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# From death to birth: how osteocyte death promotes osteoclast formation

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Bone remodeling is a dynamic and continuous process involving three components: bone formation mediated by osteoblasts, bone resorption mediated by osteoclasts, and bone formation-resorption balancing regulated by osteocytes. Excessive osteocyte death is found in various bone diseases, such as postmenopausal osteoporosis (PMOP), and osteoclasts are found increased and activated at osteocyte death sites. Currently, apart from apoptosis and necrosis as previously established, more forms of cell death are reported, including necroptosis, ferroptosis and pyroptosis. These forms of cell death play important role in the development of inflammatory diseases and bone diseases. Increasing studies have revealed that various forms of osteocyte death promote osteoclast formation via different mechanism, including actively secreting pro-inflammatory and pro-osteoclastogenic cytokines, such as tumor necrosis factor alpha (TNF- $\alpha$ ) and receptor activator of nuclear factor-kappa B ligand (RANKL), or passively releasing pro-inflammatory damage associated molecule patterns (DAMPs), such as high mobility group box 1 (HMGB1). This review summarizes the established and potential mechanisms by which various forms of osteocyte death regulate osteoclast formation, aiming to provide better understanding of bone disease development and therapeutic target.

## KEYWORDS

osteocyte, necrosis, apoptosis, necroptosis, ferroptosis, pyroptosis, osteoclast, osteoimmunology

## 1 Introduction

Bone remodeling is a dynamic and continuous process that ensures skeletal integrity by balancing bone formation and resorption. This process is essential for maintaining bone homeostasis, repairing microdamage, and adapting to mechanical forces. Bone remodeling involves three major cell types: osteoclasts, which mediate bone resorption; osteoblasts, which mediate bone formation; and osteocytes, which are embedded within the bone matrix and serve as key regulators of the remodeling process (1–3). Osteocytes, accounting for over 90% of bone cells, act as the important mechano-sensors and orchestrators of bone remodeling by balancing the activities of osteoclasts and osteoblasts (4, 5). Unbalance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation will lead to abnormal bone remodeling, which, if the unbalance prolongs, will further lead to various inflammatory bone diseases, such as osteoporosis, periodontal disease and rheumatoid arthritis (6). These diseases are partially characterized by excess osteoclastic activity. Osteoclasts differentiate from mononuclear/macrophages (7). This process is regulated by various signals from immune cells and bone cells. These interaction between bone cells and immune cells are recently referred as “osteimmunology” (8). Among these osteoimmune signals, receptor activator of nuclear factor-kappa B ligand (RANKL) is the predominant cytokine and is crucial in osteoclast formation (9). Studies have reported that osteocytes are an essential source of RANKL (10, 11), making themselves a crucial regulator of osteoclast formation and activation. Under physiological condition, osteocytes primarily exert inhibitory effects on osteoclast formation, while their death turns off the inhibition and exert pro-osteoclastogenic effect in multiple ways (12). Like death of other cell types, osteocyte death also triggers immune reaction, but in its unique way which ultimately recruits osteoclasts. Studies found that apoptotic osteocytes could promote osteoclast formation by increasing expression of pro-osteoclastogenic cytokines, such as RANKL, and releasing pro-inflammatory damage associated molecular patterns (DAMPs), such as high mobility group box 1 (HMGB1) (13, 14). In addition to apoptosis, studies have revealed novel types of cell death, such as necroptosis and ferroptosis. Osteocytes undergoing these types of cell death are reported to induce inflammation by releasing pro-inflammatory factors and DAMPs, which participate in the development of bone diseases (15, 16). Given the important role of osteocyte-osteoclast crosstalk in bone remodeling and bone diseases, it is necessary to understand whether and how osteocytes undergoing various types of cell death are involved in regulation of osteoclast formation. This review summarizes various types of cell death in osteocytes and their potential mechanism to regulate osteoclast formation, with the aim to further understand the development of bone disease and to explore potential therapeutic target.

## 2 Osteoclast formation and regulation

### 2.1 A brief overview of osteoclast formation and activation

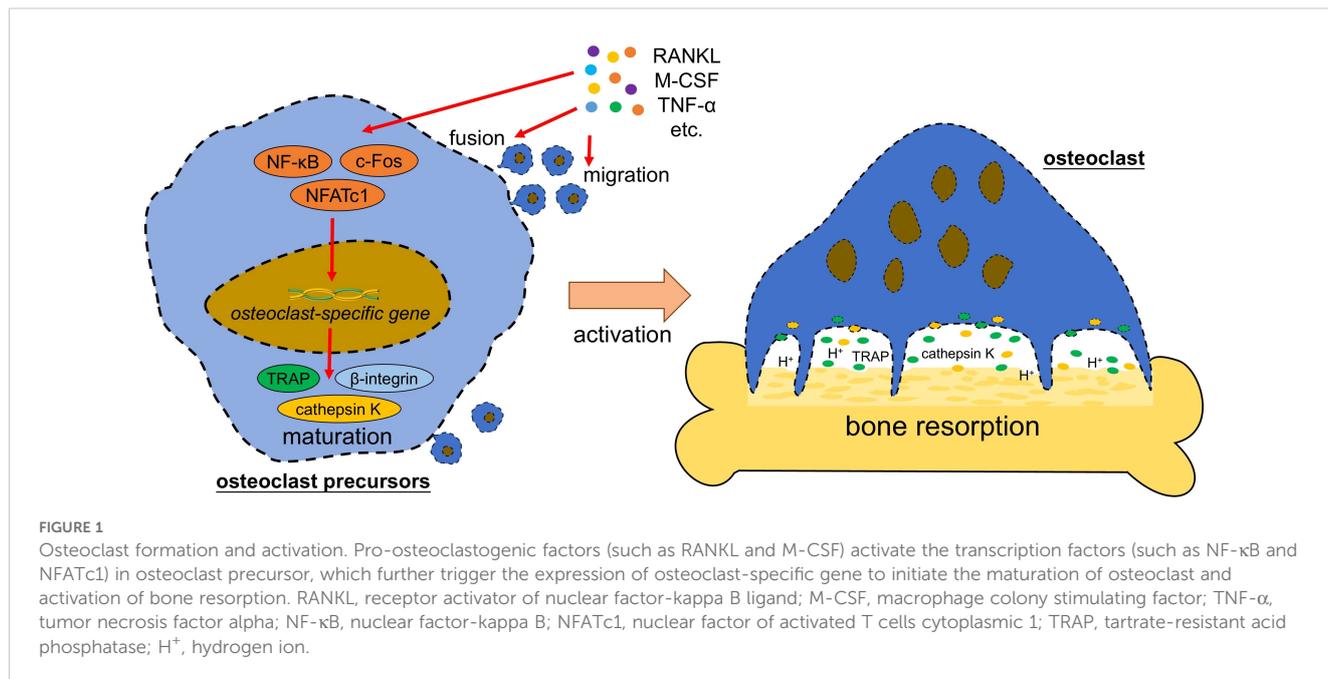
Osteoclasts originate from mononuclear phagocytes, which comprise monocytes/macrophages and dendritic cells (DC) (7, 17). These mononuclear cells with potential to form osteoclasts are called osteoclast precursors (OCPs) and located in multiple tissue, including bone marrow, spleen, thymus, or peripheral blood (18). The differentiation of OCPs to mature osteoclasts requires macrophage colony stimulating factor (M-CSF) and RANKL, which induce the expression of osteoclast-specific genes, including those encoding tartrate-resistant acid phosphatase (TRAP),  $\beta$ -integrin and cathepsin K (Figure 1). These are key proteins that lead to the maturation of osteoclasts (6). Mature osteoclast is the multinucleated cell with at least three nuclei. Its cytoplasm contains large amounts of vesicles that are filled with TRAP and cathepsin K. When activated by extracellular signals of bone remodeling, osteoclast undergoes polarization and reconstruction via the rearrangements of actin cytoskeleton, to form a sealed compartment between the bone surface and basal membrane of osteoclast. Then acids and lysosomal enzymes (TRAP, cathepsin K, etc.) are secreted into this sealed compartment to initiate bone resorption (19).

### 2.2 Signal molecules from osteocytes and immune cells that promote osteoclast formation

Osteoclast formation is triggered by various signal molecules from osteocytes and immune cells, such as RANKL and TNF- $\alpha$  (Table 1). Some of these signal molecules are recognized as DAMPs, which refer to intracellular contents released from damaged osteocytes into extracellular matrix, such as HMGB1 and adenosine triphosphate (ATP). DAMPs can interact with downstream pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), and cause the aseptic inflammatory responses (20–23). These signal molecules exert directly or indirectly regulating effects on osteoclasts in an interacting and complicated manner, and they are to be introduced as follow.

#### 2.2.1 RANKL

Receptor activator of nuclear factor kappa- $\beta$  ligand (RANKL) is a cytokine belonging to TNF superfamily. Its receptor, RANK, is a member of TNF receptor superfamily. RANKL can be expressed by various types of cells, such as osteocytes, osteoblasts, B lymphocytes, T lymphocytes, and periodontal ligament cells (11, 24–26). Among these cells, osteocytes are found to express high levels of RANKL



and have greater capacity to promote osteoclastogenesis *in vitro* (11). Studies have shown that mice with gene deletion of RANKL in osteocytes exerted phenotype of osteopetrosis due to the defects in osteoclast formation, indicating that osteocytes is an essential source of RANKL (10, 11).

RANKL play crucial role in osteoclastogenesis. Binding of RANKL with RANK in OCPs, with co-stimulation of M-CSF, induces osteoclast differentiation, fusion, and activation. When

binding to RANKL, RANK recruits a variety of multifunctional adaptor proteins, including the TNF receptor-associated factors (TRAFs) (18). RANK also recruits kinases, such as TGFβ activated kinase-1 (TAK1), to sequentially activate nuclear factor-kappa B (NF-κB), c-Fos, and nuclear factor of activated T cells cytoplasmic 1 (NFATc1), three transcriptional factors that are essential for osteoclastogenesis (27). NFATc1 is the master transcription factor regulating osteoclastogenesis (28, 29). Over-expression of NFATc1

**TABLE 1** Signal molecules from osteocytes and immune cells that promote osteoclast formation.

Signal molecule	Source	Pro-osteoclastogenic effects
RANKL	Viable/apoptotic/ferroptotic Ocy; OB, BC, TC, etc.	Activating NFATc1 to initiate the expression of osteoclast-related gene in OCPs
TNF-α	Apoptotic/ferroptotic Ocy; Mφ, etc.	Increasing expression of RANKL, IL-1β, etc; Mediating the switching of M2 to M1 macrophages; Directly act on OCPs to promote osteoclastogenesis
IL-1β	Viable/apoptotic/ferroptotic/pyroptotic Ocy; Dendritic cells, monocytes, TC, Mφ, etc.	Increasing expression of RANKL; Inhibiting OPG expression; Directly act on OCPs to promote osteoclast migration, formation and activation
IL-6	Apoptotic/ferroptotic Ocy; Monocytes, Mφ, TC, BC, fibroblasts, etc.	Mediating the transmigration of OCPs from blood to bone marrow; Increasing expression of RANKL
Sclerostin	Ocy, etc.	Increasing expression of RANKL; Inhibiting OPG expression;
VEGF	Viable/apoptotic Ocy; OB, chondrocytes, Mφ, etc.	Directly act on OCPs to promote osteoclastogenesis
HMGB1	Viable/apoptotic/necrotic <sup>Pt</sup> /necroptotic <sup>Pt</sup> /ferroptotic <sup>Pt</sup> /pyroptotic <sup>Pt</sup> Ocy; Osteoclasts, OB, etc.	Increasing expression of RANKL, TNF-α, and IL-6; Directly act on OCPs to promote osteoclastogenesis
ATP	Apoptotic/damaged Ocy, etc.	(low concentration) Promoting osteoclastogenesis; (high concentration) Inhibiting osteoclastogenesis <sup>Pt</sup>

Ocy, osteocytes; OB, osteoblasts; BC, B lymphocyte; TC, T lymphocyte; NFATc1, nuclear factor of activated T cells cytoplasmic 1; OCPs, osteoclast precursors; Mφ, macrophages; <sup>Pt</sup> Potentially.

results in the differentiation and fusion of OCPs without stimulation of RANKL (28, 29). In addition to the common OCPs, studies found that RANKL could induce B lymphocytes to differentiate into osteoclasts (30–32), indicating a wide range of pro-osteoclastogenic effect of RANKL.

RANKL-RANK interaction can be blocked by osteoprotegerin (OPG). OPG is a soluble decoy receptor of RANKL, with capability of preventing RANKL from binding RANK and thus inhibiting osteoclast formation and bone resorption (33). Studies have shown that genetic deletion of OPG could lead to osteoporosis in mice (34, 35), and homozygous deletion of OPG gene in human results in juvenile Paget's disease, a disease characterized by osteopenia and fractures (36).

### 2.2.2 TNF- $\alpha$

TNF- $\alpha$  is a potent pro-inflammatory cytokine secreted by various cells, such as macrophages and osteocytes. TNF- $\alpha$  is central to the pathogenesis of inflammatory diseases, such as rheumatoid arthritis (RA) (37). Our previous studies revealed that TNF- $\alpha$  also plays important role in the development of postmenopausal osteoporosis (15). It has been widely reported that TNF- $\alpha$  plays crucial role in regulating osteoclast formation and activation (38). TNF- $\alpha$  exerts pro-osteoclastogenic effects in two ways: acting on osteocytes and on osteoclasts. TNF- $\alpha$  can increase the expression of RANKL in osteocytes. Marahleh et al. found that primary osteocytes stimulated by TNF- $\alpha$  showed significantly higher RANKL mRNA expression (39). They also found that mouse with TNF- $\alpha$  injected into the calvaria generated increased number of RANKL-positive osteocytes and osteoclasts. A further study revealed that TNF- $\alpha$  induced sclerostin expression in osteocytes, which further triggered RANKL expression in osteocytes, thereby enhancing osteoclastogenesis (40). On the other hand, TNF- $\alpha$  directly acts on OCPs to trigger osteoclastogenesis. It has been found that TNF- $\alpha$  could work synergistically with RANKL and M-CSF to trigger osteoclastogenesis via NF- $\kappa$ B and phosphatidylinositol 3 kinase (PI3k)/AKT pathway (38). Moreover, Zhao et al. found that TNF- $\alpha$  could mediate the M-CSF-induced switching of M2 to M1 macrophages, which have higher potential to differentiate into osteoclasts (41). While it was previously considered that RANKL was essential for TNF- $\alpha$ -induced osteoclastogenesis (42, 43), a recent study showed the opposite result. O'Brien et al. found that TNF- $\alpha$  plus interleukin-6 (IL-6) could induced osteoclast formation in mice with *Rank* deficient (44), indicating that TNF- $\alpha$  is able to induce osteoclastogenesis independent of RANKL. Together, TNF- $\alpha$  is capable of inducing osteoclastogenesis, by acting directly or indirectly on OCPs, in a manner dependent or independent on RANKL/RANK pathway.

### 2.2.3 IL-1 $\beta$

Interleukin-1 $\beta$  (IL-1 $\beta$ ) is an important pro-inflammatory cytokine, normally produced by multiple cells including dendritic cells, monocytes, T lymphocytes, osteocytes and macrophages (45). IL-1 $\beta$  is widely involved in various inflammatory response and recently increasing studies have revealed its role in inflammatory

bone diseases, such as rheumatoid arthritis and osteoporosis (46–48). It is reported that IL-1 $\beta$  promoted RANKL expression in MLO-Y4 osteocyte-like cells, while inhibiting OPG expression. This dual effects further increase the formation of osteoclasts (49). Moreover, IL-1 $\beta$ , in combination of other exogenous cytokines, such as IL-6 and TNF- $\alpha$ , is found to upregulates IL-1 $\beta$  expression in human osteocyte, forming a positive feedback loop to intensify the inflammation (50). IL-1 $\beta$  is also able to act on OCPs and osteoclasts directly. IL-1 $\beta$  promotes RANKL-induced osteoclast differentiation via the activation of NF- $\kappa$ B (51). IL-1 $\beta$  also increases the expression of C-C motif chemokine receptor 7 (CCR7), the receptor of C-C chemokine ligand 19 (CCL19) and CCL21, to promotes osteoclast migration and activation (52). Osteoclast activation requires proteolytic enzymes like plasminogen and collagenases to break down bone matrix proteins. It is reported that IL-1 $\beta$  could enhance the expression of these proteolytic enzymes (53). Together, these results suggest a pro-osteoclastogenic role of IL-1 $\beta$ .

### 2.2.4 IL-6

IL-6 is a proinflammatory cytokine produced by various cells including monocytes, macrophages, T lymphocytes, B cells, fibroblasts (54). IL-6 is also found to derive from apoptotic osteocytes (55). IL-6 plays important roles in inflammation, autoimmunity, injury, rheumatoid arthritis, and cancer (56, 57). It is also reported to involve in bone diseases, such as osteoporosis. Tanaka et al. found that IL-6 regulated sphingosine-1-phosphate receptor (S1PR) on the OCPs. They found IL-6 upregulated the expression of S1PR2, which mediated the transmigration of OCPs from blood to bone marrow, while downregulated the expression of S1PR1, which mediated the transmigration in opposite direction (58). Besides, IL-6 inducing RANKL expression in osteoblasts and osteocytes via the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) pathway, indirectly promoting osteoclast-mediated bone resorption (59–61).

### 2.2.5 Sclerostin

Sclerostin is a glycoprotein expressed predominantly by osteocytes that is best known to play negative role in bone formation by suppressing the Wnt signaling pathway, a canonical pathway that promotes osteogenic differentiation and osteoblast maturation and survival (62). In addition to its inhibiting effect of osteogenesis, studies also reveal its promoting effect of osteoclastogenesis and bone resorption. Wijenayaka et al. found that sclerostin could increase RANKL expression in MLO-Y4 osteocyte-like cells, while downregulating OPG, which promoted osteoclast formation and bone resorption (63). Then Otori et al. further found that sclerostin-induced RANKL expression in primary osteocytes could be enhanced by TNF- $\alpha$  (40), indicating a complicated crosstalk among pro-osteoclastogenic cytokines.

### 2.2.6 VEGF

The vascular endothelial growth factor (VEGF) family comprises five proteins: namely VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF), among which

VEGF-A is the most extensively studied member. As a primary regulator of blood vessel formation and permeability, VEGF-A is involved in both physiological and pathological conditions, such as wound healing cancer (64). Recent studies reveal that VEGF also participate in bone remodeling by regulating osteoclast formation and activation. VEGF can be secreted by bone-related cells, such as osteocytes, osteoblasts, chondrocytes and macrophages, and this secretion can be enhanced by apoptotic osteocytes in the neighborhood (65). Study found that VEGF can promote osteoclast differentiation and bone resorption, which is sufficient to take the place of M-CSF (66). Yang et al. further found that VEGF could bind to VEGFR2 in OCPs and promote osteoclast formation through PI3K/Akt and MEK/ERK signaling (67). These results indicate a novel role of VEGF in osteoclast regulation.

### 2.2.7 HMGB1

High mobility group box 1 (HMGB1) is a proinflammatory cytokine expressed and released by various cell types, including osteoclasts, osteoblasts, osteocytes, which exerts various cellular compartment-specific functions (68). After secretion by active cells or release from damaged cells, HMGB1 serves as a protein of DAMPs and triggers a series of cellular processes, including differentiation, proliferation, apoptosis, and autophagy (68, 69). This process is mediated by its interaction with two receptors: toll-like receptor 4 (TLR4) and receptor for advanced glycation end products (RAGE) (70). It has been reported that HMGB1 plays important role in regulating bone tissue homeostasis (13, 68, 69). Studies found that damaged MLO-Y4 osteocyte-like cells released HMGB1, which induced expression of RANKL, TNF- $\alpha$ , and IL-6 in other stromal cells and macrophages (65, 71). These cytokines are crucial for inducing osteoclastogenesis. HMGB1 can also directly act on OCPs and osteoclasts to induce osteoclast differentiation through RAGE and TLR4 activation (13). When activated by HMGB1, TLR4 triggers multiple signaling pathway, such as NF- $\kappa$ B signaling and integrin signaling, to initiate osteoclast-related gene expression at early stage and actin ring formation at later stage (72).

### 2.2.8 ATP

Adenosine triphosphate (ATP) is a multifunctional signaling molecule, which plays a crucial role in the metabolism of cellular energy. It is recently reported that elevated extracellular ATP engages in the development of inflammatory disease, such as osteoporosis (73). Under pathological condition such as fracture, apoptotic or damaged osteocytes release ATP, which induces bone remodeling by interacting with osteoblasts and osteoclasts (73, 74). Extracellular ATP mainly interacts with P2X purinergic receptors that are widely expressed in various kinds of bone cells. Among P2X receptors, P2X7 receptors are considered to play important role in osteoclast formation (75, 76). Activated P2X7 receptor induces the fusion of mononuclear precursors osteoclast into multinucleated osteoclast (76). Besides, P2X7 receptors activated by extracellular ATP have been found to exert effects on osteoclasts resorption by inducing the formation of resorption area on the bone surface and the secretion of osteolytic molecules, such as cathepsin K (77).

These pro-osteoclastogenic effects are considered to result from the ATP-mediated rise of calcium ions in osteoclasts, which further upregulates the expression of NFATc1, the key pro-osteoclastogenic transcription factor (76). A recent study by Lu et al. revealed another mechanism that P2X7 receptors promote osteoclast formation and bone resorption via PI3K-Akt-GSK3 $\beta$  signaling pathway (75). While studies reported the pro-osteoclastogenic effects of ATP, some studies found the opposite results. Miyazaki et al. found that treatment of high concentration (100  $\mu$ M and 150 $\mu$ M) of ATP analogue showed strong inhibitory effects on morphology, survival, and the bone-resorbing activity of mature osteoclasts (78). This result is consistent with a previous study revealing that extracellular ATP at low concentrations (0.2~2  $\mu$ M) could promote osteoclast formation and resorption, while extracellular ATP a higher concentration (20~200  $\mu$ M) might exert inhibitory effects on osteoclasts (79). These results indicate a complex role of ATP in osteoclast formation and activation. More studies are required to understand the role of extracellular ATP.

To summarize, these signal molecules are secreted by viable osteocytes, immune cells and other cells and exert regulating effects on osteoclast formation. Studies have revealed that their secretion do not cease when osteocytes die. Instead, dying or impaired osteocytes continue to regulate osteoclast formation by actively secreting or passively releasing signal molecules, which act on osteoclasts directly or indirectly. It is important to understand how osteocyte death regulates osteoclast formation.

## 3 Osteocyte death and osteoclast formation

Cell death is defined as an irreversible loss of plasma membrane integrity (80). In 2005, Nomenclature Committee on Cell Death (NCCD) classified cell death based on the morphological characteristics and specified 3 forms of cell death: apoptosis, autophagy, and necrosis (81). As more and more forms of cell death have been identified, previous classification become inapplicable. Currently, cell death is classified into two main forms: non-programmed cell death (i.e. necrosis) and programmed cell death (PCD), which is further divided into apoptosis, pyroptosis, necroptosis and ferroptosis, etc., based on different pathological process (82). Unlike accidental and unregulated process of necrosis, PCD is a spontaneous and programmed process of cell death under the regulation of certain gene and protein, which can be blocked by the corresponding inhibitors (83). Studies have revealed the crucial role of cell death in the development of diseases (84, 85). Various forms of osteocyte death, including apoptosis, necroptosis, ferroptosis and pyroptosis, have been reported to participate in multiple bone diseases, especially inflammation and bone loss, where osteoclasts are excessively generated and activated (86). As previous studies have reported that death of osteocytes recruit osteoclasts and promote bone resorption (87), it is important to sort out the mechanisms of various osteocyte death to regulate osteoclast. Some mechanisms have been verified by current studies, while some remain unclear.

These mechanisms, established or potential, are discussed as following (Table 2).

### 3.1 Necrosis

Necrosis is a uncontrolled and pathological form of cell death that is caused by supraphysiological conditions, such as mechanical force, heat, or cold, and morphologically characterized by plasma membrane rupture and dilatation of cytoplasmic organelles, particularly mitochondria (88). Rupture of cell membrane leads to a release of intracellular contents (i.e. DAMPs) and induces aseptic inflammatory responses (20–23).

Osteocyte necrosis participates in the regulation of osteoclast formation and development of multiple bone diseases. Andreev et al. found that osteocyte necrosis, rather than apoptosis, significantly increased osteoclastogenesis and promoted bone loss (89). They further found that osteoclasts sensed DAMPs from necrotic osteocytes by macrophage-inducible C-type lectin (Mincle), a member of pattern recognition receptors (PRRs) that were subsequently found to upregulate the expression of gene related to oxidative phosphorylation and calcium signaling in osteoclast. However, they did not identify the specific molecules of DAMPs in the regulation of osteoclastogenesis. Gkouveris et al. reported that osteocyte death led to release of HMGB1 in mice model of medication-related osteonecrosis of the jaw (MRONJ) and blocking HMGB1/RAGE interaction would reduce the incidence of MRONJ (90). Therefore, necrotic osteocytes might regulate osteoclastogenesis via the interaction between DAMPs released from dead osteocytes, such as HMGB1, and PRRs on osteoclasts, such as MINCLE. It is also reported that DAMPs released from necrotic osteocytes, such as HMGB1, S100 family molecules, and heat-shock proteins, could bind to PRRs, such as TLR2, TLR4, and RAGE, on

immune cells including macrophages and dendritic cells, and promote their production of pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-6, which further induces RANKL expression in osteoblast lineage cells or promotes osteoclastogenesis directly (91). Meanwhile, necrotic osteocytes might indirectly promote osteoclastogenesis by exerting pro-neovascularization effect. Osteonecrosis of the femoral head (ONFH) is a bone disease with a progressive pathological process of femoral head ischemia, involving not only osteocyte necrosis, but hyperactive osteoclasts (92, 93). Varoga et al. found VEGF expression in osteocytes was upregulated and immature vessels increased in penumbra area of ONFH, where osteocytes are still alive (94). This result suggests that necrotic osteocytes might promote VEGF expression in nearby surviving osteocytes and thereby trigger neovascularization, which might promote signal transduction and recruit osteoclasts. Meanwhile, given that VEGF is currently found to participate in the regulation of osteoclast formation as discussed previous, it is potential that necrotic osteocytes regulate osteoclast formation via the direct effects of VEGF on osteoclasts. However, a recent study found that sclerostin was significantly upregulated while VEGF decreased in necrotic area of the femoral head induced by glucocorticoid, and silencing sclerostin of osteocytes rescued the expression of osteogenic and angiogenic gene (95). Thus, it requires further studies to verify the effects of necrotic osteocytes on angiogenic gene expression. To summarize, necrotic osteocytes can promote osteoclast formation by releasing DAMPs which directly stimulate osteoclasts or trigger the expression of pro-osteoclastogenic cytokines in related cells.

### 3.2 Apoptosis

In contrast to necrosis, apoptosis is a form of programmed cell death that is best studied as a vital physiological process critical for

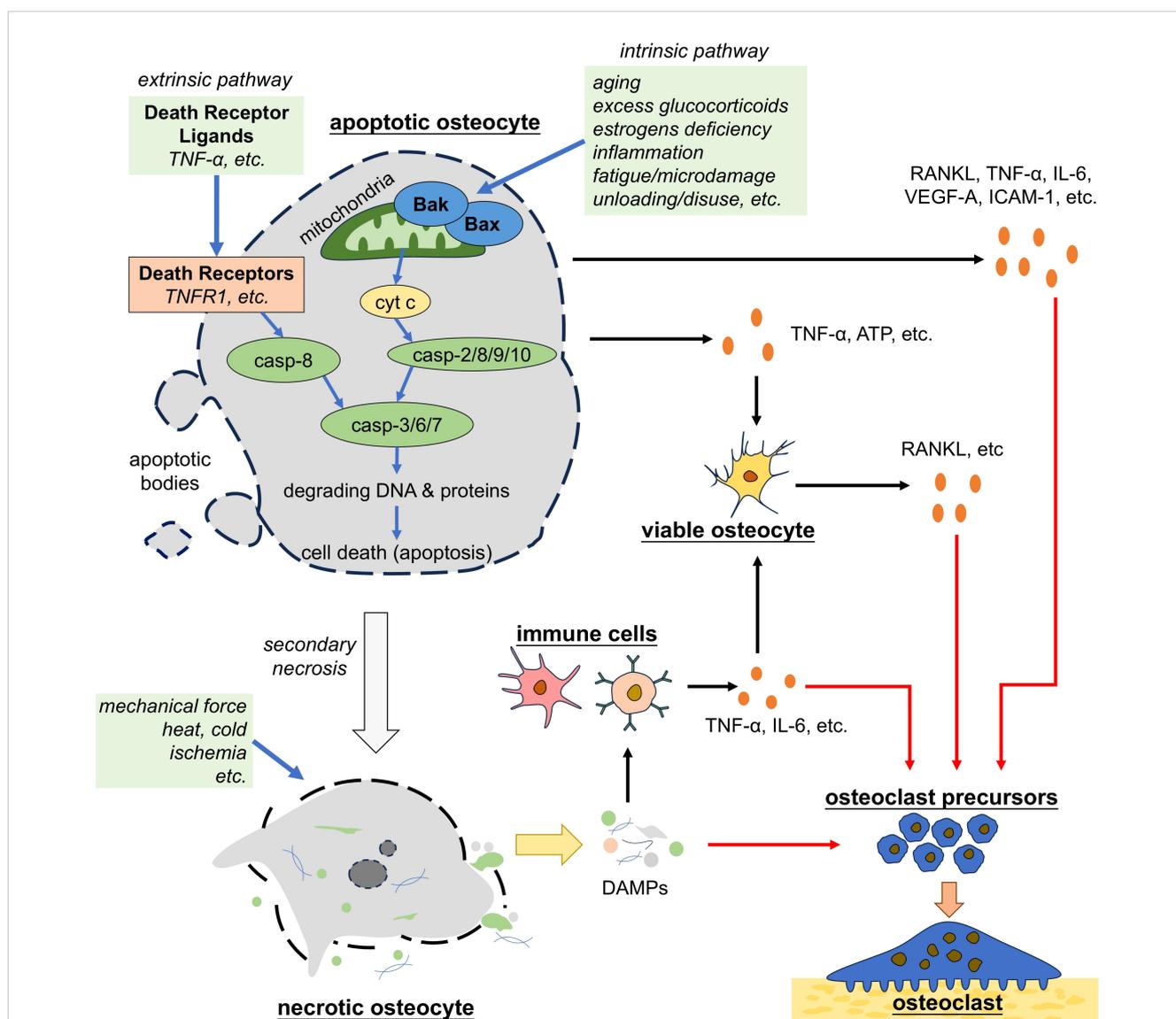
TABLE 2 Comparison of various types of osteocyte death.

Osteocyte death	Triggering factors	Key pathway	Outcome	Released pro-osteoclastogenic factors
Necrosis	Mechanical force, heat, cold, ischemia, etc.	No	Cell membrane rupture; Release of cellular contents; Triggering inflammation	HMGB1 <sup>pt,d,id</sup> , S100 family molecules <sup>id</sup> , heat-shock proteins <sup>id</sup>
Apoptosis	Aging, excess glucocorticoids, estrogens deficiency, inflammation, fatigue/microdamage, unloading/disuse, death receptors ligands, etc.	Caspase activation cascade	Secondary necrosis	RANKL <sup>d</sup> , TNF- $\alpha$ <sup>d,id</sup> , IL-6 <sup>id</sup> , VEGF-A <sup>d</sup> , ICAM-1 <sup>d</sup> , HMGB1 <sup>d,id</sup> , ATP <sup>id</sup>
Necroptosis	Death receptors ligands, etc.	RIPK1/RIPK3/MLKL	Cell membrane rupture; Release of cellular contents; Triggering inflammation	DAMPs (HMGB1 <sup>pt</sup> ) <sup>d,id</sup>
Ferroptosis	Iron overload, etc.	Fe <sup>2+</sup> /ROS	Peroxidation of lipid, DNA, proteins; Cell membrane rupture <sup>pt</sup>	RANKL <sup>d</sup> , M-CSF <sup>d</sup> , IL-1 $\beta$ <sup>d,id</sup> , IL-6 <sup>d,id</sup> , TNF- $\alpha$ <sup>d,id</sup> , DAMPs (HMGB1 <sup>pt</sup> ) <sup>d,id</sup>
Pyroptosis	PAMPs, DAMPs, LPS, etc.	Caspase/GSDMD	Cell membrane rupture; Release of cellular contents; Triggering inflammation	IL-1 $\beta$ <sup>id</sup> , IL-18 <sup>id</sup> , DAMPs (HMGB1 <sup>pt</sup> ) <sup>d,id</sup>

HMGB1, high mobility group box 1; RANKL, receptor activator of nuclear factor-kappa B ligand; TNF- $\alpha$ , tumor necrosis factor alpha; IL, interleukin; VEGF, vascular endothelial growth factor; ICAM-1, intercellular cell adhesion molecule-1; ATP, adenosine triphosphate; RIPK, receptor-interacting serine/threonine protein kinase; MLKL, mixed lineage kinase domain-like; DAMPs, damage associated molecule patterns; ROS, reactive oxygen species; M-CSF, macrophage colony stimulating factor; PAMPs, pathogen-associated molecular patterns; LPS, lipopolysaccharides; GSDMD, gasdermin D; <sup>d</sup> directly promoting osteoclastogenesis; <sup>id</sup> indirectly promoting osteoclastogenesis; <sup>pt</sup> Potentially.

maintaining cellular homeostasis across tissues, characterized by cell shrinkage, chromatin condensation, and membrane blebbing (96). Apoptosis is a caspase-dependent form of programmed cell death that is activated by two main pathways: the intrinsic and extrinsic pathways (Figure 2) (65). Intrinsic pathway is triggered by pathological conditions, such as DNA damage, hypoxia and loss of integrin signaling and begins with the activation of Bax and Bak, the pro-apoptotic members of the Bcl-2 family of proteins. Activation of Bax and Bak leads to a conformational change that exposes cryptic epitopes at their N-termini, which promotes the formation of Bax and Bak homo-oligomers or hetero-oligomers within the outer mitochondrial membrane (96, 97). These oligomers form channels that release cytochrome c from the mitochondria into the

cytoplasm. Then cytochrome c activates latent caspases, a family of endopeptidases, among which the initiator caspases (caspase-2/8/9/10) are primarily activated and the effector caspases (caspase-3/6/7) are subsequently activated. Effector caspases are responsible for demolition of the cell. Caspase-3/6/7 degrade critical intracellular proteins, and activate other enzymes including DNase (98, 99). Extrinsic pathway of apoptosis is triggered by the interaction between death receptors ligands (such as TNF- $\alpha$ ), and death receptors (such as tumor necrosis factor receptor 1, TNFR1), which recruit and activate caspase-8, the key protein for the following caspase activation cascade as introduced above (100). Apoptotic cells eventually break apart into vesicles, which are called apoptotic bodies and are usually phagocytosed by macrophages or



**FIGURE 2** Apoptotic and necrotic osteocytes promote osteoclastogenesis. Osteocytes that undergo apoptosis by either the intrinsic or extrinsic pathways trigger the secretion of pro-osteoclastogenic factors by nearby osteocytes or directly promote osteoclastogenesis. Apoptotic osteocytes eventually undergo secondary necrosis. Necrotic osteocytes, either derived from apoptotic osteocytes or induced by environmental disadvantages, lose the integrity of cell membrane and release the cellular contents (i.e. DAMPs), which trigger inflammation and osteoclastogenesis. TNF- $\alpha$ , tumor necrosis factor alpha; casp, caspase; ATP, adenosine triphosphate; RANKL, receptor activator of nuclear factor-kappa B ligand; IL-6, interleukin 6; VEGF, vascular endothelial growth factor; ICAM-1, intercellular cell adhesion molecule-1; DAMPs, damage associated molecule patterns.

other cell types. Phagocytosis requires the guidance of “find-me” signals and “eat-me” signals. Apoptotic cells actively release soluble molecules, such as Sphingosine-1-phosphate (S1P), ATP, uridine triphosphate (UTP), Lysophosphatidylcholine (LPC) and C-X3-C motif chemokine ligand 1 (CX3CL1). These molecules attract macrophages to the site of cell death, thus known as “find-me” signals (101). Macrophages in proximity to apoptotic cells recognize “eat-me” signals displayed on the surface of apoptotic cells, such as Fc and phosphatidyl serine (PS) (101). During apoptosis, the PS molecules within the phospholipid bilayer of cell surface membrane undergo a translocation from inner to outer surface of the bilayer, which are recognized by macrophages and then facilitate phagocytosis (65). Following this regulated process, apoptosis ensures the removal of damaged or unnecessary cells without inciting inflammatory responses (96).

Osteocyte apoptosis is recognized as a pivotal event in bone remodeling. Recent research has highlighted that osteocyte apoptosis is triggered by pathological conditions such as aging, excess glucocorticoids, estrogens deficiency, inflammation, fatigue/microdamage, and unloading/disuse (65). Aging increased oxidative stress and mitochondrial dysfunction, driving osteocyte apoptosis and subsequently diminishing the structural integrity of bone (102). Excessive glucocorticoid use and estrogen deficiency are reported to induce osteocyte apoptosis and cause bone loss (103, 104). Under inflammatory condition, inflammatory cytokines, particularly TNF- $\alpha$ , increase osteocyte apoptosis and lead to osteoporosis, a disease characterized by elevated bone fragility (105). In addition to systemic factors, local mechanical unloading or disuse significantly promotes osteocyte apoptosis (106).

Normally, apoptotic bodies are engulfed by macrophages timely and therefore not inducing wide range of inflammatory response, but osteocytes are located within bony lacunae, making apoptotic osteocytes unapproachable for macrophages (89). Under physiologic condition, osteocytes connect to bone surface and each other via the canalicular network. However, study found that few apoptotic bodies could traverse this canalicular network occupied by the dendritic processes of surrounding viable osteocytes, although it is theoretically accessible (107). As many studies have indicated that apoptotic osteocytes promote osteoclast mediated bone resorption (10, 108), there must be non-contacting approach by which apoptotic osteocytes recruit and activate osteoclast, since their apoptotic bodies are unapproachable for macrophages, at least at the early stage.

Current studies have revealed that apoptotic osteocytes regulate osteoclast formation in both active and passive way. Osteocytes undergoing apoptosis increase the secretion of the pro-osteoclastogenic factors, including RANKL, TNF- $\alpha$ , IL-6, VEGF-A, intercellular cell adhesion molecule-1 (ICAM-1) (13, 14, 55, 109, 110). These factors exert dual effects on osteoclasts directly or indirectly. As discussed in previous passage, RANKL, TNF- $\alpha$ , IL-6, VEGF-A act on OCPs and promote their differentiation to mature osteoclasts as well as the subsequent activation of bone resorption. IL-6 and ICAM-1 are reported to promote the adhesion of OCPs to endothelial cells (55). Besides, apoptotic osteocytes can signal to nearby osteocytes and macrophages to stimulate their secretion of

pro-osteoclastogenic factors, such as RANKL, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and VEGF (65, 111). It is reported that dead osteocytes accounted for only about 15~20 percent even in the regions showing the highest apoptosis induced by estrogen loss (112). Verborgt et al. found that osteocytes surrounding the apoptotic osteocyte at microdamage sites upregulated the expression of apoptosis-inhibiting gene (113), showing that surviving osteocytes could detect apoptotic osteocytes and potentially continue to exert regulating effects on bone remodeling. TNF- $\alpha$  secreted by apoptotic osteocytes acts on TNFRs in viable osteocytes in the neighborhood, which further activates mitogen-activated protein kinases (MAPKs) phosphorylation and the NF- $\kappa$ B pathway to increase expression of RANKL and sclerostin, both of which subsequently initiate osteoclast formation (39, 40). Additionally, studies found that apoptotic osteocytes induced by fatigue loading released ATP, which triggered nearby osteocytes to express RANKL and induced osteoclast-mediated bone resorption (114, 115). To summarize, apoptotic osteocytes actively regulate osteoclast mediated bone resorption in both direct and indirect way. However, it requires more studies to reveal the mechanism by which these signal molecules are upregulated in apoptotic osteocytes and further explore the strategy to block the secretion of pro-osteoclastogenic factors by apoptotic osteocytes.

Apoptotic osteocytes also regulate osteoclast-mediated bone resorption in a passive way. Free from phagocytosis by macrophages, apoptotic osteocytes undergo secondary necrosis, leading to the release of DAMPs, including HMGB1, which further trigger the release of pro-osteoclastogenic cytokines in osteoblast and stromal cells (116). As discussed previously, HMGB1 stimulates the synthesis of RANKL, TNF- $\alpha$ , and IL-6, but inhibits the production of OPG in bone marrow-derived stromal cells (65, 69, 71). Besides, HMGB1 can also directly act on TLR4 in OCPs and osteoclasts to trigger osteoclast-related gene expression at early differentiation stage and actin ring formation at later stage (13, 72).

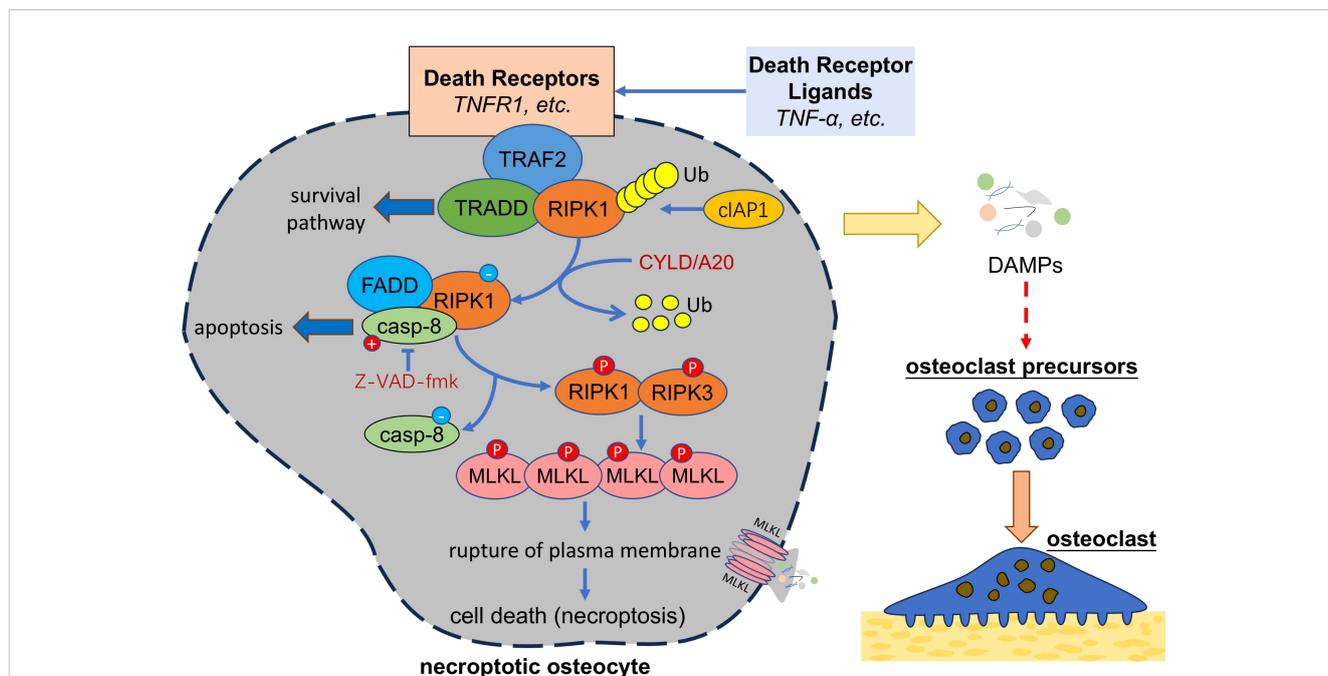
In conclusion, osteocyte apoptosis plays important role in osteoclast regulation. However, Plotkin et al. found that inhibition of osteocyte apoptosis was not sufficient to prevent the osteoclast increase or bone mass decrease induced by unloading (117), indicating another advantaged mechanism of regulating osteoclasts. He et al. found that compared with apoptosis, necroptosis, a novel form of programmed cell death, took more responsibility for osteocyte death in ovariectomy (OVX)-induced osteoporosis (118). This may account for the insufficiency of inhibiting osteocyte apoptosis to prevent osteoclast increase. Thus, focus should be put not only on osteocyte apoptosis, but also on other types of programmed osteocyte death.

### 3.3 Necroptosis

It was thought that apoptosis was the only form of programmed cell death. In 2005, Degterev et al. identified a form of cell death which was characterized by necrotic cell death morphology but could be inhibited by necrostatin-1 (Nec-1). They termed it as “necroptosis”

and classified it as a novel form of programmed cell death which is distinct from apoptosis (119). Necroptosis proceeds in a programmed way, rather than in an unregulated way as happened in necrosis, and undergoing in a Caspase-independent manner, rather than in a Caspase-dependent manner as happened in apoptosis (Figure 3). Necroptosis can be triggered by TNF family, Fas ligands, interferon, and RNA or DNA sensors (120). TNF- $\alpha$  and FAS, two death receptor ligands, are the best studied inducers of necroptosis. Activated by TNF- $\alpha$ , TNFR1 on the cell membrane recruits receptor-interacting serine/threonine protein kinase 1 (RIPK1), TNFR-associated death domain (TRADD), TNFR-associated factor 2 (TRAF2) and form complex I (120), which is involved in the survival pathway and contributes to cell survival. This process is dependent on the ubiquitination of RIPK1 by cellular inhibitor of apoptosis protein 1 (cIAP1), so when RIPK1 is deubiquitinated by Cyldromatosis (CYLD) or deubiquitination enzyme A20, complex I will be converted into complex II, which is composed of Fas associated via death domain (FADD), RIPK1 and caspase-8 (121). Activated caspase-8 in complex II degrades RIPK1 and initiates apoptosis, but when caspase-8 is inhibited (eg, inhibited by Z-VAD-fmk), RIPK1 recruits RIPK3 and the interaction between RIPK1 and RIPK3 further recruits and phosphorylates mixed lineage kinase domain-like (MLKL) (122). Phosphorylated MLKL oligomerizes and translocates into the plasma membrane, leading to the rupture of plasma membrane and cell death (123). As happened in necrosis, cell necroptosis ultimately causes a release of DAMPs and induces inflammatory responses.

Osteocyte necroptosis is recently found involved in the development of bone diseases like osteoporosis (124). It is reported that both necroptosis and apoptosis were found responsible for death of osteocytes and bone loss in OVX mice, the model of postmenopausal osteoporosis, but necroptosis might hold more responsible on the death of osteocytes than apoptosis (118). As osteoclasts were found increased in OVX mice (125), it might be a relation of osteocyte death, especially necroptosis, to osteoclastogenesis. Although a few studies have revealed how osteocyte necroptosis triggers osteoclastogenesis, given that necroptosis of osteocytes ultimately leads to the rupture of cell membrane and release of DAMPs, it is potential that DAMPs released from necroptotic osteocytes recruit OCPs and trigger osteoclastogenesis, as happened in necrosis or apoptosis of osteocytes. HMGB1 has been identified as one of the DAMPs released from necroptotic cells (126). As discussed previously, impaired osteocytes release HMGB1 and trigger osteoclastogenesis. It is potential that necroptotic osteocytes release HMGB1 as the major regulator of osteoclastogenesis. Besides, Zhu et al. found that knockdown of RIPK1, one of the key regulators of necroptosis, down-regulated VEGF-D gene and protein levels in astrocytes (127). Similar to necrosis, it is potential that necroptotic osteocytes regulate osteoclast formation via VEGF related signaling pathway. To summarize, necroptotic osteocytes might promote osteoclast formation by releasing DAMPs or upregulating the expression of pro-osteoclastogenic cytokines. However, it should be reminded that in necroptosis-inducing environment, high level of TNF- $\alpha$  might induce RANKL production in osteocytes ahead of the complete



**FIGURE 3**  
 Necroptotic osteocytes promote osteoclastogenesis. Necroptosis of osteocytes can be triggered by death receptor ligands, which activate a series of signal transduction and activate RIPK1 and RIPK3 under certain condition. RIPK1-RIPK3 interaction can activate MLKL, which oligomerize and damage the integrity of plasma membrane, leading to cell death and release of cellular contents (i.e. DAMPs) that might further trigger the osteoclastogenesis. TNF, tumor necrosis factor; TRAF2, TNFR-associated factor 2; RIPK, receptor-interacting serine/threonine protein kinase, TRADD, TNFR-associated death domain; cIAP1, cellular inhibitor of apoptosis protein 1; CYLD, Cyldromatosis; FADD, Fas associated via death domain; casp-8, caspase-8; MLKL, mixed lineage kinase domain-like; DAMPs, damage associated molecule patterns.

death, which would trigger osteoclastogenesis (39). Thus, studies are required to distinguish between osteoclastogenesis triggered by TNF- $\alpha$ -induced RANKL secretion and by osteocyte necroptosis-induced DAMPs release.

Though it remains uncertain how necroptotic osteocytes induce osteoclast formation, studies have revealed the potential target of osteocyte necroptosis as clinical application for relevant bone diseases. Cui et al. found the treatment of Nec-1, an inhibitor of RIPK1, alleviated bone loss in OVX mice, indicating the potential application of Nec-1 in the treatment of postmenopausal osteoporosis (128). Similar result was found in the rat of glucocorticoid induced osteoporosis (129). In addition to RIPK1 inhibitors, there are other inhibitors of necroptosis, such as RIPK3 inhibitor GSK'872 and MLKL inhibitor Necrosulfonamide (124). However, their efficacy in the treatment of relevant bone diseases and safety for human being requires further investigation.

### 3.4 Ferroptosis

In addition to apoptosis and necroptosis, more forms of programmed cell death are identified. In 2003, Dolma et al. found that the small molecule erastin can induce a novel form of cell death, which was further found unable to be reversed by neither apoptosis inhibitors nor necroptosis inhibitors, while iron-chelating agent deferoxamine mesylate (DFO) could inhibits such form of cell death. Thus, a new form of iron-dependent cell death was identified

and named as ferroptosis (130). Different from apoptosis and necroptosis, ferroptosis is characterized by the increase of the mitochondrial membrane density with mitochondrial shrinkage and triggered by the dysregulation of iron homeostasis and lipid metabolism (Figure 4) (131, 132). Normally, extracellular Fe<sup>3+</sup> binds to transferrin (TF), which can transport Fe<sup>3+</sup> into cells through TF receptor 1 (TFR1). Intracellular Fe<sup>3+</sup> is then reduced to Fe<sup>2+</sup> by iron reductase, such as six-transmembrane epithelial antigens of the prostate 3 (STEAP3) and divalent metal transporter 1 (DMT1) (133). Fe<sup>2+</sup> is unstable and able to generates high level of reactive oxygen species (ROS) via the Fenton reaction (134). Iron overload leads to excessive ROS, which, if the antioxidant capacity of cells is insufficient, further causes oxidative damage to polyunsaturated fatty acids (PUFAs) in cell membrane, DNA, proteins, and ultimately cell death (i.e. ferroptosis) (134, 135). Dysregulation of lipid metabolism can also cause ferroptosis, where acyl-CoA synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3) play important roles (136). ACSL4 promotes the conversion of free PUFAs to PUFA-CoAs and then LPCAT3 catalyze the formation of phospholipid-PUFAs (PL-PUFAs) from PUFA-CoAs. PL-PUFAs are highly prone to be oxidized into lipid peroxides, thereby making cells susceptible to ferroptosis. Ferroptosis is regulated by several signaling pathway, including system Xc<sup>-</sup>/glutathione (GSH)/glutathione peroxidase 4 (GPX4) axis (137). System Xc<sup>-</sup> is located on the cell membrane and composed of the transporter protein solute carrier family 7 members 11 (SLC7A11)

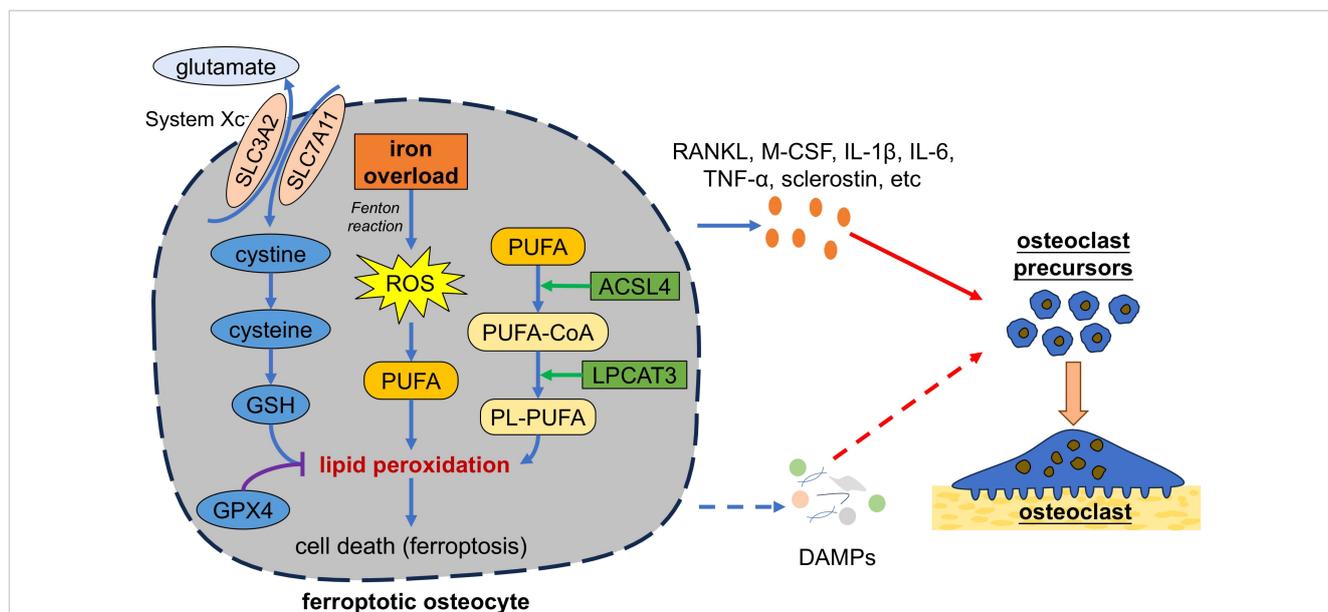


FIGURE 4

Ferroptotic osteocytes promote osteoclastogenesis. Iron overload causes excessive generation of ROS in osteocytes. Excessive ROS can cause oxidative damage to membrane lipids and ultimately lead to cell death. Dysregulation of lipid metabolism mediated by ACSL4 and LPCAT3 can also cause ferroptosis. Ferroptosis can be regulated by several signaling pathway, such as system Xc<sup>-</sup>/GSH/GPX4 axis. Ferroptotic osteocytes secrete pro-osteoclastogenic factors to trigger osteoclastogenesis. Additionally, ferroptotic osteocytes might release cellular contents (i.e. DAMPs) and thereby trigger the osteoclastogenesis. SLC3A2, solute carrier family 3 members 2; ROS, reactive oxygen species; GSH, glutathione; GPX4, glutathione peroxidase 4; PUFAs, polyunsaturated fatty acids; ACSL4, acyl-CoA synthetase long-chain family member 4; LPCAT3, lysophosphatidylcholine acyltransferase 3; PL, phospholipid; RANKL, receptor activator of nuclear factor- $\kappa$ B ligand; M-CSF, macrophage colony stimulating factor; TNF- $\alpha$ , tumor necrosis factor alpha; IL, interleukin; DAMPs, damage associated molecule patterns.

and regulator protein SLC3A2. System  $X_c^-$  is responsible for exporting intracellular glutamate in exchange for extracellular cystine, which is further reduced to cysteine for the synthesis of GSH, the main antioxidant in cells (138). GSH is an essential cofactor for GPX4, which reduces the toxicity of lipid peroxides by converting peroxide (R-OOH) into alcohol (R-OH), thereby protecting cells from ferroptosis (132). Erastin can inhibit system  $X_c^-$  and induce ferroptosis (139).

Increasing studies have revealed the important role of iron overload and ferroptosis in bone diseases like postmenopausal osteoporosis, where increased oxidative stress and iron accumulation is found (16). While previous studies focused on ferroptosis of osteoblasts in bone diseases, a recently increasing number of studies have paid attention on osteocytes. Yang et al. reported that iron overload induced by ferric ammonium citrate (FAC) could lead to osteocyte apoptosis and conditioned medium from iron-overload-induced osteocytes could promote osteoclast differentiation and activity of bone resorption. They further revealed that iron overload significantly increased the gene expression and protein secretion of RANKL in osteocytes, which promoted osteoclast differentiation and activation (140). Similar results were found by Ma et al. and Guo et al (141, 142). Ma et al. found that osteocyte apoptosis induced by iron overload led to an increasing expression of RANKL and sclerostin with a decreased expression of OPG both *in vivo* and *in vitro*, while Guo et al. reported that iron overload upregulated RANKL expression with no significant impact on OPG and sclerostin levels *in vivo*. These results suggest that iron overload could induce osteocyte death and promote osteoclast differentiation and activation by increasing RANKL expression. However, as increasing studies have revealed the relation between iron overload and ferroptosis (137), these studies that focused on osteocyte apoptosis might neglect the significance of osteocyte ferroptosis in osteoclast regulation. A recent study by Jiang et al. reported that iron overload induced osteocyte death in OVX mice, which could be decreased by ferroptosis inhibitor, but not by apoptosis inhibitor, neither by necroptosis inhibitor, therefore confirming that ferroptosis, instead of apoptosis or necroptosis, was the predominant form of death among osteocytes in a high-iron environment (16). They also found that osteocytes induced by iron overload significantly increased the secretion of RANKL, M-CSF, IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , which could be inhibited by ferroptosis inhibitor liproxstatin-1 (Lip-1) and ferrostatin-1 (Fer-1), indicating that osteocyte ferroptosis could promote osteoclast formation by secreting pro-osteoclastogenic cytokines. Consistently, they found that iron overload could lead to bone loss, which could be suppressed by ferroptosis inhibitor. Their further study revealed the crucial role of nuclear factor erythroid derived 2-related factor-2 (Nrf2) in the regulation of osteocyte-ferroptosis-induced osteoclastogenesis, as inhibition of Nrf2 downregulated DNA methyltransferase 3a (Dnmt3a)-mediated the methylation levels of RANKL promoter and thereby reduced the expression of RANKL. These results provide a novel insight for therapy of postmenopausal osteoporosis by targeting osteocyte ferroptosis. Similar results were found in the studies of periodontitis. Li et al. identified the occurrence of alveolar osteocyte

ferroptosis in diabetic periodontitis mice (143). Their further *in vitro* study revealed the increased protein expression of TNF- $\alpha$  and IL-1 $\beta$  in osteocytes, as well as the upregulated mRNA expression of proinflammatory cytokines, including sclerostin, IL-6, TNF- $\alpha$  and RANKL, which could be reversed by ferroptosis inhibitor. These results are consistent with study by Tang et al (144).

There are some studies implying the potential mechanism by which ferroptosis osteocytes regulate osteoclasts. Li et al. found triggering the ferroptosis of pancreatic ductal adenocarcinoma cells would induce the release of HMGB1 (145), which facilitated the pro-inflammatory M1 polarization of macrophages via HMGB1/TLR4/STAT3 axis (146). Wei et al. found the similar result that ferroptosis of acute myeloid leukemia cells could lead to the release of HMGB1 (147). Additionally, it is reported that cancer cells undergoing ferroptosis released proteoglycan decorin, which triggered the production of pro-inflammatory cytokines in macrophages via NF- $\kappa$ B axis (148). These results indicate that ferroptosis osteocytes might also release DAMPs that participate in osteoclast regulation, which requires further studies to explore.

To summarize, iron overload-induced osteocyte ferroptosis plays important role in the regulation of osteoclast formation and bone resorption activity. However, it should be taken into consideration that high-iron environment could act on osteoclasts directly. Osteoclast differentiation and activation require energy provided by mitochondria, while mitochondrial biogenesis requires iron. Thus, high level of iron might promote osteoclast differentiation and activation, thereby contributing to bone resorption (149). Das et al. found that suppressing iron uptake of osteoclast attenuated their mitochondrial metabolism and cytoskeletal organization, leading to decreased bone resorption (150). This result indicates the important of role of iron in the regulation of osteoclast-mediated bone resorption and is consistent with the study by Li et al. reporting that isobavachin, a RANKL inhibitor, could suppress RANKL-induced osteoclastogenesis by promoting iron efflux and inhibiting mitochondrial biogenesis (151). Therefore, a comparison between the effect of osteocyte ferroptosis and iron overload on regulating osteoclast differentiation and bone resorption is required to clarify the exact role of osteocyte ferroptosis in osteoclast regulation, which would help to provide an osteocyte ferroptosis-targeting insight for therapy of osteoclast-mediated bone diseases.

### 3.5 Pyroptosis

Pyroptosis is another form of programmed cell death, which is triggered by infections or inflammatory signals and results in the rupture of the cell membrane. Two pyroptosis pathway have been identified: Canonical pathway and noncanonical pathway (Figure 5). Canonical pyroptosis is triggered by the interaction between PRRs and pathogen-associated molecular patterns (PAMPs) or DAMPs, which induces the formation of inflammasome that consists of nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3),

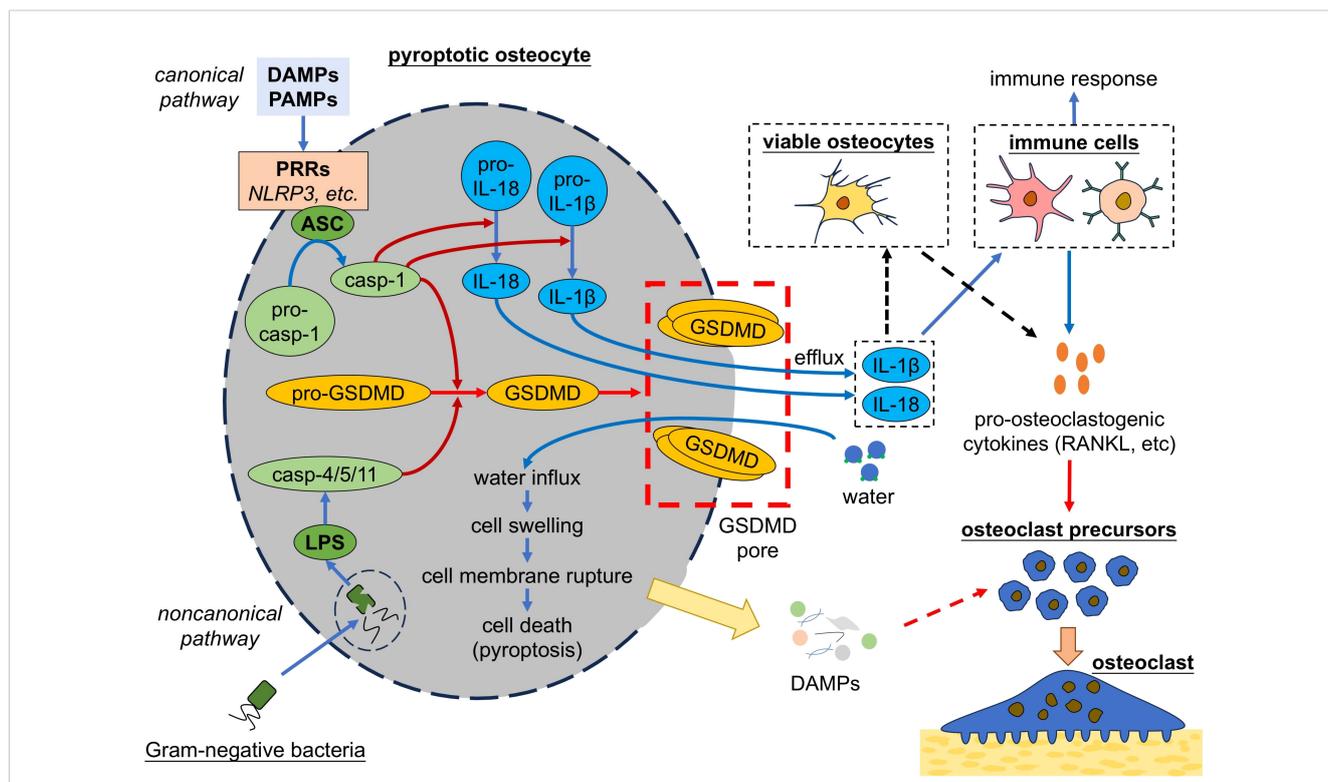


FIGURE 5

Pyroptotic osteocytes promote osteoclastogenesis. Osteocytes undergo pyroptosis by either canonical pathway or noncanonical pathway, both of which lead to the activation of GSDMD. GSDMD form pores in cell membrane, leading to cell membrane rupture and cell death. Pyroptotic osteocytes release pro-inflammatory cytokines, which act on immune cells to trigger the immune response and secretion of pro-osteoclastogenic cytokines. Additionally, pyroptotic osteocytes might release cellular contents (i.e. DAMPs) and thereby trigger the osteoclastogenesis. DAMPs, damage associated molecule patterns; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; NLRP3, nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3; ASC, apoptosis-associated speck-like protein containing a CARD; casp, caspase; IL, interleukin; LPS, lipopolysaccharides; GSDMD, gasdermin D; RANKL, receptor activator of nuclear factor- $\kappa$ B ligand.

apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase-1 (152). Inflammasome activates caspase-1, which cleaves gasdermin D (GSDMD), pro-IL-1 $\beta$  and pro-IL-18 into their mature forms. Then GSDMD form pores in cell membrane that allow water influx and induce cells to swell until the cell membrane ruptures, ultimately leading to the release of cellular contents and triggering strong inflammatory response (153). GSDMD-pores also allow efflux of pro-inflammatory factors, such as IL-1 $\beta$  and IL-18 (154). Noncanonical pyroptosis is triggered by lipopolysaccharides (LPS) of Gram-negative bacteria and characterized by the activation of caspase-4/5/11, which activate GSDMD and lead to cell death as happened in canonical pyroptosis (155). Cell pyroptosis induces strong immune reactions in response to host defenses infection, but excessive pyroptosis can lead to multiple inflammatory diseases, including bone diseases like osteoporosis (156).

A few studies have focused on relation of osteocyte pyroptosis to osteoclast regulation. Zhao et al. found that osteocyte pyroptosis promoted osteoclast formation and bone resorption (157). Although their study did not further explore the mechanism, they found that osteocyte pyroptosis led to upregulated secretion of IL-1 $\beta$ , an inflammatory cytokine that can increase RANKL expression and thereby promote osteoclastogenesis (156). Similarly, Zhang et al. reported pyroptosis of MLO-Y4 osteocyte-like cells led to

increased release of lactate dehydrogenase (LDH), IL-1 $\beta$  and IL-18 (158). IL-18 is reported to upregulate RANKL in T lymphocytes, indirectly stimulating osteoclast formation (159). These results suggest that osteocyte pyroptosis can promote osteoclastogenesis and bone resorption, probably by release of pro-inflammatory cytokines including IL-1 $\beta$  and IL-18, which induced upregulation of RANKL expression. There are multiple studies on how pyroptosis of various cells regulate osteoclasts, which may help to understand the relation of osteocyte pyroptosis and osteoclast regulation. It is reported that osteoblast pyroptosis induced by IL-17 promoted the release of IL-1 $\beta$  and RANKL, which further promoted osteoclastogenesis and bone resorption (160). Similarly, pyroptosis of periodontal ligament cells was found to promote osteoclast-mediated bone resorption, probably by IL-1 $\beta$ -induced upregulation of RANKL (161, 162). These results indicate the important role of pro-inflammatory cytokines including IL-1 $\beta$  and IL-18 in osteoclast regulation, while there might be other potential mechanism. As presented above, pyroptosis of MLO-Y4 osteocyte-like cells released LDH (158). However, such a large molecule is unlikely to pass through GSDMD pores formed during the process of pyroptosis, but possibly through cell membrane rupture sites at the later stage of pyroptosis (163), suggesting that pyroptotic cells may undergo cell membrane

rupture and release DAMPs. Li et al. reported that pyroptotic renal tubular cells released HMGB1 and then triggered inflammatory responses (164). It is possible that pyroptotic osteocytes promote osteoclast formation and bone resorption by releasing DAMPs, such as HMGB1, which need more studies to explore.

## 4 Conclusion and prospects

Viable osteocytes serve as a crucial regulator of bone remodeling by translating environmental alterations into cellular signals which are further transmitted to related cells including osteoblasts and osteoclasts to initiate their activity of bone formation and resorption, respectively. This regulatory effect of osteocytes does not vanish when osteocytes die; instead, dying or dead osteocytes continue to regulate osteoclast formation in various ways and thereby affect bone remodeling. As multiple forms of osteocyte death have been identified in various bone diseases, their roles on osteoclast regulation are also revealed by current research. This review summarizes the established and potential mechanisms by which osteocyte death, including necrosis, apoptosis, necroptosis, ferroptosis and pyroptosis, regulate osteoclast formation, which might provide better understanding of pathological bone remodeling and the development of bone diseases. Whereas, there are still some questions needed to be answered: Among multiple forms of cell death, which one hold the predominate responsibility for the development of different bone diseases? How to effectively block the pro-osteoclastogenic effects of osteocyte death and how to translate into clinical application? It has been revealed that osteocyte death exerts regulatory effects on osteoclast formation, but some mechanisms remain unclear and require more studies to explore. Meanwhile, some novel forms of cell death are recently revealed, such as cuproptosis, and might participate in bone remodeling under specific conditions (165). In addition, various forms of cell death do not act in an isolate way, but interact with each other. Thus, a novel concept of integrated cell death emerges and are named as PANoptosis, which refers to the cell death modality involving the interactions among pyroptosis, apoptosis, and necroptosis (166). Besides, studies also revealed the interaction between ferroptosis and other cell deaths, such as apoptosis and pyroptosis (149). Together, there might be an intracellular network composed of various death signaling pathways which is activated under pathological conditions. A comprehensive understanding of cell death in osteocytes and their role in osteoclast regulation is needed and requires more studies to reveal.

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WZ: Conceptualization, Supervision, Visualization, Writing – original draft, Writing – review & editing. JQ: Writing – original draft, Writing – review & editing. JL: Writing – original draft, Writing – review & editing. TS: Writing – original draft. XD: Writing – original draft. YF: Writing – original draft. XL: Visualization, Writing – original draft. HC: Conceptualization, Supervision, Writing – original draft.

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## Conflict of interest

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