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Bone and the Innate Immune System

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Abstract

The immune system and bone are intimately linked with significant physical and functionally related interactions. The innate immune system functions as an immediate response system to initiate protections against local challenges such as pathogens and cellular damage. Bone is a very specific microenvironment in which infectious attack is less common but repair and regeneration are ongoing and important functions. Thus in the bone the primary goal of innate immune and bone interactions is to maintain tissue integrity. Innate immune signals are critical for removal of damaged and apoptotic cells and to stimulate normal tissue repair and regeneration. In this review we focus on these innate immune mechanisms that function to regulate bone homeostasis.

Keywords

Bone; Innate immunity; Osteoclast; Osteomac; Osteocyte; Apoptosis; Autophagy; Necroptosis

Introduction

The recognition that immune cell function directly influences bone remodeling with reciprocal support and influence over immune cells by bone cells has led to the active research field of osteoimmunology. In addition to providing a structural support for the body and a reservoir for calcium, bone encases the bone marrow, a primary site for hematopoiesis and immune system development. Immune system influences on bone are diverse and have been the topic of several recent reviews, which have highlighted the effects of activation of the adaptive immune system on bone in the setting of infection, osteoporosis, cancer and autoimmunity (1–5). In this review, we address the impact of the innate immune system on

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Conflict of Interest

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Human and Animal Rights and Informed Consent

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bone, particularly with respect to the role of the innate immune system in tissue repair and homeostasis.

The innate immune system: source of cytokines

The innate immune system provides an immediate, invariant response to infection and injury with the goals of limiting the spread of infection, initiating the adaptive immune response and initiating tissue repair. Innate immune cells such as macrophages and dendritic cells (DC) utilize pattern recognition receptors (PRR) to recognize local “danger signals” such as DAMPs (damage-associated molecular patterns) released by tissue damage or PAMPS (pathogen associated molecular patterns) displayed by infectious organisms. Activation via PRRs leads these cells to produce inflammatory cytokines such as TNF α , IL-1, IL-6 and interferon- γ (IFN- γ). These inflammatory cytokines have direct effects on both osteoclasts (OC) and osteoblasts, as well as indirect effects on osteoclasts via osteoblast upregulation of RANKL, the key cytokine for osteoclastogenesis, which has been the topic of recent reviews (6–8). Other innate immune cells, natural killer cells and neutrophils, are reported to produce RANKL in inflamed synovial tissue and in response to LPS, respectively, but the physiologic significance compared to osteoblast, osteocyte and T cell derived RANKL is unclear (9, 10).

Bone resorption: an innate immune function?

Myeloid progenitors for osteoclasts can also differentiate to DC and macrophages (11, 12), suggesting the close relationship of osteoclasts to cells of the innate immune system. Interestingly, both human and mouse CD11c⁺ DC transdifferentiate into osteoclasts in vitro (13, 14) and mouse DC can contribute to osteoclast formation *in vivo* (15). It does not appear DC are critical for normal bone turnover as DC deficient mice do not have a bone phenotype (16), but it is possible that DC contribute to pathologic bone loss as transdifferentiation is promoted by rheumatoid synovial fluid, inflammatory cytokines and in multiple myeloma (13, 17).

Classic stimuli for innate immune cells also directly regulate osteoclast differentiation. For example, PAMPs can directly regulate osteoclasts via osteoclast toll like receptors (TLR), although the effect of TLR activation depends on the stage of osteoclast differentiation (18, 19). TLR activation inhibits differentiation of early osteoclast precursors but can stimulate late osteoclast differentiation and increases survival of mature osteoclasts. Activation of TLRs in osteoblasts or stromal cells stimulates production of osteoclastogenic cytokines, such as RANKL and TNF- α . Similarly, Fc γ RIV crosslinking by immune complexes enhances osteoclast differentiation in vitro and osteoclast specific deletion of Fc γ RIV partially protects mice from bone erosion in inflammatory arthritis (20). Osteoclasts are regulated by a number of other innate immune receptors and are able to rapidly initiate bone remodeling and calcium mobilization in response to microenvironmental signals. Thus, it can be argued that osteoclasts are innate immune cells of the bone (21).

In the bone microenvironment, infectious insults are less common but repair and regeneration are ongoing and critical functions. Ongoing bone resorption is important for bone homeostasis. Bone remodeling occurs continuously to adapt to changes in load and to

replace damaged bone (22). The absence of osteoclast function results in osteopetrosis, but the resulting bone may be compromised due to lack of repair functions (23). Tissue repair and homeostasis requires an innate immune response, which is of particular relevance in bone.

Innate Immunity and Tissue Homeostasis

In addition to its well-recognized function as the first line of defense against pathogens, the innate immune system plays a crucial role in tissue homeostasis and repair. Tissue resident macrophages are increasingly recognized as important sources of trophic factors in tissue homeostasis and repair (24). A further critical function of the innate immune system in tissue homeostasis is active surveillance and eradication of damaged or dying cells in the local microenvironment. Cell death can occur in several ways and the innate immune system responds differently to each type of cellular damage. Macrophages are the primary scavengers removing apoptotic cells, which have undergone programmed cell death during tissue remodeling or in response to stress. During injury, necrotic cells release DAMPs, initiating inflammatory cytokine release and influx of inflammatory cells. Resolution of inflammation is a prerequisite for tissue repair, and the innate immune system contributes in the form of alternatively activated (M2) macrophages and myeloid derived suppressor cells (MDSC) among other mechanisms. Autophagy, the degradation of cytoplasmic components in response to nutritional stress, may be the most primitive form of innate immunity and is crucial for cellular homeostasis. Autophagy can function both in direct microbial elimination and is activated by stimulation by DAMPs as well as PAMPS in the setting of infection (25). Thus, innate immune stimuli in injury or infection stimulate homeostatic mechanisms at both the cellular and tissue level.

The recent identification of bone resident tissue macrophages distributed throughout the periosteum and associated with sites of bone modeling in vivo suggests that in bone, as in other tissues, resident macrophages may contribute to tissue homeostasis. Consistent with this, these “osteomacs” promote osteoblast differentiation and mineralization in vitro and their depletion in vivo rapidly destroyed maintenance of the osteoblast bone modeling surface (26). More recently, osteomacs were shown to promote bone matrix deposition and mineralization in a bone injury model (27), suggesting that these innate immune cells are important for both basal bone homeostasis and tissue repair.

Innate Immune Recognition of Cell Death and Bone

Cell turnover is an important aspect of bone remodeling and apoptosis of osteoclasts and osteoblasts has long been suggested to contribute to bone mass regulation (28). *Apoptosis* or programmed cell death is an essential process that organisms use to remove unwanted or defective cells during development and tissue remodeling. Factors that activate the apoptotic death pathway include DNA damage, hypoxia and activation of death receptors. Apoptotic-inducing stimuli initiate a caspase activation cascade and increased permeability of the outer mitochondrial membrane. Apoptotic cells must be cleared by the immune system and are considered immunologically silent because they do not generally stimulate production of inflammatory cytokines (29). Macrophages and/or stromal cells remove apoptotic cells by

phagocytosis and failure of this removal is a factor leading to chronic inflammation and autoimmunity. A number of receptors have been implicated in engulfment of apoptotic cells by macrophages, dendritic cells and non-professional phagocytes, including CD36 and the scavenger receptors (29). Mice deficient in the scavenger receptor type A locus show increased bone mass, increased femur length and decreased osteoclasts (30, 31). In contrast a low bone mass phenotype is seen in *Cd36*^{-/-} mice with diminished osteoblast differentiation (32). Thus, it is likely that defects in removal of apoptotic cells effects bone homeostasis but the roles of specific receptors remains to be established.

Apoptosis of osteocytes, the mechanosensors of the bone, has been suggested to target osteoclastogenesis and initiate bone remodeling at specific areas of bone damage (33). Osteocyte apoptosis is induced in areas surrounding fatigue-loading microcracks, where bone remodeling is localized (34, 35) and caspase inhibition prevented fatigue induced cortical remodeling (36). During skeletal unloading, osteocyte apoptosis is induced and precedes osteoclastogenesis and bone loss (37, 38). The finding that a caspase inhibitor that blocks osteocyte apoptosis in mice abrogates bone loss due to skeletal unloading further suggests that osteocyte apoptosis is required to initiate skeletal unloading-induced bone loss (39). Other studies showed that blocking osteocyte apoptosis also blocks increased cortical bone resorption in mice after ovariectomy (40). Several studies recently demonstrated the importance of osteocyte produced-RANKL in cancellous bone remodeling. Mice deficient in RANKL specifically in osteocytes are markedly osteopetrotic despite production of RANKL by other cell types (41, 42). One of these studies also demonstrated that osteocyte produced RANKL was required for skeletal unloading induced bone loss (42). Interestingly, this latter finding suggests a role for osteocyte apoptosis in stimulating osteocyte production of RANKL. Studies of microcracks suggest that RANKL is expressed by healthy neighboring osteocytes rather than the apoptotic cells themselves (34). Thus, osteocyte apoptosis appears to be a key feature of bone response to diverse injuries.

Rapid clearance of apoptotic cells is implicated in maintaining tissue health and failure of clearance has been associated with autoimmunity, neuropathy, and atherosclerosis (43). Embedded in their matrix lacunae, apoptotic or dysfunctional osteocytes are inaccessible to macrophages and other professional phagocytic cells. Thus degradation of matrix by osteoclasts may be needed to remove damaged osteocytes. Osteoclasts also express engulfment receptors and are able to phagocytose apoptotic cells in vitro (44) and in vivo (45). Thus osteoclast activation in response to osteocyte apoptosis may serve to the dual function of clearance of apoptotic osteocytes and maintenance of bone homeostasis. Induction of osteocyte apoptosis in pathological bone turnover states such as estrogen deficiency, glucocorticoid use or skeletal unloading likely coopts the same cell death removal pathway and initiates osteoclast activation.

More recently an alternate type of programmed cell death, termed regulated necrosis or *necroptosis*, was described as a highly regulated cell death pathway, that leads to release of DAMPs and an inflammatory response (46). Necroptosis can be defined as a caspase-independent pathway to cell death that is dependent on the receptor-interacting protein kinase 1 (RIPK1)-RIPK3 complex (47). Necroptosis can be initiated by inflammatory or injury signals including TNF α , Fas ligand, TRAIL ligand, double-stranded RNA (dsRNA),

IFN- γ , ATP depletion, ischemia-reperfusion injury, and pathogens (48). Necroptosis leads to rapid plasma membrane permeabilization and release of cell contents with resultant exposure and release of damage-associated molecular patterns (DAMPs) (46). While the role of necroptosis has not been directly examined in bone cells, the known effects of the initiating factors such as TNF α on bone suggest that responses to necroptosis are likely also significant in regulating bone function. Due to the regulated nature of necroptosis, it will be of interest to determine if specific components of this pathway are required in distinct types of pathologic bone loss.

In contrast to controlled cell death, *necrosis* is considered accidental cell death due to extreme cellular physicochemical stress, damage or injury such as heat, osmotic or mechanical shock. Necrosis involves loss of membrane integrity and sudden release of intracellular content. Cellular damage releases DAMPs, which include HMGB1, IL-1 α , uric acid, DNA fragments, mitochondrial content, and ATP. The release of DAMPs to the extracellular space where they stimulate other cells leads to initiation of an acute inflammatory response such as seen with sepsis, ischemic injury or trauma (49).

During bone regeneration following injury, the inflammatory cytokines and macrophages have increasingly been recognized as critical elements during normal repair. Inflammatory cytokines TNF α , IL-1, and IL-6 are expressed at the fracture site within 24 hours of injury in mouse models of bone repair (50). During early fracture repair, levels of inflammatory cytokines are biphasic with acute rise of TNF α and IL-1 at initiation and a later peak at the transition from chondrogenesis to osteogenesis during endochondral maturation (50). TNF α receptor (p55^{-/-}/p75^{-/-}) knockout mice demonstrate delayed endochondral maturation in a delayed fracture repair model and IL-6 deficiency leads to delayed callus remodeling and mineralization (51, 52). Other acute inflammatory mediators such as the complement cascade have also been suggested to be important in bone regeneration (53). Complement components have been shown to regulate osteoclastogenesis however all the roles of complement in bone are not yet fully understood (54, 55). Thus, acute inflammation has an essential role in initiating repair, though persistent inflammation of this type is likely detrimental to bone homeostasis (56).

HMGB1 has also been implicated in stimulating tissue repair through regulation of stem cell trafficking and may similarly contribute to tissue repair in bone. During endochondral ossification HMGB1 has been shown to act as a chemotactic agent to osteoclasts and osteoblasts (57). Osteoblasts release HMGB1 in response to parathyroid hormone and recombinant HMGB1 treatment stimulates RANKL, TNF α and IL-6 expression by stromal cells and osteoblasts, thus potentially increasing the resorptive process contributing to bone repair (58–61). Consistent with this, deficiency of a HMGB1 receptor RAGE (receptor for advanced glycosylated end products) causes increased BMD and decreased osteoclast function (62, 63). RAGE deficiency also abrogates bone loss following ovariectomy (62).

Inflammasome activation in prosthetic osteolysis

Resolution of the initial inflammatory response is necessary for optimal wound healing and tissue repair. Prolonged inflammation is a factor in bone loss due to aging, estrogen

deficiency and autoimmune disease (64, 65). Pathologic bone loss due to prolonged inflammation is also seen in models of prosthetic osteolysis (66–68). Recent studies demonstrated that orthopedic particulate debris activates myeloid cells via both TLR signaling and inflammasome activation (69, 70). Production of pro-inflammatory cytokines especially IL-1 β and IL-18 is activated by a proteolytic cleavage cascade dependent on caspase-1. Activation of caspase-1 requires oligomerization of signaling proteins into a common structure termed the inflammasome. Thus, Inflammasomes are multi-protein complexes that respond to a variety of DAMPS and PAMPS by activating maturation of IL-1 (49, 71).

Human monocytes and mouse macrophages produce IL-1 in response to polymethylmethacrylate (PMMA) particle phagocytosis (70). In polyethylene (PE) particle-induced osteolytic lesions, TLR-2 and TLR-4 expression is elevated with a decrease in TLR-9 and both polymethylmethacrylate (PMMA) and PE particles activate macrophages via the TLR pathway, initiating secretion of TNF α and IL-6 (72, 73).

DAMP stimulation can activate the inflammasome cascade in macrophages and leads to production of IL-1. In a recent study by Burton and colleagues, the NALP3 inflammasome was found to be integral to the development of peri-implant osteolysis in mouse models. Cells from mice deficient in NALP3, ASC, or caspase-1 showed a decreased cytokine response to PMMA phagocytosis. PMMA particles implanted over calvaria of caspase-1 deficient mice also showed a reduced in vivo osteolytic response. The effect on bone resorption is secondary to inflammatory changes because absence of NALP3 inflammasome components does not cause a cell autonomous defect in osteoclastogenesis (70). Inflammasome activation in prosthetic osteolysis models likely contributes to osteoclast activation and the development of aseptic loosening (69, 70, 74). Similarly, activation of the inflammasome pathway has been shown to be important in the inflammatory responses to gout, CPPD and silica crystals (71, 75). These data suggest that orthopedic wear debris induce and activate components of the innate immune system, leading to production of inflammatory cytokines, and downstream effects on bone homeostasis.

Innate immunity, bone and resolution of inflammation

An important component of the homeostatic response to cellular damage is to protect tissue integrity. Thus innate immune mechanisms must also be in place to lead to resolution of inflammation following cellular injury or stress (76). Apoptosis of inflammatory cells is a non-inflammatory mechanism of cell removal and plays a critical role in successful resolution of the inflammatory response. Bone matrix resorption can release TGF β (77), which is a potent suppressor of classical macrophage activation and an important mediator of tissue repair (78). Tissue damage can promote production of glucocorticoid hormones which affects osteocyte viability, osteoclastogenesis and monocyte/macrophage polarization towards an anti-inflammatory state (79).

Inflammatory cytokines such as TNF α have long been known to increase osteoclast precursors in the bone marrow and periphery which has been thought to contribute to bone loss in inflammatory arthritis and other states of chronic inflammation (80). Interestingly,

we recently found that osteoclast precursors in the bone marrow are phenotypically the same as cells previously described as monocytic myeloid derived suppressor cells (M-MDSC) (11). In mice, we identified a myeloid precursor population in the normal bone marrow with osteoclast and suppressor cell potential that expanded during inflammatory arthritis. The osteoclast precursors could suppress CD4⁺ and CD8⁺ T cell proliferation in vitro and in vivo. It seems likely that the expansion of these osteoclast precursors actually represents a homeostatic mechanism in response to chronic inflammation to restrain T cell expansion. However, the MDSC mechanism is unfortunately ineffective in the inflammatory arthritis microenvironment, and the expanded pool of precursors is available for osteoclastogenesis. Recent studies by others have similarly shown that MDSCs expanded in response to a tumor environment can also differentiate into mature and functional osteoclasts in vitro and in vivo. Studies in both a xenograft model of myeloma and a mouse model of metastatic breast cancer suggest that tumor-induced MDSCs can promote lytic lesions and bone destruction by directly serving as osteoclast precursors (81, 82). Human differentiated osteoclasts have also been suggested to suppress T cell proliferation in vitro (83). Thus, an innate immune response to dampen inflammation can have a significant effect on bone through expansion of the shared myeloid precursor for osteoclasts and monocytic myeloid derived suppressor cells.

Autophagy for Cellular Homeostasis in Bone

Autophagy is thought to be a primordial form of innate immune pathogen elimination that has acquired a function in cellular homeostasis by enabling cells to survive stress or nutrient deprivation through the self-consumption of damaged cellular organelles (25). Autophagy is also important for the clearance of protein aggregates, targeting them for degradation (84). The process of autophagy initiates autophagosome formation and proteolytic degradation of cellular organelles and protein aggregates by lysosomes, freeing energy that can be used by the cell. Autophagy is also thought to regulate apoptosis (85), thus autophagy has both pro-survival and pro-death functions. Recent studies examining autophagy in bone cells and the requirement for autophagic components in osteoclast function have highlighted the interactions between these pathways, as discussed below.

Due to the physical structure of bone, autophagy is of particular relevance for osteocytes, cells embedded in bony matrix and cartilage which both lack direct access to vascular supplies of nutrients and oxygen. The importance of autophagy in osteocytes is seen in mice with deficiency of Atg7, a critical protein for autophagy, only in osteocytes using a DMP1-cre for tissue specific expression. Lack of Atg7 in osteocytes prevents autophagy, which leads to increased oxidative stress in the bones of young mice to levels of aged mice. At 6 months of age conditional Atg7 knockout mice had low bone mass at all skeletal sites, similar to elderly animals (86). The osteocyte specific Atg7 deficient animals showed abnormal bone turnover with decreased osteoclasts and decreased bone formation. Thus taken together the deficiency of autophagy in osteocytes led to skeletal changes of early aging, suggesting that autophagy may play a role to inhibit skeletal aging (86). Osteocyte autophagy is also likely to be an important contributor to pathologic changes in bone from glucocorticoid exposure. Osteocytes exposed to low dose glucocorticoids have been

demonstrated to undergo autophagy(87), however osteocytes exposed to high dose glucocorticoids or glucocorticoids for a prolonged period of time undergo apoptosis (88).

Interestingly, differentiation of myeloid precursors to functional osteoclasts utilizes components of the autophagy pathway. DeSelm and colleagues demonstrated that proteins essential for autophagy, including Atg5, Atg7, Atg4B, and LC3, are important for the development of the secretory function of osteoclasts (89). Localization of Rab7, which is required at the osteoclast ruffled border occurs in an Atg5-dependent manner. Formation of the external phagolysosome utilizes autophagy proteins to generate polarized secretion of lysosomal contents that contributes to functional bone resorption *in vitro* and *in vivo*. Mice deficient in *Atg5* in myeloid cells showed inhibited osteoclastogenesis and bone resorption and were partially protected from ovariectomy - induced bone loss (89).

The pathways of autophagy and osteoclastogenesis also converge on the scaffolding protein p62, also known as sequestosome 1 (SQSTM1), which colocalizes with the autophagic marker light chain 3 (LC3) to link polyubiquitinated protein aggregates to the autophagic machinery (90). Mutations in SQSTM1 are a known defect in some forms of familial Paget's, a disease of abnormally large and active osteoclasts (91, 92). SQSTM1/p62 is upregulated by RANKL stimulation and complexes with TRAF6 and the atypical PKC (93). Genetic deficiency of *p62* in mice leads to impaired osteoclastogenesis *in vitro* and *in vivo* (93), whereas *p62* mutations seen in Paget's disease increase osteoclast sensitivity to pro-osteoclastogenic cytokines (94). The shared usage of intracellular signaling components further suggests the linkage of the homeostatic functions of autophagy and bone resorption.

Conclusions

Innate immune mechanisms are required for maintenance of bone homeostasis which requires clearance of apoptotic cells, utilization of autophagy and resolution of inflammation in response to cellular damage. Likely due to their common goals, bone and immune cells utilize multiple shared pathways and precursors. These interactions are necessary for normal bone development, turnover and remodeling. Dysregulation of innate immune homeostatic pathways has been implicated in a wide variety of disease states such as autoimmunity, aging and atherosclerosis and the recognition of their importance should be extended to include multiple types of pathological bone loss.

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