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***Aggregatibacter actinomycetemcomitans*, a potent immunoregulator of the periodontal host defense system and alveolar bone homeostasis**

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Summary

Aggregatibacter actinomycetemcomitans is a perio-pathogenic bacteria that has long been associated with localized aggressive periodontitis. The mechanisms of its pathogenicity have been studied in humans and pre-clinical experimental models. Although different serotypes of *A. actinomycetemcomitans* have differential virulence factor expression, *A. actinomycetemcomitans* cytolethal distending toxin (CDT), leukotoxin, and lipopolysaccharide (LPS) have been most extensively studied in the context of modulating the host immune response. Following colonization and attachment in the oral cavity, *A. actinomycetemcomitans* employs CDT, leukotoxin, and LPS to evade host innate defense mechanisms and drive a pathophysiologic inflammatory response. This supra-physiologic immune response state perturbs normal periodontal tissue remodeling/turnover and ultimately has catabolic effects on periodontal tissue homeostasis. In this review, we have divided the host response into two systems: non-hematopoietic and hematopoietic. Non-hematopoietic barriers include epithelium and fibroblasts that initiate the innate immune host response. The hematopoietic system contains lymphoid and myeloid-derived cell lineages that are responsible for expanding the immune response and driving the pathophysiologic inflammatory state in the local periodontal microenvironment. Effector systems and signaling transduction pathways activated and utilized in response to *A. actinomycetemcomitans* will be discussed to further delineate immune cell mechanisms during *A. actinomycetemcomitans* infection. Finally, we will discuss the osteo-immunomodulatory effects induced by *A. actinomycetemcomitans* and dissect the catabolic disruption of balanced osteoclast-osteoblast mediated bone remodeling, which subsequently leads to net alveolar bone loss.

Keywords

Aggregatibacter actinomycetemcomitans; periodontitis; inflammation; osteoclast; osteoblast

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Introduction

Aggregatibacter actinomycetemcomitans is a Gram negative capnophilic coccobacillus, which is commonly isolated from the oral cavity of adolescents and young adults afflicted by aggressive periodontal disease states (Newman *et al.*, 1976; Slots, 1976; Slots *et al.*, 1980). In youth, localized aggressive periodontitis, formally known as localized juvenile periodontitis (Armitage, 1999), was classically described as affecting preferentially the succedaneous 1st molars and incisors (Van der Weijden *et al.*, 1994). In addition, *A. actinomycetemcomitans* has been implicated in extra-oral infections, including infective endocarditis (Nakano *et al.*, 2007), bacterial arthritis (Cuende *et al.*, 1996), pregnancy associated septicemia (Shalini *et al.*, 1995), cerebral abscesses (Stepanovic *et al.*, 2005; Zijlstra *et al.*, 1992), and osteomyelitis (Antony *et al.*, 2009). The primary source however is ultimately the oral cavity (Fine *et al.*, 2007; van Winkelhoff *et al.*, 1993; Zambon, 1985), thus requiring translocation of *A. actinomycetemcomitans* from the oral cavity to extra-oral infection sites.

This review will critically evaluate host immune response mechanisms to *A. actinomycetemcomitans* in the following categories (Fig. 1):

- I. Response of initial non-hematopoietic host barriers to *A. actinomycetemcomitans*: epithelium and fibroblast function
- II. Response of cells of hematopoietic origin to *A. actinomycetemcomitans*: myeloid and lymphoid lineages
- III. *Aggregatibacter actinomycetemcomitans* induced osteo-immunomodulatory effects impacting osteoclast-osteoblast mediated alveolar bone remodeling

In periodontal health the oral cavity is colonized by the oral commensal (non-pathogenic) flora. Under physiological states the normal oral flora stimulates the innate immune defense system in the periodontium, which controls bacterial colonization of periodontal tissues in close proximity to the gingival sulcus. In this periodontal microenvironment, *A. actinomycetemcomitans* infection induces a supra-physiological immune-inflammatory response state, which disrupts normal periodontal tissue homeostasis in the gingiva, periodontal ligament (PDL), cementum, and alveolar bone, ultimately promoting tooth loss. Clinical treatment of aggressive periodontal disease historically proves to be challenging. *Aggregatibacter actinomycetemcomitans* depletion is poorly achieved by scaling and root planing (Christersson *et al.*, 1985; Kornman *et al.*, 1985; Renvert *et al.*, 1990). However, scaling and root planning with concomitant systemic antibiotic therapy or surgical intervention supports the realization of desirable microbiological and clinical outcomes (Christersson *et al.*, 1985; Kornman *et al.*, 1985). While there are six serotypes of *A. actinomycetemcomitans*, serotypes a, b, and c have been isolated most often from periodontal diseased sites (Bandhaya *et al.*, 2012; Fine *et al.*, 2007; Kawamoto *et al.*, 2009). Different serotypes possess differential virulence factor expression, making some serotypes more pathogenic than others. For example concerning leukotoxin (LtxA), a surface-associated and secreted protein by *A. actinomycetemcomitans* (Kachlany *et al.*, 2000), levels are variable on serotypes a-c, but higher concentrations may enhance *A. actinomycetemcomitans* pathogenesis with the potential to exacerbate periodontal disease

progression. To date, animal experimental periodontal disease models have been the primary means used to advance the understanding of cellular mechanisms mediating *A. actinomycetemcomitans* pathogenesis.

Aggregatibacter actinomycetemcomitans virulence factors interact with host cells to initiate an aberrant inflammatory response in the periodontal gingival tissues. While it has been reported that trans-epithelial migration of polymorphonuclear leukocytes (PMNs) into the gingival sulcus results in a formed pseudo-barrier, which is several cell layers thick between the plaque and junctional/sulcular epithelium surface (Garant, 1976), this review considers the gingival epithelium to be the initial barrier to *A. actinomycetemcomitans*. First responders are non-hematopoietic resident cells: gingival fibroblasts and epithelium cells. *Aggregatibacter actinomycetemcomitans* stimulates the host responses via exotoxin and endotoxin virulence factors, activating superficial epithelial cells and underlying fibroblast cells. *Aggregatibacter actinomycetemcomitans* can effectively migrate through the gingival epithelium (Fives-Taylor *et al.*, 1999), and once *A. actinomycetemcomitans* bypasses these initial barriers, a host inflammatory response is initiated. Once *A. actinomycetemcomitans* penetrates deeper in the subgingival tissues, a broader host immune response is activated.

Following the initial colonization, innate immune cells derived from the myeloid hematopoietic origin such as monocytes, neutrophils, and dendritic cells (DCs) are recruited into the periodontal microenvironment. Notably and highly central to its pathogenicity, *A. actinomycetemcomitans* has intrinsic defense mechanisms against host cells. Leukotoxin and cytolethal distending toxin (CDT) are *A. actinomycetemcomitans* exotoxins that contribute to its pathogenesis. CDT was detectable in a majority of *A. actinomycetemcomitans* isolated from Swedish (Ahmed *et al.*, 2001) and Brazilian cohorts (Fabris *et al.*, 2002). *Aggregatibacter actinomycetemcomitans* strains with leukotoxin are also associated with aggressive periodontal disease states (Bueno *et al.*, 1998; Haraszthy *et al.*, 2000). Leukotoxin is thought to be the main *A. actinomycetemcomitans* constituent that causes cell death of monocytes (Taichman *et al.*, 1980), PMNs (Taichman *et al.*, 1980), and T cells (Mangan *et al.*, 1991), and facilitates *A. actinomycetemcomitans* evasion of host defense mechanisms.

Aggregatibacter actinomycetemcomitans perpetuation of the host immune system promotes adaptive immune responses by T and B cells that are derived from lymphoid progenitors in the hematopoietic system. Initial responding resident tissue macrophages and DCs secrete cytokines and chemokines to promote activation and recruitment of B and T cells, which are found at high density in periodontal disease afflicted sites (Lin *et al.*, 2011; Page *et al.*, 1976). Patients with localized aggressive periodontitis had circulating opsonic IgG antibodies against *A. actinomycetemcomitans* (Baker *et al.*, 1989), which are secreted by mature B cells (plasma cells). *Aggregatibacter actinomycetemcomitans* also induces cytokines that polarize T helper (Th) cells, including Th1, Th2, Treg, and Th17 cells. *Aggregatibacter actinomycetemcomitans* stimulates interferon (IFN)- γ and interleukin (IL)-12 production that polarize Th1 (Garlet *et al.*, 2005; Kobayashi *et al.*, 2000) cells and IL-4 that polarizes Th2 cells (Garlet *et al.*, 2006). Thus, *A. actinomycetemcomitans* initiates a complex Th1 and Th2 response during disease progression.

Aggregatibacter actinomycetemcomitans immune stimulation in the periodontal microenvironment elicits a pathophysiologic pro-inflammatory state, which disrupts normal periodontal tissue remodeling processes ultimately promoting collateral tissue damage. In periodontal disease, the supra-physiologic level of pro-inflammatory and pro-resorptive cytokines favors alveolar bone resorption by monocyte or defined osteoclast progenitor (dOCP) derived osteoclasts, versus alveolar bone formation by mesenchymal derived osteoblastic cells. When bone resorption exceeds bone formation, an unbalanced bone remodeling process having catabolic effects on alveolar bone homeostasis, ultimately results in net alveolar bone loss (Baron *et al.*, 1978). Bone-lining tartrate resistant acid phosphatase (TRAP) positive multinucleated osteoclasts secrete bone degradation enzymes, including matrix metalloproteinases (MMPs) and cathepsin K in the acidic sealing zone microenvironment, via integrin protein adherence to the bone surface (McCauley *et al.*, 2002; Teitelbaum *et al.*, 1997). *Aggregatibacter actinomycetemcomitans* has been shown to induce osteoclast formation and bone loss in rodent animal models (Dunmyer *et al.*, 2012; Garlet *et al.*, 2005; Lima *et al.*, 2010). Pro-inflammatory cytokines with potent pro-resorptive actions, including tumor necrosis factor (TNF)- α , IL-1, and IL-6 are highly upregulated by *A. actinomycetemcomitans* and thus promote osteoclast formation and bone resorption (Assuma *et al.*, 1998; Chiang *et al.*, 1999; Hotokezaka *et al.*, 2007; Ishimi *et al.*, 1990; Zhang *et al.*, 2001). In humans, *A. actinomycetemcomitans* positive patients had significantly greater periodontal bone loss than the *A. actinomycetemcomitans* negative subjects (Fine *et al.*, 2007), supporting *A. actinomycetemcomitans*'s remarkable impact on periodontal disease associated alveolar bone loss.

I. Response of initial non-hematopoietic host barriers to *A. actinomycetemcomitans*: epithelium and fibroblast function

Non-hematopoietic barriers to *A. actinomycetemcomitans* include gingival epithelial cells, resident gingival fibroblasts, and PDL fibroblasts. *Aggregatibacter actinomycetemcomitans* has the ability to adhere to mucosal and gingival epithelial cells, which is believed to be essential for its initial colonization in the oral cavity (Arirachakaran *et al.*, 2012; Meyer *et al.*, 1994). Fibroblasts derived from a mesenchymal stem cell origin are extracellular matrix collagen building cells capable of responding to pathogenic insult. Although osteoblasts are derived from non-hematopoietic precursors, they are discussed in detail in section III of this review. See Table 1 for a comprehensive list of known non-hematopoietic cellular interactions with *A. actinomycetemcomitans*.

Gingival epithelium invasion and apoptosis—In an *in vitro* model, *A. actinomycetemcomitans* was able to migrate through three layers of human gingival epithelium cells by upregulating pro-inflammatory cytokine production of TNF- α , IL-1 β , IL-6, IL-8 and increasing cell apoptosis (Dickinson *et al.*, 2011). IL-8 in particular is a chemotactic ligand to CXCR1 and CXCR2 expressed on neutrophils, which are primary innate immune cells central to the host defense response. A study utilizing the *A. actinomycetemcomitans* rat-feeding model found that *A. actinomycetemcomitans* mediated gingival epithelium apoptosis through the caspase 3/7 pathway (Kang *et al.*, 2012). At the subcellular level, *A. actinomycetemcomitans* stimulated Smad2 phosphorylation via TGF- β 1 signaling in human gingival epithelium cells and the human epithelial cell line OBA9

leading to cleaved caspase 3 activity and subsequent cell apoptosis (Yoshimoto *et al.*, 2014). These findings indicate the potential for *A. actinomycetemcomitans* to traverse gingival epithelium through pro-apoptotic mechanisms. Disruption of the gingival epithelium results in the induction of a pathologic inflammatory periodontal microenvironment that supports recruitment of hematopoietic lineage derived immune response cells to subgingival tissues.

Fibroblasts initiate inflammation—Resident gingival and PDL fibroblasts are two distinct fibroblast subsets located in the periodontium that act as first responders to *A. actinomycetemcomitans* infection. DNA microarray analysis revealed differential gene expression of gingival fibroblasts and PDL fibroblasts (Han *et al.*, 2002). *Aggregatibacter actinomycetemcomitans* promoted secretion of IL-6 and IL-8 from human gingival fibroblasts (Belibasakis *et al.*, 2005b; Im *et al.*, 2015). Although *A. actinomycetemcomitans* did not stimulate IL-1 β and TNF- α secretion from gingival fibroblasts (Belibasakis *et al.*, 2005b), both cytokines stimulated RANKL promoter activity and cell surface expression on bone marrow stromal cells through the p38 mitogen activated protein kinase (MAPK) pathway (Belibasakis *et al.*, 2005b; Rossa *et al.*, 2006; Rossa *et al.*, 2005). Bone marrow stromal cells can differentiate into fibroblasts under defined culture conditions. IL-1 β and TNF- α cytokines also stimulated p38 and JNK phosphorylation in immortalized mouse PDL fibroblasts (Rossa *et al.*, 2005). Resident gingival and PDL fibroblasts elaborate pro-inflammatory mediators consistent with the early immune-inflammatory response during *A. actinomycetemcomitans* elicited perio-pathogenesis.

II. Response of cells of hematopoietic origin to *A. actinomycetemcomitans*: myeloid and lymphoid lineages

Host innate immune-inflammatory cells are responsible for phagocytizing, clearing, and secreting effector molecules necessary for defending against perio-pathogenic bacteria. The mechanisms mediating host inflammatory cell recruitment by *A. actinomycetemcomitans* have been studied in experimental animal models and characterized in clinical investigations of aggressive periodontal disease. Circulating host immune cells possess surface integrins and chemokine receptors that facilitate crossing the vascular endothelium in response to local chemokines present in the periodontal tissue matrix. Once inflammatory cells are recruited to the local site of infection, they employ overlapping and unique effector defense functions. Inflammatory cells identified during *A. actinomycetemcomitans* infection include, but are not limited to, myeloid-derived macrophages, neutrophils, and DCs, and B and T lymphocyte populations. See Table 1 for a more comprehensive list of known hematopoietic cellular interactions with *A. actinomycetemcomitans*.

Chemokine and chemokine receptor recruitment of inflammatory cells—

Chemokines recruit inflammatory cells to the local site of infection through cell surface chemokine receptors. Garlet *et al.* (2003, 2005) characterized chemokine/chemokine receptor signaling in clinical and experimental rodent *A. actinomycetemcomitans* infected periodontal tissues. In patients afflicted by aggressive periodontal disease, tissue biopsies had increased gene expression of CCL3 (MIP-1 α), CCR5, CXCR3, and interferon inducible protein (IP)-10 (CXCL10) (Garlet *et al.*, 2003). Both CCR5 and CXCR3 are Th1 receptors that respond to CCL3 and IP-10 respectively, but these receptors are expressed by multiple

cell types and are characterized by non-specific ligand binding. For example, F4/80+ macrophages concomitantly expressed CCR5 and CCR1 surface receptors, both of which are the receptors for CCL3, in response to oral *A. actinomycetemcomitans* inoculation in a murine periodontal disease model (Repeke *et al.*, 2010). In a similar mouse model, CCL3, CCL4 (MIP-1 β), CCL5 (RANTES), and CCL11 transcript expression was also detected early and sustained throughout the 60-day study duration (Garlet *et al.*, 2005). Interestingly, a human longitudinal study suggested that elevated CCL3 was a prognostic marker for bone loss in periodontal disease subjects colonized by *A. actinomycetemcomitans* (Fine *et al.*, 2009).

In the oral murine *A. actinomycetemcomitans* infection model, infiltrating cells assessed via flow cytometry showed that Gr1+ cell (neutrophil marker) numbers increased more significantly than F4/80+ cells (macrophage marker) 24 hours following initial inoculation (Garlet *et al.*, 2005). CXCL1, a chemo-attractant cytokine whose primary role is to recruit neutrophils through ligand binding to CXCR1 and CXCR2 receptors, was the first chemokine mRNA upregulated in response to *A. actinomycetemcomitans* inoculation (Garlet *et al.*, 2005). CD3+CD4+ and CD3+CD8+ T lymphocytes were significantly increased at 1 week post-infection (Garlet *et al.*, 2005). Lastly, CD19+ B lymphocytes began to increase after 15 days and a Th-2 type response occurred after 30 days (Garlet *et al.*, 2005). While certain chemokine receptors are more highly expressed on specific immune cell populations, it must be noted that they are not unique to specific immune cell lineages. Similarly, chemokines are secreted by many cell types and typically function as ligands having affinity for more than one receptor. The incompletely understood pleiotropism and functional redundancy associated with a non-specific immune cell chemokine-chemokine receptor interactions implicate the importance of fine-tuned regulation in the host defense response during *A. actinomycetemcomitans* infection (Garlet *et al.*, 2003). These data highlight chemokines as dynamic biologic factors defining the host cell milieu throughout progression of *A. actinomycetemcomitans* pathogenesis.

Neutrophil interactions with the host and *A. actinomycetemcomitans*—

Neutrophils (polymorphonuclear leukocytes/PMNs) are critical first responders to microbial pathogenic insult. Controlled PMN activation promotes non-oxidative proteins and reactive oxygen species (ROS) required to destroy pathogens (Miyasaki *et al.*, 1986). Non-oxidative proteases include cathepsin G and elastase. Destructive effects of oxygen-derived free radicals were demonstrated by its depolymerization of hyaluronic acid and to a lesser extent proteoglycan, which are both constituents of extracellular matrix in the periodontium (Bartold *et al.*, 1984). PMNs isolated from patients with aggressive periodontal disease with IgG titers against *A. actinomycetemcomitans* demonstrated greater PMN phagocytosis and killing compared to PMNs isolated from periodontally healthy control subjects (Guentsch *et al.*, 2009). Relative to healthy control subjects, PMNs from aggressive periodontal disease patients were characterized by a significant increase in basal activity of human neutrophil elastase (Guentsch *et al.*, 2009). *In vitro*, cathepsin G, a neutrophil-derived proteinase, had more bactericidal activity against *A. actinomycetemcomitans* than elastase (Miyasaki *et al.*, 1991). With regards to the oxidative pathway, neutrophil phagocytosis of *A. actinomycetemcomitans* is associated with ROS, as indicated by the ability of *A.*

actinomycetemcomitans to induce neutrophil activity by promoting ROS production (Guentsch *et al.*, 2009; Permpnich *et al.*, 2006). It was suggested that self-aggregating *A. actinomycetemcomitans*, possessing a rough fimbriated surface, impaired PMN phagocytosis, which perhaps is simply secondary to PMN limitations in engulfing large foreign bodies (Holm *et al.*, 1993; Permpnich *et al.*, 2006). Leukotoxin expression is another potential mechanism employed by *A. actinomycetemcomitans* to resist phagocytosis (Johansson *et al.*, 2000). However contradictory reports concerning the role of leukotoxin expression levels in *A. actinomycetemcomitans* evasion by PMNs (Permpnich *et al.*, 2006) call into question the role of leukotoxin as a virulence factor in host immune cell evasion.

Aggregatibacter actinomycetemcomitans also exhibits cytotoxic effects on PMNs. It has been reported that *A. actinomycetemcomitans* serotype b strains, including JP2, possess enhanced cytotoxicity against neutrophils (Permpnich *et al.*, 2006). JP2, a highly studied serotype b strain, is characterized by excessive leukotoxin expression relative to other *A. actinomycetemcomitans* strains due to a 530-bp deletion mutation in the operon promoter region (Brogan *et al.*, 1994). Another study reported that leukotoxin variability of *A. actinomycetemcomitans* strains did not affect PMNs ability to kill *A. actinomycetemcomitans*, and speculated that undefined *A. actinomycetemcomitans* virulence factors are responsible for strain associated variability in PMN cytotoxicity (Holm *et al.*, 1993). Interestingly, extracellular supernatant was also cytotoxic, which was attributed to secreted leukotoxin (Permpnich *et al.*, 2006). Based on the evasive and destructive nature of *A. actinomycetemcomitans*, studies have been conducted to determine the effects of antibiotics on PMNs ability to clear *A. actinomycetemcomitans*. Neutrophils with intracellular azithromycin accumulation demonstrated decreased *A. actinomycetemcomitans* survival (Lai *et al.*, 2015). Taken together, the role of *A. actinomycetemcomitans* leukotoxin expression in PMN phagocytosis evasion is unclear. Perhaps such inconsistencies in reported findings are a product of strain and serotype heterogeneity as well as inconsistent methodology (Permpnich *et al.*, 2006).

Aggregatibacter actinomycetemcomitans ligands induce TLR signaling in macrophages—Macrophages are proven key players in *A. actinomycetemcomitans* pathogenesis. Monocytes enter the local tissue site of infection by diapedesis from the circulation where they differentiate into activated macrophages or osteoclasts. Surface toll-like receptors (TLRs) recognize pathogenic constituents and have been extensively studied in macrophage function. *Aggregatibacter actinomycetemcomitans* possesses endotoxic LPS, a potent agonist recognized by TLR2, TLR4, and TLR5 (Park *et al.*, 2014), with TLR4 currently being considered the primary receptor for LPS.

Park *et al.* (2014) clearly delineated the function of TLR2 and TLR4 signaling in macrophage interactions with *A. actinomycetemcomitans*. When bone marrow derived murine macrophages from *Tlr2*^{-/-} and *Tlr4*^{-/-} mice were stimulated with *A. actinomycetemcomitans*, TLR2 and TLR4 deficient macrophages versus wild-type control macrophages, exhibited attenuated production of pro-inflammatory cytokines TNF- α and IL-6. Furthermore, double TLR2/4-deficient macrophages demonstrated a more prominent blunting of pro-inflammatory cytokine secretion. MyD88 is the adapter protein for all TLRs (except for TLR3), IL-1R, and IL-18R. MyD88 deficiency further reduced cytokine

production by *A. actinomycetemcomitans*, suggesting that while TLR2 and TLR4 are critical regulators of *A. actinomycetemcomitans* induced inflammatory cytokine production, other receptors also propagate TNF- α and IL-6 production. *Aggregatibacter actinomycetemcomitans* also stimulated IL-12p40 (IL-12B) production in macrophages, which notably was also MyD88 dependent (Park *et al.*, 2014). These findings clearly show that TLR signaling is critical for inflammatory cytokine formation, but that it is unclear how TLR4 and TLR2 dually interact with *A. actinomycetemcomitans* to modulate its periopathogenesis.

MAPK and NF- κ B intracellular signaling in macrophages—Our project laboratory and other research groups have shown that MAPK and NF- κ B signaling are largely involved in *A. actinomycetemcomitans* pathogenesis through pre-clinical rodent experimental periodontal disease models (Dunmyer *et al.*, 2012; Li *et al.*, 2011; Yu *et al.*, 2011a). A consequence of TLR activation is regulation of intracellular signaling cascades. In murine derived macrophages, the cytoplasmic TLR adaptor protein, MyD88, was essential for *A. actinomycetemcomitans* induced phosphorylation of I κ B- α , which promotes NF- κ B activation, and all three MAPKS: p38, JNK, and ERK (Park *et al.*, 2014). As shown in Fig. 2, *A. actinomycetemcomitans* LPS also led to phosphorylation of NF- κ B subunits (p65 and p105) JNK, p38, and ERK MAPKS in rat macrophages (Li *et al.*, 2011; Yu *et al.*, 2011a).

We have elaborated on the involvement of MAPK cascade proteins, MAPK-activated protein kinase 2 (MK2) and MAPK-phosphatase (MKP)-1 in *A. actinomycetemcomitans* pathogenesis. MK2 is a phosphorylation substrate of p38 α/β MAPK that is activated by *A. actinomycetemcomitans* and *A. actinomycetemcomitans* LPS in macrophages (Dunmyer *et al.*, 2012; Li *et al.*, 2011). Attenuation of MK2 in a rat oral LPS injection model led to decreased inflammatory infiltrate and bone loss, proving that MK2 is essential for upregulating the host response (Li *et al.*, 2011; Rogers *et al.*, 2007). We also investigated the role of MKP-1, an anti-inflammatory phosphatase that dampens the immune response in a similar rat model (Yu *et al.*, 2011a). MKP-1, primarily responsible for dephosphorylation of phospho(p)-p38 and p-JNK, down regulated cytokine production of IL-6, TNF- α , IL-10, and CXCL1 in macrophages. MKP-1 also decreased inflammatory infiltration *in vivo* (Yu *et al.*, 2011a). Conversely, when MKP-1 was over-expressed in the same rat model, histological findings were similar to when MK2 was down-regulated (Li *et al.*, 2011; Yu *et al.*, 2011a).

In addition to MAPK cytokine modulation, RNA binding proteins tristetraprolin (TTP) and AUF-1 post-transcriptionally destabilize pro-inflammatory cytokines activated by *A. actinomycetemcomitans*. Activation of the p38/MK2 axis leads to phosphorylation of TTP (Chrestensen *et al.*, 2004). Under normal conditions, TTP binds to AU rich elements (ARE) in cytokine mRNA transcripts, such as IL-6, TNF- α , IL-10, IL-8, and PGE₂, subsequently promoting mRNA degradation (Palanisamy *et al.*, 2012). Upon phosphorylation by activation of the p38/MK2 axis, TTP can no longer bind to the ARE, which increases cytokine translation. One study showed that TTP over expression in macrophages dampened LPS-induced inflammatory cytokine production of TNF- α , IL-6, and PGE₂ (Patil *et al.*, 2008). MKP-1 overexpression stimulated with *A. actinomycetemcomitans* LPS showed activation of AUF-1 and destabilization of TNF- α , IL-6, and IL-10 transcripts (Yu *et al.*,

2011b). Taken together activation of MAPK signaling through intermediate pathways is an essential regulator of LPS actions driving pro-inflammatory cytokine responses.

Aggregatibacter actinomycetemcomitans induces inflammasome pathways in monocytes—

Leukotoxin and CDT are inducers of the inflammasome pathway that stimulates cleavage of pro-IL-1 β and pro-IL-18 into their respective activated forms. Leukotoxin initiated IL-1 β and IL-18 production in monocytes via a caspase-1 dependent pathway (Kelk *et al.*, 2011; Kelk *et al.*, 2003). Caspase 1 enzymatically cleaves pro-IL-1 β into biologically active IL-1 β at an inflammasome complex partially composed of nod like receptor proteins (NLRPs) in monocytes (Thornberry *et al.*, 1992). In particular *A. actinomycetemcomitans* stimulated NLRP3 gene expression (Belibasakis *et al.*, 2012; Okinaga *et al.*, 2015). More recent studies show that *A. actinomycetemcomitans* (serotype a) elicited NLRP3 and conversely attenuated NLRP6 gene expression ultimately leading to a significant increase in IL-1 β secretion in human mononuclear leukocytes (Belibasakis *et al.*, 2012). Interestingly, this aforementioned study showed that IL-1 β secretion was independent of the *A. actinomycetemcomitans* virulence factors, CDT and leukotoxin (Belibasakis *et al.*, 2012). Conversely, CDT induced IL-1 β and IL-18 secretion via inflammasome proteins NLRP3, adaptor protein ASC, caspase-1, ROS, extracellular ATP, and K⁺ in a monocyte cell line (Shenker *et al.*, 2015). A proposed model for leukotoxin inducing IL-1 β and IL-18 secretion involves leukotoxin forming pores in the cell membrane that cause ATP to leak and activate the P2X₇ on the extracellular surface (Haubek *et al.*, 2014; Kelk *et al.*, 2011). This facilitates an influx of K⁺ that activates the inflammasome complex leading to mature IL-1 β and IL-18 secretion (Haubek *et al.*, 2014; Kelk *et al.*, 2011).

Nevertheless, there is conflicting evidence as to whether *A. actinomycetemcomitans* induction of IL-1 β secretion is inflammasome dependent. Contradictory findings reported by another recent investigation demonstrated that inhibition of cathepsin B and ROS attenuated *A. actinomycetemcomitans* (serotype b) mediated IL-1 β secretion, whereas inhibition of NLRP3 and caspase-1 had no effect (Okinaga *et al.*, 2015). Taking into consideration that ROS are potent inducers of the inflammasome pathway, it is plausible that in the aforementioned study siRNA mediated partial and incomplete knockdown of NLRP3 and caspase-1 resulting in IL-1 β expression (Okinaga *et al.*, 2015). These recent conflicted findings concerning *A. actinomycetemcomitans* regulatory mechanisms impacting IL-1 β secretion imply that *A. actinomycetemcomitans* actions may be strain and/or immune cell dependent. Inflammasome related ROS, NLRP, and caspase-1 are key players *in vitro* during *A. actinomycetemcomitans* stimulation of IL-1 β (Fig. 2).

Aggregatibacter actinomycetemcomitans controls macrophage cell cycle and apoptosis—

Aggregatibacter actinomycetemcomitans has a dual function in host evasion via virulence actions causing cell cycle arrest and promoting apoptosis. *A. actinomycetemcomitans* CDT was capable of binding to the cell surface of a human monocyte cell line and inducing cell cycle arrest in the G2/M phase (Mise *et al.*, 2005). A more recent study suggested that the intracellular JAK2/STAT3 signaling axis was essential for causing cell cycle arrest in the G1 cell cycle phase. *Aggregatibacter actinomycetemcomitans* induced activation of the JAK2/STAT3 pathway, which controls cell

proliferation in mouse monocyte/macrophage RAW276.4 cells leads to a decrease in cell cycle (Okinaga *et al.*, 2013). Overexpression of SOC3 normalized cell cycle in the *A. actinomycetemcomitans* treated cells via down-regulating the JAK2/STAT3 axis (Okinaga *et al.*, 2013). These findings suggest a critical role for *A. actinomycetemcomitans* in cell cycle arrest throughout phases of the cell cycle that may be promoted by different virulence factors or signaling pathways.

Leukotoxin is considered the main component that induces monocyte cell death. Relative to cells lacking human CD18, leukotoxin increased cytotoxicity in mammalian leukemia cell lines expressing CD18 (Dileepan *et al.*, 2007). Taking into account that CD18 is a subunit of LFA-1 (CD18/CD11b), the mechanism by which leukotoxin induces monocyte cell death was studied in human monocyte leukemia cell lines (THP-1, HL-60) and the human erythromyeloidblastoid leukemia cell line lacking LFA-1 expression (K562) (Kaur *et al.*, 2014). This study revealed that leukotoxin stimulated cofilin dephosphorylation, actin depolymerization, internalization of leukotoxin, and ultimate lysosomal mediated cell death that is dependent on LFA-1 (Kaur *et al.*, 2014). CD14, a TLR4 co-receptor, was also crucial for the presence of intracellular *A. actinomycetemcomitans* that caused DNA fragmentation and monocyte apoptosis (Muro *et al.*, 1997). Moreover, poly-N-acetylglucosamine (PGA), an *A. actinomycetemcomitans* exopolysaccharide, enhanced colonization (Shanmugam *et al.*, 2015), biofilm formation (Izano *et al.*, 2008), and decreased monocyte viability (Venketaraman *et al.*, 2008). Thus, monocyte surface receptors, LFA-1 and CD14, mediate *A. actinomycetemcomitans* internalization that ultimately may result in monocyte apoptosis.

Dendritic cells and the antigen response—Dendritic cells (DCs) are critical antigen presenting cells responsible for activating and driving T-cell differentiation. One study demonstrated that monocytes isolated from patients with localized aggressive periodontitis differentiated spontaneously into CD11c⁺ DCs *in vitro* (Barbour *et al.*, 2002). *Aggregatibacter actinomycetemcomitans* was the most potent inducer of IL-12p70 (a Th1 inducer) production from DCs when compared to other pathogenic bacteria, *Escherichia coli* and *Porphyromonas gingivalis* (Kikuchi *et al.*, 2004). Like macrophages, DCs possess TLRs equipped to respond to pathogenic signals. IL-12p70 was regulated by TLR4 in DCs when activated with *A. actinomycetemcomitans* LPS (Kikuchi *et al.*, 2004).

In a separate study conducted by Diaz-Zuniga *et al.* (2015b), peripheral blood mononuclear cells isolated from healthy individuals were differentiated into DCs *in vitro*. *Aggregatibacter actinomycetemcomitans* serotypes a, b or c were employed to elucidate serotype-specific actions in TLR2 versus TLR4 dependent stimulation of chemokine receptor expression and cytokine secretion. Interestingly, all serotypes induced TLR4 gene expression, but serotype b more significantly induced TLR2 expression than serotypes a and c. Furthermore, serotype b also more preferentially elicited IL-12, IL-1 β , and IL-23 protein secretion as well as CCR5 and CCR6 gene expression. IL-12, IL-23, and CCR6 gene expression proved to be solely regulated by TLR2 signaling independent of TLR4 (Diaz-Zuniga *et al.*, 2015b). Thus, *A. actinomycetemcomitans* is clearly capable of triggering DC functions through TLR2 and TLR4, which is similar to TLR signaling in macrophages described previously.

Are mast cells involved in *A. actinomycetemcomitans* pathogenesis?—There is limited knowledge about the function of myeloid derived mast cells (MCs) with regards to periodontal disease. In general MCs contain granules of histamine, proteoglycans, and proteinases that are released from the cells in response to a stimulus. These cells can also secrete pro-inflammatory TNF- α , nitric oxide (NO) (Bidri *et al.*, 1997), and fibroblast growth factor (Qu *et al.*, 1995). MCs were increased in periodontal disease tissues from patients with localized chronic periodontal disease (Batista *et al.*, 2005). No known studies have been conducted to assess infiltration or function of mast cells in aggressive periodontal disease. With regards to *A. actinomycetemcomitans*, a single *in vitro* study concluded that mouse MCs were able to phagocytize non-opsonized *A. actinomycetemcomitans* (Lima *et al.*, 2013). The current lack of published studies explaining the role of MCs suggests these cells are either not playing a critical role in the pathogenesis of periodontal disease or are alternatively being overlooked.

Lymphoid-derived B and T cells respond to *A. actinomycetemcomitans*—T and B lymphocytes are both found in periodontal lesions (Lin *et al.*, 2011; Page *et al.*, 1976). For the purpose of this review, B, T, and Natural Killer (NK) cells are considered lymphoid-derived cells, although NK cells are unique in that they have been differentiated from myeloid progenitors (Grzywacz *et al.*, 2011). Early literature reported conflicting evidence about CD4⁺/CD8⁺ T lymphocyte ratio imbalance in juvenile and rapidly progressing periodontal disease states (Kinane *et al.*, 1989; Nagasawa *et al.*, 1995). Lymphocytes have been implicated to play an integral role in adaptive immune responses directly associated with *A. actinomycetemcomitans* pathogenesis (Lin *et al.*, 2011; Underwood *et al.*, 1993). NK cells, a cytotoxic lymphocyte, were critical for DC-induced IFN- γ production by peripheral blood cells secondary to *A. actinomycetemcomitans* treatment (Kikuchi *et al.*, 2004).

Li *et al.* (2010) reported activation of the adaptive immune response in a rat periodontal disease model induced by *A. actinomycetemcomitans* feeding. The investigators recovered CD45RA⁺ B and CD4⁺ T cells from draining cervical and submandibular lymph nodes. In the *A. actinomycetemcomitans* exposed treatment groups, activated B cells, defined by the antigen presenting cell marker, MHC Class II, were also increased. The gene profile of B cells demonstrated transcriptional upregulation of bone morphogenic proteins, whereas T cells were characterized by induction of pro-inflammatory cytokines and RANKL the pro-resorptive cytokine required for osteoclast formation and resorptive function (Li *et al.*, 2010).

Earlier literature reported that serum antibodies against *A. actinomycetemcomitans* having opsonic capabilities and the potential to promote *A. actinomycetemcomitans* phagocytosis were detectable in patients with localized juvenile periodontitis (Baker *et al.*, 1989), indicating that plasma cells (mature B cells) actively secrete antibodies against *A. actinomycetemcomitans* during infection. Furthermore, monocytes control IgG2a antibody production (Ishihara *et al.*, 2000; Zhang *et al.*, 1996), which is in line with T and B cell activation by *A. actinomycetemcomitans* in advanced stages of periodontal disease progression.

Polarizing effects of *A. actinomycetemcomitans* on Helper T cells—While Th cells are delineated into 5 subsets (Th1, 2, 9, 17, and 22), historical theory emphasizes the ratio of Th1/Th2 as the critical regulator of periodontal disease progression, but has resulted in a poor understanding of the Th cell subsets in *A. actinomycetemcomitans* associated perio-pathogenesis. *Aggregatibacter actinomycetemcomitans* CdtB (part of the CdtA,B,C holocomplex) and LtxA induced CD4⁺ lymphocyte cell proliferation *in vitro* (Schreiner *et al.*, 2013). Th1 cells typically secrete IFN- γ and are classically induced by IL-12 (p70), a heterodimer of IL-12A (p35) and IL-12B (p40) (Trinchieri, 2003). Th17 cells are a pro-inflammatory Th subset that secrete high levels of IL-17 and are polarized by IL-23, which shares the IL-12B subunit with IL-12 (Trinchieri *et al.*, 2003). More recently, it has been suggested that the Th1/Th2 paradigm in periodontal disease studies should be shifted to Th1/Th2/Th17 cells due to the vast amount of research known about Th17 cells in other diseases, such as arthritis (Gaffen *et al.*, 2008; Kramer *et al.*, 2007). A recent publication suggested that the Th phenotype is serotype dependent, and Th17 polarization occurs in response to *A. actinomycetemcomitans* (Diaz-Zuniga *et al.*, 2015a). DCs treated with whole *A. actinomycetemcomitans* or isolated LPS from serotypes a-c used to stimulate CD4⁺ T cells showed that serotype b induced the most robust Th1 and Th17 response (Diaz-Zuniga *et al.*, 2015a). This indicates that *A. actinomycetemcomitans* serotype b has the most pro-inflammatory potential. A heat shock protein, GroEL, derived from *A. actinomycetemcomitans*, was also capable of polarizing human peripheral blood cells into Th1-type CD4⁺ cells that produce both IFN- γ and IL-10 (Saygili *et al.*, 2012).

On the other hand, Th2 cells secrete IL-4, IL-5, and IL-13. *Aggregatibacter actinomycetemcomitans* induced IL-10 and IL-4, which promotes Th2 polarization (Kikuchi *et al.*, 2004). Unlike pro-inflammatory Th1 and Th17 cells, Th2 and Treg cells are immunosuppressive in nature. A recent timely study by Araujo-Pires *et al.* (2015) clearly defined Treg migration in an *A. actinomycetemcomitans* murine model. Interestingly, CCR4 proved to regulate Th2 (CD4⁺IL4⁺) and Treg (CD4⁺FOXP3⁺) cell infiltration secondary to mouse oral *A. actinomycetemcomitans* inoculation (Araujo-Pires *et al.*, 2015). Furthermore, CCR4-deficient mice, IL-4 deficient mice, and mice blocked against CCL22, had decreased Tregs, enhanced inflammatory infiltrate, and exacerbated periodontal bone loss (Araujo-Pires *et al.*, 2015).

Another study suggested that CCL22, a CCR4 ligand, preferentially increased Treg associated mRNA levels of FOXP3, IL-10, and TGF- β , but not Th2-associated IL-4 during *A. actinomycetemcomitans* infection (Glowacki *et al.*, 2013). Deficiencies in CCR4, IL-4, and CCL22 led to a similar cytokine profile with significant increases in pro-inflammatory cytokines TNF- α , IL-17, and RANKL, and decreases in anti-inflammatory cytokines IL-10, TGF- β , and OPG (Araujo-Pires *et al.*, 2015). These two murine experimental periodontitis studies demonstrated that IL-4 cytokine and CCL22/CCR4 chemokine signaling are critical regulators of Treg migration, suppression of inflammation, and attenuation of periodontal bone loss in *A. actinomycetemcomitans* mediated periodontal disease progression. The T cell milieu is composed of classically considered pro- and anti-inflammatory T cell subsets that respond to *A. actinomycetemcomitans*. While the mechanisms of Th2/Treg subsets

during *A. actinomycetemcomitans* pathogenesis were eloquently studied *in vivo*, Th1/Th17 mechanistic responses have been mostly been studied *in vitro*.

III. *Aggregatibacter actinomycetemcomitans* osteoimmuno-modulatory actions alter bone remodeling processes, having catabolic effects on alveolar bone homeostasis

In a longitudinal study, 91.7% of subjects presenting with vertical periodontal bone loss detected by radiograph were *A. actinomycetemcomitans* positive, highlighting the destructive pathologic impact of *A. actinomycetemcomitans* on the alveolar bone complex (Fine *et al.*, 2013). Under physiological conditions, bone remodeling/turnover is a coupled cellular process in which balanced osteoclast-osteoblast actions renew the bone matrix, supporting skeletal tissue homeostasis in health. Alveolar bone remodeling takes place within small foci and occurs in the following sequence: Activation-Resorption-Reversal-Formation (Baron, 1973; 1977). The seminal work that defined the impact of periodontal disease on alveolar bone remodeling in the golden hamster, elucidated that periodontal disease causes net alveolar bone loss via disruption/uncoupling of normal alveolar bone remodeling processes (Baron *et al.*, 1978). Notably, it was clearly shown that periodontal disease induces catabolic effects on alveolar bone homeostasis that are secondary to not only an increase in bone resorption, but also a decrease in bone formation (Baron *et al.*, 1978). *Aggregatibacter actinomycetemcomitans* LPS, LtxA, and to a lesser extent CdtB, PGA, and OMP29 are virulence factors that have been shown to promote bone loss in pre-clinical rat models (Li *et al.*, 2011; Lin *et al.*, 2011; Rogers *et al.*, 2007; Schreiner *et al.*, 2013; Shanmugam *et al.*, 2015; Yu *et al.*, 2011a).

RANKL signaling regulates alveolar bone loss during pathogenesis—RANKL is an essential cytokine in osteoclast formation and resorptive function contributing to bone loss. This mechanism is fine-tuned by the RANKL decoy receptor osteoprotegerin (OPG), which blocks RANKL signaling through its cognate receptor, RANK. The imbalance of the RANKL/OPG ratio is thought to deregulate bone remodeling, driving bone loss when RANKL concentrations exceed OPG relative to normal physiology (Boyce *et al.*, 2008). RANKL is expressed as a surface molecule mainly on fibroblasts, osteoblasts, and T cells, but can also be secreted locally into the extracellular matrix and is found in circulation. *Aggregatibacter actinomycetemcomitans* indirectly regulates osteoclast formation through inducing RANKL expression by non-osteoclastic cells thereby stimulating RANK on dOCs to form mature osteoclasts. RANKL binding activates NFATc1, which is the considered the master transcription factor of osteoclastogenesis (Kim *et al.*, 2005). However, inflammatory chemokines and cytokines are also known to directly induce osteoclast formation independent of RANKL (Ha *et al.*, 2011; Hotokezaka *et al.*, 2007; Valerio *et al.*, 2014). Thus RANKL-RANK signaling and pro-inflammatory cytokines drive osteoclastogenesis (Fig. 3).

Aggregatibacter actinomycetemcomitans increases RANKL expression in the local inflammatory microenvironment by indirect mechanisms. More specifically, CDT, LPS, and outer membrane protein (OMP) 29 have been shown to stimulate RANKL expression (Belibasakis *et al.*, 2005a; Lin *et al.*, 2011; Wada *et al.*, 2004). One study showed that *A. actinomycetemcomitans* induced RANKL mRNA expression in human gingival fibroblasts

independent of TNF, IL-1 β , and IL-1R (Belibasakis *et al.*, 2005b). CDT upregulated RANKL on gingival fibroblasts and PDL cells (Belibasakis *et al.*, 2005a). Additionally, *A. actinomycetemcomitans* activates T lymphocytes, which express RANKL to further, promote osteoclastogenesis. Using a co-culture system, surface RANKL on T-cells primed with *A. actinomycetemcomitans*-derived OMP29 was essential for osteoclastogenesis (Lin *et al.*, 2011). Most recently, Tregs have been demonstrated to blunt RANKL mediated periodontal bone loss (Araujo-Pires *et al.*, 2015).

Aggregatibacter actinomycetemcomitans endotoxins can also promote osteoclast formation by direct interactions with dOCs or monocyte osteoclast precursors. LPS and TNF- α activated p38/JNK MAPKs and directly induced osteoclast formation from the mouse macrophage/monocyte leukemia cell line, RAW264.7 (Hotokezaka *et al.*, 2007). In the *A. actinomycetemcomitans* LPS rat injection model published from our project laboratory, MK2 attenuation decreased the number of TRAP positive osteoclast cells, whereas MKP-1 overexpression also decreased osteoclast formation and maxillary alveolar bone loss caused by *A. actinomycetemcomitans* LPS (Li *et al.*, 2011; Yu *et al.*, 2011a). Interestingly, MK2 inhibition also significantly blunted IL-1 β gene expression in *A. actinomycetemcomitans* LPS treated macrophages (Li *et al.*, 2011). IL-1 receptor (R) antagonist, which competes with IL-1 β and IL-1 α for binding to inhibit IL-1R signaling, proved to be critical for down-regulating osteoclast formation and bone loss secondary to mouse LPS challenge (Izawa *et al.*, 2014). These results suggest that LPS induction of the IL-1R axis is a critical regulator of bone loss. Furthermore, in a rat calvarial model, whole *A. actinomycetemcomitans* induced osteoclast and resorption pit formation (Dunmyer *et al.*, 2012).

To better understand the role of MAPK signaling in osteoclastogenesis, our project laboratory first showed that *A. actinomycetemcomitans* LPS promoted osteoclast formation from murine dOCs *in vitro* (Valerio *et al.*, 2014). In this same study, we found that MKP-1 signaling through chemokines CXCL1 and CXCL2 dampened LPS-induced osteoclast formation (Valerio *et al.*, 2014). We have previously shown that *A. actinomycetemcomitans* LPS signals through MK2 and regulates CXCL1 gene expression (Li *et al.*, 2011). Since TTP overexpression during LPS challenge led to attenuation of bone loss (Patil *et al.*, 2008), a possible mechanism for MK2/TTP regulation of bone loss is by CXCL1 mRNA stability (Datta *et al.*, 2008). Based on these findings, it appears that both direct and indirect signaling mechanisms of stimulation promote mature osteoclast formation in the presence of *A. actinomycetemcomitans* (Fig. 3).

Bone resorption by host matrix metalloproteinase and *A. actinomycetemcomitans* collagenase—

Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are expressed by many cell types, including monocytes, fibroblasts, and osteoclasts, all of which play a role in *A. actinomycetemcomitans* perio-pathogenesis. MMPs are enzymes that degrade collagen and solubilize matrix. Similar to the inhibiting action of OPG against RANKL signaling, TIMPs nonspecifically regulate MMPs (Baker *et al.*, 2002). MMP-13 is expressed in oral cell types, dental pulp cells, and PDL fibroblasts (Rossa *et al.*, 2005; Suri *et al.*, 2008). *Aggregatibacter actinomycetemcomitans* induced MMP-1, -2, and -9 expression in a murine infection model and was followed by upregulation of TIMP genes (Garlet *et al.*, 2006). Furthermore, secreted

MMP-1, -3, and -9 and TIMP-1 were activated by *A. actinomycetemcomitans* LPS in human peripheral blood monocytes (Serra *et al.*, 2010). Monocytes adjacent to the alveolar bone have the potential to form osteoclasts in the periodontal microenvironment. Osteoclasts respond to MMPs and have MMP activity by promoting osteoclast migration (Engsig *et al.*, 2000; Sato *et al.*, 1998). Interestingly, *A. actinomycetemcomitans* can bind to saliva coated hydroxyapatite, the main constituent of dentoalveolar tissues (Kagermeier *et al.*, 1985; Rosan *et al.*, 1988), and has been shown to have bone-degrading collagenase activity (Robertson *et al.*, 1982). Taken together, it is highly evident that *A. actinomycetemcomitans* has catabolic osteo-immunomodulatory actions promoting bone loss.

Aggregatibacter actinomycetemcomitans impairs osteoblastogenesis—Early studies assessing *A. actinomycetemcomitans* effects on bone formation revealed that *A. actinomycetemcomitans* is a potent inhibitor of bone collagen synthesis (Harvey *et al.*, 1986; Meghji *et al.*, 1992; Wilson *et al.*, 1988). It was demonstrated that LPS purified from *A. actinomycetemcomitans*, at a concentration of 1 ug/ml in mouse calvaria explant cultures inhibits DNA and collagen synthesis by 30% and 40% (Harvey *et al.*, 1986). A subsequent *ex vivo* mouse calvaria explant culture study performed by the same research group showed that isolated capsular material, free of LPS, significantly blunted synthesis of DNA and collagen at concentrations as low as 10 ng/ml (Wilson *et al.*, 1988). A follow-up investigation, evaluating the direct effect of *A. actinomycetemcomitans* derived LPS versus surface associated material on murine calvaria-derived osteoblastic cells, elucidated that *A. actinomycetemcomitans* virulence factors directly impair osteoblast proliferation and collagen synthesis (Meghji *et al.*, 1992).

Li *et al.* (2012) conducted a single investigation of *A. actinomycetemcomitans* LPS actions on osteoblast differentiation highlighting that *A. actinomycetemcomitans* LPS has a concentration dependent, inverse regulatory impact on osteoblastic cell differentiation. The noted study was an *in vitro* investigation assessing the impact of *A. actinomycetemcomitans* derived LPS on bone sialoprotein (BSP), a marker of osteoblast differentiation, in the osteoblast-like ROS17/2.8 cell line. Interestingly, 0.1 ug/mL LPS suppressed and 0.01 ug/mL LPS enhanced BSP gene transcription, which occurred via heterogeneous signaling pathways mediated through CRE, FRE, and HOX elements in the rat BSP gene promoter (Li *et al.*, 2012). *In vitro* and *ex vivo* osteoblast studies focus on *A. actinomycetemcomitans* LPS dosage dependent responses on osteoblast differentiation.

Aggregatibacter actinomycetemcomitans promotes osteoblast apoptosis—Investigations of *A. actinomycetemcomitans* inhibitory effects on osteoblastogenesis revealed that *A. actinomycetemcomitans* elicited apoptosis in osteoblastic cells through its multifactorial virulence mechanisms. *Aggregatibacter actinomycetemcomitans* capsular-like polysaccharide antigen (CPA) from serotype c (CPA-c) inhibited osteoblast cell line proliferation via a pro-apoptotic mechanism (Yamamoto *et al.*, 1999). These results indicated that CPA-c mediated apoptosis of MC3T3-E1 murine osteoblastic cells via the Fas system, independent of Bcl-2 or NO synthase (Yamamoto *et al.*, 1999). To the contrary of *A. actinomycetemcomitans* CPA-c mechanisms driving apoptosis in osteoblasts, *A. actinomycetemcomitans* derived LPS induced production of NO in the HOS human

osteoblastic cell line (Sosroseno *et al.*, 2009), which suggests that LPS may contribute to *A. actinomycetemcomitans* pro-apoptotic actions on osteoblastic cells via NO signaling.

Taking into consideration clinical studies reporting ultra-structural findings which imply that localized aggressive periodontitis is associated with bacterial invasion of the alveolar bone surface (Carranza *et al.*, 1983), it is conceivable that the inflammasome protein, NLRP3, is a critical regulator of *A. actinomycetemcomitans* induced osteoblast apoptosis (Zhao *et al.*, 2014). Zhao *et al.* (2014) infected isolated human osteoblastic MG63 cell cultures with *A. actinomycetemcomitans*, which increased cell apoptosis. *Aggregatibacter actinomycetemcomitans* infection upregulated the expression of inflammatory mediators downstream of the NLRP3 inflammasome protein, and knockdown expression of NLRP3 by siRNA attenuated apoptosis of *A. actinomycetemcomitans* infected MG63 osteoblastic cells (Zhao *et al.*, 2014). While the aforementioned study does not elucidate the role of specific *A. actinomycetemcomitans* virulence factors, findings from this *in vitro* investigation highlight that *A. actinomycetemcomitans* invasion of the alveolar bone surface may directly promote osteoblast apoptosis through the NLRP3 inflammasome (Zhao *et al.*, 2014). Furthermore, Teng and Hu (2003) identified a novel gene, the *CagE* homologue of *A. actinomycetemcomitans*, which encodes a protein associated with the bacterial type IV secretion system. Serum immune studies, demonstrating recognition in *A. actinomycetemcomitans* infected periodontitis subjects but not periodontally healthy control subjects, support CagE's contributory role in *A. actinomycetemcomitans* associated periodontal tissue destruction. Notably, the secreted CagE protein significantly induced apoptosis in isolated human primary osteoblastic cell cultures (Teng *et al.*, 2003).

The reviewed body of limited published works elucidating the inhibitory impact of *A. actinomycetemcomitans* on osteoblastogenesis highlights the need for future investigations defining the role of *A. actinomycetemcomitans* virulence factor mechanisms in the regulation of osteoblastic cell proliferation, differentiation, apoptosis, bone matrix formation, and mineralization. Baron and Saffar's (1978) seminal experimental periodontitis - alveolar bone remodeling investigation, clearly demonstrated that net alveolar bone loss in periodontal disease is secondary to both increased osteoclastogenesis mediated bone resorption and decreased osteoblastogenesis mediated bone formation. However, researchers have focused on osteoclastogenesis and neglected the study of periodontitis effects on osteoblastic cells. While future studies are needed, it has been realized that *A. actinomycetemcomitans* impairs osteoblastogenesis. Periodontal researchers must embrace that *A. actinomycetemcomitans* induced alveolar bone loss is not solely caused by increased osteoclastogenesis mediated bone resorption, but rather is secondary to altered bone remodeling, a process which is dynamically mediated by both osteoclastic and osteoblastic cell actions.

Concluding remarks—A review of the literature showed that many non-hematopoietic and hematopoietic cell types contribute to pathogenesis of *A. actinomycetemcomitans*. While numerous signaling pathways and host response mechanisms have been divulged through pre-clinical animal models, many facets of the host response are not well understood. We observed that a majority of known mechanisms of *A. actinomycetemcomitans* pathogenesis are limited to *in vitro* findings. Interestingly, there is

an overwhelming majority of experimental studies in hematopoietic cells compared to non-hematopoietic lineages, which are entirely limited to *in vitro* findings. The field could benefit from applying modern murine transgenic/knockout pre-clinical models to further elucidate immunomodulatory effects of *A. actinomycetemcomitans* on non-hematopoietic or hematopoietic immune response cells.

Aggregatibacter actinomycetemcomitans induces the activation of MAPK signaling pathways in several cell types, increasing pro-inflammatory cytokines and cell apoptosis. Chemokines are elevated during *A. actinomycetemcomitans* infection, which appears to be an integral contributor to the recruitment and expansion of host immune cell response mediating the *A. actinomycetemcomitans* induced periodontal disease state. We identified major gaps in knowledge in macrophage and T cell polarization and osteoblast signaling during *A. actinomycetemcomitans* progression. It is also unclear whether *A. actinomycetemcomitans* alone can stimulate osteoclastogenesis and/or inhibit osteoblastogenesis. Indeed, it appears the host immune response mechanisms demonstrated thus far are not only cell-type dependent but also *A. actinomycetemcomitans* strain and serotype-specific.

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Figure 1. *Aggregatibacter actinomycetemcomitans* colonizes the gingival sulcus by attachment to the sulcular/junctional epithelium cells

It subsequently invades through the epithelium via pro-apoptotic virulence mechanisms and penetrates into the subgingival connective tissue where it stimulates epithelial cells and fibroblasts to secrete pro-inflammatory cytokines (I). Neutrophils and monocytes are thereby recruited to the local site of infection and perpetuate the host inflammatory response. Subsequently, B and T cells are recruited to the diseased periodontium from the circulation (II). T cells secrete pro-resorptive factors that drive osteoclast (OC) formation and drive bone resorption. *Aggregatibacter actinomycetemcomitans* simultaneously impairs osteoblast (OB) function, perturbing bone remodeling processes, which ultimately results in catabolic alveolar bone loss (III).

Figure 2. *Aggregatibacter actinomycetemcomitans* activates macrophage cell surface TLR2, TLR4, and LFA-1

TLR2 and TLR4 activation initiates JNK, ERK, p38 MAPKs and NF-κB intracellular signaling cascades that regulate pro-inflammatory cytokine transcription. MKP-1 negatively controls MAPK signaling by dephosphorylating p-JNK and p-p38. p38 activation leads to MK2 phosphorylation as an intermediate kinase in cytokine production. LFA-1, which is a heterodimer of CD18 and CD11a, initiates the inflammasome complex consisting of NLRs, ASC, and pro-caspase 1. Once activated, caspase-1 cleaves pro-IL-1β into mature IL-1β cytokine.

Figure 3. M-CSF release from osteoblasts lining alveolar bone stimulates defined osteoclast progenitors (dOCPs) to express the RANK receptor in preparation for a RANKL signaling response

Aggregatibacter actinomycetemcomitans stimulates RANKL, which is on the cell surface and secreted by osteoblasts, T-cells, and fibroblasts differentiates dOCPs into mononuclear pre-osteoclasts (pre-OCs) that are pushed toward the osteoclast (OC) lineage. Amid RANKL signaling, in the micro-environment pro-inflammatory cytokines TNF- α additively stimulates formation of TRAP positive multinucleated functional OCs. *Aggregatibacter actinomycetemcomitans* LPS also stimulates OC formation. Combined these *A. actinomycetemcomitans*-induced drivers of OC formation lead to mature OC formation and subsequent alveolar bone loss.

Table 1
Aggregatibacter actinomycetemcomitans

Cellular Interactions and Effectors

Cell origin	Cell type	Virulence factor	Effector (s)	Reference (s)
Non-hematopoietic	Epithelial	LPS, whole bacteria	TNF- α , IL-1 β , IL-6, IL-8, IL-15	Dickinson <i>et al.</i> , 2011; Suga <i>et al.</i> , 2013
	Fibroblast	CDT, whole bacteria	RANKL, IL-6	Belibasakis <i>et al.</i> , 2005a; Belibasakis <i>et al.</i> , 2005b
	Osteoblast	LPS	NO	Sosroseno <i>et al.</i> , 2009
Hematopoietic	Monocyte/macrophage	LPS, CDT, Leukotoxin, whole bacteria	IL-6, IL-10, TNF- α , IL-12, PGE ₂ , CXCL1, L-1 β , IL-18, NO	Belibasakis <i>et al.</i> , 2012; Blix <i>et al.</i> , 1998; Kelk <i>et al.</i> , 2011; Okinaga <i>et al.</i> , 2015; Park <i>et al.</i> , 2014; Patil <i>et al.</i> , 2008; Shenker <i>et al.</i> , 2015; Yu <i>et al.</i> , 2011a
	Neutrophil	Whole bacteria	ROS	Guentsch <i>et al.</i> , 2009; Permpanich <i>et al.</i> , 2006
	Dendritic cell	Whole bacteria	IL-12, IL-10, IFN- γ	Kikuchi <i>et al.</i> , 2004
	Th cell	GroEL, OMP29	IL-10, IFN- γ , TNF- α , RANKL	Lin <i>et al.</i> , 2011; Saygili <i>et al.</i> , 2012
	Treg	Whole bacteria	IL-10, TGF- β	Araujo-Pires <i>et al.</i> , 2015
	Osteoclast	LPS	CXCL1, CXCL2	Valerio <i>et al.</i> , 2014