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Biologic and Pathologic Aspects of Osteocytes in the Setting of Medication-Related Osteonecrosis of the Jaw (MRONJ)

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Keywords

MRONJ; cell death; apoptosis; autophagy; necrosis; necroptosis; osteocyte death

Introduction

Medication-related osteonecrosis of the jaw (MRONJ) is a potentially debilitating condition seen in patients who have been treated with powerful antiresorptives (pARs) or angiogenesis inhibitors (AgIs). MRONJ is defined as exposed bone or bone that can be probed through an intra- or extraoral fistula in the maxillofacial region for more than eight weeks in patients who have been treated with pARs or AgIs and have no history of radiation therapy or metastatic disease in the jaws[1, 2].

pARs including nitrogen-containing bisphosphonates (N-BPs; e.g., zoledronic acid [ZOL], alendronate [ALN], etc.) and anti-RANKL antibodies (e.g., denosumab) are used to manage bone metastases in patients with cancer[3–6] or to prevent fragility fractures in patients with osteoporosis[7]. MRONJ associated with pARs is common in patients with cancer (1.8-5% incidence), but rare in patients with osteoporosis (0.01-0.03%)[1, 2, 8, 9]. Patients with MRONJ experience reduced oral health-related quality of life[10]. Clinical and preclinical data suggest that for MRONJ to occur, systemic risk factors (e.g., pARs or AgIs) and oral risk factors, such as tooth extraction and inflammatory dental disease (e.g., periodontitis, periapical infection) must co-occur[1, 2, 11–22]. MRONJ management can be challenging and the outcomes difficult to predict, often with problematic resolution[23–25].

Bone necrosis, the hallmark of MRONJ, is recognized histologically as an area of bone tissue with numerous contiguous empty osteocyte lacunae[26–28]. However, osteocytes die before this histologic pattern appears. Whereas the causes and mechanisms of osteocyte death have been studied in conditions like osteonecrosis of the femoral head (ONFH)[29], few studies of the causes and mechanisms of osteocyte death have been done in MRONJ. Improving the understanding of osteocyte death in MRONJ may be critical for preventing disease and developing treatment approaches. This review intends to provide insight into the

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Conflict of Interest

The authors have no conflicts of interest.

biology of osteocytes, cell death in general, and osteocyte death in particular and discuss possible mechanisms for MRONJ-related osteocyte death.

Osteocyte biology

Osteocytes are the most numerous bone cell type in the adult skeleton. Osteocytes comprise 90-95% of the bone cells, while osteoclasts and osteoblasts make up the remaining 5-10%[30]. They terminally differentiate from osteoblasts, which themselves differentiate from mesenchymal precursors residing in the bone marrow and at bone surfaces[31]. As new bone is formed, osteoblasts synthesize osteoid at existing bone surfaces and undergo cellular transformations that involve changes in shape, size, and development of dendrite-like processes that extend into the mineralizing front of the osteoid to communicate with existing mature osteocytes[31–34]. As osteoblasts differentiate into osteocytes, they become encased in their surrounding recently-mineralized bone matrix[34, 35]. Their cell bodies reside within lacunae, and their dendritic processes, ranging from 40–100 per cell[35], run through 30-300nm diameter tunnels named canaliculi. The canaliculi traverse the mineralized bone matrix allowing intercellular communication among osteocytes. The physical structure of interconnected tunnels and lacunae is known as the osteocyte lacunar-canalicular network (LCN)[31, 35] (Figure 1). The connection among osteocyte cell processes within the LCN and osteoblasts at bone surfaces is attained via gap channel junctions[36–38]. Gap channel junctions are formed by connexins (Cx)[36–38], with Cx43 being the most abundant[39, 40]. Osteocytes and osteoblasts also express functional Cx43 hemichannels[41]. Hemichannels mediate communication, not only between adjacent cells but also with the extracellular matrix as it deforms. It has been proposed that gap junctions and hemichannels contribute to maintaining bone integrity and function by permitting the exchange of bone modulators and regulating signals elicited by mechanical stimulation through influencing bone modeling and/or remodeling[42–45]. Cx43 hemichannels may also play essential roles as transducers for the anti-apoptotic signals of bisphosphonates[46, 47].

The osteocytes in the LCN form a functional syncytium with cells on the bone surfaces, including osteoblasts and lining cells, which in turn are in direct physical contact with endothelial cells of blood vessels, stromal cells, hematopoietic stem cells in the bone marrow, and nerves[48] (Figure 1). Notably, osteocyte dendritic processes can extend beyond the cells in bone surfaces to interact directly with blood vessels and cells in the bone marrow[35]. It has been proposed that this organization allows osteocytes to play a bidirectional role (receiver-transmitter system). Thus, osteocytes not only act as "*receivers*" of systemic signals (e.g., hormones, drugs) directly from blood vessels or local signals from the mineralized matrix as it is deformed, but also as "*transmitters*" of signals to the executor cells of bone modeling and/or remodeling (osteoclasts, osteoblasts, and lining cells). Hence, osteocytes are thought to operate as effective orchestrators of modeling and remodeling, integrating hormonal, pharmaceutical, and mechanical cues to regulate osteoblast and osteoclast function. It is proposed that the osteocyte's centralized role in regulating responses to mechanical stimuli allows the skeleton to meet its mechanical and calcium and phosphorus homeostatic needs[35, 49, 50].

The control of bone remodeling requires the precise regulation of both osteoclast and osteoblast cell activity. Indeed, both osteocytes and osteoblasts control osteoclastogenesis and bone resorption by regulating RANK/RANKL/OPG signaling. Osteocytes and osteoblasts synthesize RANKL[51, 52]. Cells of the osteoclast lineage express RANK on their surfaces[53]. RANKL binding to its receptor RANK promotes differentiation of osteoclast precursors and the activation of mature osteoclasts that together increase bone resorption activity[54].

Interestingly, osteocytes and osteoblasts are also a source of osteoprotegerin (OPG), a soluble decoy receptor that binds to RANKL and prevents its binding to RANK, a process which in turn inhibits osteoclast-mediated bone resorption[55–57]. Thus, osteocytes regulate bone resorption, utilizing a unique molecular system based on the differential synthesis of OPG and RANKL[58].

Osteocytes also control osteoblast activity and bone formation by regulating the bone anabolic actions of the canonical Wnt/β-catenin signaling pathway[59]. This pathway is activated when Wnt proteins bind to receptor complexes that comprise frizzled proteins (receptors) and co-receptors low-density lipoprotein (LDL) receptor-related proteins (LRP) LRP 5 and/or 6[55, 60–62]. Though LRP5 and LRP6 are the more studied, other LDL receptor family members, including LRP4[61–63] and LRP8[64, 65], also function as co-receptors for Wnt ligands in the regulation of bone homeostasis. Mature osteocytes synthesize several modulators of Wnt signaling, including sclerostin and Dickkopf-related protein 1 (DKK-1)[63, 64]. Sclerostin and DKK-1 are potent, specific inhibitors of the Wnt/β-catenin pathway that bind to LRP4, LRP5, and LRP6[65] and prevent the binding of Wnt, playing a critical role in regulating bone formation[66]. At present, it is unclear whether LRP8 is inhibited by sclerostin or DKK1. By reducing the synthesis of these Wnt pathway inhibitors, osteocytes induce the upregulation and translocation of β-catenin to the nucleus and activate gene transcription signaling in osteoblasts to increase bone formation[66–68]. In contrast, increased expression of sclerostin and DKK1 by osteocytes, as occurs in skeletal unloading, suppresses bone formation[69, 70]. Osteocytes also mediate the effects of parathyroid hormone (PTH) on bone formation. These effects are in part attributable to the suppressive effect of PTH on sclerostin synthesis by osteocytes via transcriptional downregulation of the SOST gene that encodes sclerostin[71–73]. PTH also increases RANKL synthesis in osteocytes, indicating that PTH indirectly regulates bone resorption[74]. Furthermore, osteocytes are a source of diverse molecules that modulate bone remodeling. These molecules include mediators such as prostanoids, nitric oxide, nucleotides, and a broad spectrum of cytokines and growth factors such as insulin-like growth factor-1 (IGF-1), vascular endothelial cell growth factor (VEGF), and TGF-β[75–82]. Moreover, osteocytes are a significant source of fibroblast growth factor 23 (FGF-23), which decreases serum phosphorus levels by increasing renal phosphate excretion[83]. In addition, osteocytes are responsible for regulating the mineralization process as they become embedded in osteoid gradually mineralizing[84]. It has been proposed that the osteoid osteocyte is the cell primarily responsible for mineralization instead of osteoblasts on the bone surfaces. The SIBLING proteins DMP1 and MEPE and the protein PHEX are highly expressed in osteocytes[85–90] and are identified as essential molecules for bone mineralization[30, 91, 92]. DMP1 is expressed during the initial stages of mineralized

matrix formation in bone and dentin[93]. It is expressed along and in the canaliculi of osteocytes in the bone matrix at gap regions between collagen type 1 fibrils[94, 95]. As a highly phosphorylated protein, DMP1 may be involved in the regulation of hydroxyapatite formation. The effects of DMP1 in the regulation of mineralization and osteocyte maturation appear to be predominantly due to its role in phosphate homeostasis regulation because the mineralization defects and the impairment in osteocyte maturation can be rescued by restoration of the normal phosphate homeostasis[96]. MEPE interacts with DMP1 and PHEX to affect FGF23 expression, regulating phosphate mineralization and bone turnover[97]. PHEX was initially described on the plasma membrane of osteoblasts and osteocytes[88], and loss-of-function mutations in this gene resulted in X Linked hypophosphatemic rickets[98].

Osteocytes of the craniofacial skeleton

Since MRONJ affects the jaws, it is pertinent to consider the possibility that the biology and regulation of osteocytes in the jaws might differ from that in non-jaw skeletal sites. The craniofacial skeleton differs in several ways from the better-studied postcranial skeleton, particularly concerning the embryologic origin, molecular regulation during skeletogenesis, and structural organization. The craniofacial skeleton has two embryonic origins[99]. The majority of the craniofacial bones, namely all facial bones and most cranial bones, including the maxilla and mandible, are derived from the cranial neural crest (NC). In contrast, the parietal and occipital bones of the calvarium are derived from the paraxial mesoderm[100–102]. Cranial NC cells originate from the anterior-dorsal aspect of the developing neural tube, contributing to most of the cartilage and bone of the cranial region[103, 104]. Most rostral cranial NC cells arise from the diencephalic and mesencephalic neural tube and form the skull's frontonasal skeleton and membranous bones. The posterior cranial NC cells, coming from the posterior mesencephalon and hindbrain, occupy the pharyngeal arches and form the mandible, maxilla, middle ear bones, and hyoid bone.

Maxillae and mandibles develop from tissues of the first pharyngeal arch[104]. The mandible develops from the mandibular process and the maxilla within the maxillary process that expands from it. Though mandibular and maxillary primordia originate from similar NC cells, they develop into very different structural entities[104]. Once the positional identities of bone progenitor cells are defined by a unique combination of homeodomain transcription factors, the NC-derived mesenchymal stem cells differentiate into osteoblast lineage cells through the upregulation of BMPs and osteoinductive factors similar to those in the postcranial skeleton[103, 104].

Most craniofacial bones, such as the calvaria, some facial bones, and the mandible (except its condylar process), are formed through intramembranous ossification[105, 106]. On the other hand, the cranial base, the supporting platform for the development of the brain, is formed by endochondral ossification in the same manner as the appendicular skeleton and vertebrae[107].

It has been assumed that the biology of NC-derived osteoblasts is similar to that of mesoderm-derived osteoblasts. However, some studies have shown differences among

osteoblasts from the two embryologic origins. Osteocytes derived from calvaria are commonly used for *in vitro* differentiation assays. As described above, these cells originate from NC or mesenchymal stem cells. Thus, their progenitors possess different bone-forming abilities[108]. Calvarial osteoblasts from the frontal bone, derived from NC stem cells, have better intrinsic osteogenic and tissue regeneration capacity than mesoderm-derived calvarial osteoblasts from the parietal bone[108, 109]. Furthermore, NC–derived frontal bone cells display a superior capacity to undergo osseous healing compared to the mesoderm-derived parietal bone cells due to greater activation of the canonical Wnt signaling pathway[109]. These studies suggest NC-derived osteoblasts and mesoderm-derived osteoblasts have different biologic features. However, it is unknown whether NC-derived osteocytes from the maxilla and mandible resemble NC-derived osteocytes from the frontal bones or mesoderm-derived bones[110]. Furthermore, it is unknown whether the regulatory mechanisms, biologic responses to cell survival signals, and cell death responses of NC-derived osteocytes are different from those in mesoderm-derived osteocytes.

Cell Death

Cell death is a terminal biologic event in which the affected cell ceases to carry out its functions. Dying cells are involved in a process that is reversible until the first irreversible event or "*point-of-no-return*" occurs[111]. In the absence of an accepted view of biochemical events considered as the point-of-no-return, the Nomenclature Committee on Cell Death (NCCD) recommended that a cell be considered dead when any of the following morphological criteria are met: a) permanent loss of the plasma membrane barrier; b) breakdown of a cell into discrete fragments, which are commonly referred to as apoptotic bodies; or c) engulfment of the cell by dedicated phagocytes or other cells with phagocytic activity[112]. Since this initial NCCD recommendation, additional cell death modalities have been described[111, 113, 114]. Subsequent reports from the NCCD have recommended limiting the definition of "dead" exclusively to cells that either exhibit irreversible plasma membrane permeabilization or have undergone complete fragmentation[115].

Although cell death can occur due to overwhelming damage, most cell death occurs actively through specific signaling pathways. Cell death has been operationally classified into two broad categories: "*accidental*" or "*regulated*"[115]. Accidental cell death (ACD) is caused by severe insults, including physical, chemical, and mechanical stimuli[115]. When exposed to extreme physicochemical or mechanical insults, cells die uncontrollably, losing their structural integrity and releasing damage-associated molecular patterns (DAMPs), endogenous molecules with immunomodulatory and sometimes, cytotoxic activity[116, 117]. Regulated cell death (RCD), in contrast, involves a genetically encoded molecular machinery[113, 118]. RCD occurs not only as a consequence of microenvironmental perturbations but also during post-embryonic development, immune responses, and inflammation[119]. While ACD is challenging to control, RCD can be modulated by inhibiting the transduction of death signals and enhancing the capacity of cells to mount adaptive responses to stress[115]. Indeed, the course of RCD can be modified by pharmacologic and/or genetic interventions that target its key components.

Cell death can also be formally classified into three different forms based on morphologic features and mechanisms by which dead cells and their fragments are removed: a) ***type I cell death*** (***apoptosis)***, b) ***type II cell death (autophagy)***, and c) ***type III cell death (necrosis)*** [111, 120]. Although these morphological classifications have limitations and caveats, they are extensively employed.

Type I cell death (apoptosis):

the term "*apoptosis*" was initially coined by Kerr *et al*.[121] to describe a RCD form with specific morphologic features that include cell shrinkage, retraction of cellular pseudopodia, reduction of cellular volume (pyknosis), chromatin condensation (karyopyknotic), nuclear fragmentation (karyorrhexis), little or no ultrastructural modifications of cytoplasmic organelles, and plasma membrane blebbing while the cell maintains its integrity until the final stages of the process[111]. Cells that die through apoptosis end by forming intact small vesicles, known as apoptotic bodies, which are phagocytosed by neighboring cells and degraded within lysosomes. There are several subtypes of apoptosis that are triggered through different biochemical routes, for instance, through "intrinsic" or "extrinsic" pathways[122, 123]. The subtypes of apoptosis are thoroughly reviewed elsewhere[124].

Type II cell death (autophagy):

the term "autophagy" derived from the Greek meaning "*eating of self*" was first coined by Duve[125]. It is an essential cellular mechanism for balancing energy sources at critical times during development or in response to nutrient stress or starvation[126]. Autophagy also plays a housekeeping role in removing misfolded or aggregated proteins, clearing damaged organelles, such as mitochondria, endoplasmic reticulum, peroxisomes, and eliminating intracellular pathogens[126]. Though autophagy is generally associated with survival mechanisms, its deregulation has also been linked to a non-apoptotic RCD mechanism. Morphologically, cell death by autophagy occurs in the absence of chromatin condensation and involves massive autophagic vacuolization of the cytoplasm[126, 127]. In contrast to apoptotic cells, whose clearance is ensured by phagocytosis and lysosomal degradation by neighboring phagocytic cells, cells that die by autophagy have little or no association with neighboring cells[128, 129]. Autophagy relies on the formation of autophagosomes and activation of the autophagic machinery. The fusion of autophagosomes with lysosomes generates autolysosomes, in which acidic lysosomal hydrolases degrade their luminal content.

Type III cell death (necrosis):

The term "necrosis," from the ancient Greek νέκρωσις, nékrōsis, "death," has been used for centuries to define drastic tissue changes visible to the naked eye[130]. Pathologists use this term to describe the presence of dead tissues, representing the sum of changes that occur in cells and tissues after they have died, regardless of the pre-lethal processes[131, 132]. Though the term has been typically used with this meaning, several investigators believe that necrosis is not a form of cell death but only refers to features that become apparent after cell death, which can occur by any cell death form, including apoptosis and autophagy[130, 132, 133]. However, with no consensus, the NCCD recommends that the term necrosis be

used to mean a type of cell death that involves rupture of the plasma membrane without the hallmarks of apoptosis or autophagy[111–113, 115]. Different organelles and cellular processes are implicated in necrotic cell death than in apoptosis and autophagy. These primarily include a gain in cell volume (*oncosis*); swelling of the endoplasmic reticulum, mitochondria, and Golgi apparatus; increases in the cytosolic concentration of calcium (which results in mitochondrial overload and activation of non-caspase proteases); freed lysosomal hydrolases; degradation of nucleic acids, proteins, and lipids (e.g., calpains and cathepsins); and ultimately rupture of the plasma membrane and release of cellular content into the extracellular space causing inflammation[111, 134, 135]. Since oncosis precedes cell death and is accompanied by cellular swelling, organelle swelling, blebbing, and increased membrane permeability, it was proposed as the term to define the pre-lethal portion of the necrosis pathway[130, 136].

Necroptosis and the interrelationship with apoptosis and inflammation

For many years, necrotic cell death was considered only as a form of ACD. However, accumulating evidence showed that necrotic cell death could result from finely regulated sets of signal transduction pathways and catabolic mechanisms[135, 137–142]. The form of RCD displaying a necrotic cell death phenotype was named "*necroptosis*"[143]. A variety of triggers initiates necroptosis, including tumor necrosis factor-alpha (TNFα), other cytokines of the TNF superfamily, such as TNF-Related Apoptosis-Inducing Ligand (TRAIL/TNFSF10) and Fas Ligand (FasL/TNFSF6), interferons (IFNs), pathogen-associated molecular patterns (PAMPs), lipopolysaccharides (LPS), dsRNA, DNA damage, viral infections, anti-cancer drugs, etc. (Figure 2). These ligands bind to specific receptors, including FAS, TRAILR 1/2, and Toll-like receptors 2 and 4 (TLR2/4), respectively[144]. Most of the knowledge gained about necroptosis is based on TNFα-TNFR1 signaling[140].

When TNFα binds to TNFR1, it causes trimerization of TNFR1 and the activation of death domains in the intracellular site via removal of SODD (Silencer of death domain). TNFR1 activation induces the formation of Complex I, which includes the receptor-interacting serine/threonine-protein kinase (RIPK1), adaptor proteins TNFR1-associated death domain (TRADD) and TNF receptor-associated factors 2 and 5 (TRAF2/TRAF5), and E3 ubiquitin ligases (cIAP1/cIAP2, and LUBAC complex)[145] (Figure 2). RIPK1 is regulated by multiple posttranslational modifications, being ubiquitination one of the most critical regulatory mechanisms. cIAP1/2 are recruited into Complex I, which with the help of TRAF2/5, mediate RIPK1 K63 ubiquitination. K63 ubiquitination of RIPK1 by cIAP1/2 facilitates the recruitment of the LUBAC complex, which performs further ubiquitination of RIPK1[146].

RIPK1 was the first protein demonstrated to be essential for TNFα-, Fas-, and TRAIL-induced necroptosis. The removal of ubiquitin chains from RIPK1 leads to its interaction with FADD, TRADD, RIPK3, and caspase-8, resulting in Complex II formation[138] (Figure 2). Under conditions of caspase-8 inactivation/depletion or cIAP deficiency, RIPK1 and RIPK3 are not cleaved and become phosphorylated. RIPK1 is activated via deubiquitination mediated by cylindromatosis (CYLD), which destabilizes Complex I and promotes activation of a cytosolic necrosome complex, also known as Complex IIb[147,

148] (Figure 2). This complex contains a hetero-oligomer of RIPK1 and RIPK3, which interact through their cognate RHIM domains[149–152]. Activated RIPK1 undergoes autophosphorylation at Ser14/15, Ser20, and Ser161/166[153]. Ser166 phosphorylation has emerged as a biomarker of RIPK1 activation[153–155]. RIPK1 autophosphorylation is then followed by RIPK3 autophosphorylation in the necrosomes on Ser227 (Thr231/Ser232 for mouse RIPK3)[140, 156]. Upon RIPK3 autophosphorylation, the mixed lineage kinase domain-like pseudokinase (MLKL) is phosphorylated at segment residues Thr357/Ser358[157, 158] (Ser 345 for mouse MLKL)[159, 160], causing pore-forming oligomers that puncture cell membranes, inducing cell death by necroptosis[138, 139, 158, 159, 161] (Figure 2). MKLK, which is downstream of kinases RIPK1 and RIPK3, is considered a more specific kinase for the necroptosis pathway[158, 159].

TNFα-TNFR1 signaling and RIPK1 are not exclusive to necroptosis since they are also involved in inflammation pathways, via kinase-dependent and independent functions, and in apoptosis[155, 162–166] (Figure 2). Indeed, polyubiquitinated RIPK1 in Complex I promotes the recruitment and activation of TAK1 kinase through the polyubiquitin binding adaptors TAB2/TAB3 and recruitment of the IKK complex, leading to NF-kB activation, gene expression of pro-inflammatory cytokines, and inflammation[167–169] (Figure 2). Furthermore, TRADD and RIPK1 can become modified and dissociate from TNFR1. The liberated death domain (DD) of TRADD (and/or RIPK1) binds to FADD, resulting in RIPK cleavage, caspase-8 recruitment (forming complex IIa), activation of caspase-8, which results in activation of caspase-3, and cell death by apoptosis[170–172] (Figure 2).

Unlike apoptosis[121], necroptosis can trigger or amplify inflammation[140, 173–175] and mediates a variety of inflammatory conditions, including periodontal, autoimmune, infectious cardiovascular, and pulmonary diseases[176–184], strongly suggesting that necroptosis plays a critical role in many other different disease processes[138–141, 161, 169, 176, 185].

Osteocyte Death

Osteocytes, as postmitotic cells, cannot replicate. However, they have developed adaptative mechanisms to ensure their survival under stressful conditions, such as immobilization, hypoxia, and disease[35]. However, when their survival capacity is overwhelmed, osteocytes can die. Osteocyte death has been associated with pathological conditions including osteoarthritis[186], inflammatory skeletal diseases[185, 187, 188], metastatic bone disease[189, 190], aging[191, 192], osteonecrosis of the femoral head[29], osteoradionecrosis[193], periodontitis[194–196], and MRONJ[2, 197].

All three forms of cell death (apoptosis, autophagy, and necrosis)[120] have been recognized in osteocytes. Osteocyte apoptosis was demonstrated under different conditions, including skeletal immobilization due to oxygen deprivation[198], osteonecrosis of the femoral head (ONFH)[29], estrogen withdrawal[199–201], and bone microcracks after bone fatigue[202–204]. It has also been associated with the natural process of aging, after menopause and bone unloading/weightlessness[205, 206]. Furthermore, increased osteocyte apoptosis plays an essential role in the decreased bone strength observed with glucocorticoid (GC)

treatment[207]. Notably, treatment with N-BPs reduces osteocyte apoptosis in response to fatigue loading[208] and protects against GC-induced apoptosis by transiently increasing ERK phosphorylation[209]. A similar effect was observed with calcitonin and mechanical stimulation[210]. Mechanical stimulation also prevented osteocyte apoptosis[210], and treatment of OVX mice with a pan-caspase inhibitor inhibited OVX-induced osteocyte apoptosis and reduced bone resorption[201]. A study showed that young mice lacking FGFR1/FGFR2 or only FGFR1 are phenotypically normal. However, at age 6-12 weeks, mice developed a high bone mass phenotype and increased porosity preceded by a striking peak in osteocyte death, particularly by apoptosis[211]. The study identified a role for FGFR1 signaling in osteocytes and mature osteoblasts, which is required for osteocyte survival and the regulation of bone mass.

Osteocytes can also undergo autophagy[212–215]. GCs activate the autophagosomal pathway in osteocytes, increasing markers of autophagy[212]. This mechanism could be beneficial to repair damaged organelles or cell membranes. However, dexamethasone also reduced the number of metabolically normal osteocytes. This effect was augmented when autophagy was suppressed, suggesting that autophagy is an adaptative mechanism used by osteocytes to attenuate the impact of GCs[212]. The cell protective function of autophagy is likely to occur under short or moderate stress conditions. However, higher or more prolonged stress may result in an accumulation of autophagosomes and cell death[213]. This is not surprising since autophagy previously was suggested to act as a “double-edged sword” involved in both cell protection and cell death[216, 217]. Mechanical compression forces were also found to activate autophagy in osteocytic cells (MLO-Y4) *in vitro* and osteocytes *in vivo,* as demonstrated in an orthodontic tooth movement model[214]. Notably, suppression of osteocyte autophagy caused skeletal changes similar to those caused by aging, including decreased bone mass and strength[215].

Osteonecrosis implies the death of bone cells. It can be caused by disease or trauma, such as a fracture, which negatively affects the blood supply to the bone. Osteonecrosis can also be idiopathic, but the pathological picture and resultant early clinical course are quite stereotypical[218]. The term osteonecrosis for certain skeletal conditions, such as aseptic, avascular, or ischemic necrosis, may be technically inaccurate, as it has not been demonstrated that the bone cells die by necrosis[29]. Komori[219] proposed that any form of osteocyte death, such as apoptosis or autophagy, ultimately results in secondary necrosis because dead osteocytes encased in the bone matrix cannot be immediately reached by phagocytic scavenger cells[220].

Necrosis ultimately leads to the rupture of the osteocyte cytoplasmic membrane, with most of the intracellular content being released into the extracellular environment[221]. Dying osteocytes release large amounts of DAMPs into the lacuna and adjacent canaliculi, including the histone deacetylase complex subunit SAP130, released and degraded cartilage matrix constituents, S100 family molecules, the high-mobility group box 1 (HMGB1) protein, purine metabolites, heat-shock proteins, and uric acid[185, 220]. DAMPS released into the canaliculi reach bone surfaces and vascular canals, initiating inflammatory responses by binding to various PRRs, such as the macrophage inducible C-type

lectin receptor Mincle, TLR2/4, and RAGE on osteoclasts, macrophages, dendritic cells, monocytes, neutrophils[222–225].

Notably, necroptosis has also been identified as a RCD form in osteocytes under certain conditions[226–228]. Indeed, in addition to apoptosis, necroptosis was found in osteocytes under conditions of estrogen deficiency in OVX rats, suggesting the involvement of osteocyte necroptosis in the pathophysiology of postmenopausal osteoporosis[226]. Furthermore, necroptotic osteocytes and trabecular bone deterioration are related to the production of TNFα in OVX ratsl[227]. Besides apoptotic osteocytes, necroptotic osteocytes were also found in rats with GC-induced osteoporosis[228]. Notably, necrostatin-1 (Nec-1), a specific RIPK1 inhibitor that inhibits TNF-α induced necroptosis[143], ameliorated the skeletal effects of GCs[228]. The coexistence of apoptotic and necroptotic osteocytes is not surprising since it was previously suggested that apoptosis and necroptosis could co-occur[229].

Osteocyte death in MRONJ

Though bone necrosis is the hallmark of MRONJ, little attention has been paid to investigating the type of cell death afflicting osteocytes in MRONJ. Early studies[230] found focal areas of bone matrix necrosis in the mandible of dogs treated for three years with ALN. It has been shown that osteocyte death occurs as a physiologic end-stage of the skeleton's life cycle[231], and that the prevalence of osteocyte death increases with skeletal aging[232, 233]. Investigators might assume from these findings that a systematic process for removing dead osteocytes, perhaps based on bone resorption, exists in the adult skeleton. Based on these ideas, the authors[230] suggested that jaw bone necrosis associated with ALN treatment resulted from dead osteocyte accumulation caused by the suppression of bone resorption by ALN.

In contrast, it was suggested that the necrotic alveolar bone would have been efficiently removed in the absence of an N-BP and a normal bone turnover rate, particularly in jawbones that appear to have higher basal bone turnover than the postcranial skeleton[234, 235]. These authors[230] also proposed an alternate theory in which ALN could have directly affected osteocyte viability, decreasing their lifespan and increasing the rate of bone necrosis. However, in contrast to this theory, preclinical *in vivo* studies have shown that clinical doses of N-BPs positively affect osteocyte viability, preventing osteocyte apoptosis induced by GCs in mice[209], or by fatigue cyclic loading in rats[208]. Supra-clinical doses of ALN have cytotoxic *in vitro* effects on fibroblasts[236] and endothelial cells[237]. Since N-BPs accumulate in the skeleton in a dose-dependent manner, these authors[230] also suggested that with prolonged exposure to ALN, the local bone accumulation of drug could reach levels that cause cytotoxic effects on osteocytes. However, a cytotoxic effect of N-BPs on osteocytes has never been proven.

Sustained activation of Nod-like receptor (NLR) family, pyrin domain-containing protein 3 (NLRP3) inflammasome contributes to persistent inflammation and impaired cutaneous wound healing in diabetic mice and humans[238]. One study[239] showed that macrophages at MRONJ-like lesions of diabetic mice harbor an up-regulated expression of NLRP3

inflammasome components and that ZOL augmented the persistent NLRP3 activation in diabetic macrophages, which may have contributed to the impaired oral socket wound healing and increased incidence and severity of MRONJ-like lesions in the diabetic mice. Though the study showed increased caspase-1 expression in cells within the MRONJ-like lesion, it did not specifically investigate cell death types and mechanisms in the osteocytes.

Herein, we propose a model that in the presence of systemic risk factors (e.g., pARs), inflammation associated with oral risk factors sustains molecular signaling pathways, largely TNFα/TNFR1 signaling, that enhance RCD-related osteocyte death, particularly necroptosis and apoptosis. These, in turn, promote the propagation of inflammatory signaling, accelerating soft and hard tissue necrosis to induce MRONJ (Figure 3).

Clinical and preclinical data indicate that for MRONJ to occur, systemic risk factors (e.g., pARs and AgIs) and oral risk factors, such as tooth extraction and inflammatory dental disease (e.g., periodontitis, periapical infection) must co-occur[1, 2, 11–22]. Oral risk factors associated with inflammation and/or infection induce local production of pro-inflammatory cytokines, such as TNF-α and IL-1, which stimulate inflammation and osteocyte death in alveolar bone[194–196]. Cell death associated with tissue infection and inflammation is linked to ACD[240]. However, as described earlier, strong evidence suggests that biomolecules that activate inflammation, like TNF-α and others, simultaneously activate cell death by RCD mechanisms[114, 143, 161, 174, 241–243], including necroptosis[138, 140, 141, 161, 169, 176, 244] and apoptosis[245, 246], and also stimulate inflammation[173] (Figure 3).

In cells that die by apoptosis, the apoptotic cell bodies are quickly taken up by neighboring cells and degraded within phagolysosomes. Therefore, and in contrast to necrosis, apoptosis might not induce an inflammatory reaction harmful to the host[247].

However, if cells at the terminal phases of apoptosis are not immediately engulfed by phagocytes, they can undergo secondary necrosis[130, 248]. Secondary necrosis has been suggested to occur in osteocytes dying by apoptosis and autophagy[219, 220]. In patients taking antiresorptives, the removal of necrotic bone is delayed or suppressed. Thus, it is possible that osteocytes dying by apoptosis or autophagy remain longer in the LCN of the persistent necrotic bone, increasing the probability of secondary necrosis[219, 220].

Since empty osteocyte lacunae are the distinctive histologic feature of MRONJ, the cellular remnants resulting from apoptosis, autophagy, or necrosis have to undergo a process of clearance from the LCN. Mobilization of apoptotic bodies, DAMPs, or cellular debris from the bone necrotic sites would be limited by the low permeability of the LCN to the movement of large solutes[249, 250]. Typical diameters of lacunae and canaliculi are ~10μm and 0.03-0.3 μm, respectively[251–254]. The annular fluid space surrounding the osteocyte cell processes inside canaliculi is much smaller, ~50–100 nm wide[249, 254, 255], and is filled with a gel-like matrix composed of proteoglycans and other matrix molecules[256]. Besides providing resistance to fluid flow[255, 256], the pericellular matrix of the LCN modulates solute transport behaving as a molecular sieve[250, 257]. Selective *in vivo* perfusion studies with various sized tracers demonstrate that the cut-off size of the

LCN in adult bone lies between 7-12 nm[250, 254]. Interestingly, it is well accepted that convection due to mechanical loading augments solute diffusion in the bone as demonstrated in theory[258] and observed on histological sections[259, 260]. Apoptotic bodies range from 50 to 5000 nm in diameter[261]. Thus, even under mechanical loading conditions, they appear too large to circulate through the LCN[80]. As mentioned earlier, osteocytes dying by apoptosis, autophagy, or necrosis can undergo autolytic changes that result in the formation of DAMPs[219, 220, 262] (Figure 3). Andreev et al.[185] confirmed that high molecular weight DAMPs can circulate through the LCN. Thus, if DAMPs can circulate through the LCN, it is reasonable to suggest that dead osteocytes can be removed from the LCN in the form of DAMPs. Furthermore, DAMPs release into the canaliculi could reach bone surfaces and adjacent bone postcapillary venules, activating PRRs on osteoclasts, pericytes, and other types of phagocytic cells[222–225], enhancing innate immune responses and inflammation[263]. In addition, DAMPs could advance into the blood circulation, activate PRRs on immune cells in the circulation or lymphoid organs, and be cleared by macrophages in the red pulp of the spleen[264–267].

As seen in necrosis, necroptotic cells also manifest loss of membrane integrity and release of the cellular content, which function as DAMPs[140, 268] (Figure 3). Recently, Mincle was recognized as the PRR that more specifically senses another DAMP, SAP-130[269]. Dying osteocytes release SAP-130, and Mincle is highly expressed at skeletal sites of osteocyte death[185]. Mincle is specifically upregulated in osteoclasts in a RANK-RANKL-independent fashion, and its signaling appears to target bone resorption upon osteocyte death[185]. In patients taking N-BPs, though bone resorption is inhibited, the number of osteoclasts at bone surfaces does not decline[270]. When necrotic bone persists due to the inhibition of bone resorption by pARs, DAMPs, including SAP-130, would accumulate in the necrotic alveolar bone, suggesting that Mincle expression in N-BP-treated patients would be chronically elevated. Indeed, Mincle is highly expressed in necrotic bone areas of patients with MRONJ[185], and these authors suggested that SAP-130 and Mincle could be potential early markers for MRONJ. The DAMP molecule HMGB-1 activates TLR-2 and TLR-4, triggering an immune system response and inflammation in the extracellular milieu[271]. The pathophysiological significance of elevated expression of DAMPs and PRRs in the context of impaired bone resorption, as occurs in pAR-treated patients, has not been directly investigated.

After discovering necroptosis, several inhibitors of kinases involved in necroptosis and/or apoptosis signaling pathways, namely RIPK1, RIPK3 and MLKL, were developed[268, 272–274] (Figure 3). Necrostatins (Nec) are tryptophan-based compounds that inhibit RIPK1[153]. Nec-1 was first discovered during chemical screening for necroptosis antagonists[153]. However, Nec-1 has moderate potency, poor specificity, and poor pharmacokinetic properties[275, 276]. GSK2982772 is a highly selective inhibitor of RIPK1, being developed to treat chronic inflammatory diseases characterized by necroptosis and apoptosis[140]. GSK2982772 is currently being tested in clinical trials for psoriasis, rheumatoid arthritis, and ulcerative colitis. GSK’547[277] is a rodent-specific RIPK1 inhibitor that inhibits necroptosis, associated inflammation, and apoptosis[142, 153, 155, 272, 278]. RIPK3 provides the scaffold for RIPK1 that contributes to full caspase-8 activation independently of its kinase activity or intact RHIM domain to

induce apoptosis[279] and NF-kB mediated inflammation[280]. The RIPK3 inhibitor HS-1371 suppresses TNF-induced necroptosis but does not inhibit TNF-induced apoptosis, indicating that HS-1371 specifically inhibits RIPK3-mediated necroptosis by suppressing RIPK3[281]. Another potent RIPK3 inhibitor (Zharp-99) was recently developed to ameliorate necroptosis-associated inflammatory injury[282]. Notably, necrosulfonamide (NSA) is an MLKL inhibitor that selectively inhibits necroptosis[159]. Thus, if necroptosis and/or apoptosis are indeed involved in MRONJ pathophysiology, as well as inflammation, inhibitors for these signaling pathways[142, 268, 272, 277, 283–286] represent pharmacologic interventions that could slow/stop the progression of MRONJ. They might be applied as monotherapy in early phases of MRONJ (stage 0) or as adjunctive therapy to existing effecting practices, such as the infection control measures used in stages 1-3, or the surgical interventions used to reduce the heavy burden of necrotic bone in more advanced cases.

Conclusions

MRONJ is a potentially debilitating condition that affects patients with cancer and patients with osteoporosis who have been treated with pARs or AgIs and have concurrent oral risk factors, including tooth extraction or inflammatory dental disease. Though several mechanisms have been proposed to explain the occurrence of MRONJ, the underlying pathophysiology has not been completely elucidated.

Bone necrosis represents the hallmark of MRONJ. However, we know very little about the precise timing and mechanisms involved in osteocyte death in the context of this disease. Osteocytes are postmitotic cells that have developed adaptative mechanisms to ensure their survival under stressful conditions. However, when their survival capacity is overwhelmed, osteocyte death occurs. All the three general forms of cell death (apoptosis, autophagy, and necrosis) have been recognized in osteocytes under different pathological conditions. Osteocyte death is, in a certain way, distinct from cell death in other cell types because osteocytes are isolated in the bone matrix, meaning that osteocytes that die by apoptosis or autophagy cannot be immediately phagocytized by scavenger cells. Thus, osteocytes that die by these cell death mechanisms persist for some time in the bone matrix, possibly ending in molecular and morphologic changes of secondary necrosis[219, 220]. Necrosis leads to the rupture of the osteocyte cytoplasmic membrane, with most of the intracellular content being released into the extracellular environment. Necrotic cell death can also occur as *necroptosis,* a form of RCD[143]. Unlike apoptosis[121], necroptosis triggers or amplifies inflammation[140, 173, 174] and mediates a variety of different inflammatory conditions[138–141, 161, 169, 176–185], suggesting that it might be involved in MRONJ pathophysiology.

A proposed hypothesis for MRONJ is that signaling pathways associated with oral risk factors, particularly TNFα/TNFR1 signaling, intensify the inflammatory response and the triggering of RCD mechanisms, including necroptosis, apoptosis, or both (Figure 3). In the presence of antiresorptives, DAMPS that accumulate in necrotic bone activate various PRRs (present on osteoclasts, phagocytic and antigen-presenting cells) that amplify the inflammatory response, inducing further osteocyte cell death and soft tissue

necrosis. Several inhibitors of kinases involved in the necroptosis and/or apoptosis signaling pathways, namely RIPK1, MLKL, and RIPK3, have been developed[142, 268, 272, 277, 283–286]. Thus, if apoptosis, necroptosis, or both are involved in MRONJ pathophysiology, these inhibitors would represent pharmacologic interventions to slow/stop disease progression. In any case, improving our understanding of osteocyte death associated with MRONJ could be critical for developing more efficacious treatments.

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Abbreviations

ACD	Accidental cell death
AgIs	angiogenesis inhibitors
ALN	alendronate
BMPs	Bone morphogenetic proteins
cIAP	Cellular inhibitor of apoptosis protein
Cx	Connexins
CYLD	Deubiquitinase cylindromatosis
DAMPs	Damage-associated molecular patterns
DD	Death domain
DKK-1	Dickkopf-related protein 1
DMP1	Dentin matrix protein-1
ERK	Extracellular signal-regulated kinase
FADD	FAS-associated death domain
FasL/TNFSF6	Fas Ligand
FGFR	Fibroblast growth factor receptor
FGF23	fibroblast growth factor 23
GC	Glucocorticoid
HD	Homeodomain proteins
HMGB1	High-mobility group box 1 protein
IFNs	Interferons
IGF-1	Insulin-like growth factor-1

IKKs	IKKα and IKKβ complex
LNC	Lacuna-canalicular (network)
LRP	Low-density lipoprotein receptor-related protein
LPS	lipopolysaccharide
LUBAC	linear ubiquitin chain assembly complex
MEPE	Matrix extracellular phosphoglycoprotein
Mincle	Macrophage inducible C-type lectin receptor
MLKL	Mixed lineage kinase domain-like pseudokinase
MLO-Y4	Murine long bone osteocyte-like cell line
MRONJ	Medication-related osteonecrosis of the jaw
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
ONFH	Osteonecrosis of the femoral head
OVX	Ovariectomized
N-BPs	nitrogen-containing bisphosphonates
NCCD	Nomenclature Committee on Cell Death
NC	neural crest
Nec-1	Necrostatin-1
NLR	Nod-like receptor
NLRP3	NLR family, pyrin domain-containing protein 3
NSA	Necrosulfonamide
ONFH	Osteonecrosis of the femoral head
OPG	Osteoprotegerin
PAMPs	Pathogen-associated molecular patterns
pARs	powerful antiresorptives
PCD	Programmed cell death
PGE^2	Prostaglandin E2
PHEX	Phosphate regulating endopeptidase homolog X-linked protein
PRR	Pattern recognition receptors

PTH	Parathyroid hormone
P50	NF-κB1
P65	RelA
RAGE	Receptor for advanced glycation end-products
RANK	receptor activator of nuclear factor kappa-B
RANKL	RANK ligand
RCD	Regulated cell death
RIPK	Receptor-interacting serine/threonine-protein kinase
SAP130	Histone deacetylase complex subunit SAP130
SIBLINGs	Small integrin-binding ligand N-linked glycoproteins
SODD	Silencer of death domain
S100	Soluble 100% protein
TAB2	TGF-Beta Activated Kinase 1 (MAP3K7) binding protein 2
TAB3	TGF-Beta Activated Kinase 1 (MAP3K7) binding protein 3
TAK1	Transforming growth factor-*β*-activated kinase 1
TGF-β	Transforming growth factor-beta
TLR3/4	Toll-like receptors 3/4*FAS*: CD95/APO-1
TNFα	Tumor necrosis factor-alpha
TNFR1	TNF receptor 1
TRADD	TNFRSF1A-associated via death domain
TRAF	TNF receptor-associated factors
TRAILR1/2	TRAIL receptor 1/2
TRAIL/TNFSF10	TNF-Related Apoptosis-Inducing Ligand
VEGF	vascular endothelial cell growth factor
ZOL	zoledronic acid

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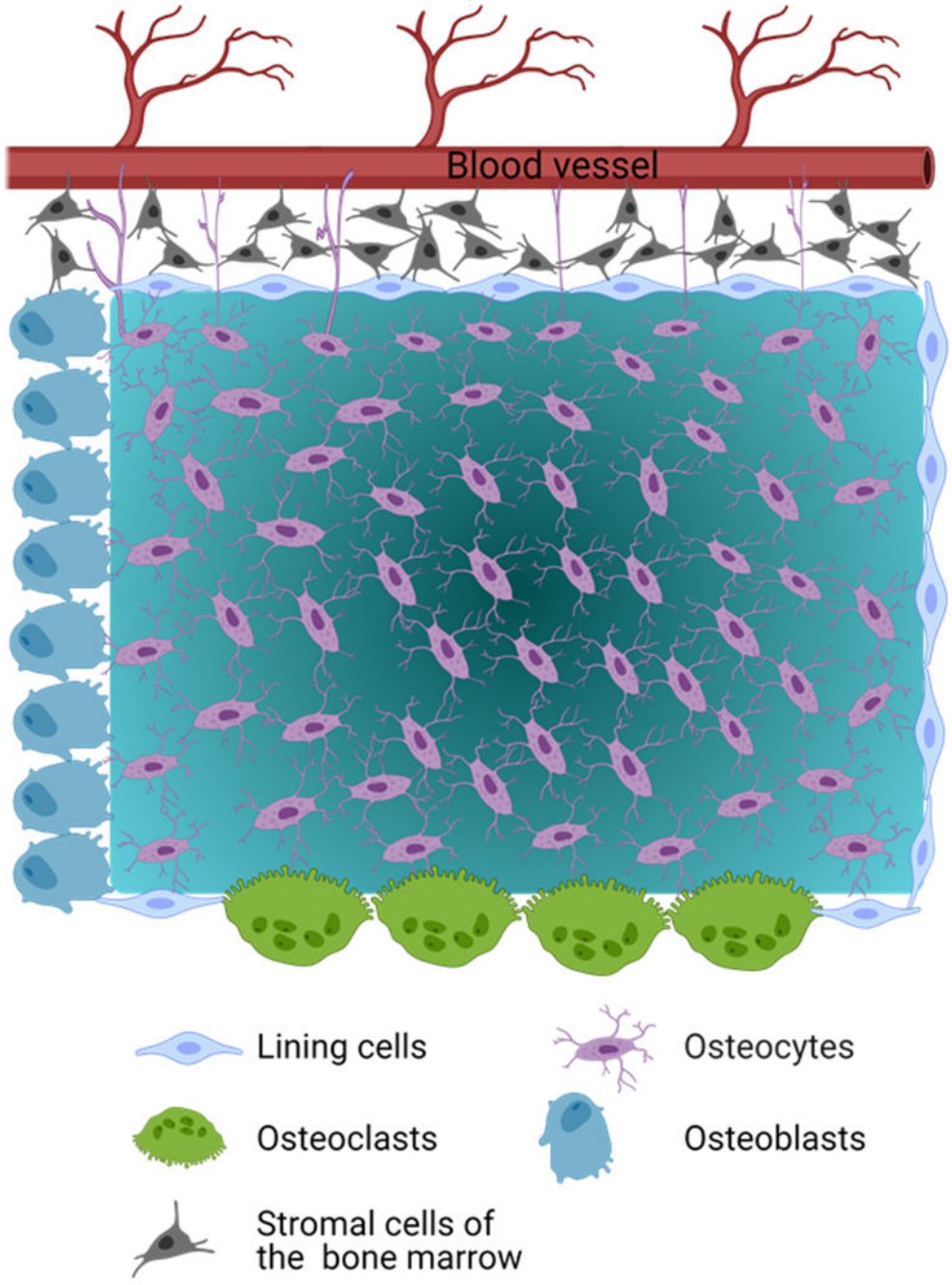

Figure 1. Cartoon depicting the lacunar-canalicular network (LCN) and the functional syncytium.

The LCN is the physical structure of interconnected tunnels and lacunae, where osteocytes reside. Osteocytes in the LCN form a functional syncytium with cells on the bone surfaces, including osteoblasts and lining cells, which in turn are in physical contact with stromal cells and hematopoietic stem cells in the marrow and endothelial cells of blood vessels. In addition, Osteocyte dendritic processes can extend beyond the cells in bone surfaces to directly interact with cells in the bone marrow and blood vessels[35], as depicted in the figure.

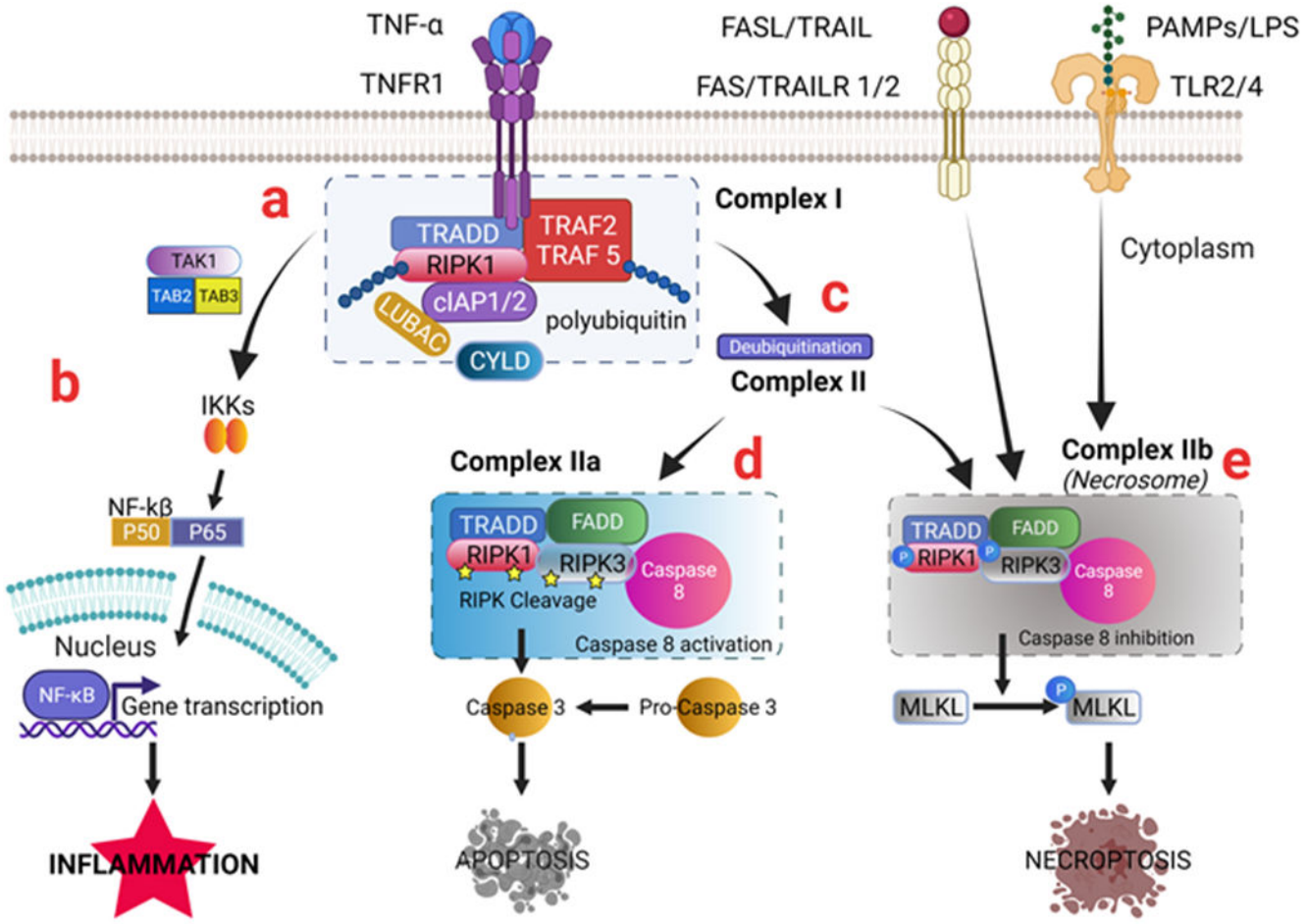

Figure 2. TNFα/TNFR1 signaling activates necroptosis and triggers apoptosis and inflammation.

TNFα/TNFR1 molecular signaling is explained in more detail. Other signals such as FASL/FAS, TRAIL/TRAIL, PAMPS, or LPS/TLR2/4 can also trigger necroptosis. TNFα is released during inflammatory conditions. (**a**) TNF*α* binds to TNFR1, inducing recruitment of TRADD, RIPK1, TRAF 2, TRAF5, cIAP 1/2, and other molecules to form *Complex I*. (**b**) Upon polyubiquitinated RIPK1 in *Complex I*, the TNFα/TNFR1 signaling can activate IKKs, which triggers the NF-*k*B signaling pathway cascade that leads to gene expression of pro-inflammatory cytokines and inflammation. (**c**) deubiquitination and activation of RIPK1 by CYLD lead to the formation of *Complex II*. (**d**) TRADD and RIPK1 become modified and dissociate from TNFR1. The liberated death domain(DD) of TRADD (and/or RIPK1) binds to FADD, resulting in RIPK cleavage, caspase-8 recruitment (forming *Complex IIa*), activation of Caspase 8, which results in Caspase 3 activation and apoptosis. **e**) Inactivation of Caspase-8 in *Complex II* leads to the phosphorylation and activation of RIPK1, RIPK3, and subsequent phosphorylation and activation of MLKL during the necrosome assembly (*Complex IIb*), oligomerization of MLKL monomer leads to induction of necroptosis. *Abbreviations*: *TNF*: tumor necrosis factor; *TNFR1*: TNF receptor 1; *FAS*: CD95/APO-1; *FASL*: FAS ligand; *TRAIL*: CD253 or TNFSF10; *TRAILR1/2*: TRAIL receptor 1/2; *PAMPs*: Pathogen-associated molecular patterns; *LPS*: lipopolysaccharide. *TLR3/4*, Toll-

like receptors 3/4; *TAK1*: Transforming growth factor-β-activated kinase 1; *TAB2*: TGF-Beta Activated Kinase 1 (MAP3K7) binding protein 2; *TAB3*: TGF-Beta Activated Kinase 1 (MAP3K7) binding protein 3; *IKKs*: IKKα and IKKβ complex; *NF-kB*: nuclear factor kappa-light-chain-enhancer of activated B cells; *P50*:NF-κB1; *P65*: RelA; *TRADD*: TNFRSF1A-associated via death domain; *RIPK*: Receptor interacting serine/threonine kinase; *TRAF*: TNF receptor-associated factors; *cIAP*: Cellular inhibitor of apoptosis protein; *CYLD*: Deubiquitinase cylindromatosis; *FADD*: FAS-associated death domain; *MLKL*: mediator mixed-lineage kinase domain-like.

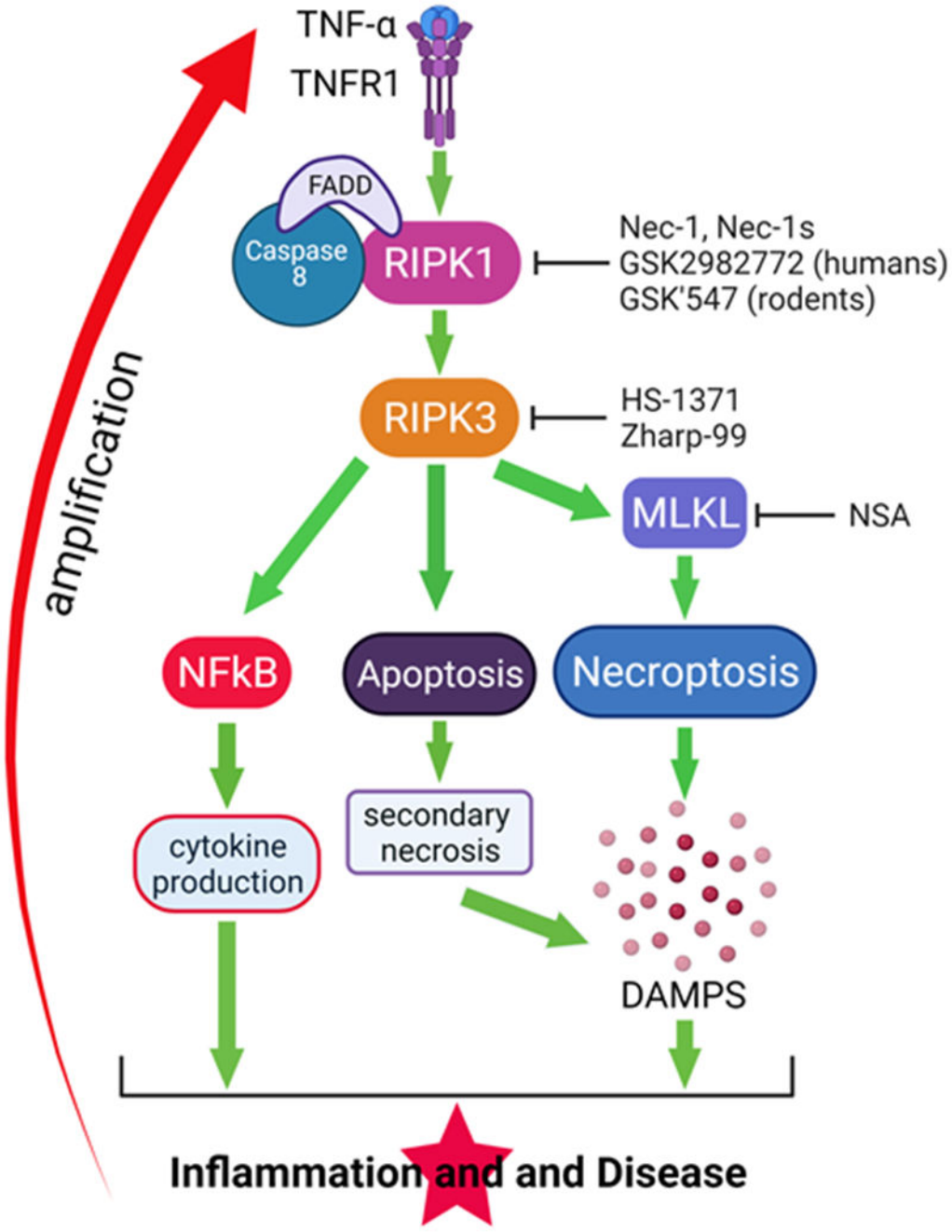

Figure 3. A proposed model for MRONJ.

In the presence of systemic risk factors (e.g., pARs or AgIs), the inflammation associated with oral risk factors induces and sustains molecular signaling pathways, largely TNF-α -TNFR1, which enhance osteocyte death particularly by necroptosis but also apoptosis. TNF-α -TNFR1 signaling also promotes the activation of the NFkB cascade with the synthesis of pro-inflammatory cytokines. pARs prevent the resorption of bone, including necrotic bone. The accumulated necrotic osteocytes generate an accrued amount of DAMPs that further stimulate inflammation. Altogether these events amplify inflammation,

oral soft and hard tissue destruction, and induction of MRONJ. Several inhibitors of RIPK1 (Nec-1, Nec-1s, GSL2982773, GSK'547); RIPK3 (HS-1371, Zharp-99); and MLKL (NSA) have been developed that, if apoptosis, necroptosis, or both are involved in MRONJ pathophysiology, will represent pharmacologic interventions to slow/*stop MRONJ progression*. *Abbreviations*: *TNF*: tumor necrosis factor; *TNFR1*: TNF receptor 1; *NF-kB*: nuclear factor kappa-light-chain-enhancer of activated B cells; *RIPK*: Receptor interacting serine/threonine kinase; *FADD*: FAS-associated death domain; *MLKL*: mediator mixed-lineage kinase domain-like; DAMPs: damage-associated molecular patterns; *Nec-1*: Necrostatin 1; *NSA*: necrosulfonamide.