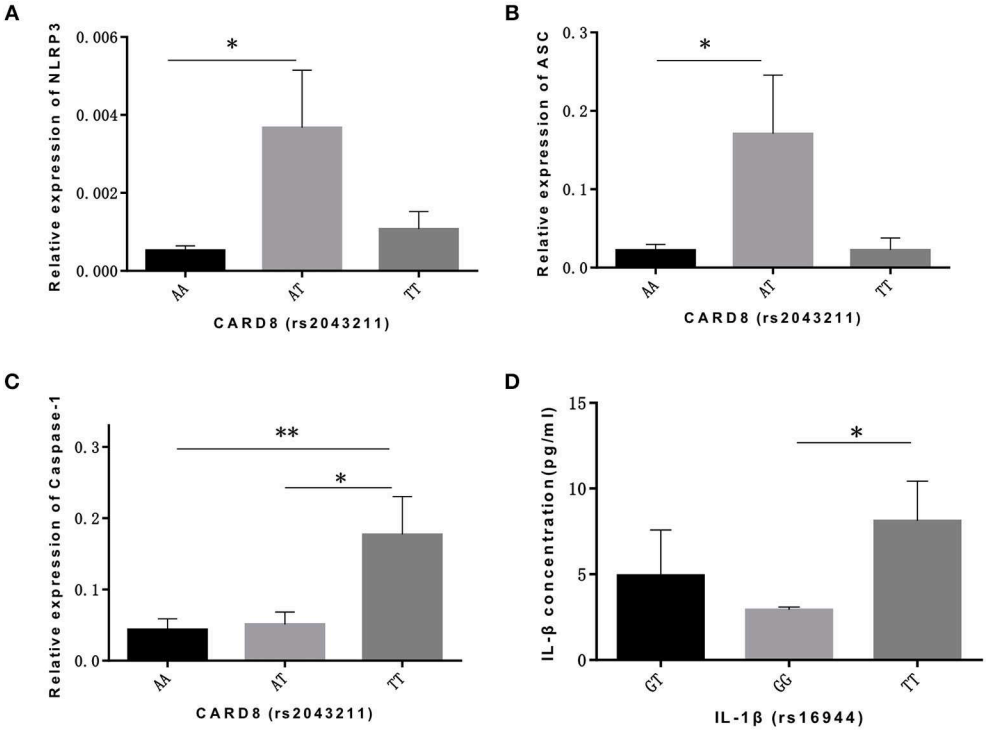

FIGURE 1 | CARD8 polymorphisms (rs2043211) was associated with the mRNA expression ofNLRP3 inflammasome related genes and its effective cytokines in ALL patients bone marrow microenvironment. AT genotype was correlated with higher mRNA expression ofNLRP3 **(A)** and ASC **(B)** compared to AA genotype, while TT genotype was correlated with higher caspase-1 **(C)** mRNA expression capared with AA and AT genotypes. TT genotype also contributed to the higher IL-18 concentration **(D)** in ALL patients bone marrow microenvironment. $^{*}p < 0.05$; $^{**}p < 0.01$.

statistically associated with hemoglobin (Hb) compared to WW/WD genotype under recessive model. Under codominant model, DD genotype ($p = 0.019$) was significantly associated with splenomegaly in contrast to WW genotype. As for the frequency of allele, compared with the−94insATTG (W) allele, the minor allele homozygotes were also associated with splenomegaly ($p = 0.045$; **Table 6**).

Under recessive model, TT genotype of IL-18 (rs1946518) was statistically correlated with both splenomegaly and AST concentration in serum compared with GG/GT genotype ($p = 0.018$, $p = 0.034$, respectively). In addition, TT genotype was also found to be related to the fasting blood-glucose under codominant model compared to GG genotype ($p = 0.047$). As for the frequency of allele, compared with the G allele, the T allele homozygote was also associated with the fasting blood-glucose ($p = 0.049$; **Table 6**).

Furthermore, the polymorphism in IL-1β rs16944 was supposed to have connection with creatinine concentration. Under recessive model, TT genotype ($p = 0.031$) was significantly associated with creatinine concentration by the contrast to GG/GT genotype. As for the frequency of allele, the G allele was also associated with creatinine concentration ($p = 0.031$; **Table 6**).

Association Between the Polymorphisms and the Expression of NLRP3 Inflammasome Related Genes

The pathogenesis and development of ALL involves a dysregulated immune microenvironment, which contains the production of multiple immunological effector proteins and the activation of the downstream pathways. To further comprehend the relationship between NLRP3 inflammasome related gene polymorphisms and its effective proteins, we use RT-PCR to examine NLRP3 inflammasome related gene expression at mRNA level, and ELISA to detect the secretion of effective proteins.

As for CARD8 (rs2043211), AT genotype showed a significant association with the higher mRNA expression levels of NLRP3 and ASC compared to AA genotype ($p = 0.017$, $p = 0.024$, respectively; **Figures 1A,B**). We also found that TT genotype was correlated with higher caspase-1 mRNA level when compared to both AA and AT genotype ($p = 0.009$, $p = 0.039$, respectively; **Figure 1C**). Moreover, TT genotype in CARD8 polymorphism was also found to be associated with IL-18 concentration in ALL bone marrow microenvironment compared to AA genotype (**Figure 1D**).

As for IL-1β (rs16944) polymorphism, GT genotype was significantly associated with IL-18 expression compared with

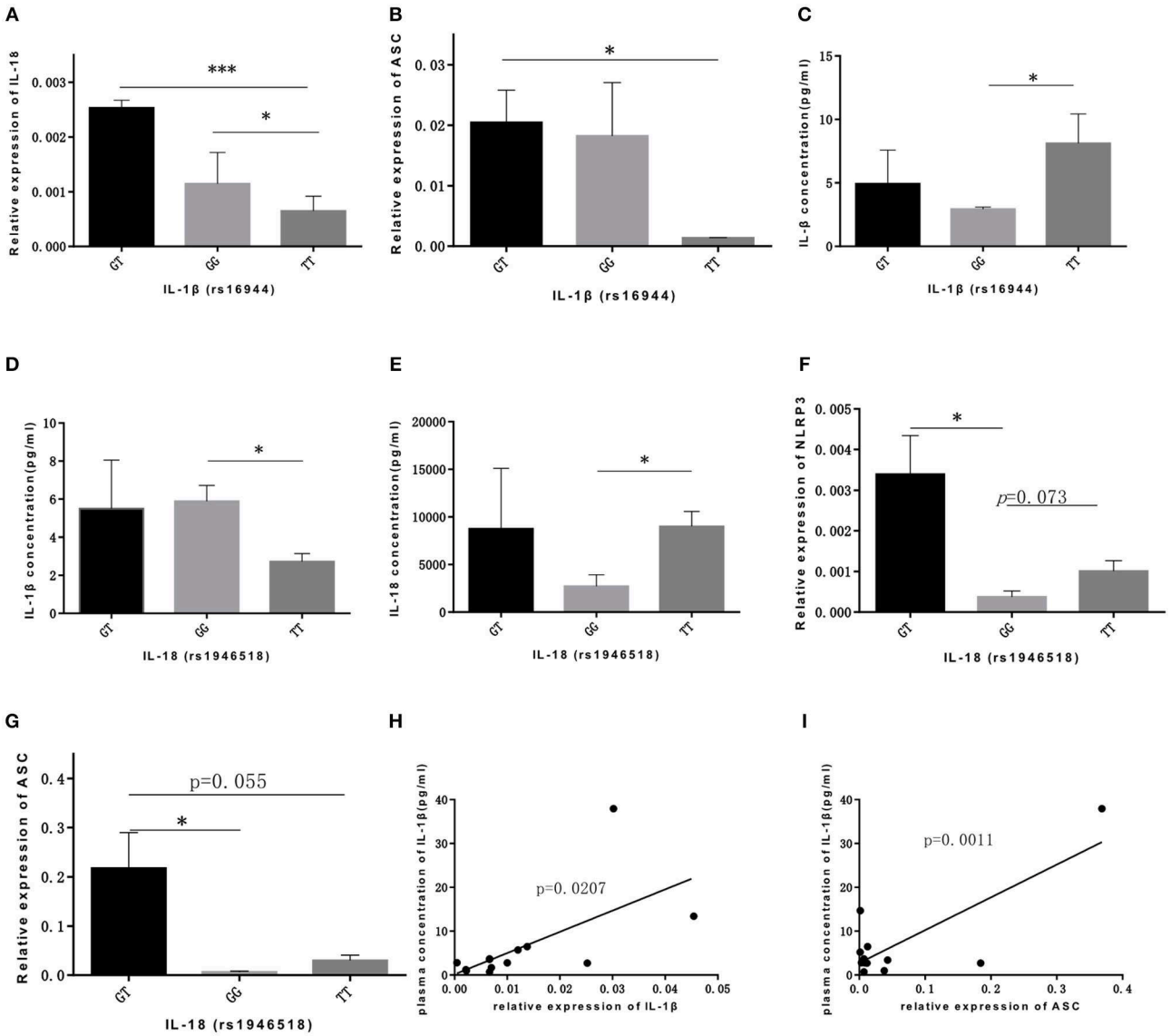

FIGURE 2 | IL-IP (rsl6944) and IL-18 (rsl946518) were associated with higher mRNA expression ofNLRP3 related genes and secreted cytokines. GT and GG genotypes of IL-IP (rsl6944) were associated with mRNA expression levels ofiL-18 **(A)** and ASC **(B)** compared to TT genotype. IL-I p concentration **(C)** was higher in ALL patients bone marrow with TT genotype. IL-I p concentration **(D)** or IL-18 concentration **(E)** were higher in GG or TT genotype of IL-18 (rs1946518) polymorphisms. NLRP3 **(F)** and ASC **(G)** mRNA expression levels were significant high in GT genotype ofiL-18 (rsl946518) polymorphisms. IL-IP concentration correlated with mRNA expression of IL-I p **(H)** and ASC **(I)**. $^{*}p < 0.05$; $^{***}p < 0.001$.

GG and TT genotype ($p = 0.021$, $p = 0.000$, respectively; **Figure 2A**), and was also associated with ASC expression compared with TT genotype ($p = 0.045$; **Figure 2B**). Our data showed that TT genotype was correlated with IL-1β concentration ($p = 0.044$; **Figure 2C**). With the similar trend, TT genotype in IL-18 (rs1946518) polymorphism was also associated with both IL-1β and IL-18 concentration compared to GG genotype ($p = 0.029$, $p = 0.022$, respectively; **Figures 2D,E**). However, GT genotype in IL-18 polymorphism was related to a higher level of NLRP3 and ASC ($p = 0.034$, $p = 0.048$, respectively; **Figures 2F,G**). Besides, we also found significant correlation between concentration of IL-1β in bone marrow microenvironment and mRNA expression level of IL-1β and ASC ($p = 0.020$, $p = 0.001$, respectively; **Figures 2H,I**). Thus, AT and TT genotype of CARD8 (rs2043211), GT and TT genotype of IL-1β (rs16944) and TT genotype of IL-18 (rs1946518) were associated with higher mRNA expression of NLRP3 related genes and secreted cytokines.

DISCUSSION

ALL is a malignant blood disease with origins from somatic genetic alterations in bone marrow microenvironment (27). Though traditional chemotherapy has made a great progress

in the last decade, ALL patients still have a poor prognosis. Compared with 90% 5-year overall survival in children, it is merely 30–40% in adult ALL. NLRP3 inflammasome systematically exists in human organism, and its activation has been identified as initiation of numerous diseases, such as immune diseases (28), cardiovascular diseases (29), gut homeostasis (30), and even malignant cancers (31). In hematological diseases, genetic polymorphisms of the NLRP3 have been reported to contribute to the pathogenesis of multiple myeloma (32) and lymphoma (33). But what is the correlation between NLRP3 inflammasome and ALL is still unknown. Therefore, our study mainly focused on the relationship between NLRP3 inflammasome and ALL in order to depict the internal communication between them.

We found that the DD genotype and D allele in NF-κB-94 ins/del ATTG was associated with a decreased risk of ALL, and had been considered as a protective factor. With the similar trend, under both recessive mode and codominant model, DD genotype exhibits as a protective factor on WBC count and Hb concentration. NF-κB-94 ins/del ATTG is considered as a protective factor for ALL patients. NF-κB is not only the downstream of NLRP3 inflammasome, but also is involved in many immune and inflammatory responses. In the resting state, NF-κB was found in cytoplasm, combined with several regulatory proteins, which inhibit its function (34, 35). After stimulating factors activate inflammatory responses, it moves into nucleus and binds to specific DNA sequences to modulate apoptosis and proliferation related genes (36). As NF-κB-94 ins/del ATTG polymorphism was determined as a protective factor in our study, this gene polymorphism may result in low affinity to nucleus; however, further studies need to be performed to illuminate its mechanisms.

On the contrary, CARD8 (rs2043211), another polymorphism we focused on, seems to increase the risk for ALL according to our study. It should be noted that TT genotype under codominant model increased the susceptibility of ALL, showed strong positive correlation with caspase-1 mRNA expression and the concentration of IL-18 in bone marrow microenvironment. CARD8 polymorphism was supposed to positively enhanced the NLRP3 inflammasome activation and the susceptibility of ALL.

Since we are aimed to provide new horizons for clinical management of ALL patients, we focused on several clinical indicators that may be related to prognosis. Though there were still many indicators we studied not listed in NCCN Guideline of prognostic indicators, they are significantly related to complications or the survival quality of ALL patients. According to our study, polymorphisms of IL-18 and IL-1β should be significantly associated with blood routine index, biochemical criterion.

In conclusion, our investigation found that NLRP3 inflammasome-related SNPs, especially NF-κB-94ins/del ATTG and CARD8 (rs2043211) genotype might serve as a novel biomarker and potential targets for ALL. Furthermore, IL-1β (rs16944) and IL-18 (rs1946518) polymorphism are able to predict the prognosis and guide the treatment of ALL. Further research with a larger sample size and in-depth mechanism study is required to validate the clinical potential of IL-1β (rs16944) and IL-18 (rs1946518) polymorphism.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of CIOMS Guideline. The protocol was approved by the Laboratory Animal Ethic Committee of Qilu Hospital of Shandong University.

AUTHOR CONTRIBUTIONS

DM and CJ conceived and designed the experiments. CZha performed the experiments. RW and FH analyzed the data. YS, CZho, and MH contributed reagents, materials, and analysis tools. XH and XZ wrote the paper. All authors read the manuscript critically and approved the submitted version.

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REVIEW
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Foxp3$^+$ Regulatory T Cells in Bone and Hematopoietic Homeostasis

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The bone represents surprisingly dynamic structures that are subject to constant remodeling by the concerted action of bone-forming osteoblasts and bone-resorbing osteoclasts - two cell subsets of distinct developmental origin that are key in maintaining skeletal integrity throughout life. In general, abnormal bone remodeling due to dysregulated bone resorption and formation is an early event in the manifestation of various human bone diseases, such as osteopetrosis/osteoporosis and arthritis. But bone remodeling is also closely interrelated with lympho-hematopoietic homeostasis, as the bone marrow niche is formed by solid and trabecular bone structures that provide a framework for the long-term maintenance and differentiation of HSCs (>blood lineage cells and osteoclasts) and MSCs (>osteoblasts). Numerous studies in mice and humans have implicated innate and adaptive immune cells in the dynamic regulation of bone homeostasis, but despite considerable clinical relevance, the exact mechanisms of such immuno-bone interplay have remained incompletely understood. This holds particularly true for CD4$^+$ regulatory T (Treg) cells expressing the lineage specification factor Foxp3: Foxp3$^+$ Treg cells have been shown to play an indispensable role in maintaining immune homeostasis, but may also exert critical non-immune functions, which includes the control of metabolic and regenerative processes, as well as the differentiation of HSCs and function of osteoclasts. Here, we summarize our current knowledge on the T cell/bone interplay, with a particular emphasis on our own efforts to dissect the role of Foxp3$^+$ Treg cells in bone and hematopoietic homeostasis, employing experimental settings of gain- and loss-of-Treg cell function. These data make a strong case that Foxp3$^+$ Treg cells impinge on lympho-hematopoiesis through indirect mechanisms, i.e., by acting on osteoclast development and function, which translates into changes in niche size. Furthermore, we propose that, besides disorders that involve inflammatory bone loss, the modulation of Foxp3$^+$ Treg cell function *in vivo* may represent a suitable approach to reinstate bone homeostasis in non-autoimmune settings of aberrant bone remodeling.

Keywords: bone disorders, bone microenvironment, lympho-hematopoiesis, osteoclasts, Foxp3$^+$ Treg cells

INTRODUCTION

In adult mammals, the bone marrow (BM) is the primary site of hematopoiesis. Hematopoietic stem cells (HSCs) reside within specific niches, which are specialized microenvironments within the BM cavity, providing HSCs with regulatory signals essential for their maintenance, proliferation and differentiation into blood and immune cells. HSCs dynamically regulate their numbers by undergoing asymmetric, self-renewing divisions depending upon cell-intrinsic and cell-extrinsic mechanisms, thereby sustaining hematopoiesis over lifetime (1, 2). Homeostasis of the bone microenvironment critically depends on bone remodeling, a tightly regulated process involving three major cell types: bone-resorbing osteoclasts, bone-forming osteoblasts and osteocytes. Osteocytes originate from mature osteoblasts, residing within the lacuna of the mineralized bone matrix, and act as orchestrators of bone remodeling (3). Under physiological conditions, resorption of damaged bone by osteoclasts is followed by the recruitment of osteoblasts and the formation of new bone, a concerted action critical for the maintenance of skeletal integrity and bone homeostasis. Defects in osteoclastic activity cause osteopetrosis, a disease associated with high bone mass, whereas enhanced osteoclastic bone resorption results in low bone mass and osteoporosis (4). While the direct impact of osteoclasts on maintenance and BM retention of HSCs remains controversial (5–7), there is accumulating evidence that osteoblast lineage cells play a crucial role in the regulation of hematopoiesis (8–10). Since the majority of the cells in the BM are hematopoietic cells, it appears likely that, in addition to stromal niche components, HSCs are regulated by their own progeny. In this context, megakaryocytes have recently been identified as direct regulators of homeostatic HSC quiescence using several mechanisms, including secretion of CXCL4 and TGF-β (11–13), while direct cell-cell interaction of megakaryocytes and bone cells have been described to affect skeletal homeostasis (14–16). Similarly, it has been suggested that the function of osteoblasts in maintaining the HSC niche is controlled by macrophages, whereas "HSC niche macrophages" have been described to govern maintenance of hematopoiesis directly (17–20). However, due to the heterogeneity of macrophages, and since the bone- and BM-residing macrophage populations, including osteoclasts, are primarily defined by their histological location, the precise identity and role of the different macrophage subpopulations in bone remodeling, regeneration, and shaping the HSC niche still need to be defined (21–23).

The impact of cells of the adaptive immune system — B and T lymphocytes — on bone remodeling under physiological conditions is still a matter of debate: while some studies using lymphocyte-deficient mouse models such as *Rag*$^{-/-}$, μMT or nude mice described low bone mass phenotypes, others did not (24–27). Given the fact that several populations of mature lymphocytes, such as long-lived memory T and B cells reside in the BM (28, 29) and share common niches with HSCs, it is reasonable to assume that these mature cells impinge on bone and hematopoietic homeostasis. In this context, it has been shown, that after allogeneic BM stem cell transplantation, T cells act as a double-edged sword as they can on the one hand promote graft-versus-host-disease (GvHD), but on the other hand can also provide beneficial effects on the engraftment (30). High-resolution imaging over time in an experimental setup of allogeneic HSC transplantation provided evidence that HSCs preferentially co-localize with CD4$^+$ regulatory T (Treg) cells, expressing the lineage specification factor Foxp3, that accumulate on the endosteal surface of the bones of non-irradiated recipient mice, enabling transplanted stem cells to evade from allogeneic rejection (31, 32). The essential role of Foxp3$^+$ Treg cells in maintaining immune homeostasis is firmly established (33, 34), but additional, non-immunological functions of Treg cells, such as controlling (i) the metabolic function in adipose tissue, (ii) regeneration of muscle cells, (iii) lympho-hematopoiesis, and (iv) osteoclast and osteoblast development and function, become increasingly apparent (35–41).

While much less is known about the interplay between Treg cells and osteoclasts/osteoblasts in bone remodeling and the formation of the HSC niche, a large body of experimental evidence focused on the immuno-bone crosstalk in rheumatoid arthritis (RA), one of the most common human autoimmune diseases. Osteoclasts play a central role in the pathogenesis of RA, where bone destruction is characterized by inflammatory osteolysis, caused by aberrant activation of the immune system, eventually leading to the destruction of surface cartilage and subchondral bone (42, 43). It has been proposed that the onset and pathogenesis of RA critically depends on the balance between two distinct CD4$^+$ T cell subsets: type 17 helper T (TH17) cells, a pathogenic subset of CD4$^+$ T cells that produces IL-17, and thereby promoting osteoclastogenesis, and Treg cells, crucial for the prevention of autoimmune diseases driven by TH17 cells (34, 44). The role of Treg cells in animal models for autoimmune arthritis has been demonstrated by amelioration of the disease following adoptive transfer of Treg cells and induction of an accelerated and more severe form of arthritis after depletion of Treg cells (45–48). Moreover, it has been described that under certain conditions of experimental arthritis a subpopulation of Foxp3$^+$ Treg cells can convert into autoreactive Foxp3$^-$ TH17 cells (49).

There is also evidence that Treg cells interact with osteoblasts. For example, studies on intermittent PTH-induced bone anabolism propose that Treg cells are involved in the up-regulation of the expression of the osteogenic factor Wnt10b by CD8$^+$ T cells, which was also suggested to be the main mechanism in the promotion of bone formation by oral supplementation with the probiotic *Lactobacillus rhamnosus* GG (50–53). On the other hand, Treg cells have been implicated to play a role in bone formation by promoting the differentiation of osteoblasts directly (54).

Although the close relationship between the bone and the immune system has long been recognized (55), the spatial relationship and the interaction between the different cell types within the bone microenvironment as well as the identity of their communication factors, in particular under physiological conditions, is still incompletely understood.

Studies on the interplay between osteoclasts/osteoblasts and Treg cells in the BM microenvironment are hampered by several unresolved issues: (a) osteoclasts are difficult to study due to the lack of reliable methods for their *ex vivo* purification, owing to their low abundance, large size, and lack of specific surface marker expression. Furthermore, the phenotypic definition of "true" osteoclast precursors and their developmental stages vary considerably; (b) constitutive Treg cell deficiency inevitably results in secondary effects due to systemic autoimmunity and increased systemic levels of inflammatory factors. Mice with constitutive Treg cell deficiency suffer from severe morbidity leading to premature death prior to completion of bone development; (c) due to the unique properties and structure of bone, it is technically challenging to assess and visualize interactions between cells in the BM niche. Thus, it will be essential to develop experimental systems and more advanced imaging that keep these limitations to a minimum.

In this review we discuss the impact of BM-residing Treg cells on the bone microenvironment, central to the development of therapeutic strategies for the treatment of bone diseases and to promote tolerance after stem cell transplantation.

LYMPHO-HEMATOPOIETIC NICHE AND FOXP3$^+$ TREG CELLS

For a long time, HSCs were considered as dormant cells but increasing evidence suggests HSCs as direct targets of inflammatory signals. Earlier studies have identified HSCs as "first responders" during inflammatory responses, e.g., during infections, later it became clear that pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF) and type I and type II interferons (IFNs), G-CSF, and Toll-like receptor (TLR) ligands regulate HSCs not only in response to stress but also under homeostatic conditions. Together with BM niche signals such as CXCL12, basal levels of inflammatory cytokines provided by T cells, NK cells, neutrophils and macrophages control the balance between HSC dormancy and lineage fate decision under homeostatic conditions, while inflammatory conditions promote HSC proliferation and differentiation at the expense of self-renewal, emphasizing the interdependency of the distinct BM niche components in health and disease (56–60). However, increasing evidence is pointing towards regulation of HSC maintenance by distal/systemic factors: in addition to the nervous system (e.g., by oscillation of CXCL12 production) and hormones such as PTH or estrogen that have been described to regulate HSCs from the outside, two recent studies demonstrate that also the liver and the intestine contribute to HSC maintenance under steady-state conditions (61–65). Given that bone remodeling is also highly regulated by systemic factors, further studies are required to dissect direct and indirect contributions of distal organs on hematopoietic and skeletal homeostasis.

In both mouse and man, the T cell compartment in the BM, which constitutes only about 5% of mononuclear BM cells, is characterized by a lower CD4/CD8 T cell ratio and notably, by substantially elevated frequencies of Foxp3$^+$ Treg cells within the CD4$^+$ T cell population compared to peripheral lymphoid organs (66, 67). Like other BM T cells, BM Treg cells exhibit a more activated/memory phenotype. Transcriptional characterization of BM Treg cells revealed a signature distinct from Treg cells in the periphery. The differential expression of cytokine/chemokine receptors such as *Il9r*, *Ccr2,* and *Cxcr3* and effector molecules such as *Il10* and *Ctla4* suggests increased suppressive capacity of BM resident Treg cells (68). The transcriptional signature is consistent with the idea that these niche-associated Treg cells contribute to the maintenance of the BM microenvironment as an immune privilege site, crucial for HSC survival (31, 32).

Interestingly, and reminiscent of the above-described observations, our temporal analysis of Treg cells in the BM of wildtype mice revealed that the proportion of Foxp3$^+$ Treg cells among CD4$^+$ T cells further increases with age. While in juvenile mice, Foxp3$^+$ Treg cells account for approximately 20% of the CD4$^+$ T cell population, their frequency has almost doubled in aged mice (**Figure 1A**). This can be recapitulated by adoptive Treg cell transfers into Treg cell-deficient mice: here we found Foxp3^{GFP+} Treg cells, which initially comprised about 10–15% of the adoptively transferred splenic bulk CD4$^+$ T cell population, to be selectively enriched in the BM of Foxp3-deficient recipient mice [up to 90% of transferred CD4$^+$ T cells (69)] and in the BM of T and B cell-deficient *Rag1*$^{-/-}$ recipient mice (up to 40% of transferred CD4$^+$ T cells; **Figure 1B**), indicating the physiological relevance of the enrichment. Overall, these observations raise the question whether this accumulation of Foxp3$^+$ Treg cells within the BM is due to preferential *de novo* induction of initially Foxp3-negative CD4$^+$ T cells or preferential recruitment of preformed cells to the bone microenvironment (70, 71).

Since CD4$^+$ Treg cells were originally identified exclusively by the constitutive expression of the IL-2 receptor alpha chain CD25, employing depleting antibodies directed against CD25 still represents the most widely used loss-of-function approach to characterize Treg cell function *in vivo* (72, 73). Whether the *in vivo* administration of anti-CD25 monoclonal antibodies (mAbs) leads to depletion or to functional inactivation of CD25-expressing Treg cells has been controversially discussed (74–77). In mouse models for RA, administration of anti-CD25 mAbs exacerbates the disease (45, 78, 79). However, interpretation of results from studies using this approach to assess the direct impact of Treg cells on bone cells is hampered due to the upregulation of CD25 expression on activated CD4 and CD8 T cells and in addition, due to the existence of CD25-negative Treg cells in particular in peripheral tissues and the BM. Consistently, anti-CD25 treatment spares Foxp3$^+$ Treg cells with a CD25$^{low/-}$ phenotype (80).

Only the identification of Foxp3 as the Treg cell lineage specification factor, necessary and sufficient for their development and function (81–83) allowed specific *in vivo* targeting of Foxp3$^+$ Treg cells and with this the generation of mouse models with abrogated or enhanced Foxp3$^+$ Treg cell activity.

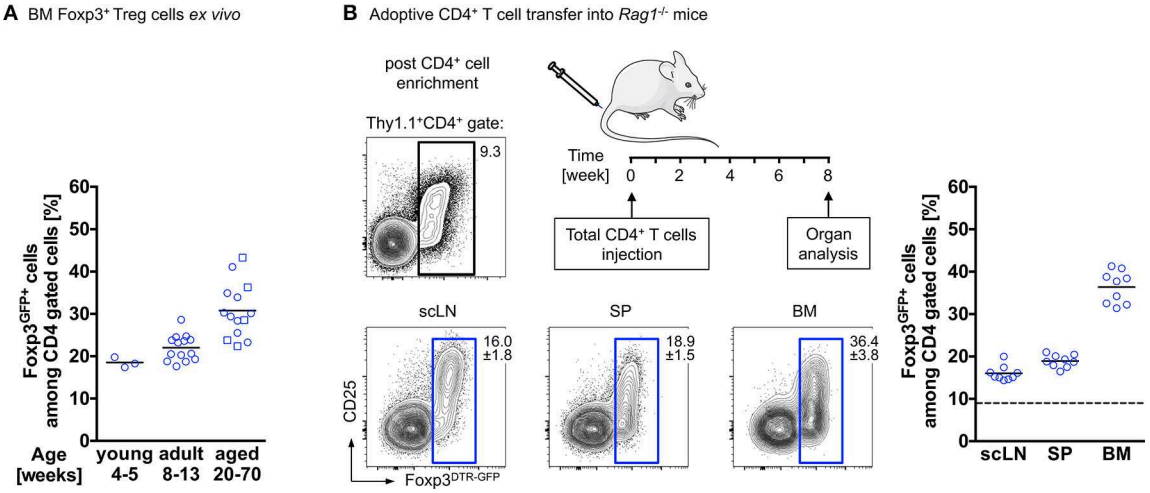

FIGURE 1 | Accumulation of Foxp3$^+$ Treg cells in the BM. **(A)** Age-related accumulation of BM-resident Foxp3$^+$ Treg cells. Flow cytometry analysis of Foxp3^{GFP+} cells within the CD4$^+$ T cell population in the BM of wildtype mice of different age groups. Each experiment was performed with at least 3 mice and symbols and lines indicate individual mice and mean values, respectively (young: 4–5 weeks; adult: 8–13 weeks; aged: circles: 20–22 weeks; and squares: 50–70 weeks). **(B)** Schematic overview of the experimental design. Four-week-old *Rag1*$^{-/-}$ mice were adoptively transferred with bulk CD4$^+$ T cells (upper plot: Foxp3$^+$ Treg cell proportion among total CD4$^+$ T cells before adoptively transferred). The distributions of gated Foxp3$^{DTR-GFP+}$ cells in various organs of the recipient mice (scLN, subcutaneous lymph nodes; SP, spleen, and BM, bone marrow) were analyzed by flow cytometry 8 weeks after transfer. Numbers in plots indicate the mean percentages ± SD of gated cells within the respective gate. Graph illustrates the accumulation of Foxp3$^+$ Treg cells in the BM. Data are collected from two independent experiments with 4–5 mice; symbols and lines denote individual mice and mean values, respectively.

Lympho-Hematopoiesis in Mice With Constitutive Treg Cell Deficiency

Scurfy mice exhibit a spontaneous loss-of-function mutation in the gene encoding Foxp3, resulting in complete absence of Foxp3 protein and functional CD4$^+$Foxp3$^+$ Treg cells. In addition to systemic autoimmunity targeting several organs such as skin, lung, stomach and liver, severe lympho-hyperproliferation represents another hallmark of the scurfy phenotype, resulting in massively increased secondary lymphoid organs and peripheral immune effector compartments (82, 84).

In initial studies in 3 to 4-week-old scurfy mice, we unexpectedly noticed that the immature B cell compartment in the spleen was essentially completely absent, which appeared to be at odds with the existing mature B cell compartments at the same anatomical site. A more systematic analysis of peripheral B cell compartments revealed even higher numbers of mature B cells in lymph nodes of scurfy mice as compared to wildtype control mice (69). These observations raised several important questions, including the origin of mature B cells in scurfy mice and the possibility that scurfy mice exhibit a heretofore-unappreciated defect in B cell development in the BM, in addition to the well-known hematopoietic bias in scurfy mice towards macrophages (85, 86). When we extended our analysis to lympho-hematopoiesis, it became clear that adolescent scurfy mice exhibit severe B cell developmental defects with near-complete absence of B lymphopoiesis in the BM. Pre-B-II and immature B cell stages were found consistently below the detection level. Several lines of evidence such as the indistinguishability of the BM microenvironment of scurfy and Foxp3-proficient mice within the first week of life with regard to the capacity to support B cell development *in vivo* argue against a constitutive lympho-hematopoietic defect, but support the assumption of an ontogenetic acquisition of B cell developmental defects in scurfy mice. The apparent inconsistency between previously described significant residual B lymphopoietic activity in the BM of 28-day-old scurfy mice (39) and the almost entire block of B cell development observed in mice of the same age in our study is most likely due to differences in the kinetics of autoimmune manifestations, perhaps due to microbial flora variations between independent colonies of Foxp3-deficient mice.

Overall, our results were further corroborated by other recent studies showing that the constitutive absence of Foxp3$^+$ Treg cells impinges on lympho-hematopoiesis in the BM although significant differences between those studies exist. In conjunction with aberrant B lymphopoiesis and in line with other reports, we detected aberrantly increased proportions and numbers of Lin$^-$Sca-1$^+$c-Kit^{hi} (LSK) cells early during ontogeny, suggesting that HSCs might be directly affected by inflammatory cytokines such as type I and type II IFNs. Analysis of *in vivo* reconstitution capacities of scurfy-derived HSCs in BM irradiation chimeras provided inconsistent results, with either substantially reduced or efficient although delayed reconstitution of lymphoid and myeloid lineages (38, 39, 87–89). Moreover, the underlying mechanism and in particular the direct and/or indirect roles of Treg cells remain a matter of debate. While it has been suggested that an altered cytokine milieu in scurfy mice promotes the differentiation of myeloid lineage cells at the expense of lymphopoiesis (87, 89, 90), indicating that the hematopoietic changes are not directly caused by the lack of Treg cells,

additional mechanisms such as significantly increased cell death of developing B cells may affect B lymphopoiesis (88).

Nevertheless, our data indicate that the observed defects may not be exclusively mediated by systemic autoimmunity that negatively feeds back to BM hematopoiesis, as neonatal adoptive Treg cell therapy suppressed exacerbated production of inflammatory cytokines such as IL-17 and IL-6, but not IFN-γ and restored defective thymopoiesis, but was ineffective in recovering defective hematopoiesis and B lymphopoiesis. One possible explanation for this might be the failure to re-establish efficient production of CXCL12 and IL-7, which was consistently decreased in the BM of untreated scurfy mice (69). Thus, it appears likely that the aberrant B lymphopoiesis in scurfy mice is a consequence of the diversion of lymphoid progenitors from the B lymphoid lineage in favor of increased myelopoiesis (85–87, 89, 90) and the corporate action of the enhanced systemic production of helper T cell cytokines and locally reduced expression of B lymphopoiesis-promoting factors in the BM (69).

Overall, our observations indicate that re-establishment of immune homeostasis is not sufficient to reinstate hematopoietic homeostasis. We speculate that the adult BM niche might be irreversibly affected by constitutive Treg cell deficiency, which is in line with the severe bone loss afflicted with pronounced autoimmune pathologies, we and others (87) detected in scurfy mice.

Lympho-Hematopoiesis in Mice With Acute Treg Cell Ablation

In contrast to the above described conventional mouse models and depletion strategies, the diphtheria toxin (DT)-mediated deletion of Foxp3$^+$ Treg cells allows for efficient, selective and temporally controlled depletion of Foxp3$^+$ Treg cells without affecting CD25$^+$ effector T cells (91, 92) and thus has provided essential information on Foxp3$^+$ Treg cell biology in health and disease.

However, striking differences exist with regard to depletion efficiency and severity of clinical symptoms after Treg cell ablation (93, 94). Two independent mouse models with an insertion of a DT receptor (DTR) into either the endogenous Foxp3 locus (91) or a Foxp3 promoter-encoding bacterial artificial chromosome [BAC; these mice were termed "depletion of regulatory T cells" (DEREG) mice, (92)] were generated in parallel. In both models, expression of the DTR under the control of the Foxp3 promoter and administration of DT leads to the induction of apoptotic cell death due to blockage of protein synthesis (95), specifically in Foxp3$^+$ Treg cells. When DT was applied to neonatal mice, both, the BAC-transgenic and the knock-in approach resulted in an autoimmune disease similar in severity to that of Foxp3-deficient scurfy mice (91, 92). However, while DT-induced Treg cell ablation in adult mice did not induce scurfy-like disease in DEREG mice (92), Foxp3^{DTR} knock-in mice succumbed to a severe lympho-hyperproliferative disorder (91), which might be explained by different efficiencies with regard to transgene expression (93). Another study assessed BAC transgenic Foxp3DTR lines (Foxp3.LuciDTR, specifically generated for bioluminescence imaging of Treg cells) for depletion efficiencies and signs of pathology and demonstrated that lines with depletion efficiencies of >95% succumbed to a wasting disease, whereas lines with depleting efficiencies <95% lacked the onset of autoimmunity (96).

To deplete Foxp3$^+$ Treg cells in adult steady-state mice, we therefore took advantage of the well-characterized DEREG mouse model (92–94, 97).

Our results show that upon administration of consecutive doses of DT, Foxp3^{GFP+} Treg cells in primary (thymus, BM) and secondary (spleen, lymph nodes, peritoneum) lymphoid organs of NOD.Foxp3$^{DTR-GFP}$ mice were highly efficiently depleted with minimal systemic effects due to rapid recovery of the depleted Treg cell compartment. In contrast to DT-treated C57BL/6 and Balb/c mice, NOD control mice did not show any DT side effects. As a read-out for the impact of Treg cell activity on the generation of adaptive immune cells from HSCs, we assessed the developmental B lymphopoietic activity in the absence and presence of Foxp3$^+$ Treg cells. Our comprehensive analysis of the BM niche revealed that acute Foxp3$^+$ Treg cell ablation in Foxp3$^{DTR-GFP}$ mice results in rapid dysregulation of lympho-hematopoiesis, as evidenced by a strongly increased HSC compartment size (**Figure 2A**) and a strong reduction of BM-residing mature B cells (**Figure 2B**). Of note, and in contrast to our results obtained in mice with constitutive Treg cell deficiency showing that B cell development was almost

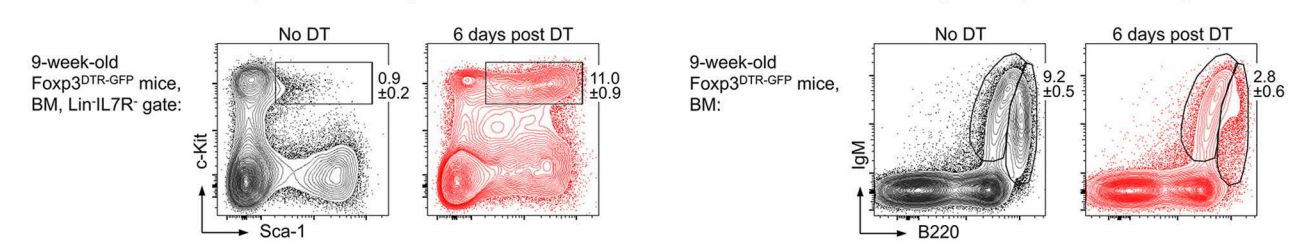

FIGURE 2 | Impact of Foxp3$^+$ Treg cell ablation on the BM niche. Foxp3$^{DTR-GFP}$ mice were *i.p.* injected with DT on 3 consecutive days and the BM was analyzed at day 6 after the first DT dose by flow cytometry. Representative plots of **(A)** LSK (Lineage$^-$IL7R$^-$Sca-1$^+$c-Kit$^+$) cells and **(B)** mature/recirculating B cells (B220^{high}IgM$^+$) in the BM of 9-week-old untreated (left) and DT-treated (right) mice.

completely abrogated (69), we observed that acute Treg cell ablation in adolescent NOD.Foxp3$^{DTR-GFP}$ mice induced only a slight reduction in B cell development. In contrast to scurfy mice, where already the earliest c-Kit$^+$ Pro/Pre-B-I cell precursors were significantly reduced, this compartment remained unaffected by the DT-mediated Treg cell depletion, emphasizing that the effects observed in scurfy mice are not directly caused by Treg cell deficiency.

The apparent discrepancy between the recently reported almost complete block in B cell development observed in C57BL/6 Foxp3^{DTR} knock-in mice (98) and the only partial block in early B cell development we observed in NOD.Foxp3$^{DTR-GFP}$ mice, could most likely be attributed to scurfy-like lethal autoimmunity and unwanted DT side effects that have been described for the former mouse model but not for the latter (91, 92, 94, 99, 100).

Overall, the prompt alteration of B lymphopoiesis in the BM upon DT-mediated Treg cell ablation in the absence of overt autoimmunity further support a potential direct role of Treg cells in hematopoiesis.

BONE HOMEOSTASIS AND FOXP3$^+$ TREG CELLS

Osteoclast Development and Treg Cells

Osteoclasts, exclusive bone-resorbing cells of hematopoietic origin, are giant multinucleated cells derived from fused precursor cells of the myeloid lineage. The development of mature osteoclasts from early myeloid progenitors is a tightly controlled process involving multiple mediators within the bone microenvironment. Osteoclastogenesis is controlled by ligation of macrophage colony-stimulating factor (c-Fms; M-CSF) and membrane receptors for receptor activator of NF-kB ligand (RANK; RANKL). Under physiological conditions, osteoblast lineage cells, which are of mesenchymal origin, seem to be the main source for M-CSF and RANKL. The central role of M-CSF in survival and proliferation of osteoclast precursors was already revealed decades ago, by studying osteopetrotic op/op mice that bear a natural mutation in the *Csf1* gene coding for M-CSF (101, 102). The discovery of the RANK/RANKL/osteoprotegerin (OPG) signaling axis represents another milestone in bone biology: the finding that RANKL-deficient mice develop severe osteoclast-deficient osteopetrosis, whereas overexpression of RANKL or the lack of the decoy receptor for RANKL, OPG, result in osteoporosis, due to excessive osteoclast formation (103–105), emphasize the critical role of these pathways in osteoclastogenesis.

RANKL promotes the differentiation of committed osteoclast precursor cells by inducing the expression of several genes, such as *cathepsin K*, *tartrate-resistant acid phosphatase* (*TRAP*) and *calcitonin receptor*, as well as transcription factors, such as NF-κB, c-Fos, and the master transcription regulator of osteoclast differentiation NFATc1 (106). Important signals downstream of c-Fms include the PI3K/Akt pathway, crucial for cell survival and the MAPK/ERK pathway essential for survival and proliferation of precursor cells (4).

The discovery that activated T cells express RANKL along with the identification of RANKL as a key differentiation factor for osteoclasts represented one of the first links between the immune and the bone system (107–109). To date, the impact of numerous inflammatory cytokines on the fate of bone cells has been investigated *in vitro* and *in vivo*, and it has been shown that for example IL-1, TNF-α, IL-17, and IL-6 promote osteoclastogenesis by either inducing RANKL expression or directly acting on osteoclast precursor cells, whereas IL-10, IL-4, IFN-γ, and GM-CSF have inhibitory effects on osteoclast differentiation and function, illustrating the tight relationship of osteoclast precursor cells with other cells in the bone microenvironment such as innate and adaptive immune cells (9).

Despite extensive research efforts, osteoclast developmental stages as well as the precise identity of bona fide osteoclast precursors have remained poorly defined and thus there is still fundamental lack of reliable methods for *ex vivo* purification of osteoclasts and their precursors. For this reason, most studies are based on *in vitro* differentiated osteoclasts derived either from macrophages expanded *in vitro* in the presence of M-CSF or from unfractionated whole BM cells cultured under osteoclastogenic conditions, i.e., in the presence of M-CSF and RANKL. However, both methods have their drawbacks since interpretation of data obtained from these cultures is hampered by the heterogeneity of the starting population of which only a minor faction represents osteoclast progenitor cells that differ in their growth kinetics and differentiation potential. Moreover, isolation of primary cells from their *in vivo* microenvironment and subsequent *in vitro* culture results in altered phenotypic and functional characteristics of these cells.

To characterize and isolate osteoclast precursor cells, we combined complex state-of-the-art experimental approaches such as osteoclast differentiation and functional assessment (110, 111) with high-end flow cytometry and cell sorting of very rare cell populations employing a comprehensive array of hematopoietic cell surface markers suggested in varying combinations in the literature (112–116). Multicolor flow cytometry allowed for identification and prospective isolation of these infrequent cell subsets, which — in some cases — account for <0.1% of total BM cells in wildtype mice. To determine the osteoclastogenic potential of the identified populations, FACS-purified cells were cultured in the presence of M-CSF and RANKL. Differentiated osteoclasts were detected as giant, multinucleated, and TRAP-positive cells.

With regard to CD11b expression on potential progenitor cells earlier studies have yielded contradictory results: while some groups described CD11b$^+$ precursor cells to exhibit high osteoclastogenic potential, others reported on CD11b$^{-/low}$ cells being more potent (114–118). In our hands, B220$^-$CD3$^-$Ter119$^-$CD11b$^+$ BM cells did not have a high osteoclastogenic potential, independent of the expression of Ly6C, whereas the B220$^-$CD3$^-$Ter119$^-$CD11b$^{-/low}$Ly6C^{hi} cell population contained the majority of osteoclast progenitors (**Figure 3**, left), which is in accordance with recent findings by Nakamura and colleagues (116). Since that study detected heterogeneous CD115 (c-Fms) and CD117 (c-Kit) expression on B220$^-$CD3$^-$Ter119$^-$CD11b$^{-/low}$Ly6C^{hi}

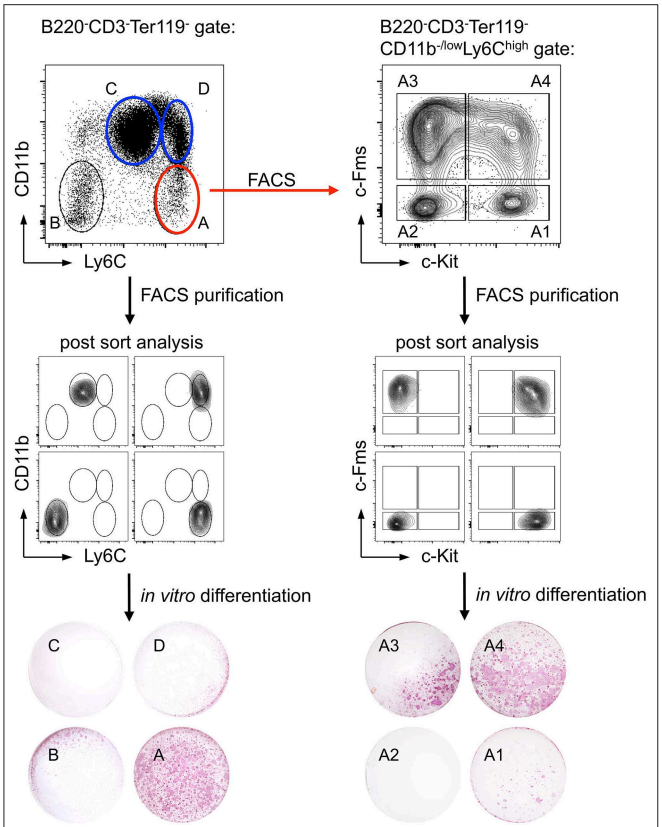

FIGURE 3 | Identification of a cell population with high *in vitro* osteoclast differentiation potential. Ly6C and CD11b expression of bead-enriched B220^{-}CD3^{-}Ter119^{-} BM cells was detected by flow cytometry. Four distinct populations (A: Ly6C^{high}CD11b$^{-/low}$; B: Ly6C^{-}CD11b^{-}; C: Ly6C^{low}CD11b^{+}; D: Ly6C^{+}CD11b^{+}) were FACS-purified and after post-sort analysis *in vitro* differentiated under osteoclast-forming conditions (cytokines: M-CSF and RANKL). The differentiated cultures were fixed and a TRAP staining was performed to identify mature osteoclasts. Additionally, population A was further subdivided by the expression of c-Kit and c-Fms (A1: c-Kit^{+}c-Fms^{-}; A2: c-Kit^{-}c-Fms^{-}; A3: c-Kit^{-}c-Fms^{+}; A4: c-Kit^{+}c-Fms^{+}). After FACS-purification and post-sort analysis, the populations were cultured and analyzed for their osteoclastogenic potential.

osteoclast progenitor cells (116) and others identified CD115^{+}CD117^{+} double positive cells as highly osteoclastogenic (113, 115), we applied in our study a combination of the surface markers used in these studies. Our results show that B220^{-}CD3^{-}Ter119^{-}CD11b$^{-/low}$Ly6C^{hi}CD115^{+}CD117^{+} BM cells efficiently differentiate into mature TRAP-expressing osteoclasts *in vitro* (**Figure 3**, right). Initial analyses of the BM niche of Foxp3$^{DTR-GFP}$ mice provided first evidence that acute Foxp3^{+} Treg cell ablation results in substantial changes in osteoclast precursors in the BM.

The sorting of low-abundance prospective primary osteoclast precursors to high purity allow for detailed *ex vivo* analysis without contamination by unwanted cells and hence represents a powerful method to evaluate how Treg cells are affecting the formation of osteoclast precursors in mouse models with abrogated or enhanced Treg cell activity.

Osteoclast Function and Treg Cells

Foxp3^{+} Treg cells control adaptive and innate immune responses by the suppression of activation, proliferation and function of various immune cell types, as for example CD4^{+} helper T cells, CD8^{+} cytotoxic T cells, B cells, NKT cells, macrophages, and dendritic cells (DCs). Multiple mechanisms of Foxp3^{+} Treg cell-mediated suppression have been proposed, involving cell contact-dependent and cell contact-independent mechanisms. The basic mechanisms used by Treg cells include (a) secretion of inhibitory cytokines, such as TGF-β, IL-10, and IL-35, (b) induction of cytolysis by for example granzymes, (c) metabolic disruption by e.g., IL-2-deprivation-mediated apoptosis, and (d) functional modification of antigen presenting cells (APCs) such as DCs. Expression of the cytotoxic T-lymphocyte antigen 4 (CTLA-4) enables Treg cells to interact with CD80/CD86 on DCs outcompeting CD28-mediated co-stimulation and thereby indirectly preventing differentiation of effector T cells (119).

In this context, it has been suggested that Treg cells have the ability to suppress osteoclastogenesis *in vitro*, but the mechanisms of suppression remain incompletely understood and controversial: while some studies identified inhibitory cytokines as key players in Treg cell-mediated suppression of osteoclasts, other reports suggested cell-cell-contact dependent mechanisms (40, 47, 48, 120–122). Most studies addressing the impact of Foxp3^{+} Treg cells on bone homeostasis *in vivo* employed mouse models with either constitutive Foxp3 deficiency or constitutive Foxp3 overexpression (48, 87, 123). As mice with constitutive Foxp3 deficiency suffer from a massive autoimmune lymphoproliferative disease and die approximately at 3 weeks of age (84) and overexpression of Foxp3 in CD4^{+} T cells *in vivo* results in reduced total numbers and functional impairment of T cells (124, 125) potential direct effects of gain and loss of Foxp3^{+} Treg cells are difficult to dissect from the impact of systemic immune dysregulation.

To our knowledge, the impact of temporally controlled, transient Foxp3^{+} Treg cell ablation (91, 92) on skeletal homeostasis under physiological conditions has not been addressed so far.

Osteoclasts and DCs share several features (126, 127). In this context, a recent study proposed that osteoclasts express the co-stimulatory molecules CD80 and CD86 and that Treg cells can regulate osteoclast differentiation via CTLA-4 (128). Moreover, it has been described that BM-residing Treg cells express higher levels of CTLA-4 than peripheral Treg cells (68). Therefore, we hypothesize that this direct cell-cell interaction may indeed play a central role in the crosstalk of osteoclasts and Treg cells.

To investigate the direct interaction of osteoclasts and Treg cells, we performed *in vitro* co-cultures of Treg cells and BM-derived precursor cells. These experiments revealed that the expression of co-stimulatory molecules on the surface of osteoclasts is down-regulated in the presence of Treg cells which leads to the suppression of osteoclast differentiation and function. To directly study cell-cell interactions, cells harvested from these cultures were subjected to simultaneous analysis by multi-parameter flow cytometry and fluorescence microscopy. Imaging flow cytometry showed the direct cell-cell interaction between individual CD11b^{+} late osteoclast precursors and Foxp3^{GFP+}

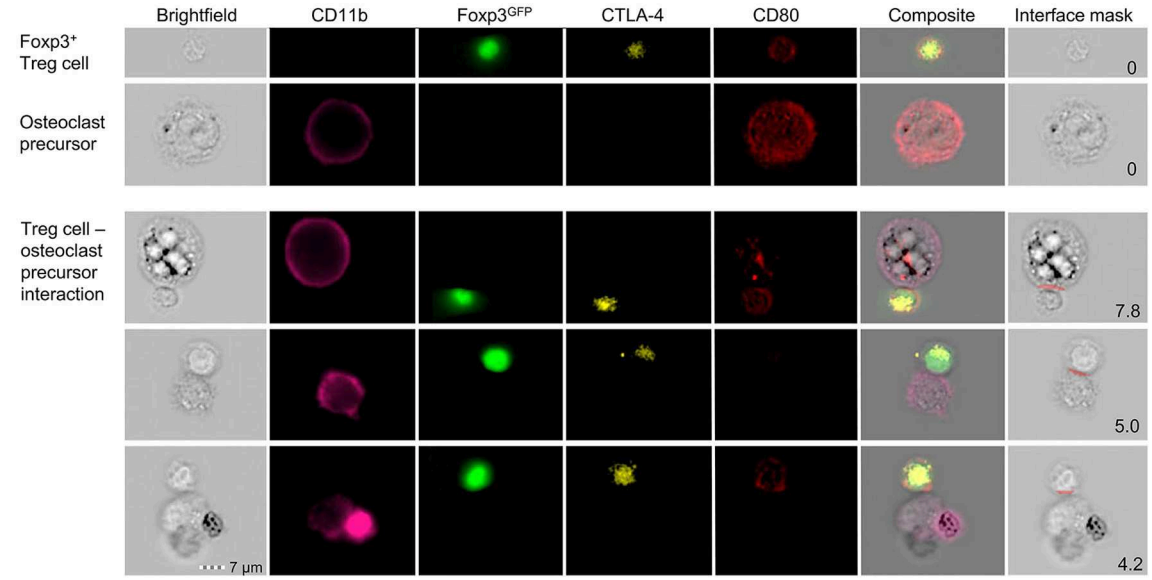

FIGURE 4 | Detailed characterization of *in vitro* Foxp3^{+} Treg cell-osteoclast precursor interaction. Representative single cell (upper panels) and cluster (lower panels) analysis by imaging flow cytometry. Stimulated FACS-purified CD4^{+}CD25^{+}Foxp3^{GFP+} Treg cells and freshly isolated irradiated T-cell-depleted splenocytes were added to the BM culture and cultured under osteoclastogenic conditions. After 48 h, Foxp3^{+} Treg cells and CD11b^{+} osteoclast precursors were analyzed for intracellular expression of CD80 and CTLA-4 by imaging flow cytometry. Cell clusters depict physical interaction of CTLA-4^{+}Foxp3^{GFP+} Treg cells and CD11b^{+} osteoclast precursors. Co-localization of CD80 in Treg cells indicates trans-endocytosis from CD11b^{+} osteoclast precursors. Numbers indicate the interface area and illustrate the overlapping fluorescence of Foxp3 and CD11b.

Treg cells which additionally exhibited intracellular red puncta (**Figure 4**), providing first direct evidence that Treg cells can remove CD80/CD86 from the surface of osteoclast precursors by CTLA-4 mediated trans-endocytosis, potentially leading to reduced co-stimulation by osteoclasts — a mechanism which has been described for the interaction of Treg cells and APCs such as DCs (129).

In sum, available data suggest that Treg cells can interfere with bone resorption by affecting osteoclast development and function at multiple stages. However, the mechanism of action is still controversial, emphasizing the complexity of this relationship.

Whether or not T cell regulation of bone and hematopoietic homeostasis requires APCs within the bone microenvironment, is still unknown, but based on the notion that osteoclasts are derived from hematopoietic precursors of the myeloid lineage, express a number of immune receptors and are regulated similarly to macrophages and DCs (130), it is a legitimate question, whether osteoclasts play a role in the active regulation of the immune system, can act e.g., as APCs. Indeed, albeit critically discussed (131), it has been suggested that, in addition to their ability to resorb bone, *in vitro* differentiated osteoclasts can function as APCs and activate CD4^{+} and CD8^{+} cells (132). In this context, it was reported that osteoclasts are capable to cross-present antigens to CD8^{+} T cells, thereby inducing Foxp3 in these cells, which were characterized as osteoclast-induced regulatory CD8^{+} T cells (133–135).

In addition, recent data suggest that osteoclasts can on the one hand participate in immunogenic T cell responses under chronic inflammatory diseases (136) and on the other hand exhibit immune suppressive functions in multiple myeloma (137, 138). Whether these proposed immune-modulating functions of osteoclasts play a role under physiological conditions or depend on the pathological conditions still needs to be defined. Data from our own group propose the concomitant up-regulation of c-Fms, RANK, and the ligand for the immune checkpoint molecule programmed death 1 protein (PD-1; PD-L1) on the surface of murine CD11b^{+} osteoclast precursors cultured under osteoclastogenic conditions, which complements the findings on human osteoclast precursor cells (137).

Overall, these observations open additional new prospects for the role of osteoclast in the intercellular interaction of bone cells and immune cells in health and disease (**Figure 5**).

CONCLUSION

The clinical relevance of the BM niche is reflected by its function as (a) the primary hematopoietic site in the adult, (b) a reservoir for memory cells, and (c) in the support of HSC transplantation. The nature of the HSC niche in the BM is defined by various components of the BM microenvironment. Several independent lines of evidence are pointing towards specialized local functions of BM-residing Treg cells in settings of unwanted (autoimmunity, transplant rejection, GvHD) and insufficient immunity (cancer, chronic infections). Modulation of Treg cell activity *in vivo* can be achieved by means such as application of IL-2/anti-IL-2 immune complexes to enhance Treg cell function, whereas depletion or other means of interference with Treg cell function promotes immune responses. This raises the question whether comparable strategies may also be suitable to modulate bone remodeling to manipulate lympho-hematopoiesis in the adult

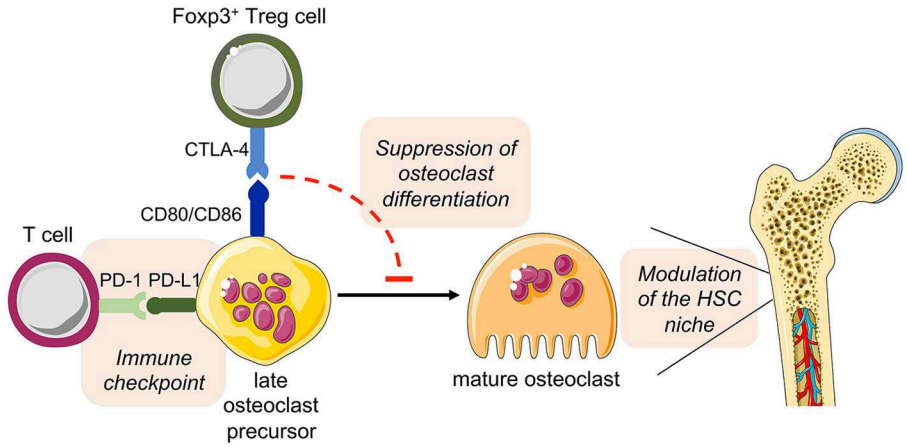

FIGURE 5 | Hypothesized direct cross-talk between osteoclast precursors and T cells and the indirect effect on hematopoiesis. The expression of the costimulatory molecules CD80 and CD86 on osteoclast precursors has been implicated in the interplay with Foxp3^{+} Treg cells (via the inhibitory molecule CTLA-4), leading to the suppression of the differentiation of osteoclast precursors into mature functional osteoclasts. As a consequence, disturbed bone remodeling results in modulation of the HSC niche. On the other hand, osteoclast precursors are able to upregulate the expression of PD-L1, whose interaction with PD-1 on T cells has immune modulatory functions, thus representing a crucial immune checkpoint. This figure was in part created with modified Servier Medical Art templates, licensed under a Creative Commons Attribution 3.0 Unported license: http://smart.servier.com.

BM. Are there niche-associated Treg cell subsets that specifically interact with bone cells such as osteoclasts? Overall, future studies are warranted to further define the molecular pathways involved in the complex intercellular communication between BM-residing Treg cells, hematopoietic stem and progenitor cells, mature immune cells, and the skeletal system. Understanding the immune regulatory mechanisms in the BM microenvironment is central for the development of effective therapeutic strategies for the treatment of bone diseases and to improve current protocols for HSC transplantation and peripheral immune reconstitution.

AUTHOR CONTRIBUTIONS

LF, CH, RK, SD, and JR designed, performed, and analyzed the experiments. KK and AG conceived the research, guided its design, analysis, and interpretation and wrote the manuscript. All authors contributed to discussions and writing of the manuscript.

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