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Aggregatibacter actinomycetemcomitans exploits and modulates the immune response by human neutrophils for survival in the anaerobic environment.

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AGGREGATIBACTER ACTINOMYCETEMCOMITANS EXPLOITS AND MODULATES THE IMMUNE RESPONSE BY HUMAN NEUTROPHILS FOR SURVIVAL IN THE ANAEROBIC ENVIRONMENT

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B.S., Universidad Ana G. Méndez, 2010
M.S., Illinois State University, 2014
M.S., University of Louisville, 2016

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A Dissertation Approved on

April 20, 2022

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DEDICATION

This dissertation is dedicated to my mother Raquel Vásquez Guzmán,

who is my example of perseverance and having an inquisitive mind. Also, I would like to

dedicate this dissertation to those who once were told they are shooting too high…

PROVE THEM WRONG.

“Great spirits have always encountered violent opposition from mediocre minds”

-Albert Einstein

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ABSTRACT

AGGREGATIBACTER ACTINOMYCETEMCOMITANS EXPLOITS AND MODULATES THE IMMUNE RESPONSE BY HUMAN NEUTROPHILS FOR SURVIVAL IN THE ANAEROBIC ENVIRONMENT

Hazel Ozuna

April 20, 2022

Aggregatibacter actinomycetemcomitans (*Aa*) is a Gram-negative facultative anaerobe and an opportunistic oral pathogen, strongly associated with localized periodontitis and other inflammatory diseases. Periodontitis is a chronic inflammation of the periodontium resulting from the inflammatory response of the host towards the dysbiotic microbial community present at the gingival crevice. The host immune response creates a hostile environment for microorganisms; therefore, it is important for *Aa* to be able to regulate the necessary genes to survive and thrive in such an environment. *Aa* expresses several virulence factors such as a cytolethal distending toxin (Cdt), lipopolysaccharide (LPS) and leukotoxin A (LtxA), that allows it to evade the immune response. For example, LtxA, considered a major virulence factor, targets leukocytes by creating pores on their membranes leading to cell lysis. LtxA has also been shown to provide *Aa* resistance against internalization and killing by neutrophils, however the effect of the toxin on neutrophils at a non-lethal level is often overlooked. At the subgingival pocket *Aa* is in a tug-of-war scenario where immune cells will secrete molecules such as

catecholamines (i.e., epinephrine) to sequester residual metals, so these are not available as nutrients. To compete for such mineral bacteria have evolved different mechanisms, such as two-component systems (TCS) that allow them to assess their environment and adjust accordingly. *Aa* expresses the QseBC TCS composed of the sensor protein QseC and the response regulator QseB. Previously, catecholamines and iron were identified as the signals that activate the QseBC TCS in *Aa*, necessary for the organism to acquire iron as a nutrient to survive in the anaerobic environment. However, the source of catecholamines had not been identified at the time. The main objectives presented in this dissertation are a) the characterization of epinephrine interaction with different components of QseBC TCS, b) to characterize the synthesis, storage, and release of catecholamines by neutrophils stimulated with *Aa,* and c) determination of the effect sublytic levels of LtxA has on neutrophils.

Previously, our group showed that QseC, primarily the periplasmic domain of QseC, is required for biofilm formation and virulence. A third gene was found to be co-expressed in the *qseBC* operon, YgiW, a protein of unknown function, but essential for biofilm growth and virulence. Additionally, it was previously shown that in the presence of Cat-Fe the expression of the enterobactin operon remained unchanged. Among the genes in this operon there is the *fepA* gene encoding for the enterobactin receptor FepA. In this work we investigated the interaction of different components of the QseBC TCS and epinephrine. Using *Aa* mutants for the different QseBC component we determined that FepA, QseC and the QseC periplasmic domain are necessary for *Aa* to interact with epinephrine (Chapter 3). In contrast, YgiW was not required for epinephrine to interact with *Aa*. Due to the high infiltration of neutrophils that is characteristic of periodontitis we

proposed neutrophils as the epinephrine source for *Aa*. We present evidence of the ability of human neutrophils to release epinephrine upon *Aa* challenge (Chapter 4). In addition, we demonstrated that epinephrine is stored in azurophilic granules and proposed that *Aa* gains access to it by inducing degranulation. To connect our findings, we demonstrated that chemically define media (CDM) supplemented with iron and epinephrine from human neutrophils promoted *Aa* growth and the induction of the *qseBC* operon.

LtxA, a major virulence factor expressed by *Aa*, targets neutrophils and induces cell lysis. However, lysis only occurs when cells are exposed to high levels of the toxin, the sublytic effects of the toxin on neutrophils remains understudied. We show that the minimally leukotoxic *Aa* strain 652 caused minimal cytotoxicity in human neutrophils, even at MOI 50 (Chapter 5). Assessment of membrane permeability confirmed that human neutrophils suffered loss of membrane integrity. However, neutrophils were functionally active and were able to internalize *Aa* at MOI 10. In contrast, *Aa* internalization did not occur at MOI 50 for both the minimally leukotoxic strain or a LtxA mutant (JP2 *ΔltxA*). *Aa* challenge induced the formation of cytotoxic vacuoles, which have previously been associated with pore-forming toxins, but we observed cytoplasmic vacuolation regardless of LtxA expression of *Aa* strains. Siglec-9, a sialic acid-binding immunoglobulin-type lectin, has been associated with cytoplasmic vacuolation. Therefore, we measured Siglec-9 as an alternative inducer of cytoplasmic vacuolation. We were able to show that *Aa* 652 enhances Siglec-9 expression in human neutrophils and that IL-8 priming before *Aa* challenge further increases expression.

The findings presented in this dissertation contribute to the current knowledge of nutrient acquisition mechanisms and provides the foundation for future studies of how *Aa* can modulates the inflammatory response of neutrophils through Siglec-9 activation.

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CHAPTER 1

INTRODUCTION

Periodontal Disease: cause and effect

Periodontal disease or periodontitis is defined as an inflammation of the gingiva caused by accumulation of oral microorganisms. Most recent statistics by the Centers for Disease Control and Prevention (CDC) report that 47.2% of adults 30 years and older are diagnosed with some type of periodontitis (1). Poor oral hygiene is the most obvious cause for periodontitis, but the risk of periodontitis also increases with age, gender, socioeconomic status and education (2). The National Health and Nutrition Examination Survey (NHANES) 2009-2012 report period found that Hispanics had the highest prevalence in periodontitis (63.5%), followed by non-Hispanic Blacks (59.1%) and Non-Hispanic Whites (40.8%). The incidence of periodontitis was higher in those with less than a high school diploma (67%) and those living under the federal poverty level (62.2%). Other risk factors such as smoking, diabetes, medications that cause dry mouth, stress, and genetics (1) can affect the oral cavity homeostasis and have a direct or indirect impact on the microbiome. This in turn can promote bacterial population shifts, favoring pathogenic bacteria.

Once host homeostasis is disrupted, bacterial communities accumulate in the sub-gingival pocket and form biofilms that lead to oral diseases such as periodontitis (3). The depth of the gingival pocket increases due to continuous periodontal ligament and alveolar

bone destruction (4). The infection starts with the formation of plaque, which accumulates and becomes tartar, serving as a reservoir for bacteria (4). Periodontal disease starts with gingivitis, characterized by gum inflammation or irritation, and it can be easily reversed by improved oral hygiene. If untreated, this is followed by bone resorption and deepening of the sub gingival pocket, distinctive of early periodontitis, allowing bacteria to accumulate creating a biofilm (4). Progression of periodontitis is based on further deepening of the sub gingival pocket leading to additional bone loss. Although, prevention by means of good oral hygiene is the best choice of treatment, dental cleanings are encouraged and for those with more serious dental needs a more extensive treatment is recommended. Such treatment consists of deep cleaning of the tooth roots and below the gum (scaling and root planing), or removal of the affected teeth, and medication (5). Other types of therapies include topical use of antibiotics or anti-inflammatories, host modulation therapy and probiotic therapy, yet the benefits have been limited due to the multispecies organization of oral communities (5, 6)

Periodontitis is a chronic inflammation of the periodontium caused by the inflammatory response of the host to plaque biofilm. Recurring inflammation of the periodontium has been associated with the initiation, exacerbation, and pathogenesis of several other inflammatory diseases (Figure 1.1). Epidemiological data and experimental evidence show that periodontitis plays a role in the development and progression of atherosclerosis cardiovascular disease (7-9). Oral bacteria associated with periodontitis, such as *Porphyromonas gingivalis*, *Streptococcus sanguis*, *Aggregatibacter actinomycetemcomitans (Aa)* and *Tannerella forsythia*, have been isolated from atheromatous plaque (10-12). In addition, a two-way relationship exists between

periodontitis and rheumatoid arthritis (RA), where RA can be a result of periodontitis or RA exacerbates periodontitis (13). Furthermore, *P. gingivalis* has been isolated from joints of RA patients and is believed to promote RA through molecular mimicry (14). *P. gingivalis* and gingipains have also been identified in the brain of Alzheimer's Disease patients (15-19). Recently, Dominy *et al* (17) used gingipain inhibitors in mouse brains to successfully block neurodegeneration induced by gingipains. This in turn reduced *P. gingivalis* bacterial load and significantly decreased the immune response against *P. gingivalis* brain infection. Finally, increased levels of pathogenic oral bacteria, in particular *Fusobacterium nucleatum*, have been associated with a risk of developing pre-cancerous gastric lesions and ultimately colorectal cancer (20-22).

The tipping point: Microbial dysbiosis and the host immune response during periodontitis

Periodontal disease is a multifactorial disease, that in addition to the external risks discussed previously, requires the invasion of bacteria into the gingiva epithelium and bacterial persistence to drive chronic inflammation. In contrast to other diseases, periodontitis is not caused by one bacterium but by a heterotypic microbial community working together to destabilize homeostasis. Under normal conditions, commensal bacteria are organized into compatible communities, and any detour from homeostasis is efficiently cleared by the host inflammatory response, returning to the "surveillance inflammatory state". Any disturbance to this balance such as changes in diet, smoking habits, genetic factors, or stress promotes changes of pH, redox potential, and nutrient availability in the oral microenvironment, leading to a dysbiotic environment (23). Microbial shifts or dysbiosis of the bacterial population can result in a shift from beneficial symbionts (commensal bacteria) to predominantly pathogenic bacteria. During dysbiosis there is

increased inflammation, the local environment becomes anaerobic and is enriched with by-products of the immune response, such as plasma proteins and hemoglobin from bleeding. These conditions select for anaerobic Gram-negative, proteolytic bacteria that use essential amino acids, and hemin as an energy source and provide an environment that favors growth and persistence of specific subsets or consortia of oral pathogenic bacteria (24, 25). Although it has been thought of as being a shift from primarily Gram-positive bacteria to mostly Gram-negative bacteria, dysbiosis or bacteria alone is not sufficient to cause periodontitis (26). This is exemplified by a mouse model study (26) that demonstrated that few oral commensals invaded the gingiva in the absence of oral pathogens, with minimal recruitment of immune cells. In this scenario commensals were quickly cleared, and homeostasis restored. However, the presence of periodontal pathogens with superior invasive ability promoted a high infiltration of immune cells. The oral pathogens persisted by resisting clearance, leading to chronic inflammation. These observations go in hand with the current accepted model for the etiology and pathogenesis of periodontitis: the Polymicrobial Synergy & Dysbiosis (PSD) Model (23). The PSD model postulates that disease is not caused by an individual pathogen but by a synergistic polymicrobial community with specific elements, a combination of functional genes, and where each member fulfills roles that help give shape and stabilize the dysbiotic microbiota (5, 23, 27-29).

The development of the diverse dysbiotic community stimulates inflammatory responses that recruits a large infiltration of neutrophils, macrophages, and lymphocytes. This leads to deterioration of the soft tissue around the teeth, destruction of the periodontal ligaments and gingival fibers, and loss of attachment to the teeth. The tissue damage caused

by the aggravated immune response provides by-products that serve as a source of nutrients supporting the dysbiotic community. As part of the host immune response, gingival epithelial cells release IL-8 which guides neutrophils from the circulation through the junctional epithelial to the site of infection. Neutrophils form a protective wall between the plaque and gingival epithelium, release antimicrobial peptides (α-defensins and LL-37) and actively phagocytose adjacent bacteria (30, 31). Gingival epithelial cells also release antimicrobial peptides, i.e., β-defensins and LL-37 (32). However, periodontal bacteria have evolved mechanisms to evade or modulate the host immune response. For example, *Aa* lipopolysaccharide (LPS) induces the expression of IL-1β and RANKL, activating osteoclasts and promoting bone resorption (33, 34).

Neutrophils in the spotlight: effector functions and role in periodontitis

Neutrophils comprise of 40-60% of peripheral blood leukocytes in humans and are a major player of the innate immune response (35). The importance of neutrophils is exemplified by predisposition to life-threatening bacterial and fungal infections, loss function to regulate inflammation and leukocyte adhesion deficiency in individuals with neutropenia or genetic defects of neutrophil anti-microbial capabilities (36-38). Neutrophils have granules packed with enzymes and other antimicrobials such as lactoferrin and transferrin (39). There are three granules: specific, gelatinase and azurophilic and secretory vesicles. Granules are released in a controlled and hierarchical manner determined by the intensity of the stimulus received (40-42). Secretory vesicles are the first granule to be mobilized, followed by gelatinase granules (40, 41). Stronger stimulation is required to mobilize specific and azurophilic granules, for example,

experimentally such a stimulus would be pre-treatment with latrunculin A to disrupt the cytoskeleton and then stimulate with fMLF.

Under homeostatic conditions, mature neutrophils exit the bone marrow and enter the circulation (43). Low concentrations of IL-8 stimulate the expression of neutrophil-specific adhesion molecules on the blood vessel wall (44-46). This interaction, in addition to IL-8 gradient (among other chemoattractants), promotes the mobilization of secretory vesicles. Once neutrophils cross through the endothelium, they continue to receive signals such as an increasing IL-8 gradient released by gingiva epithelial cells, formyl peptides (i.e. fMLF) produced by invading bacteria, the anaphylatoxin C5a (47), resulting in mobilization of gelatinase granules. These different signals "prime" the neutrophils, by initial activation that has them in "alert mode" that upon a second stimulus, will enhance the response of the neutrophil. The primed neutrophil arrives and forms a defensive wall against the tooth-associated-biofilm to prevent bacteria from reaching deeper into tissue (48). In the infected subgingival pocket, neutrophils are exposed to an overwhelming number of inflammatory signals that induce the release of specific and azurophilic granules. Granules also play a key role in neutrophil phagosome maturation. Upon internalization of a particulate stimuli, like a bacterium, recruitment of specific and azurophilic granules to the phagosome is a key event to acquire phagosome maturation generating a very toxic environment due to the release of antimicrobial peptides, enzymes such as myeloperoxidase (MPO) and generation of reactive oxygen species (ROS) (49-51). Finally, some neutrophils undergo apoptosis and are cleared by macrophages or might form neutrophil extracellular traps (NETs). NETS arise from the release of decondensed

chromatin histones and granule contents into the extracellular space as a last resort to capture and or kill microorganisms (52).

This oral mucosa environment promotes a primed and hyperactivated phenotype in neutrophils which could be potentiated by the ability of oral pathogens to modulate their effector functions. As a result, neutrophils have been suggested to promote disease progression, which may explain why neutrophil counts in periodontitis is correlated with disease severity (48, 53-56).

Siglecs and their role in periodontal disease

Neutrophils can experience cytoplasmic vacuolation when under stress and is often accompanied by cell death (57-59), but the functional role of vacuolization remains unclear. Cytoplasmic vacuolation occurs when a cell is exposed to a certain inducer and its effects can be transient or irreversible depending on the concentration and time of exposure (60-62). Bacterial, viral pathogens or natural and artificial low molecular weight compounds serve as inducers of vacuolization. Further, cell death associated with cytoplasmic vacuolization in neutrophils has been found to be mediated by sialic acid-binding Ig-type lectin (Siglecs) in a caspase-independent manner (63) and is dependent on the proinflammatory cytokine environment (64). In addition, delayed neutrophil apoptosis is associated with acute and chronic inflammatory diseases (i.e. periodontitis) and is influenced by the overexpression of neutrophil survival cytokines such as G-CSF (64, 65).

Siglecs are type I transmembrane proteins present in the membrane of most white blood cells and have an N-terminal Ig domain that recognizes sialic acid-containing glycans. These are a built-in surveillance system that aids in distinguishing from self and non-self, and helps regulate unwanted immune responses (66-70). These proteins have

conserved cytoplasmic tyrosine-based motifs, that consist of a membrane-proximal immunoreceptor tyrosine based inhibitory motif (ITIM) and a membrane-distal ITIM-like motif (71). Inhibition of immune responses normally occurs via the recruitment of tyrosine phosphatases, such as SHP1 and SHP2, by their cytoplasmic ITIM domain (72, 73). Siglecs identified in both humans and mice and what cells express them are shown in Figure1.2.

Human neutrophils express only Siglecs-5/14, which are expressed as polymorphic paired receptors, and Siglec-9 (64). Siglecs-5/14 paired expression is believed to be an evolutionary response to counteract pathogens that cloak themselves in sialic acid-containing glycans in order to suppress immune response (70). Different members of the *Pasteurellaceae* group, such as *Haemophilus influenzae*, *Aa* and *Pasteurella haemolytica* have been found to possess sialic acid on their LPS structures (74, 75) and may use this as a disguise to modulate and evade the host immune response. For example, *H. influenzae* is known to engage Siglec-5 to suppress cytokine production by myeloid cells (76). However, because Siglec-5 and Siglec-14 transduce opposing signals, when sialylated-adorned-bacteria binds Siglec-5 (inhibitory) it will also bind Siglec-14 (activating) and initiate the p38 mitogen-activated protein kinase (MAPK) and AKT signaling pathways (77). The importance of this paired expression is exemplified in individuals with a *SIGLEC14*-null polymorphism, where only Siglec-5 is expressed. In these individuals, bacteria escape surveillance and successfully suppress phagocytic killing by neutrophils (78). Further, different genome wide analysis studies (GWAS) done with samples groups from Chinese Han, German, Netherland, Turkey, northwestern Europe and British origins coincided in reporting that *SIGLEC5* and *DEFA1A3* loci were associated with both aggressive and chronic periodontitis (79-82).

It has been shown previously that Siglec-E (Siglec-9 mouse homolog) is involved in modulating neutrophil functions, including induction of apoptosis, inhibition of cellular activation, suppression of migration, modulation of oxidative stress, and regulation of inflammatory cytokines secretion (83). Recent work demonstrated that Gram-negative bacteria enhance Siglec-E activation in murine neutrophils and in turn dampens the innate response toward Gram-negative bacteria (84). Siglec-9 has also been shown to negatively regulate the innate immune response against sialylated bacteria (71). A role for Siglec-9 in periodontitis has been highlighted in a recent review by Sudhakara *et al.* (85) where sialic acid adorned *P. gingivalis* interacts with Siglec-9 and attenuates neutrophil inflammatory signaling. However, further studies will be required to confirm this activity. Siglec-9 has been also associated with vacuolization or the formation of cytoplasmic vacuoles (63). Ligation of Siglec-9 in a pro-inflammatory environment diverts to a caspase-independent cell death, ROS production and vacuole formation (86). Further, neutrophils from RA patients, a disease associated with periodontitis and *Aa*, demonstrated increased Siglec-9 expression (64). Additionally, both Siglec-5 and Siglec-9 have been shown to suppress NETosis by modulating neutrophils activation and ROS generation (87).

A. actinomycetemcomitans contribution to periodontal disease and host immune response modulation

Aggressive periodontitis is one of the three sub-classifications of periodontal disease and is characterized by rapid loss and destruction of the bone (4, 88, 89). In some cases, it has been associated with abnormalities with phagocyte function and strongly associated with infection of *Aa* (88, 90, 91). *Aa* is a Gram-negative opportunistic oral pathogen, facultative anaerobe, and non-motile coccobacillus of the *Pasteurellaceae* family (92).

Other members of the *Pasteurellaceae* family include *Haemophilus influenzae* and *Actinobacillus pleuropneumoniae*. In addition to being associated with aggressive periodontitis, *Aa* has been associated with other diseases, like cardiovascular diseases, atherosclerosis, urinary tract infections, brain abscesses, among others (93-96).

The main *Aa* strain used in this work was the afimbriated, smooth-colony-morphotype strain 652, serotype c which has been previously referred as a minimally leukotoxic strain of *Aa* (97). There are five serotypes (a-e), with variation in the global population in serotype burden, with a, b, and c being globally dominant, while d and e are rare in humans (98, 99). However, Fine *et al.* (100) showed that *Aa* 652 cultured under anaerobic conditions has increased cytotoxicity due to increased leukotoxin A production. The *Aa* 652 serotype c has been isolated from both healthy and periodontitis positive patients and is considered an opportunistic pathogen (100, 101). Finally, there is strong racial tropism in the distribution of *Aa* strains among periodontitis positive individuals (100, 102-104) where ancestry and socioeconomic status plays a major role. The low leukotoxic strains of *Aa* are found mostly in individuals of Caucasian ancestry compared to the more leukotoxic strains found in those of African ancestry (100, 102, 105-107).

In periodontitis *Aa* contributes to tissue inflammation, destruction, and bone resorption by expressing several virulence factors (Table 1.1). As discussed above, *Aa* expresses and secretes a 116 kDa leukotoxin A (LtxA) of the repeats-in-toxin (RTX) family of pore-forming bacterial toxins that target neutrophils (Figure 1.3), monocytes and lymphocytes (108, 109). In neutrophils, LtxA induces the release of granules (110) or when in large concentrations, cell death (111). JP2 is a highly leukotoxic strain of *Aa*, considered a pathogenic strain that expresses 8 to 10-fold more LtxA than the 652 strain. JP2 *ltx* operon

is transcribed from two promoters in comparison to 652 which has a 530bp region present in its promoter which contains a potential attenuator, resulting in reduced transcription of the *ltx* operon (112, 113). Individuals infected with the highly leukotoxic strain JP2 have early onset periodontitis (97, 114).

The cytolethal distending toxin (Cdt) is a genotoxin and it has three subunits: CtdA, CdtB and CdtC. CdtA and CdtC subunits are required to bind the holotoxin to the plasma membrane of the target cell and gain entry as a dimer. CdtB as its active unit translocates to the nucleus and using its DNase activity induces DNA lesions that lead to cell cycle arrest at the G_2/M interphase. (115). CdtB can also induce cell-cycle arrest and apoptosis through its phosphatase activity (116). CdtB induces apoptosis in T cells, monocytes, and gingival epithelial cells and promotes the release of IL-1β, IL-6, IL-8 by peripheral blood mononuclear cells (PBMCs), monocytes and macrophages (117-119). At high concentrations, CdtB promotes a decrease of IL-12 and IL-10 and increases IL-1β and TNFα, thus impairing phagocytosis and nitric oxide (NO) production by macrophages (120).

Outer membrane vesicles (OMVs) play a very important role in *Aa* ability to evade complement. These vesicles serve as a decoy that triggers complement activation through LPS and take in complement components (121). In turn, LPS of some *Aa* strains (*i.e. A. actinomycetemcomitans* Y4) can bind strongly to C3b, blocking the interaction between complement-derived opsonins with LPS, decreasing the neutrophil complement-dependent response (122). OMVs also contain outer membrane proteins (Omp) (123) such as Omp29 that bind C4-binding protein, inhibiting the activation of the classical and mannose-binding lectin (124) complement pathways (125). Further, Omp29 (Figure 1.3) is associated with

entry into gingival epithelial cells by upregulating F-actin rearrangement. A third Omp, Omp100 (ApiA), is produced by *Aa* in response to the presence of H_2O_2 (126). Omp100 captures the alternative complement pathway negative regulator, Factor H, and deposits it at the cell surface to modify C3b into an inactive form (127). Additionally, Omp100 aids in *Aa* adhesion and invasion of keratinocytes (127, 128). The VT745 strain of b serotype of *Aa* resists phagocytosis killing by blocking complement access to LPS through serotype-b-specific polysaccharide antigen (129).

In early stages of periodontal disease, *Aa* makes use of lactic acid produced by *Streptococcus sp*. as a nutrient to increase its population (Figure 1.3). Production of H_2O_2 by *Streptococcus sp*. and iron limitation serves as an environmental cue for *Aa* to activate the *fur* transcriptional regulator which induces the expression of dispersin B (*dspB*) to facilitate the release of *Aa* from the biofilm and migrate deeper into the gingival pocket (130). This environmental cue also activates the oxygen resistance transcription regulator (*oxyR*), which regulates the expression of Omp100 and catalase (KatA) (126). Catalase aids in the degradation of hydrogen peroxide produced by neutrophils and streptococci (131), protecting *Aa* from oxidative damage. This in turn increases oxygen availability allowing *Aa* to shift from fermentative metabolism to respiratory metabolism.

Iron transport systems

Iron limiting conditions occurs during an active infection, where host molecules such as lactoferrin or transferrin (produced by leukocytes) sequester free iron, so it is not available to bacteria. To counter this, bacteria express high affinity iron scavenging molecules, known as siderophores (132). Enterobacteria (e.g., *Echerichia coli*) encode a siderophore known as enterobactin to sequester iron in the host environment (133-136).

Salmonella typhimurium and *E. coli* express a siderophore receptor, the enterobactin receptor, along with an ATP-binding cassette (ABC) transporter (134, 135) necessary for iron uptake. On the other hand, *Bordetella* uses catecholamines already in the environment (from the host) to scavenge for iron and catecholamines are recognized by a siderophore receptor on its membrane (137-140). Interestingly, *Aa* is not able to produce siderophores (141), but it does express an enterobactin receptor and an ABC transporter (91). This enterobactin operon in *Aa* consists of four open reading frames (ORFs) encoding the ABC transporter, permease, periplasmic – binding proteins and FepA (enterobactin/siderophore receptor) (142).

Alternatively, iron uptake may be carried out by the ferrous iron transport system (Feo) and periplasmic-binding protein-dependent transport (PBT) (143-146). This system may represent the predominant iron transport system at low oxygen conditions when ferrous iron is stable and is more prevalent than ferric iron (147). In contrast, when ferric iron is predominant, it is chelated by ferric iron reductases and subsequent uptake is mediated by ferrous permeases (132, 148, 149) .

QseBC two-component system and its importance for iron transport in *A. actinomycetemcomitans*

In order to persist and colonize in a given niche is crucial for bacteria to adapt quickly to environmental changes. Bacteria has evolved mechanisms such as two-component systems (TCS) that allows them to sense external changes and regulate gene expression accordingly. TCS (Figure 1.4) are phosphorelay systems that in most cases, sense an outer cell signal and subsequently regulate the expression of targeted genes inside the cell (150-152). TCS may respond to several stimuli such as pH, temperature, and ion

concentration (i.e., zinc and iron). The prototypic TCS consists of two components, as the name entails, a sensor, and a response regulator protein. The sensor protein is a histidine kinase (HK) homodimer comprising a sensor domain and a cytoplasmic kinase domain. Interaction of the sensor domain with its cognate signal induces a conformational change that activates the kinase domain. The kinase domain phosphorylates the response regulator which in turn functions as a DNA binding protein or transcription factor, promoting gene regulation.

The QseBC TCS is associated with quorum sensing and is highly conserved in *Enterobacteriaceae* and *Pasteurellaceae* (89) but the signal recognized by members of these bacterial families differs among species. The QseC (sensor) homolog in *H. influenzae,* FirS, is activated by ferrous (Fe^{2+}) iron and zinc (153), whereas catecholamines and autoinducer-3 (AI-3) can activate QseC in *E. coli* (89, 154). The QseC signaling cascade in *Enterobacteriaceae* is more complex than that of *Pasteurellaceae.* In *E.coli* QseC serves as a global virulence regulator as it not only phosphorylates its cognate response regulator QseB, but also the non-cognate response regulators QseF and KdpE (155). Each of these response regulators control the expression of the virulence factors, shiga toxin and LEE-encoded regulator (Ler), respectively, integrating the QseBC signaling cascade to the QseEF and KdpDE TCS. (88), demonstrated that mutation of the *qseC* gene resulted in a decrease in biofilm biomass and mice inoculated with the *qseC Aa* mutant showed no bone resorption compared to wild type, highlighting the requirement of QseC for *Aa* biofilm growth and virulence.

In *Salmonella enterica,* the *ygiW* (renamed virulence and stress-related periplasmic protein (*visP*)) gene was identified upstream of the *qseBC* operon (156). VisP (a precursor

to YgiW in *E. coli*) binds to the sugar moiety of the peptidoglycan, inhibiting the Fe^{2+}/α-ketogluterate-dependent dioxygenase (LpxO) resulting in decreased LPS modification and increased resistance to stressors when bacteria were internalized and replicating within the macrophage vacuoles (156). On the other hand, in the same study using a murine colitis model VisP functioned independently of LpxO and provided resistance against cationic antimicrobials peptides. In *E.coli,* YgiW was shown to play a role in stress response by providing resistance against hydrogen peroxide, cadmium and acid stress (157). The transcription of *ygiW* has been shown to be regulated by QseB in *S. enterica* (158), *H. influenzae* (153) and *Aa* (92). In *E.coli,* YgiW was shown to play a role in stress response by providing resistance against hydrogen peroxide, cadmium and acid stress (157).

In *Aa*, the *qseBC* operon encodes the genes for QseC, a sensor molecule, and QseB the response regulator (88). In *Aa*, QseC phosphorylates the only response regulator identified to date, QseB, and this results in upregulation of genes associated with anaerobic metabolism and respiration (91), whereas genes involved in iron uptake and transport are downregulated (91). Deletion of the *qseB* gene in *Aa* demonstrated that a functional QseB protein is necessary for the expression of the *ygiW-qseBC* operon, suggesting that *qseBC* is auto-regulated (92). Weigel *et al.* (91), showed that in *Aa* both catecholamines (epinephrine and norepinephrine) and iron (ferrous and ferric iron) are the signals that activate QseC (Figure 1.5) and promote *qseBC* expression and bacterial growth. Novak *et al.* (88), demonstrated that mutation of the *qseC* gene resulted in biofilm biomass decrease and mice inoculated with a *qseC Aa* mutant showed no bone resorption compared to wild type, highlighting the requirement of QseC for *Aa* biofilm growth and virulence. The *qseBC* operon of *Aa* shares 70% sequence similarity with *E. coli;* however, the organization

of the operon is different (89). In both bacteria, the *qseBC* locus is associated with the *ygiW* gene, which encodes a putative periplasmic protein of the bacterial oligo-nucleotide/oligosaccharide-binding (OB)-fold (BOF) protein family (159, 160), but it differs in transcription of *ygiW* relative to the *qseBC* operon, due to the attenuator stem loop found in the intergenic region between *ygiW* and *qseBC* (92). Real time PCR of *qseB*, *qseC* and *ygiW*, in *Aa*, showed that all three genes were transcribed in a primary transcript (92). Promoter mapping supported this by indicating that *ygiW* gene is part of the *qseBC* operon (92). Although, no known function has been identified for YgiW in *Aa* member of the BOF protein family can act as scaffolds in ligand binding (160). Although, little characterization of BOF proteins exist, they have been linked to stress responses (161, 162).

Interkingdom signaling: catecholamines as the common language

Catecholamines (e.g., dopamine, epinephrine (adrenaline) and norepinephrine (noradrenaline)) are hormones made in the adrenal glands and are released upon physical or emotional stress. These are also known as monoamine neurotransmitters, which structure comprises a catechol and side chain amine. As a defense mechanism by the host, catecholamines are also used as antimicrobials to sequester any free iron in the environment so it is not available to pathogens (90) and "starve" microbes, this is known as nutritional immunity. These molecules also serve a role in proliferation, differentiation, apoptosis, and cytokine production in lymphocytes and PBMCs (90). Additionally, it has been shown that catecholamines enhance neutrophil activity, modulate cytokine and adhesion molecule expression, and can regulate phagocytosis (163-165). In mice, macrophages and

neutrophils are known to release catecholamines when stimulated with LPS (166). Therefore, the inflamed subgingival pocket is a catecholamine rich environment for *Aa*.

The newly emerging field of microbial endocrinology (167, 168) studies the communication or inter-kingdom signaling (155) that has evolved between microorganisms and hosts. The study of inter-kingdom signaling includes the study of hormonal communication, where microorganisms respond to the host neurohormones as environmental cues to regulate the expression of genes necessary for virulence and survival (167, 169-173). It has been shown that in chemically defined medium, where catecholamines presence and concentration are controlled, catecholamines activate signaling pathways that promote the expression of genes required for bacterial iron uptake and growth (154, 174, 175). Gram-negative bacteria such as *Yersinia enterocolitica*, *Pseudomonas aeruginosa* and *E. coli* respond to epinephrine, norepinephrine, dopamine and dopa in the environment by regulating genes necessary for bacterial growth (176). Catecholamines have also been shown to be used as cues by pathogenic and probiotic strains of *Enterococcus faecalis* to regulate the expression of genes involved in biofilm formation and adhesion (177).

In both *Bordetella bronchiseptica*, *Bordetella pertussis* epinephrine, norepinephrine and dopamine induced *bfeA* transcription, resulting in BfeA receptor production (outer membrane receptor) and increased bacterial growth (137, 138). Further, it was shown that norepinephrine facilitates *B. bronchiseptica* iron sequestration from iron-loaded transferrin (137, 138, 154) Freestone *et al*. (154) demonstrated that norepinephrine complexed with host iron-loaded transferrin and lactoferrin and that *E. coli* can bind and use this as a source of iron. Their findings suggested that transferrin and lactoferrin affinity

to iron decreased when in complex with noerpinephrine, due to conformational changes induced by the complex or by norepinephrine having a higher affinity for iron than lactoferrin and transferrin (154). This was studied and confirmed by Sandrini *et al.* (178), that structurally demonstrated that catecholamines, have the ability to interact with iron-loaded transferrin or lactoferrin and remove iron, making it available for bacterial iron uptake systems. Although, providing iron to bacteria is not the intended function for catecholamines, microbes have found a way to exploit this and use it to their advantage.

Rationale and Significance

Previously, Cat-Fe was identified as the signals that activate QseBC, yet a source for catecholamines in the oral cavity has not been identified. Neutrophils are found in large numbers at periodontitis active sites, and it has been shown that murine neutrophils release catecholamines and these in turn can sequester free ions such as iron and make them unavailable to bacteria (154, 166, 178). However, bacteria can exploit catecholamines from the host to obtain iron as a nutrient and regulate genes involve in virulence and growth (137, 138, 178). In the presence of Cat-Fe *Aa* growth is increased as well as the regulation of genes involved with iron storage and metabolism in the anaerobic environment. Therefore, we hypothesize that *Aa* utilizes catecholamines from the host environment and via QseBC, primes metabolism for growth in the anaerobic sub-gingival environment.

Additionally, *Aa* produces a pore forming toxin known as LtxA, these types of toxins are associated with the formation of cytoplasmic vacuoles (58). *Aa* has also been shown to express sialic acid on its LPS structure like other members of the *Pasteurellaceae* family (85, 179) and recently, in neutrophils Gram-negative bacteria have been demonstrated to exploit the Siglecs surveillance system by increasing Siglec-9 expression,

dampening the neutrophil inflammatory response (72, 83, 84). Thus, we propose that *Aa* induces cytoplasmic vacuolation independent of LtxA expression and enhances Siglec-9 expression to dampen the inflammatory response of neutrophils.

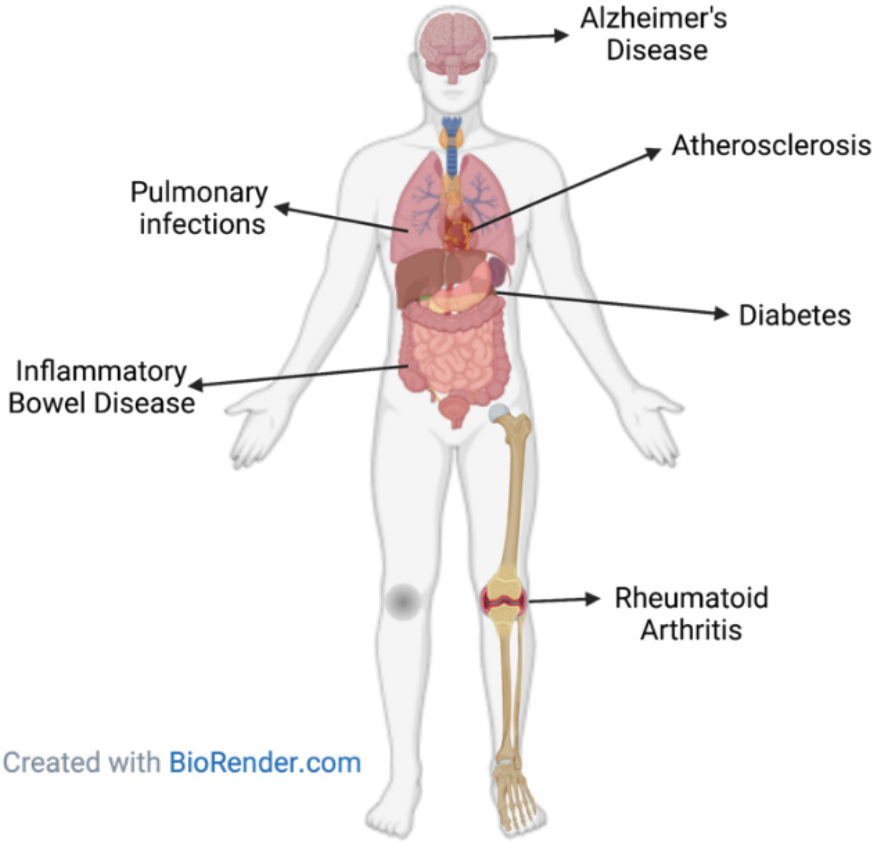


Figure 1.1. Periodontitis plays a role in several diseases at distal sites

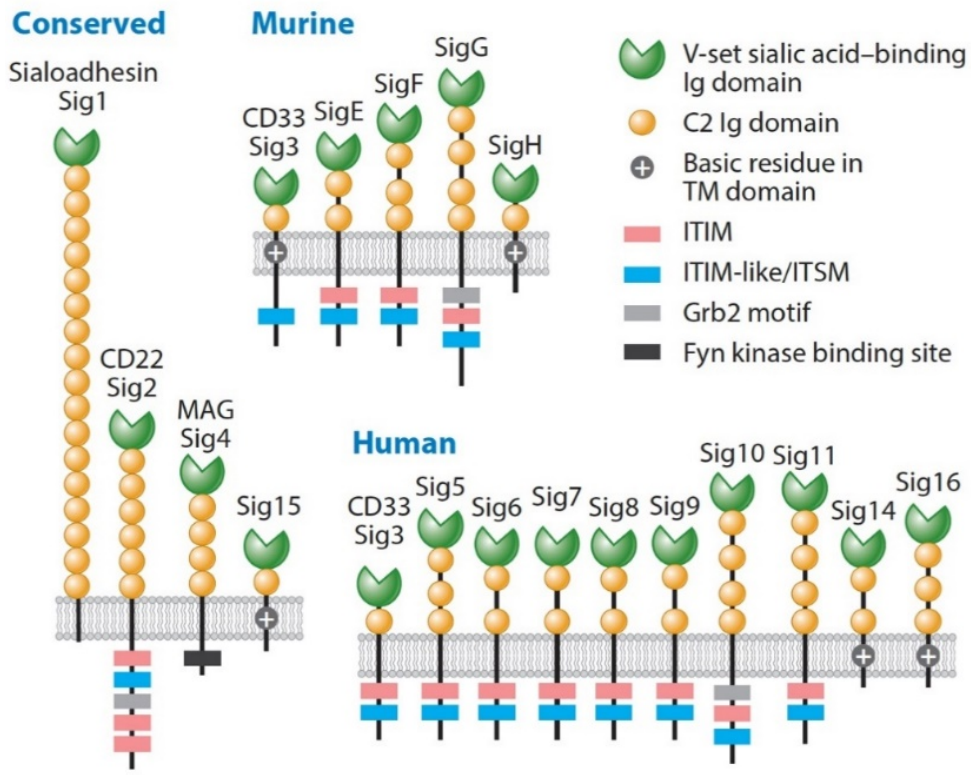


Figure 1.2. Identified Siglecs in humans and mice and their structures (70)

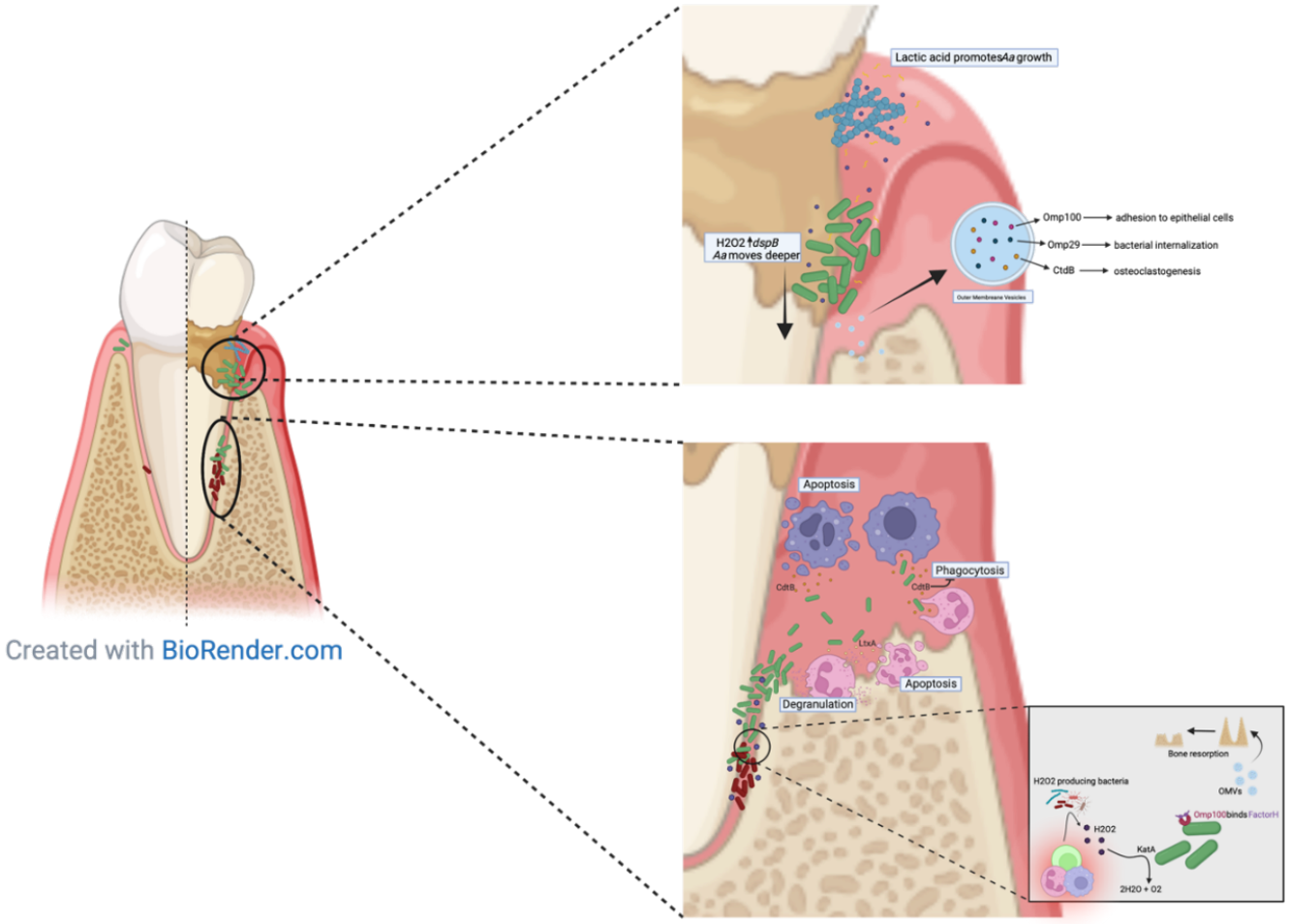


Figure 1.3. *A. actinomycetemcomitans contribution* to periodontal disease

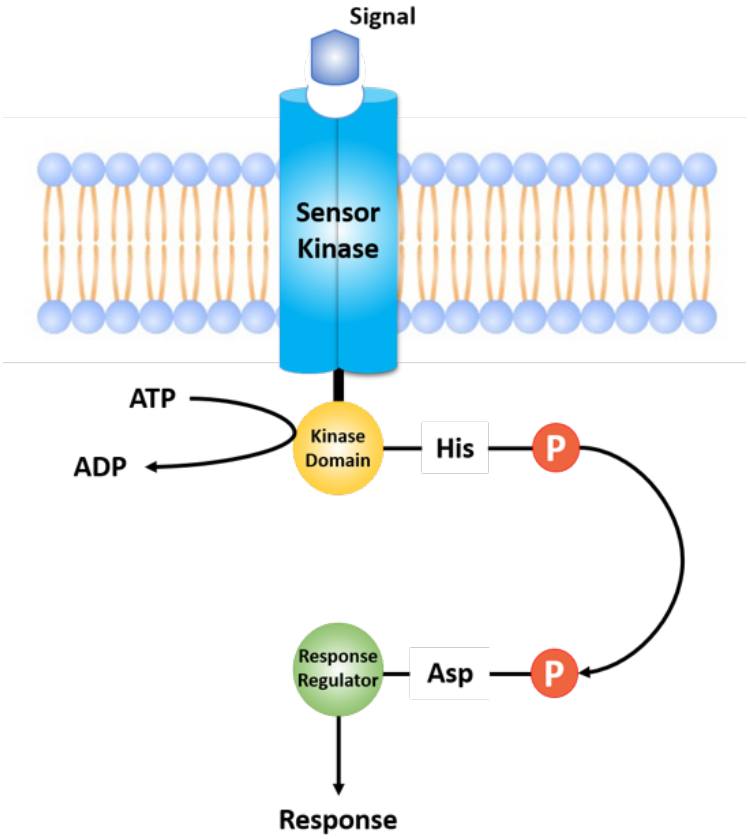


Figure 1.4. General schematic of a two-component system

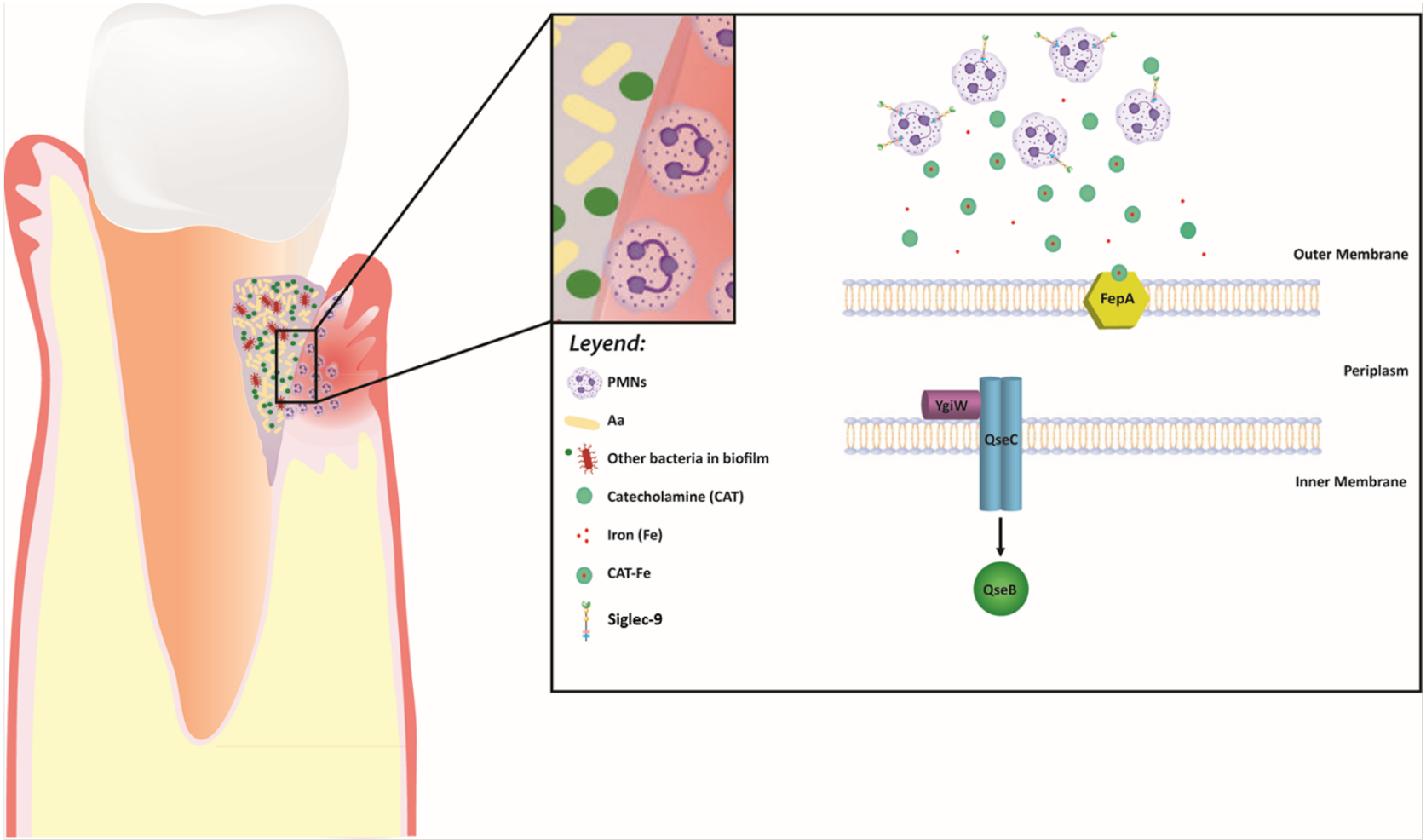


Figure 1. 5. Model proposed where neutrophils serve as a source of catecholamines for *A. actinomycetemcomitans* to acquire iron to regulate the expression of the *qseBC* operon

Table 1.1. Virulence factors of *A. actinomycetemcomitans*

Virulence Factor	Functions and immune evasion	Refs
Virulence determinants in *Aa*		
Leukotoxin A	Important for initial binding to host cells. Induces cell death of leukocytes and release of IL-1β and IL-8 by monocytes/macrophages. Induces rapid release of lysosomal enzymes and MMPs from PMNs.	(180-184)
Cytolethal Distending Toxin	Genotoxin, CdtB (active unit) has DNase and phosphatase activity. Induces cell-cycle arrest and apoptosis in T cells, monocytes, and gingival epithelial cells. Induces the release of IL-1β, IL-6, IL-8 by PBMCs, monocytes and macrophages. At high concentrations it ↓IL-12 and IL-10 vs ↑IL-1β and TNFα. Impairs phagocytosis and NO production by macrophages.	(115, 118, 119, 185, 186)
Lipopolysaccharide	Induces bone resorption, tolerance in macrophages and release of IL-1α, IL-1β and MMP9. Stimulates the expression of MIP-1α, MIP-1β, MIP-1γ, RANTES, MIP-2, IP-10 and MCP-1 in macrophages.	(187-193)
Outer Membrane Vesicles	Vehicle to traffic protein factors into host cells, including microRNAs, exRNAs and virulence factors CdtB, LtxA and LPS. ExRNAs promote TNFα production in macrophages and can cross BBB. OMVs can internalize into host cells and act as decoys triggering complement through Omps that binding to C4-binding protein or C3b inhibiting the complement pathways. Omp29 is associated with entry into gingival epithelial cells by upregulating F-actin rearrangement and Omp100 aid in Aa adhesion and invasion of keratinocytes.	(121, 127, 185, 194-198)

CHAPTER 2

MATERIALS AND METHODS

Human neutrophil isolation and purification

Recruitment of human donors, blood draws, and use of required materials were done in agreement with guidelines approved by the Institutional Review Board of the University of Louisville. Human neutrophils were isolated from whole blood of healthy donors using plasma-Percoll gradients as previously described (199). When necessary, neutrophils were further purified to obtain >99% pure population. Purification was carried by negative magnetic selection using the Easy Sep Human neutrophil isolation kit (Stemcell Technologies, Vancouver, BC, Canada). Cell purity was assessed by simultaneously staining with FITC-conjugated anti-CD66b (clone G10F5; BioLegend, San Diego, CA, USA) and APC-conjugated anti-CD16 (clone CB16; eBioscience, San Diego, CA, USA) antibodies and determining the percentage of $CD66b^{+}CD16^{+}$ cells using a BD Celesta flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo for analysis (FlowJo, LLC, Ashland, OR, USA). Both pure (>90–95%) and highly pure (>99%) neutrophils were cultured in RPMI-1640 medium without phenol red (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and 5% human serum (Atlanta Biologicals, Flowery Branch, GA, USA).

Bacteria strains and media

In this study we utilized the *Aa* 652 serotype c strain, a minimally leukotoxic, afimbriated, smooth colony-morphotype variant. Considered an opportunistic pathogen, this strain has been isolated from healthy and in large numbers in periodontitis positive individuals (100, 101). *Aa* strains (Table 2.1) were propagated in brain-heart infusion (BHI, Difco, BD Biosciences, Franklin Lakes, NJ, USA) BHI broth or BHI agar (1.5% agar) supplemented with $NaHCO_3$ (0.2 μM), bacitracin (50 μg/ml) and vancomycin (50 μg/ml), unless indicated otherwise. *Aa* was grown at 37°C under microaerophilic conditions in a closed tube, unless indicated otherwise. The *Aa* mutant strains (all in *Aa* 652 background, see Table 2.1) used for experiments were previously constructed by Juarez-Rodríguez *et al* (92): *Aa ΔqseC* (non-polar *qseC* gene deletion mutant, spectomycin 50 μg/ml), *Aa qseCΔpr* (QseC sensor protein with an in-frame deletion of the periplasmic sensor domain, spectomycin 50 μg/ml), *Aa ΔqseC-comp* (non-polar *qseC* gene deletion mutant complemented with a single genomic copy of the *qseC* gene, spectomycin 50μg/ml), *Aa ΔfepA* (in-frame deletion in the *FepA* gene, spectomycin 50 μg/ml), *Aa ΔfepA-comp* (in-frame deletion in the *FepA* gene complemented with a single genomic copy of the *fepA* gene, spectomycin 50 μg/ml), and *Aa ΔygiW* (in-frame deletion in the *ygiW* gene, spectomycin 50 μg/ml). *Escherichia coli* (*E. coli*) BL21 cultures (Table 2.1) containing the plasmids pDJR1 and pDJR3 (Table 2.2) were cultured in Luria-Bertani broth (LB) or propagated in LB agar plates (1.5% agar) supplemented with tetracycline (50 μg/mL), kanamycin (25 μg/mL). The *Aa* strain containing the plasmid pDJR29 (Table 2.1), which contains the *lacZ* gene controlled by the QseBC operon promoter (200), was used in β-galactosidase assays and was grown under anaerobic conditions in chemically define media

(CDM, Table 2.3) (201), supplemented with kanamycin (25 μg/ml). The JP2 and JP2 *Δ*ltxA of serotype b, is a pathogenic wild type strain of *Aa* and considered a highly leukotoxic strain (produces 8-10 times more LtxA than *Aa* 652). These strains were grown under the same conditions as the *Aa* 652 strain. All reagents used in anerobic experiments were oxygen-depleted.

Filifactor alocis (*Fa*) ATCC 38596 was cultured in BHI broth supplemented with 20 mg/mL yeast extract, L-cysteine (0.1%), and arginine (0.05%) for 7 days anaerobically at 37°C as previously described (Armstrong, 2018 #749;Armstrong, 2016 #159;Socransky, 1985 #630). *Peptoanaerobacter stomatis* (*Ps*) strain CM2 was cultured anaerobically at 37°C in tryptic soy broth supplemented with 20 g/liter yeast extract, 1% hemin, and 1% reducing agent (37.5 g/liter NH_4Cl, 25 g/liter $MgCl_2 \cdot 6H_2O$, 5 g/liter $CaCl_2 \cdot 2H_2O$, 50 g/liter l-cysteine HCl, 5 g/liter $FeCl_2 \cdot 4H_2O$), as previously described (202).

<u>*A. actinomycetemcomitans* challenge, epinephrine detection and neutrophil viability</u>

Human neutrophils (3 x 10^6 cells/ml) were challenged in suspension with *Aa* at a multiplicity of infection (203) of 50 at 37°C in a shaking water bath, unless indicated otherwise. As a positive control for epinephrine release, neutrophils were treated with latrunculin A (1μM, Sigma-Aldrich, St. Louis, MO, USA) for 30 mins, followed by stimulation with N-formylmethionyl-leucyl-phenylalanine (fMLF, 300nM, Sigma-Aldrich, St. Louis, MO, USA) for 5 mins at 37°C in a shaking water bath. At the end of incubation samples were centrifuged at 6,000 xg for 30 secs, supernatants were collected and supplemented with 100X Halt Protease and Phosphatase inhibitor single-use cocktail (Thermo Fisher Scientific, Waltham, MA, USA). The pelleted cells were lysed with ice-cold 1X lysis buffer (10 μl per 1 x 10^6 of cells; [20 mM Tris-HCl [pH 7.5], 150 mM NaCl,

1% [vol/vol] Triton X-100, 0.5% [vol/vol] Nonidet P-40, 20 mM NaF, 20 mM $NaVO_3$, 1 mM EDTA, 1 mM EGTA, 5 mM phenylmethylsulfonyl fluoride [PMSF], 2 mM diisopropylfluorophosphate [DFP], 21 μg/ml aprotinin, and 5 μg/ml leupeptin]). Lysates were centrifuged and cell lysate was supplemented with 100X Halt Protease and Phosphatase inhibitor single-use cocktail (Thermo Fisher Scientific, Waltham, MA, USA). Epinephrine was measured in supernatants and cell lysate with a commercially available Adrenaline/Epinephrine ELISA kit (Cat. No. E4359, BioVision Inc., Milpitas, CA, USA).

Neutrophil viability when challenged with *Aa* was determined by Trypan Blue exclusion and cytospin microscopy imaging. For Trypan Blue exclusion neutrophils were diluted 1:20 in Trypan blue at 0h, 4h, 8h and 24h and live cells were counted using a hemacytometer. For cytospin images, cells were centrifuge at 6,000 xg for 30 secs and wash twice with RPMI-1640 (no phenol red, Sigma-Aldrich, St. Louis, MO, USA) to remove bacteria. Neutrophils were resuspended at 1 x 10^5 cells in 200μl of RPMI-1640 and added 5μl of human serum (Atlanta Biologicals, Flowery Branch, GA, USA). The cell suspension was loaded into the funnel chamber that is assembled on the cytocentrifuge clip, with slide and filter. Cytocentrifuge clip was centrifuge for 5 mins at 800 rpm (Shandon Cytospin 3, Thermo Fisher Scientific, Waltham, MA, USA). The microscope slide was removed from cytocentrifuge clip and fixed and stained using the Hema 3 Protocol staining kit (Thermo Fisher Scientific, Waltham, MA, USA). Slides were visualized by light microscopy.

Lactate Dehydrogenase Assay

The CytoTox 96 non-radioactive cytotoxicity assay (Promega, Madison, Wisconsin USA) was used to measure neutrophils cytotoxicity when challenged with *Aa* strains.

Briefly, positive control was prepared by exposing 1 million neutrophils to 1% Triton X and incubated for 2 hrs in the water bath at 37˚C. Following, the supernatant was collected after centrifuging at 6,000 xg for 30 secs at 23˚C. Samples were added in triplicates of 50µl of each sample to a flat bottom 96 well plate. A volume of 50µl of CytoTox 96 Reagent was added to each well, cover from light and incubated for 30 min at room temperature. Reaction was stopped by adding 50µl of Stop Solution to each well. Absorbance was read at 490 nm within 1 hr after adding Stop Solution. Cytotoxicity percentage was calculated using the following equation:

$$\% \text{ Cytotoxicity} = 100\left(\frac{Experimental\ LDH\ Release - Vehicle\ LDH\ Release}{Max\ LDH\ Release}\right)$$

Determination of membrane integrity of neutrophils when challenged with *A. actinomycetemcomitans*

Prior to neutrophil challenge, *Aa* was labeled with carboxyfluorescein succinimidyl ester (CFSE) as follows: Bacteria were suspended in dH_2O to $2x10^9$ cfu/ml. Equal volumes of CFSE (1 µg/ml) and bacteria in dH_2O were mixed thoroughly, covered with foil, and incubated at room temperature for 30 min on a rocker platform. Labeled bacteria were washed twice with 1ml of 1XPBS and the optical density at 600nm (OD_{600}) of samples was measured. Subsequently, human neutrophils ($4x\ 10^6$ cells/ml) were challenged with wild type (WT), or heat killed (HK) *Aa* (MOI 50) in suspension for 5, 15, 30 and 60 min at 37 ˚C with shaking in a water bath. Membrane integrity of neutrophils was determined by two different methods: wheat germ agglutinin (WGA) membrane labeling and TO-PRO-3 nuclear staining.

WGA labeling of neutrophil membranes: After the bacterial challenge, samples were added onto 24-well plates containing coverslips pre-treated with 50% of human serum and centrifuged for 4 min at 600 xg with maximum braking. Supernatants were aspirated and cells were fixed onto the coverslips with 4% paraformaldehyde (PFA) for 10 min, followed by two washes of HBSS washes. Neutrophils were incubated with 5 µg/ml of WGA (Life Technologies, Carlsbad, CA) Alexa Fluor 647 conjugate in HBSS for 10 min at room temperature, then washed twice with PBS for 30 secs. Finally, cells were counter-stained with DAPI (300 nM) for 5 min and then washed once with 500 µl 1XPBS for 5 min each at room temperature. Coverslips were mounted with 4 µl Prolong Gold Antifade reagent and edges were sealed with clear nail polish. Slides were visualized using a Fluoview FV1000 confocal microscope and analyzed by FV-10ASW software.

TO-PRO-3 staining of neutrophils nucleus: At each time point, 200 µl of each sample was collected and placed on ice, protected from light. Collected samples were centrifuged at 6,000 xg for 30 secs. Cells were fixed by resuspending in 200 µl of 4% PFA and incubated for 10 min covered with foil at room temperature. Samples were washed by centrifugation as before with PBS. Neutrophils were stained with TO-PRO-3 (1µM) labeling solution in 1X PBS for 15 min at room temperature, protected from light. Following, samples were washed once with 1X PBS and the resuspended in 200 µl of 1X PBS and analyzed using flow cytometry imaging (AMNIS ImageStreamX MKII, Liminex, Austin, TX).

Phagocytosis of *A. actinomycetemcomitans* by neutrophils visualized by confocal microscopy

Human neutrophils (4x 10^6 cells/ml) were challenged with WT *Aa*, HK *Aa*, JP2 *ΔLtxA* (MOI 50) or *Fa* (MOI 10) in suspension for 15, 30 and 60 min at 37 ˚C shaking in a water bath. After challenge, cells were centrifuged at 6000 xg for 30 secs and resuspended in 500 µl 1X PBS. This volume was then applied to pre-treated as before coverslips in a 24-well plate. The plate was centrifuged for 4 min at 600 xg at 14 ˚C. Supernatants were aspirated, and cells were fixed cells using 4% PFA for 10 min. Supernatants were aspirated and 200 µl of 3% BSA in PBS was added to each well for blocking. Then, plates were incubated at room temperature for 1 hr on a slow rocker (if necessary, it was left at 4˚C overnight) and the blocking solution was removed by aspiration. To stain extracellular *Aa*, 200 µl of mouse polyclonal anti-*Aa* (1 µg/ml) primary antibody in 3% BSA-PBS was added to each well and incubated at room temperature for 1 hr on a slow rocker. Coverslips were washed twice with 1X PBS then 200 µl of goat anti-mouse Alexa Fluor 647 (Cell Signaling, Danvers, MA) secondary antibody in 3% BSA-PBS was added to each well. Plates were incubated at room temperature for 1 hr on a slow rocker, followed by two washes with 1X PBS. Antibody linkages were fixed by adding 200 µl of 4% PFA and incubating for 10 min. To stain internalized *Aa*, neutrophils were permeabilized with 3% BSA-PBS and 0.02% saponin and blocked by adding 200 µl of 3% BSA in PBS with 0.02% saponin. Plates were incubated at room temperature for 1 hr on a slow rocker. After aspiration, 200 µl of mouse anti-*Aa* primary antibody in 3% BSA-PBS with 0.02% saponin was added to each well. Plate was incubated at room temperature for 1 hr on a slow rocker and washed twice with 1X PBS. Following, wash was aspirated and 200 µl of goat anti-mouse Alexa Fluor 488 (Cell Signaling, Danvers, MA) secondary antibody in 3% BSA-PBS with 0.02% saponin was added to each well. Plates were incubated at room temperature for 1 hr on a

slow rocker and washed twice with 1X PBS. For nuclear staining we used DAPI at a final concentration of 300 nM for 5 min and washed twice with 500 µl 1X PBS for 5 min each at room temperature. Fixed coverslips were mounted with 4 µl of Prolong Gold Antifade reagent. The edges of coverslips were sealed with clear nail polish. Slides were visualized and Z-stack images were taken using the Leica TCS SP8 MP Confocal Microscope. Image analysis was done using LAS X software. Quantification was performed by counting the total viable and nonviable bacteria both intracellularly and extracellularly from 100 neutrophils in 3 independent experiments.

Measurement of Siglec-9 expression on human neutrophils by flow cytometry

Human neutrophils (4x 10^6 cells/ml) were challenged with WT *Aa* (MOI 50), *Fa* (MOI 10) or *Ps* (MOI 10) in suspension for 2 hrs at 37˚C shaking in a water bath. After challenge, 200 µl of each sample was collected and transferred into newly labeled tubes, 0.5 µl of Fc block was added and incubated on ice for 15 mins. Subsequently, Human Siglec-9 PE conjugated antibody (10 µl/10^6 cells, Biotechne FAB1139P-100, Minneapolis, MN) was added to each sample and incubated covered on ice for 1hr. As a control, additional samples were stained with Mouse Ig2A PE conjugated antibody isotype control (20 µl/10^6 cells, Biotechne IC003P, Minneapolis, MN), washed twice with 0.05% sodium azide and fixed by resuspending in 1% PFA. Samples were run using a BD FACSCelesta Flow cytometer and analyzed with FlowJo software.

Catecholamine Metabolism enzyme-linked immunosorbent assay (ELISA)

Cell lysates collected from neutrophils challenged with *Aa* at 2h, 4h, 8h and 24h were tested for the presence of enzymes involved in catecholamine metabolism. The levels of tyrosine hydroxylase (Cat. No. NBP3-06920, Novus Biologicals, CO, USA), dopamine

β-hydroxylase (Cat. No. NBP2-67945, Novus Biologicals, CO, USA), catechol-o-methyltransferase (Cat. No. OKBB00966, Aviva Systems Biology Corp., San Diego, CA, USA) and monoamine oxidase-A (Cat. No. OKEH02825, Aviva Systems Biology Corp., San Diego, CA, USA) were measured following the indicated protocols supplied by the kit manufacturer.

Neutrophil granule exocytosis and exocytosis inhibition

Neutrophils (4×10^6 cells/ml) were challenged with fMLF (300 nM, 5 min), latrunculin A (1 μM, 30 min) + fMLF (300 nM, 5 min), or *Aa* at various time points from 5 min to 24h at 37°C in a shaking water bath. The exocytosis of secretory vesicles, specific granules and azurophilic granules was determined by measuring the plasma membrane increase of granule markers using fluorescein isothiocyanate (FITC)-conjugated anti-CD63 (for azurophilic granules, Ancell 215-040, Stillwater, MN, USA), FITC-conjugated anti-CD66b (for specific granules, Biolegend 305104, San Diego, CA, USA), and phycoerythrin (PE)-conjugated anti-CD35 (for secretory vesicles, Biolegend 333406, San Diego, CA, USA) by FACSCalibur flow cytometer as previously described (McLeish, 2013 #1148;Uriarte, 2011 #212). Following antibody incubation, cells were washed with 0.5% sodium azide (S2002, Sigma, St. Louis, MO, USA) in FTA buffer (211248 BD, Franklin Lakes, NJ, USA) and fixed with 1% paraformaldehyde (PX0055-3, EMD, Darmstadt, Germany). Gelatinase granule exocytosis was determined by measuring the release of matrix metallopeptidase 9 (MMP-9) by ELISA (Cat. No. ab100610, Abcam, Cambridge, MA, USA). TAT-SNAP23 and TAT-Syntaxin 4 were used to inhibit neutrophil granule exocytosis as previously described by Uriarte *et al* (Uriarte, 2011 #212) and McLeish *et al* (McLeish, 2013 #631). Briefly, neutrophils (4×10^6 cells/ml) were

pretreated with TAT-Syntaxin 4 (0.8 μg/ml), TAT-SNAP23 (0.8 μg/ml) or TAT-control (1 μg/ml) for 15 mins, followed by challenged with *Aa* for 15min, 2h, 4h and 8h at 37°C in a shaking water bath. Granule exocytosis was measured by increase plasma membrane expression of CD35 (secretory vesicles), CD66b (specific granules), CD63 (azurophilic granules) by flow cytometry as described in previous paragraph. In addition, cell supernatants were collected to measure release of secretory vesicle content by ELISA for albumin (Cat. No. EHALB, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), gelatinase content by ELISA for MMP-9, specific granule content by ELISA for lactoferrin (Cat. No. ab108882, Abcam, Cambridge, MA, USA) and azurophilic granule content by ELISA for myeloperoxidase (Cat. No. ab119605, Abcam, Cambridge, MA, USA).

Recombinant epinephrine interaction with *A. actinomycetemcomitans*

Increasing colony forming units (CFUs) of *Aa* (0.0 - 4.0 x 10^8 CFUs) were incubated with 250 pg/ml of recombinant epinephrine (BioVision Inc., Milpitas, CA, USA) for 15 mins at 37°C in a shaking water bath. As a control, wells with epinephrine alone were run in parallel. Samples were centrifuged at 6,000 xg for 5 minutes and supernatants were collected. Immediately after, epinephrine was measured from supernatants with a commercially available Adrenaline/Epinephrine ELISA kit (Cat. No. E4359, BioVision Inc., Milpitas, CA, USA). For analysis, interpolated values were obtained from recombinant epinephrine standard curve, following manufacturer instructions. The amount of epinephrine associated with *Aa* was determined by subtracting the interpolated values bacteria-containing of wells from the values obtained for control wells.

Epinephrine interaction with *A. actinomycetemcomitans* mutant strains

Mutant strains of *Aa* (*ΔqseC, qseCΔpr* and *ΔqseC-comp, ΔygiW, ΔfepA*) were incubated at 3.0 x 10^7 CFUs with increasing concentrations (0 - 500 pg/ml) of recombinant epinephrine (BioVision Inc., Milpitas, CA, USA) for 15 mins at 37°C in a shaking water bath. Wells containing increasing concentrations of recombinant epinephrine served as control. Following, samples were centrifuged as above, and supernatants were collected. Immediately after, epinephrine was measured and analyzed as described above. The amount of epinephrine associated with *Aa* was determined by subtracting the interpolated values of *Aa* containing wells from the values of epinephrine control.

A. actinomycetemcomitans growth kinetics

Aa wild type was inoculated into BHI broth supplemented with bacitracin (50 μg/ml) and vancomycin (50 μg/ml) and grown under microaerophilic conditions at 37°C to OD_{600} of 0.3 – 0.4. Cells were subcultured in fresh BHI broth at a 1:30 dilution and grown as described above to an OD_{600} of 0.5–0.6. Cells were washed with chemically define media (CDM, Table 2.3) and inoculated into CDM supplemented as above at a 1:30 dilution. Cultures were supplemented individually or with a combination of 100 μM $FeCl_2$, 50 μM epinephrine, supernatant or cell lysate collected from neutrophils stimulated with latrunculin A and fMLF (final epinephrine concentration of 30 pg/ml). The OD_{600} was measured at various time points using a Bio- Rad SmartSpec Plus uV-vis spectrophotometer (Bio-Rad, Hercules, CA, USA).

β-galactosidase assay

Quantitative evaluation of β-galactosidase activity was determined using permeabilized cells incubated with *o*-nitrophenyl-*b*-D-galactopyranoside (ONPG) substrate (Sigma, St Louis, MO, USA) as previously described (Miller, 1972 #632).

Briefly, a primary culture of *Aa* (pDJR29) was grown at 37°C under microaerophilic conditions in a closed tube overnight in BHI broth. The culture was subcultured at a 1:30 dilution in BHI broth and grown as described in the previous paragraph for 24h. Subsequently, the secondary overnight culture (OD_{600} of 0.3–0.4) was diluted 1:30 into CDM and grown in an anaerobic chamber for 24 h at 37°C. An aliquot of 0.1 ml was then used to determine the OD_{600} of the culture at 24h and triplicate aliquots of 0.1 ml were used to measure β-galactosidase activity.

Expression and isolation of QseC and YgiW proteins

E. coli BL21 (DE3) strains (Table 2.1) containing the pDJR1 QseC and pDJR3 YgiW constructs (Table 2.2) were grown in Luria broth (LB, Difco, BD Biosciences, Franklin Lakes, NJ, USA), supplemented with respective antibiotics ($1:10^3$) with shaking at 37˚C for 2-3 hrs. Cultures were then diluted to initial OD_{600} of 0.1 in LB and incubated until an OD_{600} of 0.6 was attained. Following, isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1mM and incubated to induce protein expression. At 4hrs post-supplementation with IPTG, 1mL aliquots of *E. coli* cultures were used to measure OD_{600}. Cultures were centrifuged (4,000 xg for 20 minutes at 4°C) and supernatant was discarded. Following, pellets were resuspended in Lysis Buffer NTI-10-G (Thermo Fisher Scientific, Waltham, MA, USA), then centrifuged as before and supernatant was collected and transferred to a new tube as the soluble fraction. To obtain the insoluble fraction, pellets were resuspended in lysis buffer containing lysozyme, benzonase nuclease and n-dodecyl-β-D-maltoside (DDM). Cells were disrupted using sonication, centrifuged (20,000 xg for 1 hr at 4°C) and supernatant was collected as the insoluble fraction.

Visualization and quantification of QseC and YgiW proteins

Proteins QseC-pr and YgiW were expressed as GST and HIS fusion protein respectively. Approximately 1 μg of protein of each fraction was loaded on a NuPAGE Novex 4-12% Bis-Tris Protein gel (ThermoFisher, Waltham, MA) and electrophoresed for 30 minutes at 200V. The gel was stained for one hour with shaking with AquaStain (Bulldog Bio, Portsmouth, NH), then 1hr with dH_2O. Protein bands from PAGE gel were transferred into a PVDF membrane by western blot. QseC-pr was detected using a primary antibody rabbit anti-GST (ThermoFisher, Waltham, MA) and a secondary antibody anti-rabbit-HRP (Cell Signaling, Danvers, MA). To detect YgiW, we used a primary antibody mouse 6X-HIS Monoclonal (CloneTech, Mountainview, CA) and a secondary antibody anti-mouse-HRP (Cell Signaling, Danvers, MA). Crude samples corresponding to QseC-pr were bound to a GraviTrap GST Column (Sigma, St. Louis, MO) or those corresponding to YgiW were bound to a His60 Ni Gravity Column Set (Takara Bio USA) and eluted with elution buffer provided with manufacture's kit materials. The fractions containing the appropriate bands identified by SDS-PAGE and western blot using the previously described antibodies. Protein concentration was obtained by NanoDrop One Spectrophotometer (ThermoFisher, Waltham, MA).

Microdialysis assay of QseC or YgiW interaction with Cat-Fe

Interaction of QseC and YgiW with epinephrine or iron was done using Micro Dialyzer (Harvard Apparatus) equilibrium two chamber systems with cellulose membranes. A fixed concentration of epinephrine (50 μM) or iron (100 μM) was added to one chamber and the same concentration of protein was added to the second chamber. Dialysis was left to reach equilibrium for 24hrs. A sample was collected from each chamber

and the presence of iron or epinephrine was measured using Iron colorimetric assay (BioVision, San Francisco, CA) and Adrenaline/epinephrine ELISA (BioVision, San Francisco, CA).

Construction of *ΔfepA* complement

Cultures of XL1 *E.coli* containing the pJT3 plasmid (Table 2.1, Figure 2.1) were grown overnight. Plasmid was isolated using the Plasmid Miniprep kit (Qiagen, USA) and plasmid concentration was determined using the NanoDrop One (ThermoFisher, Waltham, MA). Isolated plasmid was first digested with *Bam*HI-*Xba*I (New England BioLabs, Ipswich, MA) to remove the *lacZ* region. The digested sample was run on an agarose gel and the upper band (~5.8kb) was extracted from the gel using the Qiagen QIAquick gel extraction kit (Qiagen, USA) and cleaned with Zymo Research DNA Clean and Concentrator Kit (Irvine, CA). Using *Aa* chromosomal DNA as a template, the *tonB* gene was PCR amplified using a high fidelity Taq polymerase (Invitrogen, Waltham, MA) and primer set: WW175 and WW176, respectively. The primers were flanked with restriction sites as shown in Table 2.2. The PCR product was visualized on a 1% agarose gel, excised, and digested with *Bam*HI-*Xba*I. Following, the PCR fragment was cleaned using Zymo Research DNA Clean and Concentrator Kit. The pJT3 *Bam*HI-*Xba*I fragment was ligated into the digested PCR product (*tonB*) *Bam*HI-*Xba*I to make pJT3/*tonB* plasmid. The *ptrC* promoter region was amplified from pJT1 plasmid (Table 2.1, Figure 2.1) DNA using the primer set WW86 and WW87 (Table 2.2), digested with *Kpn*I-*Bam*HI, cleaned with Zymo Research DNA Clean and Concentrator Kit. The *ptrC* promoter region was ligated into the pJT3/*tonB* fragment to make the pJT3/*tonB*/*ptrC* plasmid. The ligation reaction was transformed into XL1 blue *E.coli* cells by electroporation at 100V for 30 minutes and

incubated for 1 hour in SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, and 20 mM glucose) with shaking. Aliquots of 50 µl were then plated on LB agar plates supplemented with 50 µg/mL of tetracycline and 25 µg/mL kanamycin and incubated overnight. Colonies were collected and cultured in BHI, plasmid was extracted using the Plasmid Miniprep Kit (Qiagen, Germany) and confirmed by sequencing using the University of Louisville Sequencing Core to confirm correct insertion. After confirmation, 25 µg of plasmid was transformed into *Aa* 652 by electroporation. Transformed cells were cultured on BHI plates supplemented with kanamycin and resulting colonies were selected for plasmid purification, digestion with *Bam*HI-*Xba*I followed by digestion with *Kpn*I. Digestion sample was run in a 1% agarose gel with the same conditions as before. The presence of three bands 2.4kb (pJT1 without *sacB*) of 2.1kb (*tonb*) and 81bp (*ptrC*)).

Construction of *ΔfepA*-expressing the pDJR29 plasmid

Cultures of XL1 *E.coli* containing the pDJR29 plasmid (Table 2.1) were grown overnight as previously described. Plasmid was isolated using the Plasmid Miniprep kit (Qiagen, USA) and plasmid concentration was determined with a NanoDrop One (ThermoFisher, Waltham, MA). Following, 25 µg of plasmid was transformed into *Aa* 652 by electroporation. Transformed cells were cultured on BHI plates supplemented with kanamycin and resulting colonies were selected for plasmid purification. To confirm the plasmid insertion, plasmid was digested with *BamHI-XbaI* (New England BioLabs, Ipswich, MA) and ran on an agarose gel.

Statistical Analysis

Unless otherwise noted, statistical experimental conditions and time points were analyzed by an ordinary one-way ANOVA, followed by *post hoc* Tukey's multi-comparison test using GraphPad Prism Software (GraphPad PRISM v9, San Diego, CA, USA). Differences were considered significant at $P < 0.05$.

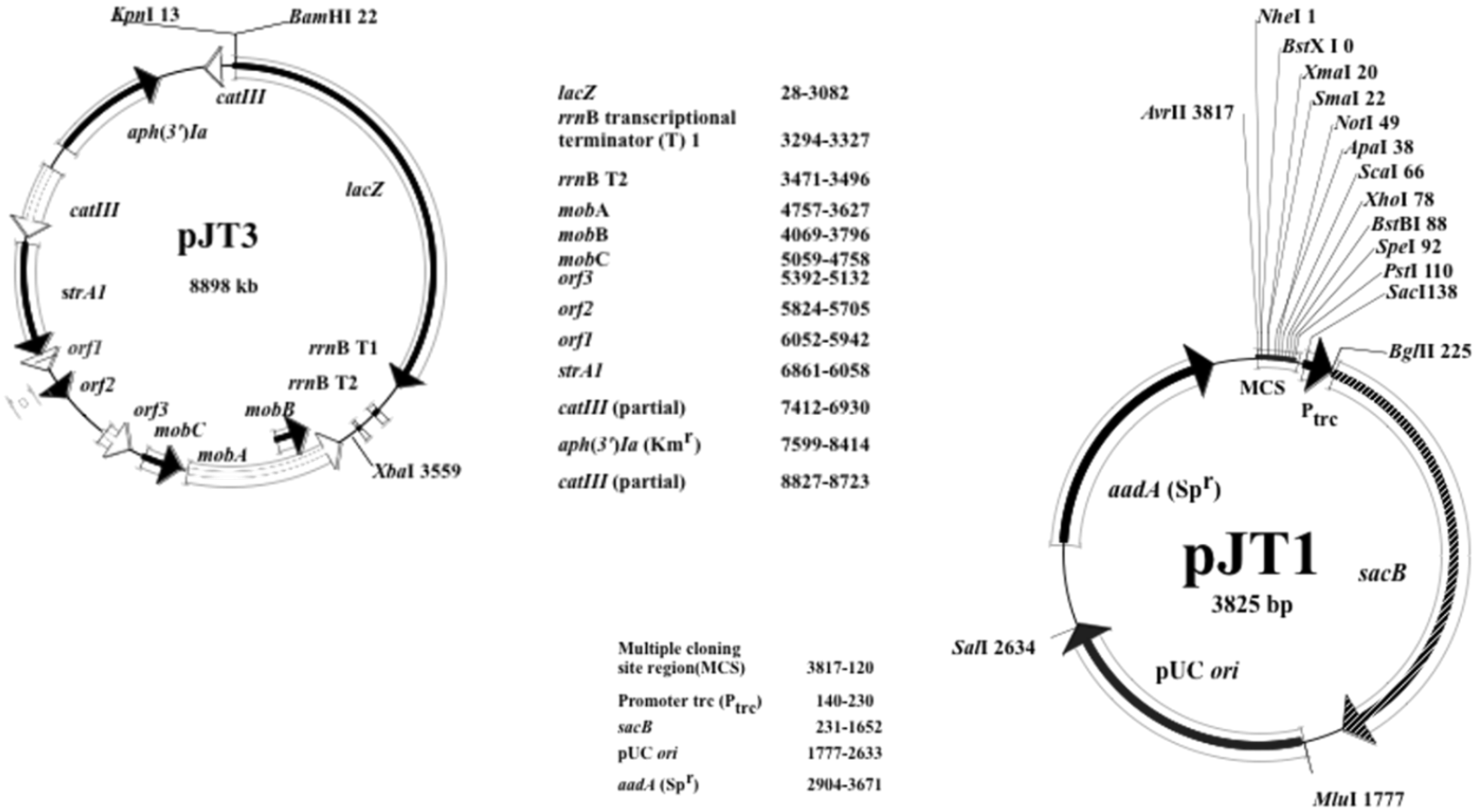


Figure 2.1. Maps of plasmids used in the construction of *ΔfepA*-complement plasmid. Modified from Juarez-Rodriguez *et al* (92).

Table 2.1. Strains and plasmids used in this project

Strain or plasmid	Derived/characteristics*	Source/Reference
	A. actinomycetemcomitans	
652	Wild type, serotype c (Vanco^r, Baci^r)	Laboratory stock
652-JR23	*qseCΔpr* (135)	(92)
652-JR38	Δ*qseC* (135)	(92)
652-JR38	Δ*qseC-comp* (135)	(92)
652-JR25	*ΔygiW* (135)	(92)
652(pDJR29)	*ygiW-qseBC* promoter $_{-1\ to\ -372}$-*lacZ* (Km^r)	(200)
652(*ΔfepA*)	*ΔfepA*(135)	(204)
652(*ΔfepA*-comp)	*ΔfepA-comp*(135)	This study
652(*ΔfepA/*pDJR29*)*	*ΔfepA*, *ygiW-qseBC* promoter $_{-1\ to\ -372}$-*lacZ* (Km^r)	This study
JP2	Wild type, serotype b, highly leukotoxic strain	(205)
JP2 (ΔLtxA)	Gene deletion mutation	DR Demuth, unpublished
	E. coli	
E. coli BL21	(DE3$^-$ *ompT hsdSB*(rB$^-$ mB$^-$)*gal dcm* rne131 BL21(DE3)	Novagen
E. coli XL1	Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F proAB. lacIqZΔM15 Tn10 (Tcr)]	Stratagene
	Other strains used	
F. alocis	Wild type	ATCC
P. stomatis	Wild type	(202)
	Plasmids	
pJT1	Sp^r; suicide vector	(200)
pJT3	Km^r; pYGK*, promoterless lacZ reporter vector	(200)
pDJR29	pJT3, $P_{ygiW372}$-lacZ (Km^r)	(92)
pDJR1	Amp^r; pGEX-4T-1; GST : : *qseC* periplasmic region	(92)
pDJR3	Km^r; pET28a+ygiW-AU1-66His	(92)

* Vanco^r – vancomycin resistance, Baci^r – bacitracin resistance, Km^r - kanamycin resistance, Sp^r - spectinomycin resistance, Amp^r – ampicillin resistance

Table 2.2. Primers used in this project

Primer	Description	Sequence	Source
WW86	ptrC F	GCGCTTATTGCTGGTACCATTCTGAAATGAGCTG	(204)
WW87	ptrC R	CATAGATCTGGATCCCATTTTCCTGTGTGAAATTGTTATCC	
WW175	tonB receptor complement F	CAAGGACGTACCATGGGATCCAAAAAACTC	
WW176	tonB receptor complement R	GCACTTTCTTATATCATTCATCTAGATTAGAAACTGTATTTCAG	

Table 2.3. Composition of chemically defined media (CDM)

Amino Acid Stock							
Hydrophilic Amino Acids		**Hydrophobic Amino Acids**		**Purine/Pyrimidine**		**L-cystine**	
10mL	H_2O	15mL	H_2O	10mL	H_2O	5mL	H_2O
2.5mL	2M NaOH	3.125mL	2M NaOH	1.25mL	2M NaOH	0.625mL	2M NaOH
499.2mg	L-glutamic Acid	200mg	L-alanine	25mg	Adenine	10mg	L-cystine
200mg	Glycine	200mg	D-alanine	20mg	Guanine		
200mg	L-Threonine	200mg	L-leucine	27mg	Cytosine hydrochloride		
200mg	L-Serine	200mg	L-valine	20mg	Thymine		
200mg	L-lysine hydrochloride	200mg	L-Tryptophan	20mg	Xanthine		
242mg	L-arginine hydrochloride	200mg	L-methionine	20mg	Hypoxanthine		
270.2mg	L-histidine hydrochloride	200mg	L-isoleucine	20mg	Uracil		
200mg	L-glutamine	200mg	L-phenylalanine				
227.2mg	L-asparagine	40mg	L-tyrosine				
200mg	L-proline						
200mg	L-aspartic acid						
40mg	L-ornithine hydrochloride						
40mg	L-hydroxyproline						
Combine hydrophobic, hydrophilic, purine & pyrimidine and L-cystine to make amino acid stock.							
Inorganic Salt Stock		**Vitamin Stock**		**Pimelic Acid/Biotin**		**1L CDM**	
99.5mL	H_2O	20mL	H_2O	5mL	H_2O	10mL	$CaCl_2$ (10mg/mL)
10mg	$MnSO_4$	1000mg	Choline chloride	5mL	Ethanol	50mL	Amino Acid Stock
200mg	NaCl	200mg	β-alanine	1mg	Pimelic Acid	50mL	Inorganic Salt Stock
400mg	K_2HPO_4	20mg	Pyridoxal	1mg	D-Biotin	1mL	Vitamin Stock
2000mg	KH_2PO_4	20mg	Pyridoxine-HCl	**Lipoamide**		50mL	$NaHCO_3$ (20mg/mL)
200mg	KNO_3	20mg	Pyridoxamine-di-HCl	1mL	β-mercaptoethanol	10mL	Cysteine (65mg/mL)
0.1mg	KI	20mg	Spermidine-tri-HCl	9mL	Ethanol	10mL	$MgSO_4$ (70mg/mL)
0.065mg	$CuSO_4$:$5H_2O$	20mg	Nicotinic Acid	1mg	Lipoamide	1mL	Pimelic acid/Biotin
0.5mg	Boric Acid	20mg	Calcium pantothenate			10mL	Riboflavin (0.1mg/mL)
0.7mg	$ZnSO_4$:$7H_2O$	20mg	Spermine-tetra-HCl	**Folic Acid**		1mL	Lipoamide
0.5mg	Sodium molybdate	20mg	Thiamine-HCl	9.975mL	H_2O	1mL	Folic Acid
		200mg	Myo-Inositol	25uL	NH_4OH		
		20mg	NAD	10mg	Folic Acid		
		2mg	*p*-aminobenzoic acid				
		.00005mg	Vitamin B12				

CHAPTER 3

TRIALS AND TRIBULATIONS: INTERACTIONS OF EPINEPHRINE WITH COMPONENTS OF THE QSEBC TWO-COMPONENT SYSTEM

Introduction

Bacteria constantly assess their surroundings and make use of the information acquired to regulate the expression of targeted genes for growth, survival, and virulence. In turn, during infection, host cells have evolved mechanisms to limit microbial access to necessary nutrients in the environment. For example, molecules like lactoferrin and transferrin are known to be released by leukocytes to sequester iron, making it unavailable to bacteria (124). However, bacteria have evolved sensing mechanisms involving two-component regulatory systems (TCS) to survey their environment and regulate the expression of genes required to adapt accordingly. In general, these systems consist of two components, as the name entails, a sensor and a response regulator protein. The kinase domain of the sensor protein phosphorylates the response regulator, which in turn will act as a DNA binding protein and transcription factor, promoting or inhibiting gene regulation. *Aa* encodes the QseBC (quorum sensing in *E. coli*) TCS, highly conserved in *Enterobacteriaceae* and the *Pasteurellaceae*, and present in organisms such as *E. coli* (206, 207), *S. Typhimurium* (158, 208, 209). The *qseBC* operon encodes the genes for QseC, a sensor kinase, and QseB, a response regulator (88). The *qseBC* operon in *Aa* exhibits 70% nucleotide similarity to *E.coli qseBC*, however this operon is organized differently in *Aa*. A third gene is also found in the *qseBC* operon, *ygiW*, it encodes a periplasmic solute

binding protein in the OB fold family of proteins (159, 160). Real time PCR of *qseB*, *qseC* and *ygiW* in *Aa*, showed that all three genes were transcribed in a primary transcript (92). Promoter mapping (Figure 3.1) supported this by indicating that the *ygiW* gene resides upstream of *qseBC* and that an attenuator region is found between *ygiW* and *qseBC* (92, 153). Although, YgiW mechanisms or functions remain unknown, knockout of the *ygiW* gene resulted in increased biofilm biomass (88, 92). Interestingly, decreased biofilm growth was observed after an in-frame deletion of the periplasmic domain of QseC (QseC-pr) in *Aa* (92). The opposing effects of these genes deletions, suggest that two independent signals are recognized by YgiW and QseC to control biofilm formation (92). Furthermore, deletion of the *qseB* gene in *Aa* demonstrated that a functional QseB protein is necessary for the expression of the *ygiW-qseBC* operon, suggesting that *qseBC* is auto-regulated (92).

Previous work demonstrated that QseBC is activated by catecholamine hormones and iron, and induces the expression of genes associated with anaerobic metabolism and respiration (91). Similar to *Bordetella*, *Aa* does not express siderophores to sequester nutrients such as iron (141) but instead uses catecholamines from the host to scavenge for iron (210), and catecholamines are recognized by a siderophore receptor on its membrane (137-140). Microarray data of *Aa* grown in chemically defined media (CDM) supplemented with epinephrine and iron showed genes involved in iron uptake were downregulated in a QseBC dependent manner, but the expression of the enterobactin (*ent*) operon remained unchanged (91). This enterobactin operon (Figure 3.2) in *Aa* consists of four open reading frames (ORFs) encoding the ABC transporter, a permease, periplasmic – binding proteins and FepA, an enterobactin/siderophore receptor (142). These observations suggest that *Aa* may exploit the catecholamine rich environment in the sub

gingival pocket to acquire iron in the host environment. For example, catecholamines released by neutrophils may function as pseudo-siderophores and sequester iron from the environment or from iron binding proteins such as lactoferrin. Subsequently, catecholamines may be bound by the *Aa* enterobactin receptor or other cell receptors for transport which may result in activation of QseBC. To further explore this hypothesis, in this chapter we describe optimized protocols for expression and isolation of the periplasmic domain of the sensor protein QseC and the periplasmic solute binding protein YgiW and approaches utilized to determine the interactions of these proteins with epinephrine and/or iron.

Results

YgiW is not required for *A. actinomycetemcomitans* to interact with epinephrine

Previous work in our lab demonstrated that QseBC was required for *Aa* biofilm growth and virulence (88) and that the periplasmic domain of QseC was required for activation of the TCS by catecholamines and iron (91). Further, we recently demonstrated that QseC and QseC-pr are required for *Aa* to interact with epinephrine (210). To determine if YgiW is required for *Aa* to interact with epinephrine, we performed epinephrine interaction assays using *Aa ΔygiW* strain (3.0×10^7 CFUs) with increasing concentrations (0 - 500 pg/ml) of recombinant epinephrine for 15 mins at 37°C in a shaking water bath. As a control, wells with only increasing concentrations of recombinant epinephrine were used. Epinephrine levels were measured by ELISA. We show that the epinephrine interaction of *Aa ΔygiW* was not different to that of wild type *Aa* (Figure 3.3). This suggests that YgiW is not required for *Aa* to interact with epinephrine.

FepA plays a role in the interaction of *A. actinomycetemcomitans* with epinephrine

Microarray data from our lab showed that expression of the genes in the enterobactin operon remained unchanged in the presence or absence of Cat-Fe (91). The *ent* operon in *Aa* is composed of the ABC transporter, permease, periplasmic-binding proteins and the enterobactin receptor FepA (D11S_1354) (141). Since *Aa* does not produce enterobactin to sequester iron, FepA was considered a potential receptor for Epi-Fe (91). We hypothesized that FepA interacts with Epi-Fe from the host environment and transports iron using its ABC transporter. To test this hypothesis, we performed an epinephrine interaction assay as described above and compared *Aa* to heat killed *Aa* and an *Aa* strain deficient in FepA *(ΔfepA)*. Interaction of epinephrine was significantly decreased by deletion of the *fepA* gene compared to wild type *Aa* (Figure 3.4, $p = 0.0312$). Interestingly, heat killed *Aa* 652 interaction with epinephrine was significantly decreased compared to live bacteria (Figure 3.4, $p = 0.0078$), suggesting that heat treatment may denature or inactivate FepA. Further, transportation through FepA is TonB energy dependent (91), which requires viable bacteria. These observations suggest that FepA interacts with epinephrine, but because this interaction was not completely ablated, our results suggest that epinephrine may interact with other adrenergic receptors. Further studies are needed for the identification of the potential receptors.

Discussion

Weigel *et al.* (91) identified catecholamine (epinephrine/norepinephrine) and iron as the signal detected by QseC and required to induce the *qseBC* operon. As initial experiments to further characterize this interaction, we performed equilibrium dialysis experiments with only epinephrine or iron only in MOPS buffer. However, when trying to establish optimal microdialysis conditions, we faced with numerous obstacles, such as high

levels of background in the control reactions. For this reason, we approached the problem from another angle, where different *Aa* strains were incubated with recombinant epinephrine. Recently we demonstrated that QseC and QseC-pr are required for *Aa* to interact with epinephrine (210). Conversely, the interaction of epinephrine with *Aa ΔygiW* was not different to that of wild type (Figure 5.5). These results show that *Aa* interaction with epinephrine occurs independently of YgiW expression. YgiW is a member of the bacterial oligonucleotide/oligosaccharide-binding (OB)-fold (BOF) family. Their structure consists of five antiparallel β-sheets that form a closed or partially opened barrel-like structure, allowing them to act as scaffolds in ligand binding (160). Although, little characterization of BOF proteins exist, these proteins have been linked to stress responses (161, 162). CusF, a BOF protein was shown to bind copper and serve as a shuttle of copper to an efflux system, increasing the accessibility of copper in the periplasmic space (211). While we do not fully understand the role of YgiW in *Aa* at this time, is likely that *Aa* shares similarities in metal binding with other BOF proteins involved in stress responses and be involved in metal binding, but this requires further exploration.

Microarray data from our lab showed that genes found in the enterobactin operon, including the enterobactin receptor FepA remained unchanged in the presence or absence of Cat-Fe while iron uptake genes were downregulated upon QseBC activation (91). We tested an *Aa* mutant strain of FepA (*ΔfepA*) to determine if FepA is required for *Aa* interaction with epinephrine. We found that the interaction of epinephrine was decreased in the *ΔfepA* strain compared to wild type, suggesting that FepA interacts with epinephrine. Similarly, heat killed *Aa* interaction with epinephrine decreased, suggesting that heat treatment may denature or inactivate FepA. *Aa* is particularly heat sensitive and by

increasing the temperature to 65^0C for 5 mins, proteins such as FepA and the TonB energy system required for transport through FepA may be denatured. The interaction of *Aa* with epinephrine was not completely inhibited in the *ΔfepA* strain, suggesting that FepA may not be the sole transport system for Cat-Fe in *Aa*. A potential alternative mechanism for *Aa* to acquire iron at low oxygen levels is the Feo iron transport system (145, 146, 212), which does not require TonB. However, it is not known if the Feo system can transport catecholamines. Further investigation is required to identify potential players.

In this chapter we show that *Aa* interaction with epinephrine requires FepA and not YgiW, contributing to previous findings were QseC and QseC-pr were shown to be required for this interaction. In conclusion, our results suggest that epinephrine interacts with FepA on the bacterial membrane. Furthermore, if YgiW shares function with other BOF proteins, it is possible that it may interact with iron in the periplasmic space and transport it to QseC, thus activating the TCS.

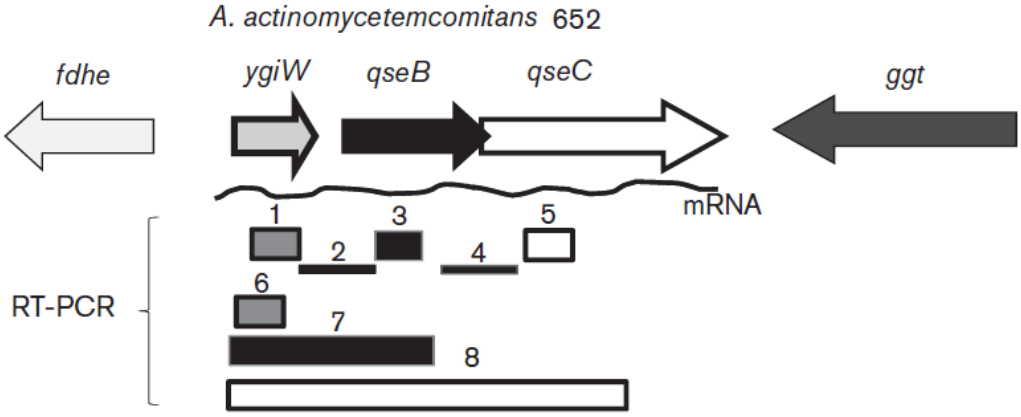


Figure 3.1. Structure of the *ygiW-qseBC* operon in *Aa*, modified from Juarez-Rodriguez *et al.* (92)

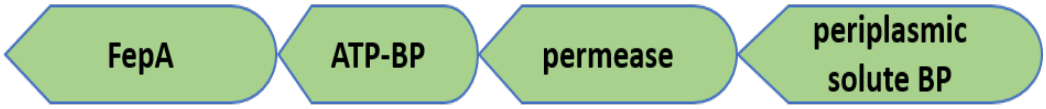


Figure 3.2. Structure of the enterobactin operon in *Aa*, based on D11S_1354-1357 (Kegg Genome Database).

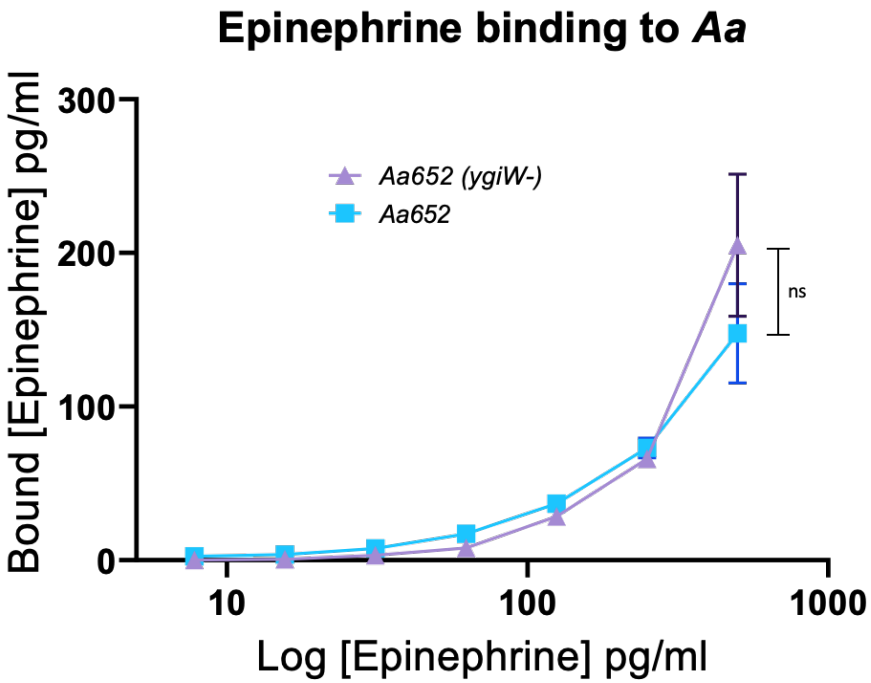


Figure 3.3. YgiW is not required for *A. actinomycetemcomitans* interaction with epinephrine. *Aa* (*ΔygiW*) was incubated at 3.0 x 10^7 CFUs with increasing concentrations (0 - 500 pg/ml) of recombinant epinephrine for 15 mins at 37°C in a shaking water bath. As a control, wells with only increasing concentrations of recombinant epinephrine were used. Epinephrine levels were measured by ELISA. The amount of epinephrine associated with *Aa* was determined by subtracting the interpolated values of *Aa* containing wells from the values of epinephrine control. The mean epinephrine concentration was plotted with standard error bars. Statistical differences among experimental conditions were analyzed by two-way ANOVA, followed by Tukey's post-test. ns = not significant.

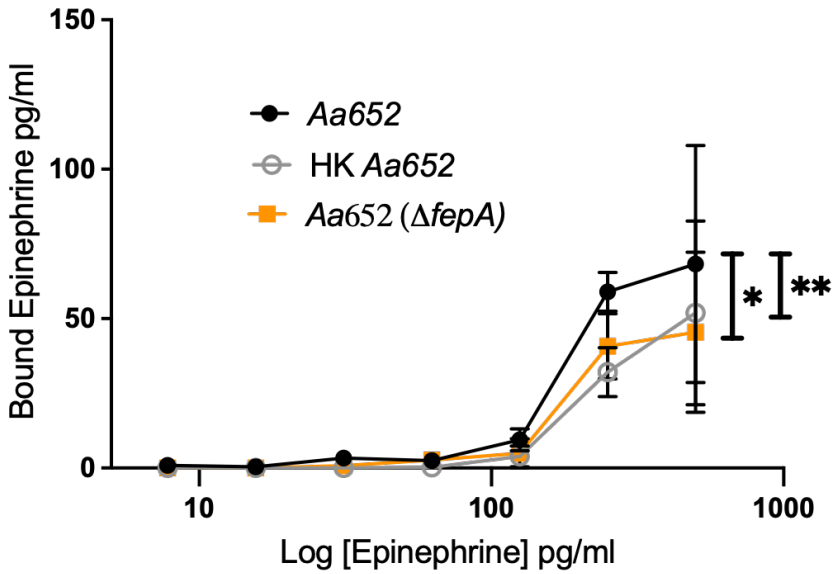


Figure 3.4. FepA is required for *A. actinomycetemcomitans* interaction with epinephrine. *Aa* (*ΔfepA*) was incubated at 3.0 x 10^7 CFUs with increasing concentrations (0 - 500 pg/ml) of recombinant epinephrine for 15 mins at 37°C in a shaking water bath. As a control, wells with only increasing concentrations of recombinant epinephrine were used. Epinephrine levels were measured by ELISA. The amount of epinephrine associated with *Aa* was determined by subtracting the interpolated values of *Aa* containing wells from the values of epinephrine control. The mean epinephrine concentration was plotted with standard error bars. Epinephrine levels were measured by ELISA and plotted as the mean ± SD of 5 independent experiments. Statistical differences among experimental conditions were analyzed by two-way ANOVA, followed by Tukey's post-test. *$p < 0.05$, **$p < 0.01$.

CHAPTER 4

THE HUNGER GAMES: *AGGREGATIBACTER ACTINOMYCETEMCOMITANS* EXPLOITS HUMAN NEUTROPHILS AS AN EPINEPHRINE SOURCE FOR SURVIVAL

*This chapter has been modified from recent publication Ozuna *et al.* (210)

Introduction

In the oral cavity, when host microbe homeostasis is broken, bacterial communities accumulate at the sub-gingival pocket and form biofilms that lead to oral diseases such as periodontitis (3). Periodontitis consists of a chronic inflammation of the periodontium caused by the inflammatory response of the host to plaque biofilm. The disease is characterized by the progressive deepening of the sulcus and loss of attachment between the bone and gingival tissue leading to bone loss. Recurring inflammation of the periodontium has been associated with the initiation, exacerbation, and pathogenesis of several other inflammatory diseases (213). *Aa* is a non-motile Gram-negative facultative anaerobe of the *Pasteurellaceae* family (34, 214, 215). *Aa* has been strongly associated with periodontitis and other diseases such as rheumatoid arthritis, cardiovascular diseases, atherosclerosis, urinary tract infections and brain abscesses (93-96). *Aa* contributes to tissue inflammation, destruction, and bone resorption by expressing several virulence factors such as cytolethal distending toxin, leukotoxin A of the RTX family of bacterial toxins and collagenase (97, 112, 117, 216).

In the subgingival pocket *Aa* must compete for nutrients, such as iron, to survive. As part of the host immune response iron is kept unavailable to bacteria by being sequestered by catecholamines (i.e. stress hormones: epinephrine, norepinephrine and dopamine) or leukocyte-produced molecules like lactoferrin and transferrin (154, 168). However, some bacteria have evolved to subvert this mechanism by producing iron scavenging molecules, known as siderophores (132, 147, 212) or using host-derived catecholamines (154). Stress is considered as an essential factor in development and progression of periodontal disease (217-219). Further, catecholamine stress hormones have been not only detected in saliva of periodontitis patients but is has been shown to increase under stress (220, 221). Catecholamines have been implicated as causative or contributory agents of periodontitis (174, 222, 223). Interestingly, *Aa* does not produce siderophores (141), but expresses the QseBC (quorum sensing in *Escherichia coli*) two-component system (TCS) that is activated in the presence of both catecholamines and iron (91). Two component systems are phosphorelay systems that sense an outer cell signal that will then translate into regulation of targeted genes inside the cell (150-152). This system consists of two components, as the name entails, a sensor and a response regulator protein. The sensor protein is a histidine kinase (HK) homodimer which sensor domain sits on the inner membrane. The kinase domain of the HK phosphorylates the response regulator which in turn will act as a DNA binding protein or transcription factor, promoting gene regulation. The *qseBC* operon, encodes the genes for *qseC*, a sensor molecule and *qseB,* the response regulator (92). Novak *et al* (88), demonstrated the requirement of QseC for *Aa* biofilm growth and virulence. Further, Weigel *et al.* (91), subsequently showed that *Aa* growth in a chemically define media (CDM) supplemented with both epinephrine or norepinephrine

and ferrous (Fe^{2+}) or ferric (Fe^{3+}) iron increased *qseBC* promoter activity and bacterial growth, indicating that both catecholamines and iron are required for the activation of QseBC. Additionally, microarray analysis showed that activation of QseBC induced the expression of genes associated with anaerobic metabolism and respiration, and reduced expression of genes involved in iron uptake and transport. However, the source of catecholamines remained to be determined.

The recent field of microbial endocrinology studies the interkingdom signaling that exists between microbe and host because of coexistence (171). Microbes have evolved systems to detect or sense host-associated signals such as stress hormones (168). This allows them to scan their environment and regulate required genes (167, 169-173). In periodontitis neutrophils are found in large numbers at the gingival crevice (35). Murine neutrophils have been shown to release epinephrine or norepinephrine when stimulated with LPS (166). Furthermore, increased levels of the enzymes (tyrosine hydroxylase and dopamine β-hydroxylase) required for the synthesis of catecholamines, and their inactivation (catecholamine-O-methyltransferase and monoamine oxidase) enzymes has been observed in murine phagocytes and human lymphocytes (166, 224). Nonetheless, the presence of catecholamines in the host environment has also been shown to promote bacterial iron uptake and growth in media limited conditions (154, 174, 223). Host-derived catecholamines have a higher affinity to iron than antimicrobials like lactoferrin and transferrin (154, 178) serving as excellent pseudo-siderophores (175) to bacteria. Therefore, we proposed that human neutrophils serve as a catecholamine source for *Aa* to sequester iron, leading to QseBC activation and bacterial growth.

In this chapter we demonstrate that human neutrophils have significant levels of tyrosine hydroxylase, monoamine oxidase A and catecholamine-O-methyltransferase, validating that neutrophils participate in catecholamine metabolism. In addition, we present evidence that human neutrophils store epinephrine in the azurophilic granules and that interaction with *Aa* induces exocytosis of azurophilic granules and release of epinephrine. Furthermore, we show that treatment with latrunculin A followed by fMLF stimulation, which is known to induce mobilization and content release of azurophilic granules, induces epinephrine release in human neutrophils. The interaction of released epinephrine with *Aa* activates QseBC and induces *Aa* growth under anerobic conditions. These results suggest that neutrophils may serve as an epinephrine source for *Aa*, to facilitate growth and prime anaerobic metabolism in the subgingival pocket. The findings presented here help shed light into the crosstalk that exits between bacteria and the host endocrine system by providing an example of the role that stress hormones play in periodontal disease and potentially other chronic inflammatory diseases.

Results

A. actinomycetemcomitans challenge induces epinephrine release, and promotes de novo catecholamine synthesis in human neutrophils

It has been previously shown that murine neutrophils stimulated with LPS can release catecholamines (166). As an initial study we replicated these findings and confirm that LPS induced the release of epinephrine by murine neutrophils and that this release increased with time (Figure 4.1.A). However, there is as of yet no clear evidence of human neutrophils ability to release catecholamines. To determine if human neutrophils release epinephrine, cells were stimulated with fMLF, latrunculin A + fMLF, LPS or IL-8.

Supernatants and cell lysates were collected, and epinephrine levels determined by ELISA. Unlike what was described in murine neutrophils (166), LPS stimulation did not induce epinephrine release by human neutrophils (Figure 4.2.A) even at extended time points (Figure 4.1.B). Similarly, stimulation of neutrophils with fMLF or IL-8 failed to induce epinephrine release (Figure 4.2.A). Interestingly, treatment with latrunculin A followed by fMLF induced significant release of epinephrine compared to fMLF alone and basal (Figure 4.2.A). Significant levels of epinephrine were detected in the cell lysates but there was no significant difference between experimental conditions (Figure 4.1.C, 4.2.B). Following, we examined the ability of *Aa* to induce epinephrine release by human neutrophils. Cells were challenged with *Aa* at a MOI of 50 in suspension for 2h, 4h, 8h and 24h. Epinephrine levels were measured from supernatants and cell lysates by ELISA. Figure 1c shows that *Aa* challenge induced significant release of epinephrine compared to basal, with maximum levels reached at 4h. Epinephrine levels in cell lysates of human neutrophils treated with latrunculin A + fMLF had significantly reduced levels of epinephrine compared to basal (Figure 4.2.D), this correlates the significant increase release of epinephrine observed in supernatants (Figure 4.2.C). Similarly, low epinephrine levels in neutrophil cell lysates were detected after challenged with *Aa* for 2h and 4h which could be related to the release observed in the supernatants (Figure 4.2.C-D). Interestingly, epinephrine release at 2h was similar to basal (Figure 4.2.C). A potential explanation for this is that the released epinephrine could be interacting in some way with *Aa*. We repeated this experiment with highly purified (>99%) human neutrophils (Figure 4.3) and obtained similar results.

To confirm that the release of epinephrine is not caused by a cytotoxic effect of *Aa* on neutrophils we evaluated the morphology of human neutrophils with cytospin imaging and viability with trypan blue exclusion. Human neutrophils morphology was not altered with *Aa* challenge (Figure 4.4.A-B) and challenge with *Aa* extended the lifespan of neutrophils (Figure 4.4.C) up to 24h. Taken together our results demonstrate that *Aa* stimulates epinephrine release in human neutrophils, depleting storage reservoirs. In addition, the difference in released (Figure 4.2.C) versus stored epinephrine at 2h (Figure 4.2.D) suggests that epinephrine may be interacting with *Aa*. The increase of epinephrine levels in cell lysates at later time points of 8h and 24h (Figure 4.2.D) suggests that human neutrophils are capable of *de novo* catecholamine synthesis.

Next, we examined the levels of enzymes involved in catecholamine metabolism. Levels of enzymes involved in catecholamine synthesis: tyrosine hydroxylase and dopamine β-hydroxylase and levels of enzymes involved in catecholamine inactivation: catechol-o-methyltransferase and monoamine oxidase-A were measured in cell lysates of human neutrophils exposed to *Aa* at 2h, 4h, 8h and 24h by ELISA. In the absence of *Aa* tyrosine hydroxylase levels are significantly low but after *Aa* challenge levels increase significantly with time compared to basal (Figure 4.5.A). In contrast, dopamine β-hydroxylase levels decrease when *Aa* is present (Figure 4.5.B). The levels of catechol-o-methyltransferase (Figure 4.5.C) and monoamine oxidase-A (Figure 4.5.D) remained comparable to basal, only peaking at 4h. Catecholamine synthesis enzyme (tyrosine hydroxylase levels) increase at 8h and 24h (Figure 4.5.A), whereas inactivation enzymes (Figure 4.5.C-D) decrease at the same time points. These results indicate that *Aa* challenge induces *de novo* catecholamine synthesis in human neutrophils.

In the infected subgingival pocket *Aa* functions in consortium with other bacteria in bacterial communities known as biofilms and Fine *et al* showed that *Aa* positive subjects with bone loss had high levels of the oral pathogen *Filifactor alocis* (225). In addition, Wang *et al* demonstrated that *F. alocis* accumulation in the oral biofilm was stimulated by the present of specific strains of *Aa* (226). To examine if the induction of epinephrine release is specific to *Aa*, we performed a time course experiment where we investigated the ability of *F. alocis* to induce epinephrine release in human neutrophils. Human neutrophils were challenged with *F. alocis* at a MOI of 10 at 1h, 2h and 4h incubation and epinephrine detection were performed as described above. *F. alocis* failed to induce significant release of epinephrine (Figure 4.6.A). Consistent with this, no significant difference in epinephrine content was detected in cell lysates among experimental conditions (Figure 4.6.B). In addition, human neutrophils were challenged with LPS up to 24h and no significant epinephrine release or difference in epinephrine content was observed (Figure 4.6.C-D).

A. actinomycetemcomitans access epinephrine by inducing exocytosis of human neutrophil granules

In Figure 4.1 we showed that treatment with latrunculin A followed by stimulation with fMLF, which is known to induce the exocytosis of gelatinase and azurophilic granules, induced epinephrine release by human neutrophils, suggesting that epinephrine may be stored in one or more of the neutrophil granules. To demonstrate this, we first determined the ability of *Aa* to induce granule exocytosis. Neutrophils were challenged with *Aa* and granule exocytosis was determined by measuring increase of plasma membrane expression of secretory vesicles, specific granules and azurophilic granule markers by flow cytometry (see gating strategy and histograms Figure 4.7) and the extracellular release of MMP-9 by

ELISA. Stimulation with *Aa* induced significant secretory vesicles mobilization by 15 mins, as indicated by increased CD35 expression (Figure 4.8.A) which was similar to the exocytosis induced by the positive control fMLF. In addition, gelatinase granules exocytosis, measured by ELISA, was significantly induced after 2h of *Aa* challenge (Figure 4.8.B). Specific granule exocytosis, measured by increased CD66b expression, was significantly increased after 4h of *Aa* challenge (Figure 4.8.C). Azurophilic granule exocytosis, measured by increased CD63 expression, was significantly increased starting at 8h post bacterial challenge (Figure 4.8.D). Mobilization of azurophilic granules requires stronger stimulation compared to the other granule subtypes, our results showed that these granules continued being mobilized by *Aa* increasingly up to 24hrs (Figure 4.8.D). These results demonstrate that *Aa* challenge induced exocytosis of all four neutrophil granule subtypes.

To further characterize if epinephrine release by neutrophils was caused by *Aa* inducing granule exocytosis, we took advantage of two degranulation inhibitors, TAT-SNAP23, which selectively inhibits secretory vesicles, gelatinase granules, and specific granules (199), and TAT-Syntaxin 4 which blocks exocytosis of all four granule subtypes (227). Human neutrophils were pre-treated with TAT-Syntaxin 4, TAT-SNAP23 or TAT-control peptide fusion proteins, followed by *Aa* challenge at 15 mins, 2h, 4h and 8h. Granule exocytosis was measured by increased plasma membrane expression of CD35 (secretory vesicles), CD66b (specific granules) and CD63 (azurophilic granules) markers by flow cytometry and the extracellular release of MMP-9 by ELISA. Treatment with TAT-Syntaxin 4 reduced *Aa*-induced mobilization of secretory vesicles (Figure 4.9.A), gelatinase granules (Figure 4.9.B), specific granules (Figure 4.9.C), and azurophilic

granules (Figure 4.9.D). The *Aa*-induced mobilization of secretory vesicles, gelatinase, and specific granules (Figure 4.9.A-C) was significantly reduced by TAT-SNAP23 pre-treatment. As expected, TAT-SNAP23 failed to inhibit *Aa*-induced azurophilic granules exocytosis (Figure 4.9.D). Further, cell-supernatants were collected at each time point, and granule content release of albumin, lactoferrin and myeloperoxidase were measured by ELISA. Like the results obtained by flow cytometry, challenge of neutrophils with *Aa* for 15 min induced significant release of albumin, which was significantly reduced by pre-treatment with TAT-SNAP23 and TAT-Syntaxin 4, but not TAT-control (Figure 4.10.A). Similarly, *Aa* challenge for 4h induced release of lactoferrin and its release was successfully reduced by TAT-SNAP23 and TAT-Syntaxin 4, but not TAT-control (Figure 4.10.B). As expected, neutrophils challenged with *Aa* for 8h resulted in significant release of myeloperoxidase which it was inhibited by TAT-Syntaxin 4 but not TAT-SNAP23 or TAT-control (Figure 4.10.C).

To further characterize which granule subtype store epinephrine, supernatants were collected to measure epinephrine levels by ELISA. *Aa* challenge of human neutrophils at 15 mins showed no significant release of epinephrine similar to basal and TAT-control (Figure 4.11.A). Moreover, *Aa* challenge at 2h induced significant release of epinephrine in human neutrophils, pre-treatment of TAT-Syntaxin 4 was able to inhibit epinephrine release but TAT-SNAP23 failed to inhibit release (Figure 4.11.B). On the other hand, only pre-treatment with TAT-Syntaxin 4, but not TAT-SNAP23, blocked *Aa*-induced epinephrine released after 4h (Figure 4.11.C) and 8h (Figure 4.11.D) of bacterial challenge. Since TAT-SNAP23 does not inhibit azurophilic granule exocytosis, these results

demonstrate that epinephrine is stored in azurophilic granules. Further, this shows that epinephrine release by neutrophils is linked to granule exocytosis.

A. actinomycetemcomitans interacts with epinephrine in a QseC dependent manner

To determine if *Aa* interacts with epinephrine, increasing CFUs of *Aa* were incubated with a fixed concentration of recombinant epinephrine. Epinephrine interaction with *Aa* increased with increasing *Aa* CFUs (Figure 4.12.A). Previous work in our lab demonstrated that QseBC was required for *Aa* biofilm growth and virulence (88) and that, the periplasmic domain of QseC was required for activation of the TCS by catecholamines and iron (91). Therefore, we performed epinephrine dose interaction assays using various *Aa* strains deficient for QseC. Interaction of epinephrine was diminished by deletion of the QseC sensor (*ΔqseC)* and in the strain expressing QseC with an in-frame deletion of the periplasmic domain (*qseCΔp)* (Figure 4.12.B). The ability of epinephrine to interact with *Aa* was restored when the *qseC* gene was complemented into the QseC deletion mutant (*ΔqseC-comp)* (Figure 4.12.B). These results show that *Aa* interaction with epinephrine requires QseC and is dependent on the QseC periplasmic domain.

Epinephrine from human neutrophils promotes *A. actinomycetemcomitans* growth and *qseBC* expression.

To determine if the epinephrine levels released by human neutrophils could promote bacterial growth, the supernatants and cell lysates were collected from cells stimulated with latrunculin A and fMLF and used to assess *Aa* growth and expression of the *qseBC* operon. *Aa* was grown anaerobically in CDM alone or supplemented individually or in combination with neutrophils supernatant, neutrophil cell lysate, iron (100μM) or epinephrine (50μM). Growth in CDM supplemented with neutrophil lysate or epinephrine alone was comparable

to the unsupplemented CDM control (Figure 4.13.A). Supplementation with iron, either alone or in combination with epinephrine resulted in a significant induction of *Aa* growth (Figure 4.13.A). Further, medium supplemented with neutrophil supernatant alone promoted growth, which was increased when supplemented with iron (Figure 4.13.A). On the other hand, neutrophil cell lysate supplemented with iron promoted growth similar to epinephrine + iron or neutrophil supernatant alone (Figure 4.13.A). Supplementation with epinephrine, neutrophil supernatant or lysate alone failed to induce expression of QseBC (Figure 4.13.B). QseBC expression was significantly induced by iron and a further significant increase in expression occurred by supplementation with a combination of iron with epinephrine or neutrophil supernatant (Figure 4.13.B). Unexpectedly, supplementation with iron and neutrophil lysate suppressed QseBC expression (Figure 4.13.B). This experiment was performed under aerobic conditions with similar results (data not shown). Here, we show that *Aa* makes use of neutrophil-derived epinephrine to promote bacterial growth and expression of the QseBC in the anaerobic environment.

Discussion

The newly emerging field of microbial endocrinology (168, 170) studies the communication or inter-kingdom signaling (155) that has evolved between microorganisms and their hosts. The study of inter-kingdom signaling includes the study of hormonal communication, where microorganisms respond to the host neurohormones as environmental cues to regulate the expression of genes necessary for virulence and survival (167, 169, 171-173).There are reports of host-pathogen crosstalk involving *Aa* biofilm sequestering and taking up IL-1β (228). Additionally, it has been shown previously that *Aa* expresses a cytokine binding receptor, BilRI, that allows it to benefit from cytokine

release to promote biofilm formation and bacterial growth (229, 230). Previous work by our group demonstrated that the presence of catecholamines and iron *in vitro* promoted *Aa* growth and regulated the expression of genes necessary for virulence and survival in the anaerobic environment (91). However, those studies did not determine if similar effects occurred *in vivo*. Based on work by others it is known that there is a large infiltration of neutrophils at the subgingival pocket during periodontitis (27, 35, 231) and murine neutrophils have been shown to release and synthesize catecholamines when stimulated with LPS (166). Therefore, we considered human neutrophils as a potential source of catecholamines for *Aa*. In our *ex-vivo* studies, we showed that *Aa* induces epinephrine release in human neutrophils depleting stored catecholamine levels (Figure 4.14.A) and indirectly inducing catecholamine synthesis (Figure 4.14.E). As a novel finding we identified azurophilic granules as the storage location of epinephrine in human neutrophils and that *Aa* gains access to epinephrine by inducing granule exocytosis (Figure 4.14.B). Then, showed that QseC is required for epinephrine interaction with *Aa* (Figure 4.14.C). Finally, we proved that host-derived epinephrine promoted *Aa* growth and QseBC expression under anaerobic conditions (Figure 4.14.D).

Marino *et al* (224) detected catecholamines in human peripheral blood mononuclear cells medium but the report failed to determine if these catecholamines were released and if so, what stimuli induced release. Likewise, catecholamine release has been shown in other immune cells such as rat and human lymphocytes (232, 233). Although, there is extensive work done in murine neutrophils there was no report of human neutrophils releasing catecholamines and an identified stimulus. Here, we present for the first time that human neutrophils release epinephrine when stimulated with latrunculin A

+ fMLF and when challenged with *Aa,* but not by *F. alocis* which occupies the same oral niche as *Aa*. These observations support previous findings by Parantainen *et al* (203), the first to identify endogenous catecholamines in human neutrophils. However, at the time the source of these catecholamines was not determined or if neutrophils participated in catecholamine synthesis. Later, further evidence of catecholamines present intracellularly in human neutrophils was found and enzymes involved in catecholamine metabolism were detected by high performance liquid chromatography (HPLC) (234). In our experiments, we observed the levels of epinephrine in cell lysates increased after 8h and 24h of bacterial challenge suggesting *de novo* catecholamine synthesis by human neutrophils. This was confirmed by the presence of significant levels of tyrosine hydroxylase levels in the presence of *Aa*. In contrast, dopamine β-hydroxylase levels decreased when *Aa* was present. Tyrosine hydroxylase catalyzes the first and rate limiting step of the catecholamine synthesis pathway, explaining larger amounts of this enzyme. A potential reason for this is that dopamine β-hydroxylase catalyzes dopamine into norepinephrine, and not all dopamine will be catalyzed explaining the decrease in enzyme levels. We also found significant levels at 4h of the catecholamine inactivating enzymes catechol-o-methyltransferase and monoamine oxidase-A. This correlates with the increased epinephrine release induced by *Aa* at 4h, as epinephrine is released demand for degradation increases. Similarly, as stored epinephrine is depleted there is demand for catecholamine synthesis at later time points, and inactivation enzymes decrease at this time. A potential limitation of our study is that granule exocytosis experiments were done under aerobic conditions and not under low oxygen or anaerobic conditions. The periodontal pocket is considered a hypoxic environment, with decreasing oxygen levels as the depth of the

periodontal pocket increases, becoming increasingly hypoxic during infection due to activation of the NADPH oxidase complex (235, 236). Therefore, it is likely that neutrophils are exposed to hypoxic conditions *in vivo* when recruited to the gingival pocket. Although, we did not performed experiments with both *Aa* and neutrophils under anaerobic conditions, preliminary work in our lab with neutrophils challenged with *Fa* showed no difference in the lifespan of neutrophils grown aerobically or anaerobically. Additionally, untreated neutrophils under anerobic conditions resulted in enhanced viability compared to cells grown aerobically. Though, we infer that human neutrophils will undergo granule exocytosis under hypoxic condition, this remains to be tested in the future.

We demonstrated that *Aa* induces the mobilization of secretory vesicles, gelatinase granules, specific granules and azurophilic granules in human neutrophils. These findings align with previous observations that identified enhanced surface expression of CD63 and CD66b in oral neutrophils, characteristic of neutrophils that are undergoing active granule exocytosis (237, 238). Johansson *et al* (110) showed that purified leukotoxin A induces release of granule contents from human neutrophils, and the authors observed increased expression of CD63 and CD66b along with the release of elastase and lactoferrin. In our *ex-vivo* model we obtained similar observations by challenging human neutrophils with a low leukotoxic strain of *Aa* compared to high concentrations of purified leukotoxin. Further, through selective inhibition of granule exocytosis, we identified azurophilic granules as the storage location for epinephrine in human neutrophils. To the best of our knowledge this observation serves as the first evidence of the storage location of epinephrine in human neutrophils. Human neutrophils are recruited out of circulation by interaction with neutrophil-specific adhesion molecules on the blood vessel wall. This

interaction in addition to chemoattractants gradient such as IL-8 promotes the mobilization of secretory vesicles. Once neutrophils cross through the epithelium, they continue to receive signals such as increasing IL-8 gradient potentially released by gingiva epithelial cells and formyl peptides from invading bacteria, resulting in mobilization of gelatinase granules. The primed neutrophil arrives at the infected subgingival pocket where there are overwhelming inflammatory signals that may induce the release of specific and azurophilic granules. Alternatively, these granules can fuse with vacuoles containing phagocytized bacteria, forming a phagosome (49-51). Thus, *Aa* may induce the release of azurophilic granules at the subgingival pocket to gain access to epinephrine and in turn the release of potent antimicrobial protein contents of these granules may contribute to tissue damage and disease progression.

When human neutrophils were challenged with *Aa* for 2h we expected to see a high release of epinephrine, due to depleted epinephrine levels in cell lysate. A plausible explanation to the difference in levels of epinephrine released versus stored at 2h is that some of the epinephrine released could be interacting with *Aa* therefore no longer in the supernatant. Epinephrine interaction with *Aa* increased with increasing *Aa* CFUs. Further, *Aa* interaction with epinephrine was found to require QseC, in a QseC periplasmic domain dependent manner. This supports previous findings made by our group that identified catecholamines (norepinephrine and epinephrine) as the signal that is sensed by QseC and that the periplasmic domain of QseC is required for *qseBC* operon expression in the presence of catecholamines and iron (91). It also strengthens the importance of QseC in recognizing catecholamines for growth and virulence of *Aa* (88). The exact details of the interaction of *Aa* with epinephrine remains unclear, but the discussed results make us

conclude that the QseC periplasmic domain is required. As for a receptor for epinephrine, when *Aa* is grown in the presence of catecholamines and iron the expression of the enterobactin operon, which contains a gene for FepA, remains unchanged (91). In *E. coli*, FepA is an outer membrane protein that serves as a receptor for the ferric enterobactin siderophore (239, 240). The uptake of this siderophore into the bacterial cells is TonB-dependent (240, 241). We performed different experiments to determine if FepA was required for *Aa* interaction with epinephrine using a *ΔfepA* mutant *Aa* strain, but results were inconclusive (data not shown). Moreover, as an alternative for iron entry into the bacterial cells there is the ferrous iron transport system (Feo) and periplasmic-binding protein-dependent transport (PBT) (143-146). These are predominant iron transport systems at low oxygen conditions when ferrous iron is stable and is more prevalent than ferric iron (147). In the case the ferric iron is predominant it will be chelated by ferric iron reductases and then uptake is mediated by ferrous permeases (148, 149). Rhodes *et al* (132) demonstrated that *Aa* makes use of inorganic iron to grow under chelated conditions and proposed that *Aa* may acquire iron through the expression of systems which function independently of an outer membrane receptor or TonB dependent transport. The elucidation of which TonB and receptor independent iron transport system is used by *Aa* will require further investigation.

Under anaerobic conditions we showed that *Aa* growth in limited media is promoted in the presence of neutrophils supernatant or cell lysate supplemented with iron. Likewise, supplementation with neutrophil supernatant and iron induced expression of QseBC. Interestingly, neutrophil cell lysate alone or supplemented with iron QseBC expression was suppressed. Human neutrophils lysates contain all internal contents of

neutrophils, including components such as calprotectin. Calprotectin is a metal binding protein, abundant in neutrophils cytoplasm (242, 243). Another protein that is found in specific granules is the neutrophil gelatinase-associated lipocalin. This protein scavenges iron from siderophores, and it has been shown to remove iron from catecholamines as well (244). These proteins can be sequestering the supplemented iron competing with epinephrine present in lysate, making it unavailable to *Aa* and consequentially suppressing the induction of the QseBC.

In *Aa* positive subjects, *F.alocis* was detected at high levels (225) and *F. alocis* accumulation in the oral biofilm has been shown to be stimulated by the presence of specific strains of *Aa* (226). Based on our experiments we determined that *F. alocis* does not induce release of epinephrine in human neutrophils. This result goes in hand with previous findings that *F. alocis* does not induce the exocytosis of azurophilic granules (245, 246). In the case that *F. alocis* is found to be catecholamine-responsive it will suggest that it may be benefiting from ability of *Aa* to induce epinephrine release. However, at the present there is no report of *F. alocis* being influenced in some way by catecholamines. *Aa* has also been found associated to *Fusobacterium nucleatum*, which growth is increased by hormones like epinephrine and norepinephrine (174), *Lactobacillus spp*. expresses transporter systems for uptake of catecholamines (247) and other catecholamine-responsive species such as *Prevotella spp* and *Leptotrichia* (168, 248, 249). Some of these bacteria have been shown to lack the ability to produce siderophores as is the case of *Aa* (250, 251). Therefore, it is likely that these bacteria work as team to thrive in the subgingival pocket, where *Aa* role may be to induce catecholamine (i.e., epinephrine) release by the infiltrating human neutrophils.

In conclusion, our *ex-vivo* experiments demonstrated that human neutrophils store and release epinephrine upon stimulation with latrunculin A + fMLF. We show that *Aa* can induce epinephrine release in human neutrophils, consequently depleting epinephrine storage and promoting catecholamine metabolism. Further, one of the most important contributions of this study was the finding of azurophilic granules as the storage location of epinephrine in human neutrophils and that *Aa* gains access to it by inducing granule exocytosis. Additionally, we found that epinephrine interaction with *Aa* depends on QseC expression and requires the QseC periplasmic domain. Finally, human-neutrophil derived epinephrine supplemented with iron promoted *Aa* growth and QseBC expression under anaerobic conditions. The presented findings supply evidence towards the growing field of microbial endocrinology by contributing to the understanding of the crosstalk between bacteria and the host endocrine system. Further it expands the knowledge of the role of stress hormones in periodontal disease and potentially other chronic inflammatory diseases.

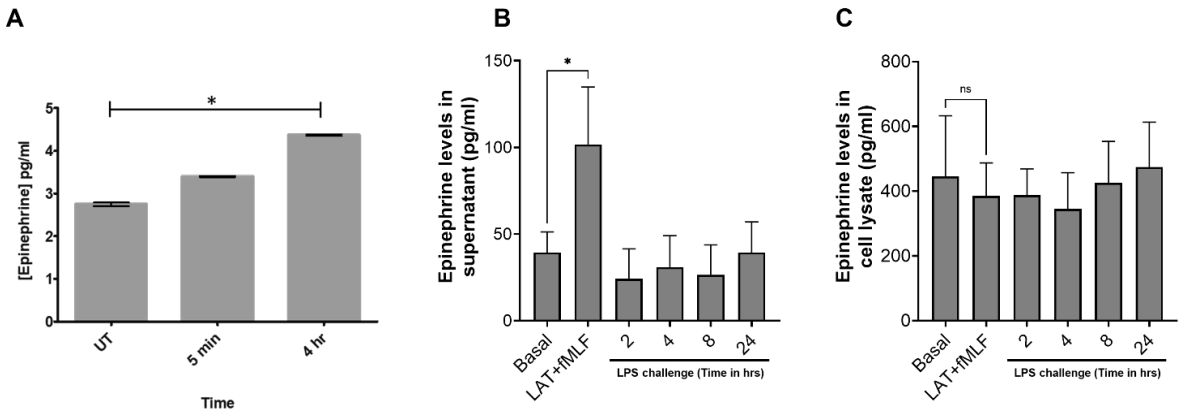


Figure 4.1. Murine neutrophils release epinephrine upon LPS challenge but human neutrophils do not. Murine neutrophils (1×10^6 cells/ml) were left untreated (UT) treated with LPS for 5 mins and 4h to replicate previous findings and induce epinephrine release **(A)**. Human neutrophils (4×10^6 cells/ml) were challenged with LPS for 1h, 2h, 4h, 8h and 24h **(B-C)**. Epinephrine content was measured by ELISA from collected supernatant LPS challenged murine neutrophils **(A)** and supernatant and cell lysate of neutrophils treated with LPS **(B-C)**. Epinephrine content was determined by ELISA. Epinephrine concentration is plotted as the mean ± SD of a N = 3 **(A)** or N = 5 **(B-C)**. Statistical differences among experimental conditions and time points were analyzed by one-way ANOVA followed by Tukey's post-test. ns = not significant, *$p < 0.05$.

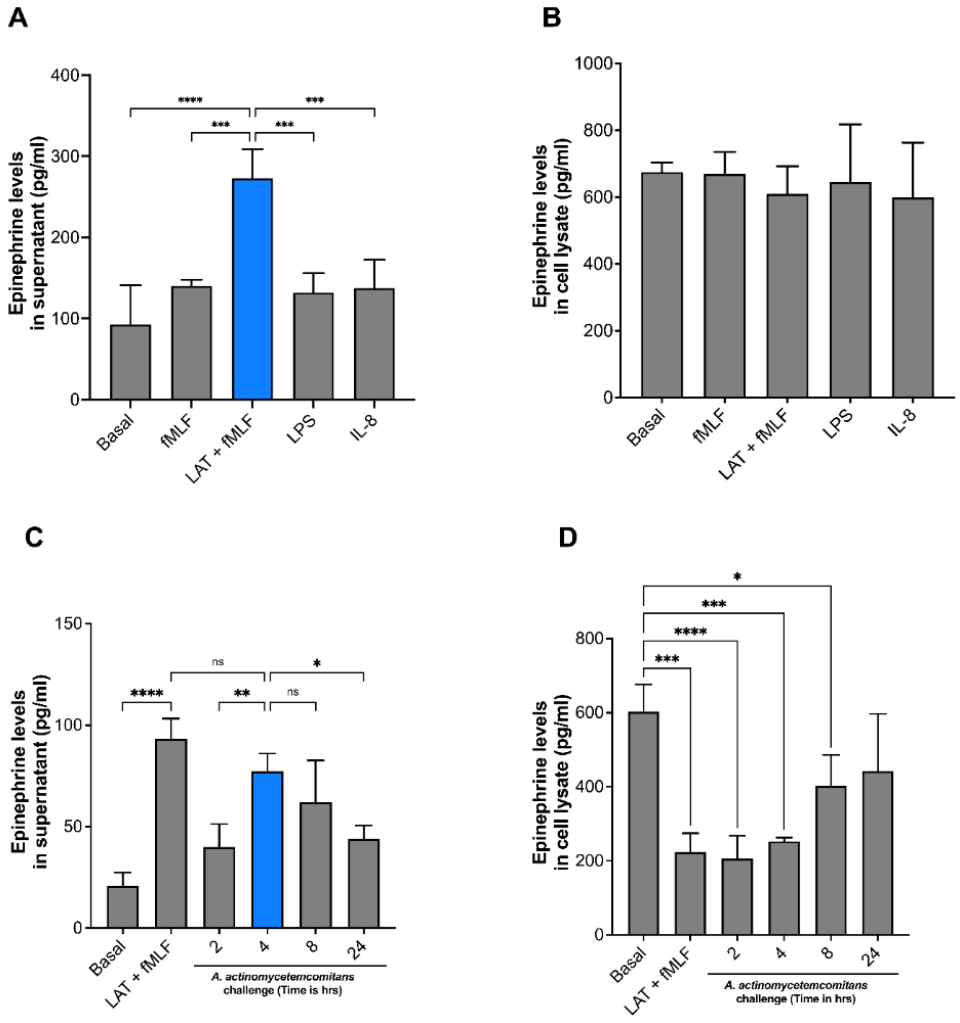


Figure 4.2. Human neutrophils release epinephrine upon *A. actinomycetemcomitans* challenge. Human neutrophils (5×10^6 cells/ml) were treated with fMLF, Latrunculin A (LAT) + fMLF, LPS or IL-8 to induce epinephrine release. Epinephrine content was determined in neutrophils supernatant **(A)** and cell lysate **(B)** by ELISA. Human neutrophils (3×10^6 cells/ml) were challenged with *Aa* (MOI 50) for 2h, 4h, 8h and 24h. At the end of each time point supernatant **(C)** and cell lysates **(D)** were collected and epinephrine content was measured by ELISA. Epinephrine concentration is plotted as the mean ± SD of 4 independent experiments. Peaked epinephrine release/content bars are in blue.

Statistical differences among experimental conditions and time points were analyzed by one-way ANOVA (A, B) or repeated measures one-way ANOVA (C, D), followed by Tukey's post-test. ns: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

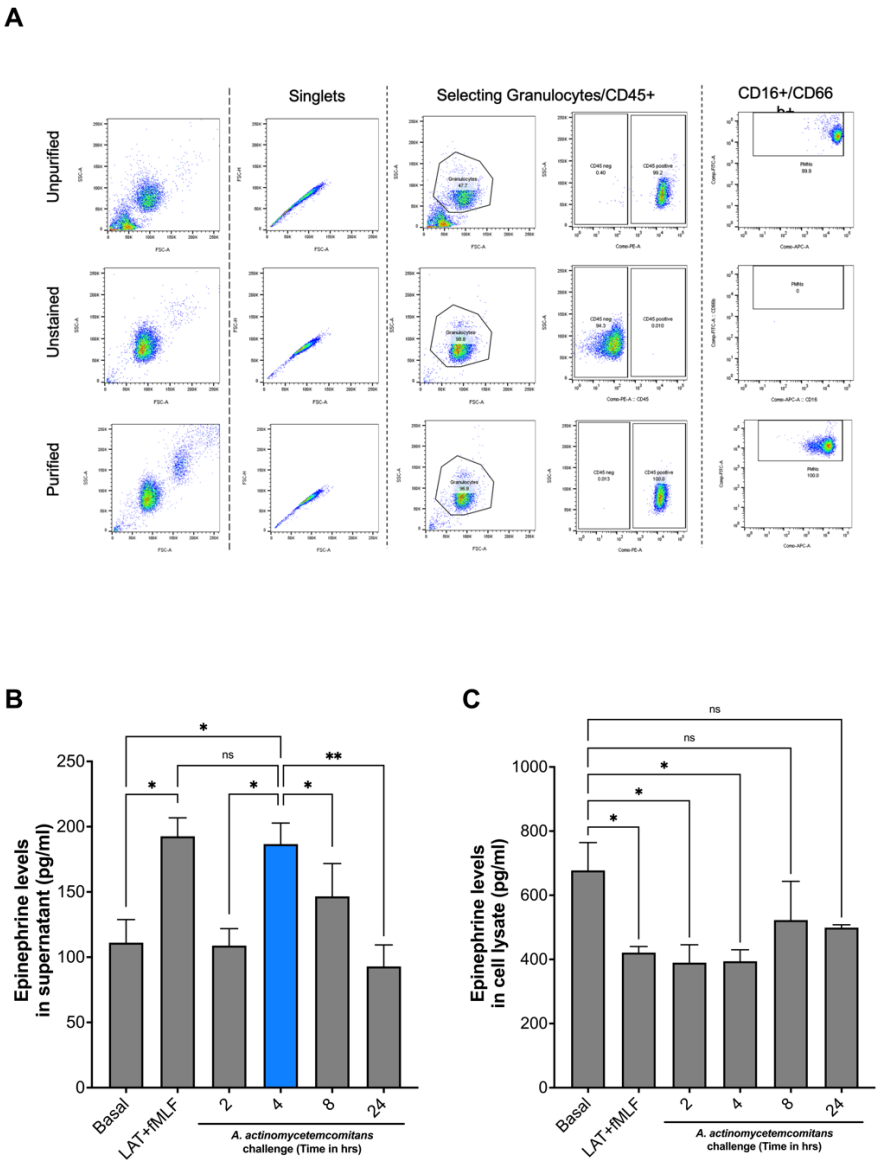


Figure 4.3. *A. actinomycetemcomitans* induces epinephrine release in highly purified human neutrophils. Isolated human neutrophils were highly purified (see Methods) starting from a >90-95% pure neutrophil isolation, confirmed by flow cytometry staining using the gating strategy shown in panel A **(A)**. and then challenged with *Aa* (MOI 50) for 2h, 4h, 8h and 24h and epinephrine content was measured by ELISA from collected supernatant **(B)** and cell lysate **(C)**. Epinephrine concentration is plotted as the mean ± SD of 3 independent experiments. Peaked epinephrine release/content bars are in blue.

Statistical differences among experimental conditions and time points were analyzed by repeated measures one-way ANOVA, followed by Tukey's post-test. ns: not significant, *$p < 0.05$, **$p < 0.01$.

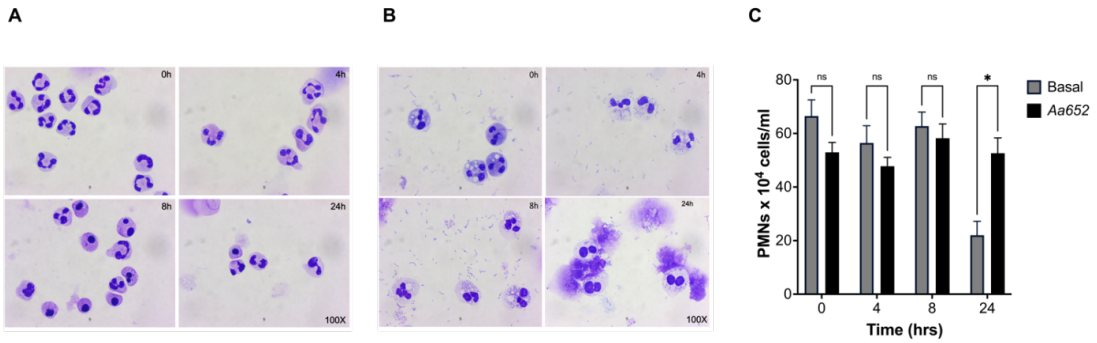


Figure 4.4. Human neutrophils are viable after 24h exposure to *A. actinomycetemcomitans.* Neutrophil viability when challenged with *Aa* was determined by cytospin imaging **(A-B)** and Trypan Blue exclusion **(C)**. Human neutrophils were challenged with *Aa* (MOI 50) for 0h, 4h, 8h, 24h, centrifuged and washed with RPMI-1640. Neutrophils were suspended at 1 x 10^5 cells in RPMI-1640 and human serum. The cell suspension was loaded into the funnel chamber and assembled on the cytocentrifuge clip, with slide and filter. Cytocentrifuge clip was centrifuged, and the microscope slide was removed from cytocentrifuge clip and fixed and stained. Images of unchallenged neutrophils **(A)** and *Aa* challenged neutrophils **(B)** are shown. Neutrophils were diluted 1:20 in Trypan blue at 0h, 4h, 8h, 24h and live cells were counted using a hemacytometer **(C)**. Viability counts are plotted as the mean ± SD of 3 independent experiments. Statistical differences among time points were analyzed by two-way ANOVA, followed by Bonferroni's post-test. ns: not significant, *$p < 0.05$

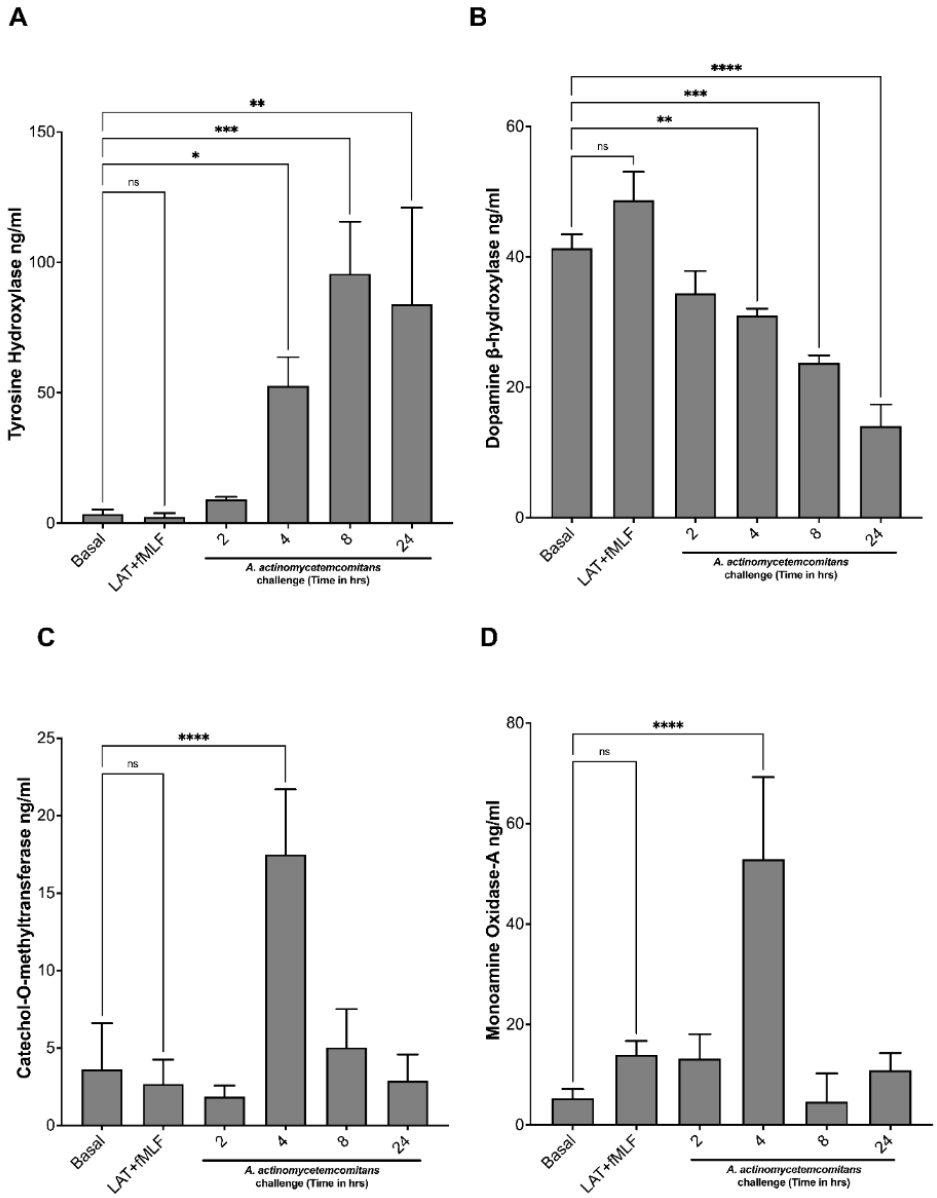


Figure 4.5. *A. actinomycetemcomitans* promotes increased levels of enzymes involved in catecholamine metabolism in human neutrophils. Human neutrophils were challenged with *Aa* (MOI 50) for 2h, 4h, 8h and 24h; cell lysates were collected and the levels of tyrosine hydroxylase **(A)**, dopamine β-hydroxylase **(B)**, catechol-O-methyltransferase **(C)** and monoamine oxidase A **(D)** were measured by ELISA. Enzymes levels are plotted as the mean ± SD of 3 independent experiments. Statistical differences among experimental

conditions and time points were analyzed by repeated measures one-way ANOVA, followed by Tukey's post-test. ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

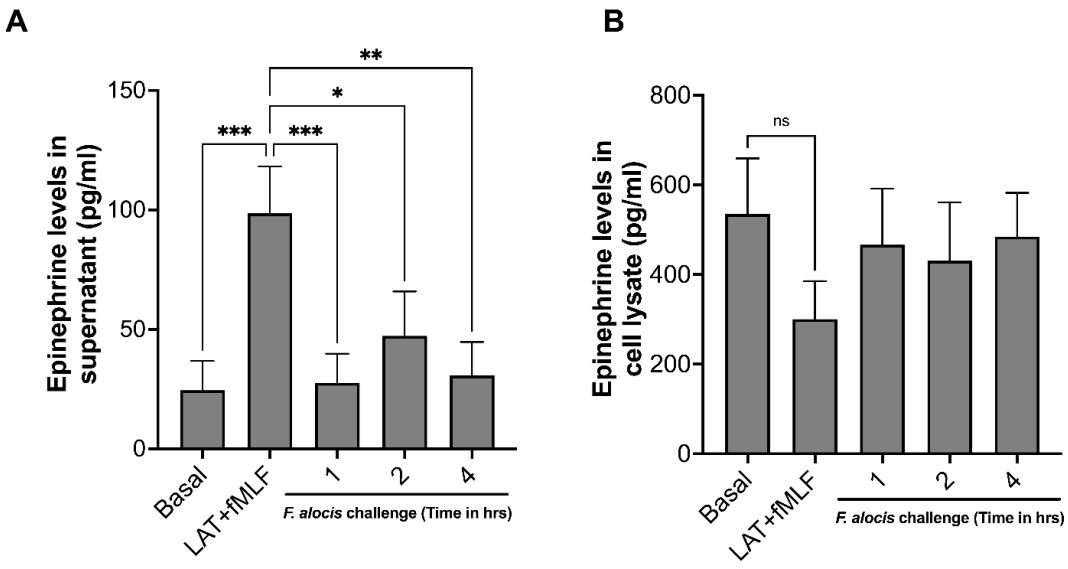


Figure 4.6. *F. alocis* is unable to induce epinephrine release in human neutrophils. Human neutrophils were challenged with *F. alocis* (MOI 10) for 1h, 2h and 4h. Epinephrine content was measured by ELISA from collected supernatant **(A)** and cell lysate **(B)** of *F. alocis* challenged neutrophils. Epinephrine concentration is plotted as the mean ± SD of 5 independent experiments. Statistical differences among experimental conditions and time points were analyzed by repeated measures one-way ANOVA, followed by Tukey's post-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

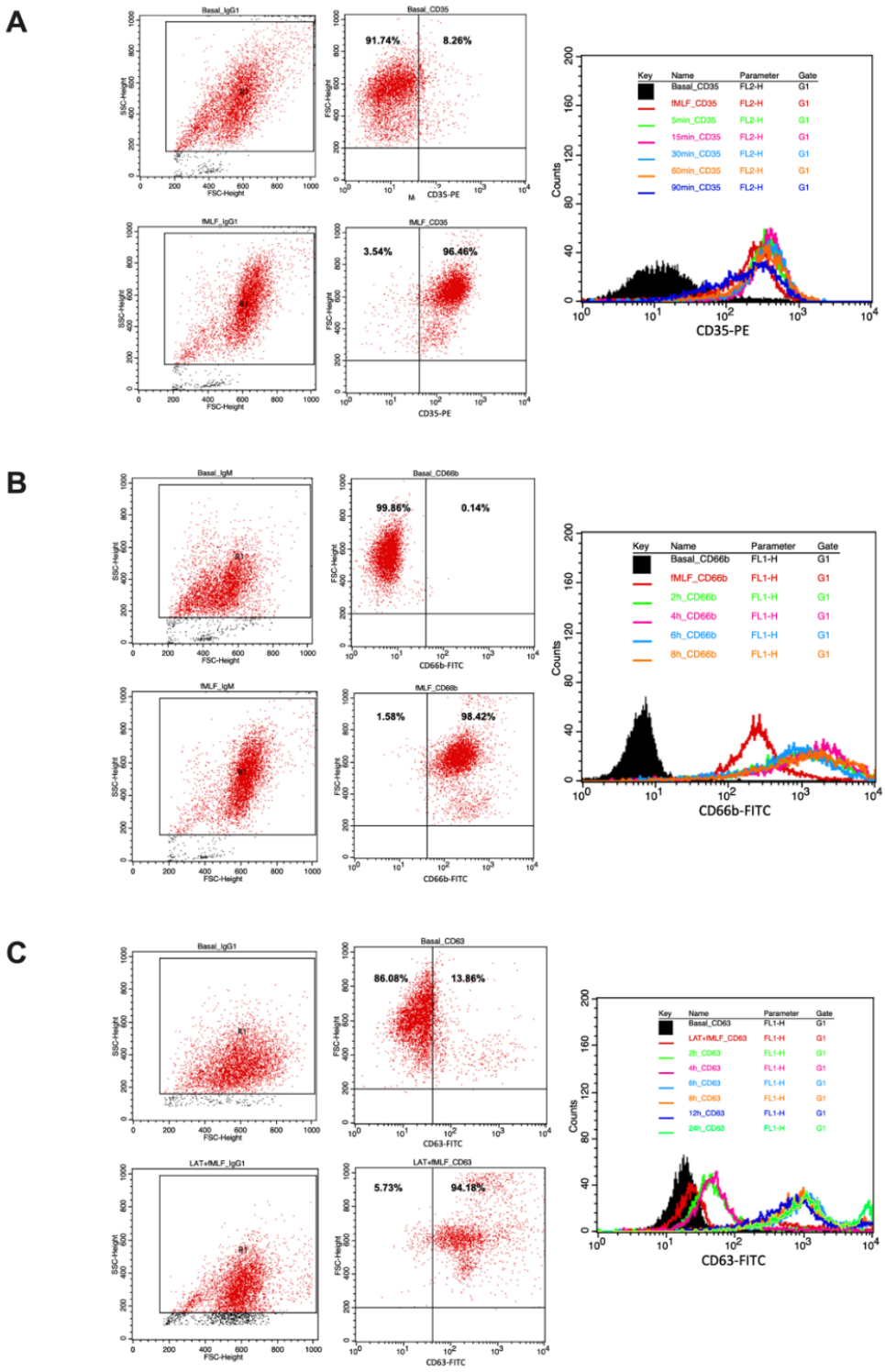

A
Basal_IgG1
Basal_CD35
91.74%
8.26%
fMLF_IgG1
fMLF_CD35
3.54%
96.46%
SSC-Height
FSC-Height
CD35-PE
Key Name Parameter Gate
Basal_CD35 FL2-H G1
fMLF_CD35 FL2-H G1
5min_CD35 FL2-H G1
15min_CD35 FL2-H G1
30min_CD35 FL2-H G1
60min_CD35 FL2-H G1
90min_CD35 FL2-H G1
Counts
B
Basal_IgM
Basal_CD66b
99.86%
0.14%
fMLF_IgM
fMLF_CD66b
1.58%
98.42%
CD66b-FITC
Key Name Parameter Gate
Basal_CD66b FL1-H G1
fMLF_CD66b FL1-H G1
2h_CD66b FL1-H G1
4h_CD66b FL1-H G1
6h_CD66b FL1-H G1
8h_CD66b FL1-H G1
C
Basal_IgG1
Basal_CD63
86.08%
13.86%
LAT+fMLF_IgG1
LAT+fMLF_CD63
5.73%
94.18%
CD63-FITC
Key Name Parameter Gate
Basal_CD63 FL1-H G1
LAT+fMLF_CD63 FL1-H G1
2h_CD63 FL1-H G1
4h_CD63 FL1-H G1
6h_CD63 FL1-H G1
8h_CD63 FL1-H G1
12h_CD63 FL1-H G1
24h_CD63 FL1-H G1

Figure 4.7. Gating strategy and histograms used in flow cytometric analysis of granule exocytosis by human neutrophils. Flow cytometry staining was performed using the shown gating strategy and examples of histogram peak shift for each granules marker CD35 (A), CD66b (B) and CD63 (C).

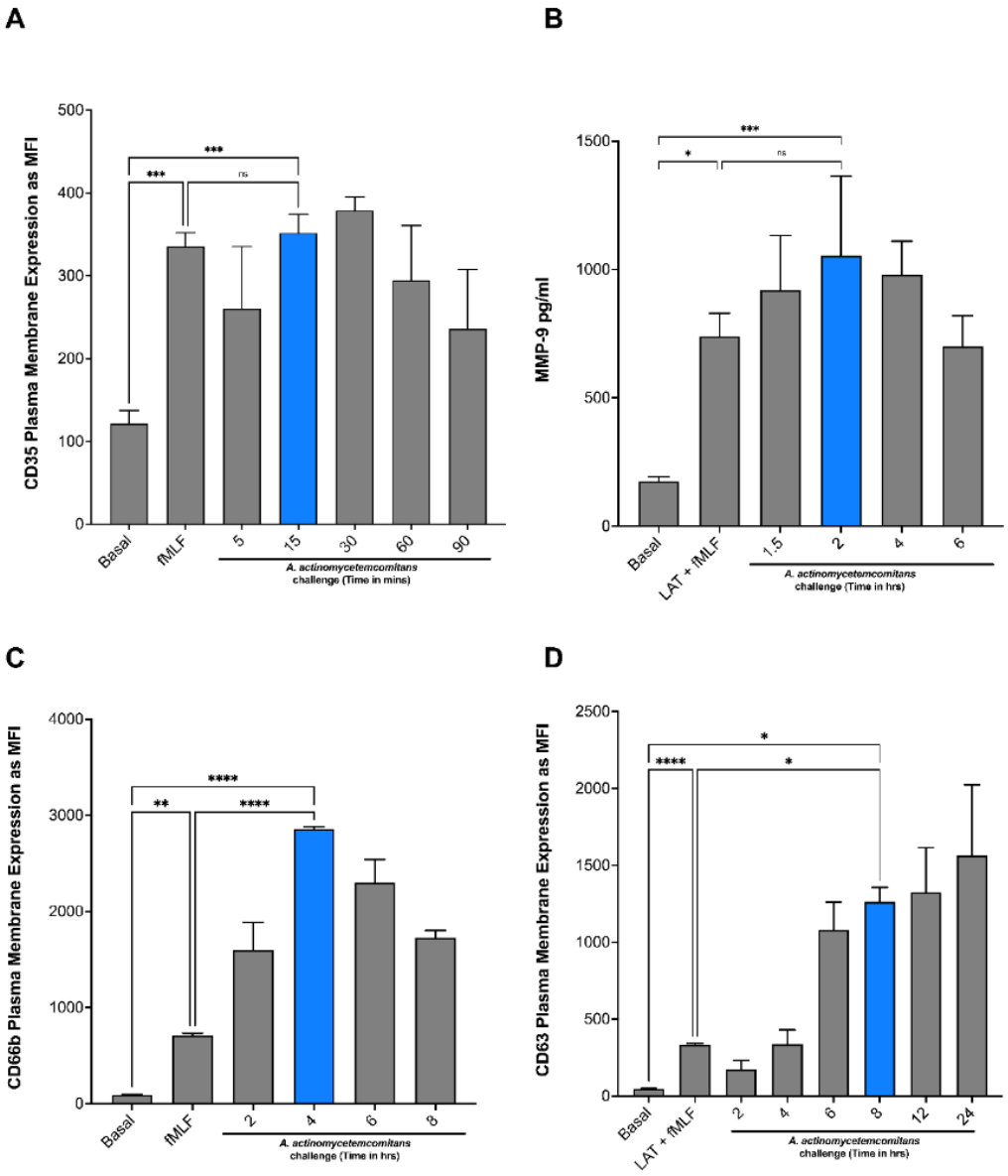


Figure 4.8. *A. actinomycetemcomitans* induces exocytosis of all four neutrophil granule subtypes. Induction of neutrophil granule exocytosis was determined by challenging human neutrophils with *Aa* (MOI 50) for 1.5h, 6h, 8h and 24h. Increase plasma membrane expression of CD35 **(A)**, CD66b **(C)**, and CD63 **(D)** was measured to define secretory vesicles, specific granules, and azurophil granule exocytosis, respectively, by flow

cytometry. Basal levels and latrunculin (Lat) + fMLF were used as negative and positive control. Granule markers are plotted as the mean channel florescence intensity (MFI) ± SEM of 3 independent experiments. For gelatinase granules exocytosis, cell supernatants were collected at basal and following each stimulation, and levels of matrix metallopeptidase 9 (MMP-9) were measured by ELISA **(B).** MMP-9 concentration is plotted as the mean ± SD of 3 independent experiments. Bars in blue are the selected time points used in the selective inhibition granule release experiments (Figure 4.9). Statistical differences among experimental conditions and time points were analyzed by repeated measures one-way ANOVA, followed by Tukey's post-test. ns: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

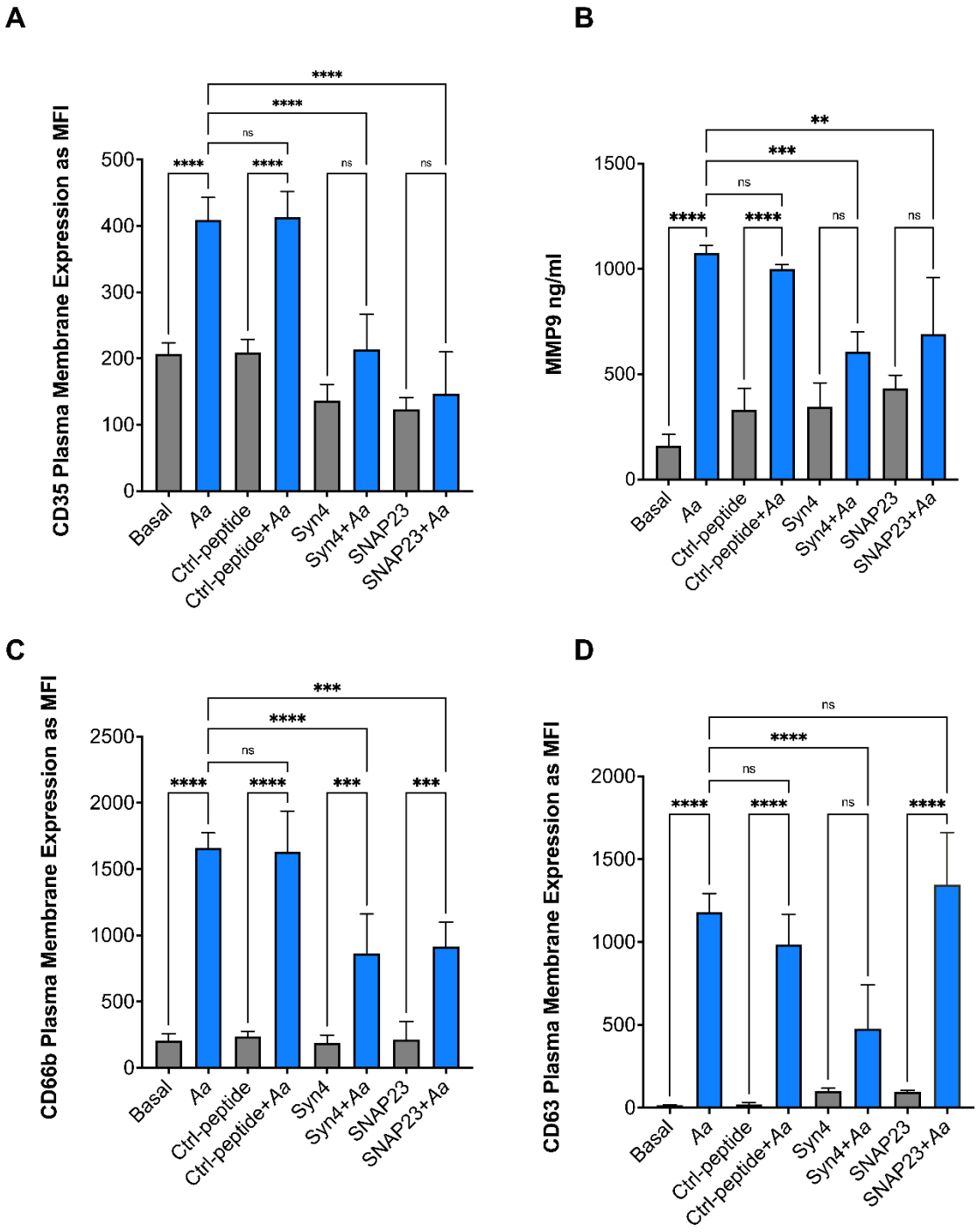


Figure 4.9. Pre-treatment with TAT fusion proteins prevented *A. actinomycetemcomitans*-induced neutrophil granule exocytosis. Human neutrophils were unstimulated, challenged

with *Aa* at MOI 50, or pre-treated with TAT-Syntaxin 4 (Syn4), TAT-SNAP23 (SNAP23), TAT-Control peptide (Ctrl-peptide) for 15 mins followed by *Aa* challenge. The peak time point of *Aa*-induced granule exocytosis was different for each granule subtype. Secretory vesicles peak was at 15 min post-bacterial challenge **(A)**, two hours for gelatinase granules **(B)**, four hours for specific granules **(C)** and eight hours for azurophilic granules **(D)**. Granule markers were measured by flow cytometry and plotted as the mean channel florescence intensity (MFI) ± SEM of 4 independent experiments **(A, C-D)**. For gelatinase granules, matrix metallopeptidase 9 (MMP-9) was measured from human neutrophils supernatant by ELISA and MMP-9 concentration is plotted as the mean ± SD of 4 independent experiments **(B)**. Bars in blue are neutrophils challenged with *Aa*. Statistical differences among experimental conditions were analyzed by ordinary one-way ANOVA, followed by Tukey's post-test. ns: not significant, **p < 0.01, ***p < 0.001, ****p < 0.0001.

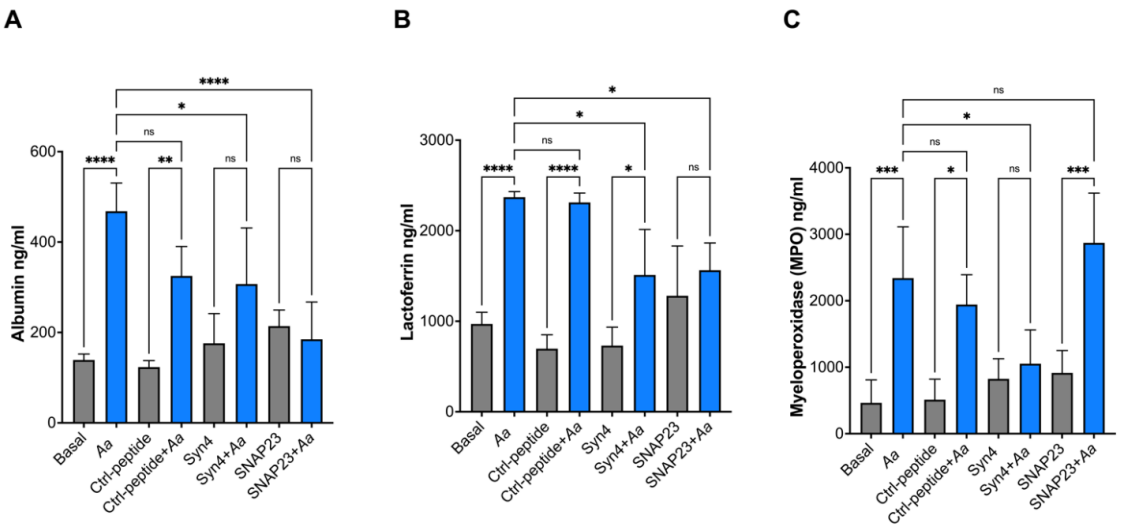


Figure 4.10. Granule content markers confirm inhibition of granule exocytosis induced by *A. actinomycetemcomitans* in human neutrophils. Granule fusion inhibition with TAT peptides was confirmed by measuring granule contents. Human neutrophils were pre-treated with Syntaxin 4 (Syn4) or SNAP23 peptide for 15 mins, then challenged with *Aa* (MOI 50) for 15 mins for secretory vesicles **(A)**, for 4h for specific granules **(B)** and for 8h for azurophilic granules **(C)**. Albumin **(A)**, lactoferrin **(B)** and myeloperoxidase **(C)** were measured from collected supernatants and detected by ELISA. The concentration of each content marker is plotted as the mean ± SD of 4 independent experiments. Bars in blue are neutrophils challenged with *Aa*. Statistical differences among experimental conditions were analyzed by ordinary one-way ANOVA, followed by Tukey's post-test. ns: not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

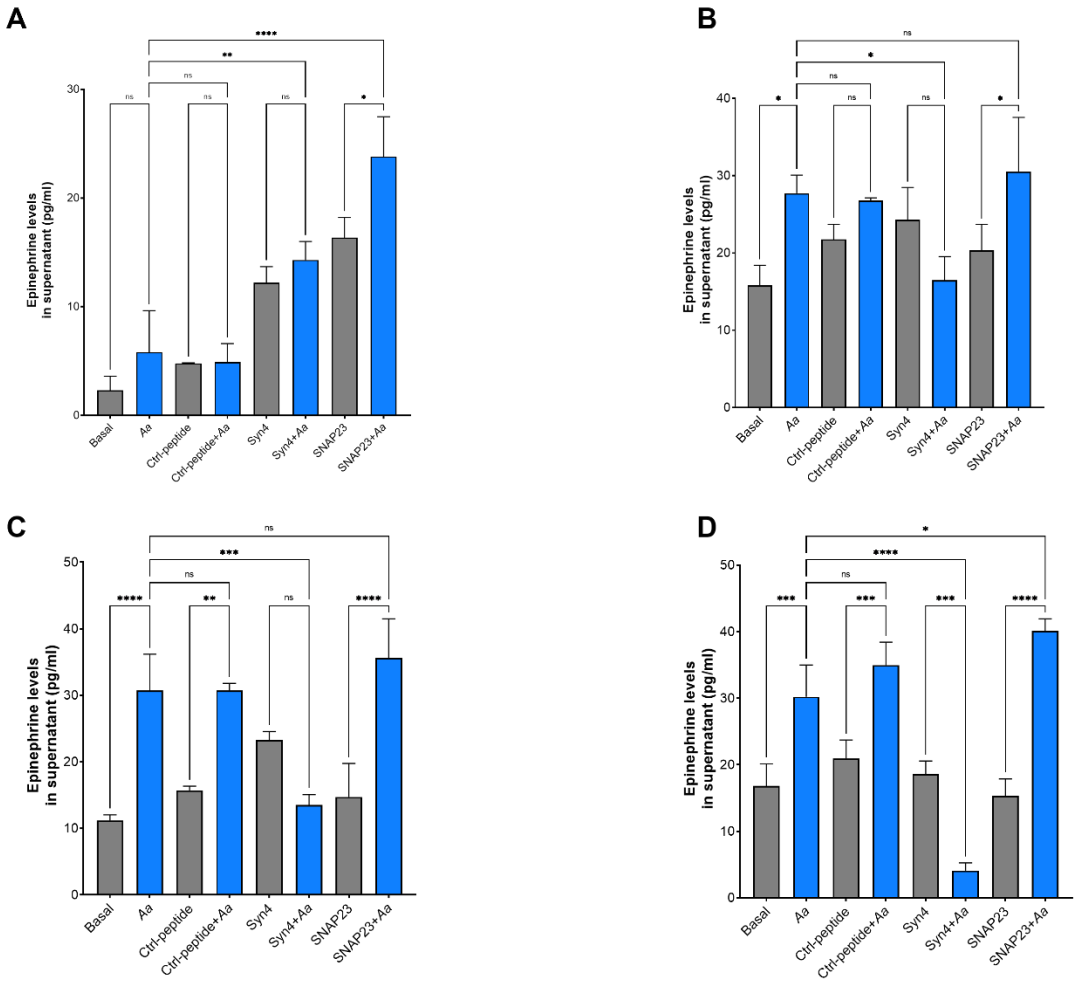


Figure 4.11. Epinephrine release of TAT peptide treated samples shows that human neutrophils store epinephrine in azurophilic granules. Epinephrine storage location was determined by pre-treating human neutrophils with Syntaxin 4 (Syn4) or SNAP23 peptide for 15 mins, then challenged with *Aa* (MOI 50) for 15 mins **(A)**, 2h **(B)**, 4h **(C)** and 8h **(D)**. Epinephrine concentrations are plotted as the mean ± SD of 3 independent experiments. Bars in blue are neutrophils challenged with *Aa*. Statistical differences among experimental conditions and time points were analyzed by ordinary one-way ANOVA, followed by Tukey's post-test. ns: not significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

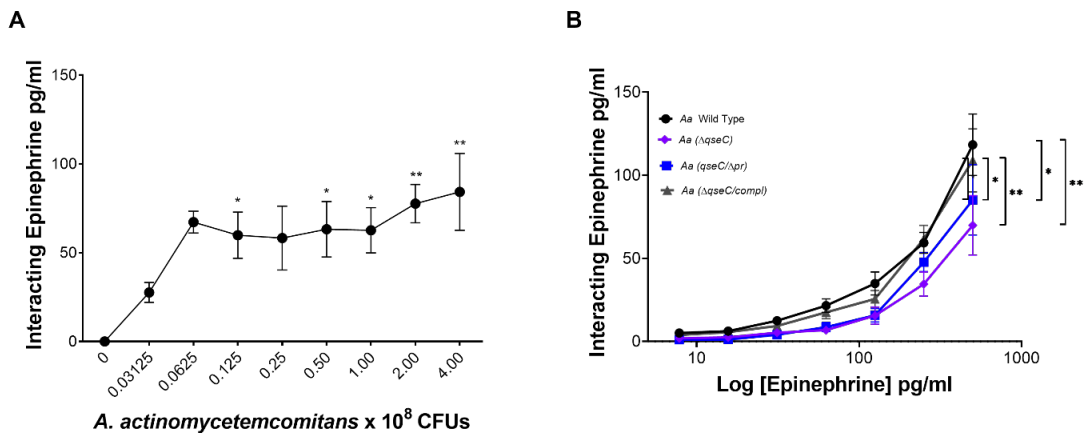


Figure 4.12. *A. actinomycetemcomitans* interacts with epinephrine in a QseC dependent manner. The interaction of epinephrine and *Aa* was determined by incubation of increasing colony forming units (CFUs) of *Aa* with 250 pg/ml of recombinant epinephrine. Associated epinephrine was plotted against increasing CFUs of *Aa* **(A)**. Requirement of QseC for epinephrine interaction with *Aa* was determined by incubation of the bacterium with increasing concentrations of recombinant epinephrine (0-500 pg/ml) **(B)**. Epinephrine levels were measured by ELISA and plotted as the mean ± SD of 5 independent experiments. Statistical differences among experimental conditions were analyzed by ordinary one-way ANOVA **(A)** or two-way ANOVA **(B)**, followed by Tukey's post-test. *$p < 0.05$, **$p < 0.01$.

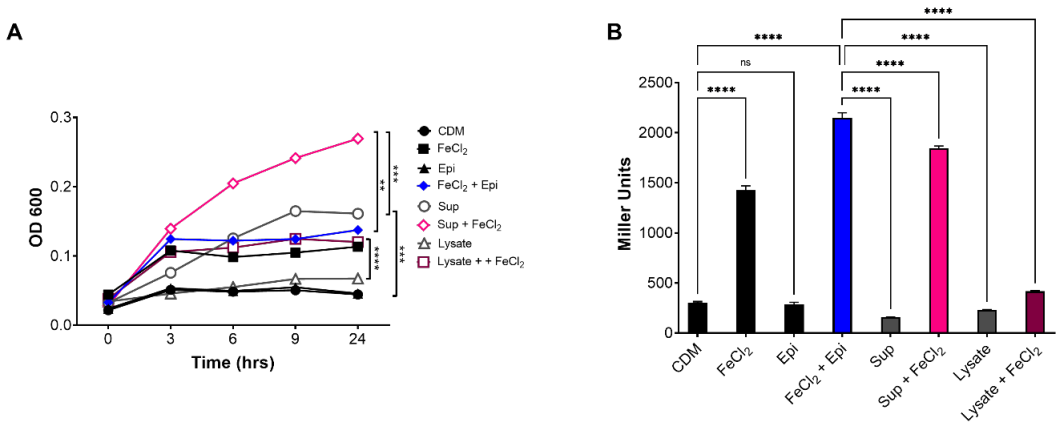


Figure 4.13. Host-derived epinephrine promotes *A. actinomycetemcomitans* growth and *qseBC* expression. The supernatants and lysates from human neutrophils stimulated with latrunculin A and fMLF were collected and used as supplement for chemically defined media (CDM). *Aa* was grown under anaerobic conditions in CDM alone or supplemented with human neutrophils supernatant (104), cell lysate (Lysate), iron ($FeCl^2$, 100µM) or Epinephrine (Epi, 50µM). At designated times growth was measured by determining the OD_{600} **(A)**. *Aa* strain (that harbors the *qseBC* promoter-*lacZ* reporter plasmid pDJR29) was grown under anaerobic conditions in CDM alone or supplemented with human neutrophils supernatant (104), cell lysate (Lysate), iron ($FeCl^2$, 100µM) or Epinephrine (Epi, 50µM). Expression of the *qseBC* operon was determine by measuring β -galactosidase activity after 24h **(B)**. The OD_{600} measurements for growth were plotted as the mean ± SEM of 3 independent experiments **(A)**. The expression of the *qseBC* operon is presented in miller units as the mean ± SD of 3 independent experiments **(B)**. Statistical differences among experimental conditions were analyzed by two-way ANOVA **(A)** or ordinary one-way ANOVA (B), followed by Tukey's post-test. ns: not significant, **p < 0.01, ***p < 0.001, ****p < 0.0001.

CHAPTER 5

LA TÓXICA: THE EFFECT OF AGGREGATIBACTER ACTINOMYCETEMCOMITANS LTXA IN HUMAN NEUTROPHILS EFFECTOR FUNCTIONS

Introduction

Periodontal disease is defined as an inflammation of the gingiva caused by accumulation of oral microorganisms. The depth of the gingival pocket increases due to continuous periodontal ligament and alveolar bone destruction (4). *Aa* is a Gram-negative opportunistic oral pathogen, facultative anaerobe and non-motile *coccobacillus* of the *Pasteurellaceae* family, associated with localized periodontitis (88, 90, 91). *Aa* contributes to tissue inflammation, destruction, and bone resorption by expressing a number of virulence factors such as leukotoxin A (LtxA) (109, 182, 252).

In periodontitis, neutrophils are the first to arrive in large numbers to the infected gingival pocket (35). Primed neutrophils arrive and create a defensive wall against the biofilm associated with the tooth to prevent bacteria from moving deeper into tissue (48). At the infected subgingival pocket (Figure 5.1), the neutrophils are exposed to an overwhelming number of inflammatory signals that induce different effector functions such as the phagocytosis of invading bacteria. Neutrophil phagosomes containing internalized bacteria can fuse with azurophilic granules to form a phagosome where engulfed microbes are exposed to antimicrobial peptides, enzymes and reactive oxygen

species (ROS) (49-51). The canonical fate of this process is complete clearance of the bacteria. However, studies on the interaction of *Aa* and neutrophils have shown inconsistent results regarding phagocytosis and killing of *Aa* by neutrophils (129). *Aa* expresses the leukotoxin LtxA, a pore forming toxin that targets leukocytes and permeabilizes the cell membrane for efflux of cytosolic potassium ions and influx of calcium (253). Although *Aa* induces the formation of ROS (254), some reports suggest that the resistance to neutrophils activity depends on *Aa* expression of LtxA and that hyper leukotoxic strains such as JP2 are highly resistant (255-259). Others studies suggest that *Aa* resists neutrophil killing regardless of leukotoxin phenotype (129, 260). *Aa* can induce granule exocytosis, releasing MMP8, epinephrine, and through LtxA, induce the release of elastase and lactoferrin (183, 210) contributing to epithelial barrier tissue damage and bacteria migration deeper into the subgingival pocket. Another effector function of neutrophils induced by *Aa* is the formation of neutrophils extracellular traps (NETs), which occurs after ROS production and is accompanied by degranulation (52, 261).

Until recently *Aa* was suggested to accelerate apoptosis (129) or induce necrosis through LtxA cell lysis (183) in human neutrophils, yet its sublytic effect is not well understood. Pore forming toxins have been associated with cytoplasmic vacuolation, which in turn is associated with non-canonical cell death types. Cytoplasmic vacuolation or vacuolization occurs when a cell is exposed to environmental stress (57-59). This environmental stress can be produced by "inducers" such as pore forming toxins (i.e., LtxA), acting as ion channels. The concentration and time of exposure to an inducer determines if a cell will commit to a cell death pathway (58, 60-62).

Sialic acid-binding Ig-type lectin 9 (Siglec-9) is associated with cytoplasmic vacuolization in neutrophils (64). Siglecs are expressed in the membrane of most white blood cells and recognize sialic acid-containing glycans. They serve as an immune-surveillance system to distinguish host cells from foreign cells, and regulate unwanted immune responses (66-70). To no surprise, pathogens have evolved mechanisms to disguise themselves by incorporating sialic acid-containing glycans on the cell surface to suppress the immune response (70). Different members of the *Pasteurellaceae* family, such as *Haemophilus influenzae*, *Actinobacillus actinomycetemcomitans* (currently known as *Aggregatibacter actinomycetemcomitans*) and *Pasteurella haemolytica* have been shown to express specialized proteases and transferases for sialic acid and to possess sialic acid on their LPS structures (74, 75). Bacteria with this characteristic are known as sialylated bacteria and Siglec-9 has been shown to negatively regulate the innate immune response against these organisms (71). Zeng *et al.* (83) demonstrated that Siglec-E (the Siglec-9 mouse homolog) is involved in modulating neutrophil functions, including induction of apoptosis, inhibition of cellular activation, suppression of migration, modulation of oxidative stress, and regulation of inflammatory cytokines secretion. More recently, Wu *et al.* (84) revealed that Gram-negative bacteria enhance Siglec-E activation in murine neutrophils and dampen the innate response toward Gram-negative bacteria. The role of Siglec-9 in periodontitis has been highlighted in a review by Sudhakara *et al.* (85) where it mentions that sialic acid adorned *P. gingivalis* interacts with Siglec-9 attenuating neutrophils inflammatory signaling. However, the role of Siglec-9 in *Aa* and its ability to induce non-apoptotic cell death in neutrophils remains unknown. In this chapter, within our study model, we characterize the effect of *Aa* and sublytic kevels of LtxA in human

neutrophils. We also determine the ability of *Aa* to induce cytoplasmic vacuoles and alter Siglec-9 expression in neutrophils. The findings presented here provide a foundation to support further study of the role that Siglec-9 plays in periodontitis and as a novel mechanism exploited by *Aa* to dampen the inflammatory response. Finally, our results contribute to current knowledge of other roles played by LtxA at sublytic levels during periodontal disease.

Results

Human neutrophils membrane integrity is compromised by *A. actinomycetemcomitans* challenged with minimal cytotoxicity

Given that *Aa* expresses the pore-forming toxin LtxA, that is known to target the neutrophil membrane, leading to cell lysis, we first sought to determine a multiplicity of infection (MOI) to use in experiments that will not result in neutrophil cell death. Human neutrophils ($3x10^6$ cells/ml) were exposed to increasing MOIs of *Aa* (5-50) for 0, 15, 30, 60, 120 and 240 mins shaking in water bath at 37°C. The cytotoxic effect of *Aa* on neutrophils was determined by measuring released lactate dehydrogenase (LDH) using a colorimetric assay. As expected, some toxicity was induced by *Aa,* and it increased with time but remained under 40% (Figure 5.2.A). Additionally, there was no significant difference in cytotoxicity within each time point, regardless of bacteria MOI. Consequently, MOI 50 was selected to be used in our experiments.

Next, we determined if neutrophil membrane integrity was affected by *Aa*. Human neutrophils ($3x10^6$ cells/ml) were challenged with *Aa* or heat killed (HK) *Aa* at a MOI of 50 in suspension for 15, 30 and 60 mins shaking in water bath at 37°C. Samples for each time point were mounted onto a coverslip and fixed with 4% paraformaldehyde (PFA). The

neutrophil membrane was labeled with wheat germ agglutinin (WGA) lectin which binds N-acetyl-D-glucosamine and sialic acid and *Aa* was labeled with CFSE before infection. Confocal microscopy (Figure 5.2.B-C) was used to evaluate the internalization of WGA in neutrophils challenged with *Aa*. As a positive control, neutrophils were treated with 0.1% Triton X to permeabilize cells and allow WGA to enter the cells (Figure 5.2.B). Confocal imaging showed no WGA inside the untreated neutrophils, However, when neutrophils were challenged with *Aa,* WGA accumulation was observed starting at 30 mins. On the other hand, HK *Aa* had no WGA accumulation but it did show an increased number of bacteria inside the cell (Figure 5.2.C). To quantify membrane integrity of neutrophils when challenged with *Aa* we used a vital impermeable nuclear dye, TO-PRO-3 and flow cytometry imaging. Neutrophils ($4x10^6$ cells/ml) were challenged with *Aa* (MOI 50) for 5, 15, 30, 60 and 90 mins in suspension, shaking in a water bath at 37°C. Neutrophils were stained with TO-PRO-3 and visualized by imaging flow cytometry. As positive controls, neutrophils were treated with 0.1% Triton X and neutrophils with red nuclei were considered positive and those with absence of red were considered negative (Figure 5.3.B, top). TO-PRO-3-stained nuclei of *Aa* challenged neutrophils were observed at all time points (Figure 5.3.A). In Figure 5.3.B (bottom) we show the quantification of TO-PRO-3 negative vs positive neutrophils. Untreated neutrophils did show TO-PRO-3 nuclei staining but the positivity was of 24.5% at 90 mins. In comparison, after 5 mins of bacterial challenge 56.5% of neutrophils had TO-PRO-3 labeled nuclei. Compared to TO-PRO-3 negative neutrophils, the positivity significantly increased to 88% by 15 mins ($p = 0.0021$), 89.5% at 30 mins (p-value = 0.0386), 90.5% at 60 mins (p-value = 0.0005) and to 93.5% by 90 mins (p-value = 0.0474).

A. actinomycetemcomitans at lower MOIs can be phagocytized by human neutrophils

Since our results showed decreased membrane integrity and cytotoxicity, but trypan blue staining showed that neutrophils lifespan is extended to at least 24h (Chapter 4, Figure 4.4.C) we wanted to determine if neutrophils were functionally active. To test neutrophil functionality, we determined the ability of human neutrophils to internalize *Aa*. Fresh human neutrophils ($4x10^6$ cells/ml) were challenged with *Aa* (MOI 10) and at 5, 30 and 60 min, infected cells were mounted and fixed onto coverslips. Intracellular and extracellular bacteria were differentially stained as described in the Materials and Methods. Briefly, extracellular *Aa*, was labeled with mouse polyclonal anti-*Aa* (1µg/ml) primary antibody and a goat anti-mouse Alexa Fluor 488. Antibody linkages were fixed by with 4% PFA for 10 min. To stain internalized *Aa*, neutrophils were permeabilized with 0.02% saponin and labeled with mouse anti-*Aa* primary antibody in 3% BSA-PBS with 0.02% saponin and incubated and washed as before. Finally, goat anti-mouse Alexa Fluor 647 in 3% BSA-PBS with 0.02% saponin was used as a secondary antibody. This results in differential staining of internal vs external bacteria, where bacteria in red have been internalized, whereas yellow corresponds to extracellular bacteria (Figure 5.4.A). Plates were incubated at room temperature as previously described and washed twice with 1X PBS. Phagocytosis of neutrophils challenged with *Filifactor alocis* (*Fa*), an assay that is well established in our lab, was used as a phagocytosis assay control. By 15 mins, 35% of neutrophils had internalized *Fa* (Figure 5.4.B) and by 60 mins 44% neutrophils internalized *Fa* with no significant difference throughout the length of the experiment, confirming the capacity of internalization of *Fa* by human neutrophils as previous demonstrated (246). In contrast, by 15 mins only 17.33% of neutrophils challenged with *Aa* had internalized bacteria (Figure

5.4.C), by 30 mins the percentage of neutrophils increased significantly to 20% (p-value = 0.0277) and 31% by 60 mins (p-value = 0.0202) when compared to 15 mins. Additionally, we observed that the number of neutrophils that internalized bacteria increased with time as shown by the percentage of neutrophils that internalized *Aa* from 30 mins to 60 mins (p-value of 0.0197). In summary, we demonstrated that neutrophils were functionally active, that *Aa* can be internalized at low MOIs (MOI 10) and that the number of neutrophils that internalize *Aa* increased with time.

A. actinomycetemcomitans resists internalization by human neutrophils at higher MOIs

Aa is known to resist phagocytosis and this is considered to be dependent on LtxA expression. However, previous work has been done using highly leukotoxic strains such as JP2 which produces 8-10 times more LtxA than *Aa* 652 (112), yet the ability of minimal leukotoxic strains to resist internalization is often overlooked. Therefore, we wanted to determine if *Aa* resistance to phagocytosis is due to if LtxA expression. For this experiment we used *Aa* 652 (minimally leukotoxic), JP2 (highly leukotoxic), JP2 *ΔltxA* (does not express LtxA) and as a control we used heat killed (HK) *Aa* 652. Human neutrophils ($4x10^6$ cells/ml) were challenged with *Aa* 652*,* JP2*,* JP2 *ΔltxA* at increasing MOIs of 10, 25, 50 and 100. Cells were challenged for 30 mins in suspension, shaking in a water bath at 37°C. Following, each condition was mounted and fixed into a coverslip and bacteria was differentially stained as described above. In Figure 5.5.A we observed that neutrophils challenged with *Aa* showed no change in morphology, and although there are bacteria internalized, there is no visual increase in the number of internalized bacteria. Interestingly, we observed similar results for JP2 *ΔltxA* challenged neutrophils. Neutrophils challenged with JP2, were similar to those challenged with *Aa* 652, or JP2 *ΔLtxA* at MOI 10 and MOI

25 (Figure 5.5.A), but after 30 mins of challenge, JP2 induced increased cell lysis. Similarly, at higher MOIs of 50 and 100, JP2-treated neutrophils underwent cell lysis and only cell debris was visualized. For this experiment we only quantified neutrophils challenged with *Aa* 652*,* HK *Aa* or JP2 *ΔLtxA* at MOI 50*.* Neutrophils challenged with JP2 at this MOI had undergone cell lysis and neutrophils that had internalized, associated or no bacteria could not be quantified. By 15 mins 8.3% of neutrophils had internalized *Aa* 652, by 30 mins, 6.67% and by 60 mins 7% of neutrophils had internalized *Aa* 652 (Figure 5.5.B, left panel). Similar to what we observed in confocal images (Figure 5.5.A) JP2 *ΔLtxA* behaved comparable to *Aa* 652, with a percentage of 7.3% of neutrophils that phagocytized *Aa* by 15 mins, increasing to 17% by 30 mins and 15.67% at 60 mins (Figure 5.5.B, right panel) suggesting that resistance to phagocytosis by human neutrophils is not attributed LtxA or that LtxA is not the sole player in phagocytosis resistance by *Aa*. A stunning 72% of neutrophils at 15 mins challenged with *HK Aa* 652 internalized bacteria (Figure 5.5.B, middle panel), although not a significant increase but by 30 mins 86% and by 60 mins 75.33% of neutrophils had internalized *Aa*. This suggested that phagocytosis resistance requires viable bacteria. The number of bacteria internalized was determined and most of the neutrophils that were challenged with *Aa* or JP2 *ΔLtxA* internalized one bacterium (Figure 5.5.C). In contrast, neutrophils challenged with HK *Aa* contained 1-4 bacteria and some more than 6 bacteria. Together, we show that at high MOIs of *Aa* viable bacteria can resist phagocytosis, independently of LtxA.

A. actinomycetemcomitans induces the formation of cytoplasmic vacuoles in human neutrophils independently of LtxA expression

Previous studies have shown that pore forming toxins can induce the formation of cytoplasmic vacuoles. Given that *Aa* expresses LtxA, a pore forming toxin we sought to determine if *Aa* induces cytoplasmic vacuoles in human neutrophils. Human neutrophils ($4x10^6$ cells/ml) were challenged with *Aa* 652, JP2 or JP2 Δ*ltxA* at a MOI 10 in suspension for 0h, 0.5h, 1h and 2h at 37°C shaking in a water bath. After challenge, an aliquot of 200 µl was collected and cells were mounted by cytospin and fixed on a slide, stained with HEMA3 to differentially stain neutrophil cells and visualized by a light microscope. LDH release was measured from sample supernatants and detected using a colorimetric assay and presented as percentage of cytotoxicity. We were able to identify vacuole formation in neutrophils challenged with *Aa* at all time points and on most of the cells in view (Figure 5.6.A). We challenged neutrophils with JP2 and JP2 Δ*ltxA* strains to determine if LtxA is responsible of inducing cytoplasmic vacuoles. Cytotoxic vacuoles were observed in JP2 challenged neutrophils at all time points. However, cell numbers decreased, and round nuclei (characteristic of apoptotic cells) were observed at later time points. Compared to *Aa* 652 and JP2 Δ*ltxA*, JP2 challenged neutrophils showed significantly higher cytotoxicity even at a low MOI of 10 (Figure 5.6.B). As expected, challenge with the highly leukotoxic strain JP2 for 2h at MOI 25 resulted in cell lysis (Figure 5.6.C). To our surprise, JP2 Δ*ltxA* induced cytoplasmic vacuoles comparable to *Aa* 652 (Figure 5.6.A). When neutrophils were challenged at MOI 25 with *Aa* 652 or JP2 Δ*ltxA* (Figure 5.6.A), we obtained similar observations. These results suggest that although LtxA is a pore-forming toxin, it is not required for the induction of cytoplasmic vacuolation.

A. actinomycetemcomitans enhances Siglec-9 expression in human neutrophils

Cytoplasmic vacuolation in neutrophils has been associated with Siglec-9 activation (64). In addition, Wu *et al.* (84) demonstrated in mice that Gram-negative bacteria enhance Siglec-E (the Siglec-9 murine homolog) expression. Considering that we showed that *Aa* induces cytoplasmic vacuoles in neutrophils (Figure 5.6), is a Gram-negative bacterium, and is known to add sialic acid to its LPS (74, 75) to negatively regulate the innate immune response, we tested the ability of neutrophils to increase Siglec-9 expression upon *Aa* challenge. Neutrophils ($4x10^6$ cells/ml) were challenged with *Aa* at MOI 10 (the same MOI at which cytoplasmic vacuoles were induced by *Aa*), in suspension for 2h at 37°C shaking in a water bath. To provide a state similar to the inflamed gingival pocket, a separate sample of neutrophils were primed with IL-8 (10ng/ml) for 1h prior to bacterial challenge. Briefly, cells were blocked for 15 mins, stained with human Siglec-9 PE conjugated antibody (10 µl/10^6 cells, Biotechne FAB1139P-100, Minneapolis, MN) for 1hr. As a control, samples were run in duplicates and stained with Mouse Ig2A PE conjugated antibody isotype control (20 µl/10^6 cells, Biotechne IC003P, Minneapolis, MN), washed twice with 0.05% sodium azide and fixed by resuspending in 1% PFA. Siglec-9 expression was quantified by flow cytometry using a BD FACS Celesta Flow cytometer and analyzed with FlowJo software. We observed that human neutrophils have a baseline expression of Siglec-9 when untreated (Figure 5.7.A, right panel). *Aa* challenge significantly enhanced the expression of Siglec-9 compared to UT with a p-value of 0.0049 (Figure 5.7.A). Priming of neutrophils with IL-8, a neutrophil chemoattractant, further increased Siglec-9 expression significantly compared to *Aa* challenge alone (p-value = 0.0001) and IL-8 treatment alone (p-value 0.0004). It has been previously shown that in mice, Gram-positive bacteria suppress Siglec-9 expression (84). We used two oral Gram-

positive bacteria, *Peptoanaerobacter stomatis* (*Ps)* and *Filifactor alocis* (*Fa)* and tested if Siglec-9 expression decreases or remains unchanged after bacterial challenge. In contrast to the literature, we found that neutrophils treated with IL-8 followed by *Ps* challenge (Figure 5.7.B, middle panel) showed significant increase in Siglec-9 expression when compared to UT (p-value = 0.0040), similar results were obtained when unprimed neutrophils were challenged with *Ps* alone (Figure 5.7.B, right panel, p-value = 0.0041). Treatment with IL-8 followed by *Fa* challenge (Figure 5.7.C, middle panel) also significantly increased Siglec-9 expression (p-value = 0.0467) as well as *Fa* challenge alone (Figure 5.7.C, right panel, p-value = 0.0159). However, for both *Ps* and *Fa* priming with IL-8 did not further enhance Siglec-9 expression.

To determine if there is a dose effect in Siglec-9 enhanced expression by *Aa*, we challenged neutrophils and stained for Siglec-9 as described above and included a challenge with *Aa* at MOIs 50 and 100. In these experiments, neutrophils were primed with TNF-α (2ng/ml), a strong priming agent, for 15 min prior to bacterial challenge. Siglec-9 expression of neutrophils challenged with *Aa* (Figure 5.8.B) was significantly increased at MOI 10 (p-value = 0.0048), MOI 50 (p-value < 0.0001) and MOI 100 (p-value = 0.0427) when compared to UT. Siglec-9 expression in neutrophils peaked at MOI 50 and was significantly higher than Siglec-9 expression with *Aa* 652 at MOI 10 (p-value < 0.0001) or MOI 100 (p-value < 0.0001). *Aa* 652 challenge at MOI 10, significantly increased Siglec-9 expression in human neutrophils compared to *Ps* (p-value < 0.0001) and *Fa* (p-value = 0.0004). Neutrophils challenged with *Aa* 652 at MOI 50 resulted in significant enhancement of Siglec-9 expression in comparison to *Ps* (p-value < 0.0001) and *Fa* (p-value < 0.0001). However, *Aa* 652 challenge at MOI 100 was only significantly higher

when compared to *Fa (*p-value = 0.0035). In contrast to IL-8 pre-treatment, there was no significant difference between Siglec-9 expression with *Aa* 652 alone and pre-treatment with TNF-α following *Aa* challenge (Figure 5.8.C). In comparison to our observations in Figures 5.7.B-C *Fa* and *Ps* did not increase Siglec-9 expression in human neutrophils. In *F. alocis* this difference in results can be due to its ability to activate NF-κβ through TLR2 signaling (262). NF-κβ then activates the transcriptional factor AP-1, that binds the Siglec-E (Siglec-9 homolog in mice) promoter (84). In summary, we found that human neutrophils increase Siglec-9 expression when challenged with *Aa* and priming of neutrophils with IL-8 further increased expression.

Discussion

Aa expresses LtxA, a pore-forming toxin that targets the leukocyte membrane. The cytotoxic effect of LtxA has been studied extensively, such as cell lysis and release of elastase by neutrophils (52, 183) particularly with highly leukotoxic strains. Nevertheless, the effects of *Aa* at LtxA sublytic levels is frequently overlooked. In our studies we demonstrated that neutrophils challenged with increasing MOIs (5-50) of the minimally leukotoxic strain of *Aa* 652 induced some toxicity starting at 10% and it increased with time but remained under 40%. Moreover, there was minimal variation in cytotoxicity across MOIs within each time point. Similar findings have reported that at lower bacterial numbers per neutrophil, cells can phagocytose and kill *Aa* efficiently (111, 263). This suggests that the known cytotoxic activities of LtxA are concentration dependent. LtxA mode of action involves binding to the lymphocyte function-associated antigen-1 (LFA-1), in the presence of cholesterol it leads to clustering of LFA-1 in lipid rafts (264, 265). Pore formation occurs and triggers the passive efflux of potassium ions and influx of calcium

ions, ultimately leading to membrane destabilization (263, 266). Therefore, we evaluated the membrane integrity of neutrophils using an impermeable dye and observed loss of membrane integrity during infection with *Aa*.

Quantification of dye translocation into cells resulted in 56.5% of dye positive neutrophils by 5 mins and increasing to 88% by 15 mins, but with minimal cytotoxicity. However, cell morphology was round with lobulated nucleus as visualized by confocal and flow cytometry imaging. Host cells have evolved membrane repair mechanisms as a counter defense from pore-forming toxins. Two repair mechanisms have been broadly discussed and they rely on the number and size of the pores, and cell type. The first, is known as ectocytosis, involves the externalization of microvesicles (267-270). The second is the endocytic method, where the membrane-pore containing portion is internalized and targeted to the lysosome for degradation (271, 272). It can be proposed that neutrophils can make use of one of these membrane repair mechanisms, most likely the endocytosis method due to the cytoplasmic vacuoles observed, or another similar mechanism to heal membrane injuries created by the toxins. Identifying the type of vacuoles and their contents in neutrophils challenged with *Aa* 652 will provide information of the potential use of membrane repair mechanisms and if these are used, it will shed light in what type of repair mechanisms is.

Previous studies have indicated that calcium influx induced at sublytic levels by RTX toxins promotes inflammatory responses (273-275). Particularly, at sublytic doses of RTX toxins, neutrophils are induced to undergo oxidative burst, degranulation, inhibit cell mobility, chemotaxis, phagocytosis and even killing by disruption of the phagosome (110, 182, 276). Previously, we demonstrated that neutrophils challenged with a minimally

leukotoxic strain undergo granule exocytosis and not cell lysis (210). Here, we presented evidence that neutrophils were functionally active by their capacity to internalize bacteria. *Aa* was internalized by neutrophils at MOI 10 but resisted phagocytosis at a MOI of 50. Further, we found that heat killed bacteria was readily phagocytosed by neutrophils. To our surprise, JP2 *ΔLtxA* bacteria resisted phagocytosis to a lesser extent than *Aa* 652, suggesting that resistance to phagocytosis by neutrophils is not solely due to LtxA. We also compared the effects of sublytic and hyper-leukotoxic levels of LtxA in phagocytosis. We observed that fewer JP2 cells were internalized at MOI 10 and 25, but future quantified experiments are required to support our observations. As expected, neutrophils underwent cell lysis at higher MOIs (50, 100) of JP2. Together, our findings support the previously discussed statement that *Aa* resists phagocytosis regardless of LtxA expression (129, 260). A potential scenario for *Aa* resistance of neutrophils internalization is a cooperative effect of LtxA and other virulence factors, such as complement inhibition by LPS, Omp29 and Omp100 (125, 127). Also, the expression of *tad* fimbriae (absent in lab stocks) has been shown to be important in resistance of neutrophil phagocytosis (129, 257).

We observed cytoplasmic vacuoles in neutrophils challenged with *Aa* at MOI 10 for 2h. The formation of these vacuoles has been associated with pore-forming toxins, such as *Helicobacter pylori* VacA (277, 278). The concentration and exposure time of cells to the pore-forming toxins determines if a cell commits to a cell death pathway (58, 60-62). In line with this, at MOI 10 and 25 we found vacuoles at all time points when neutrophils were challenged for, e.g., 0h, 0.5h, 1h and 2h with *Aa* 652. JP2 induced cytoplasmic vacuoles only at the lowest MOI of 10 and cell lysis, in less than 30mins, at MOI 25. Unexpectedly, JP2 *ΔLtxA* induced cytoplasmic vacuolation to a similar extent as *Aa* 652,

at both MOIs. This suggests that LtxA, although a pore-forming toxin, does not participate in the induction of cytoplasmic vacuoles in human neutrophils. To directly determine if LtxA plays a role in cytoplasmic vacuolation, neutrophils can be challenged with purified recombinant LtxA to evaluate the formation of cytoplasmic vacuoles. In the case that purified LtxA does induce cytoplasmic vacuolation, vacuolization may occur by increasing cytosolic calcium ions due to the creation of membrane pores, which promotes NLRP3 inflammasome activation (279, 280). Another way that cytosolic calcium levels can increase is through Siglec-9 activation. As shown by von Gunten *et al.* (64) inhibition of the NADPH oxidase prevents ROS production and results in reduced or no Siglec-9 activation. Therefore, Siglec-9 activation appears to activate NADPH oxidase and increase ROS production, which in turn will activate the inflammasome. Wu *et al.* (84) demonstrated this in mice; further they identified the ITIM domain of Siglec-E as critical for ROS production. This group also presented evidence that Gram-negative bacteria enhance Siglec-9 expression in murine neutrophils, whereas Gram-positive bacteria suppress expression. Supporting these findings, we demonstrated that *Aa* enhances Siglec-9 expression at MOI 10, 50, 100 in human neutrophils. A peak was observed at MOI 50 and a decrease at MOI 100, although still significant compared to UT. This suggests that neutrophils may have reached a threshold of membrane damage that they can withstand, once this threshold is crossed, cells may commit to a cell death pathway. Experiments evaluating cell death markers will aid to shed light in this. Priming of neutrophils with IL-8, but not TNF-α prior to *Aa* challenge further increased Siglec-9 expression. Both cytokines are known to be bound and internalized by the outer membrane lipoprotein bacteria interleukin receptor-1 (BilRI) in *Aa* (228). LPS from *Aa* binds IL-8 which

contributes to modifications of the composition of the extracellular matrix in biofilm and further activates neutrophils, whereas TNF-α favors LtxA induced apoptosis by upregulating LFA-1 expression (229, 230, 281). Our experiments of neutrophils challenged with the Gram-positive oral pathogens *Ps* and *Fa* were contradictory. A potential explanation for our observations is that *F. alocis* activates NF-κβ through TLR2 signaling (262). Following, NF-κβ activates the transcriptional factor AP-1, that binds to the Siglec-E (Siglec-9 homolog in mice) promoter (84). This suggests that Gram-positive bacteria suppression of Siglec-9 expression may rely on the non-canonical signaling pathway after TLR binding. Initial experiments showed that Gram positive bacteria increased Siglec-9 expression, although not to the extend induced by *Aa* 652. On the other hand, a second set of experiments showed that *Ps* and *Fa* did not induce increased expression of Siglec-9, similar to what it was shown by Wu *et al*. (84). In summary, LtxA does not play a major role in inducing cytoplasmic vacuoles in human neutrophils, but *Aa* may induce vacuole formation through Siglec-9 activation.

In conclusion, we show that LtxA alone does not confer full protection to *Aa* from phagocytosis, particularly at sublytic levels. We propose that human neutrophils can withstand a certain level of membrane injury by LtxA. However, once the threshold is crossed, the cell will commit to a cell death pathway. Given that pore-forming toxins induce cytoplasmic vacuoles, we initially hypothesized that LtxA was responsible for inducing cytoplasmic vacuoles in *Aa*, but the experimental evidence does not support this outcome. Thus, Siglec-9 activation represents an alternative pathway for inducing cytoplasmic vacuoles. We showed that *Aa* enhances Sglec-9 expression and the expression was increased when neutrophils were primed with IL-8 but not TNF-α. We propose a

scenario where at sublytic levels of LtxA, neutrophils withstand membrane injuries repaired by the endocytosis method membrane repair mechanism. *Aa* interaction with neutrophils leads to Siglec-9 activation which could involve NLRP3 activation and ROS production, resulting in cytoplasmic vacuoles formation. The findings presented in this chapter serve as a foundation for further studies of LtxA sublytic activity in neutrophils and how *Aa* modulates the inflammatory response of neutrophils through Siglec-9.

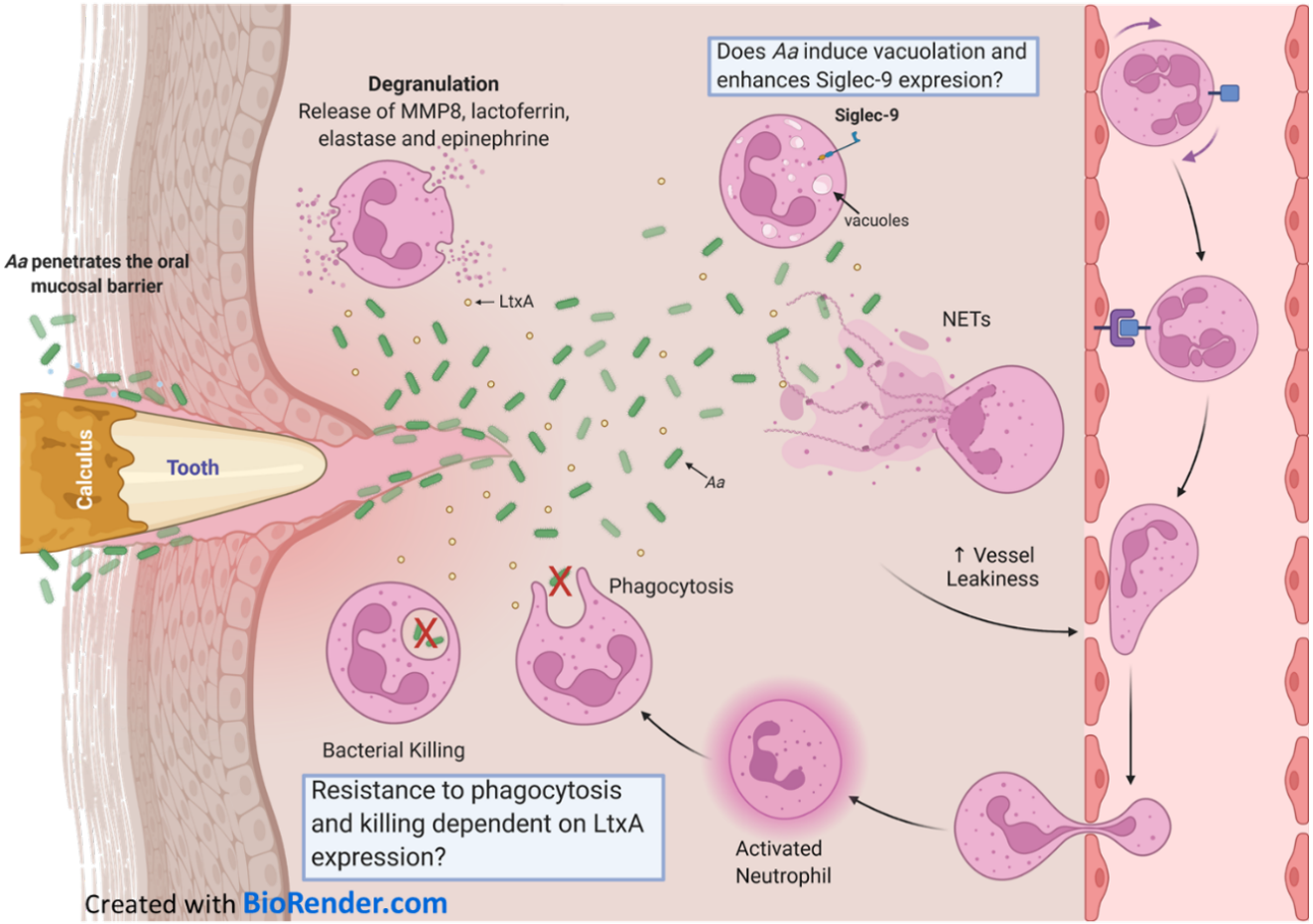


Figure 5. 1. *A. actinomycetemcomitans* evasion of neutrophils effector functions: what is known and what we are investigating

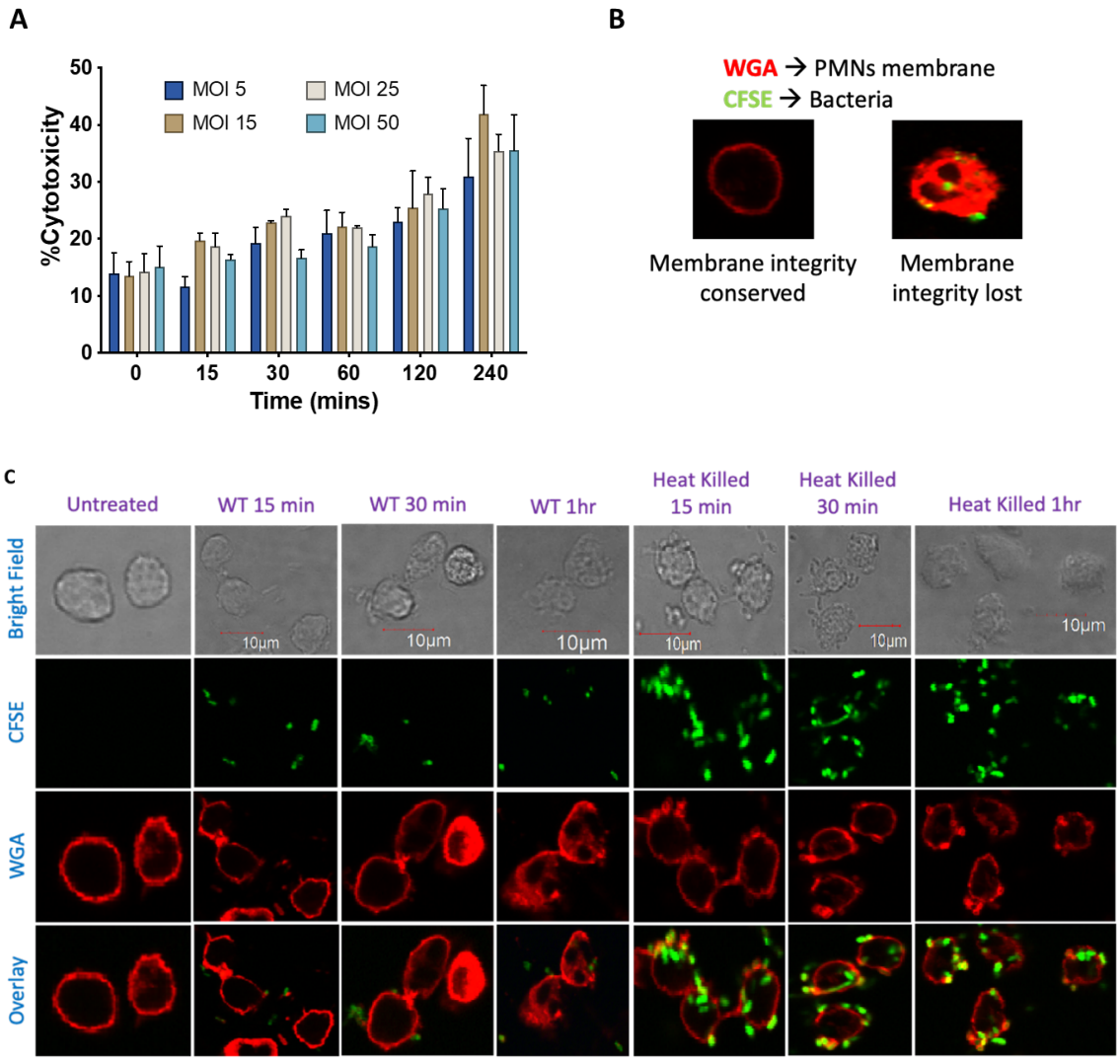
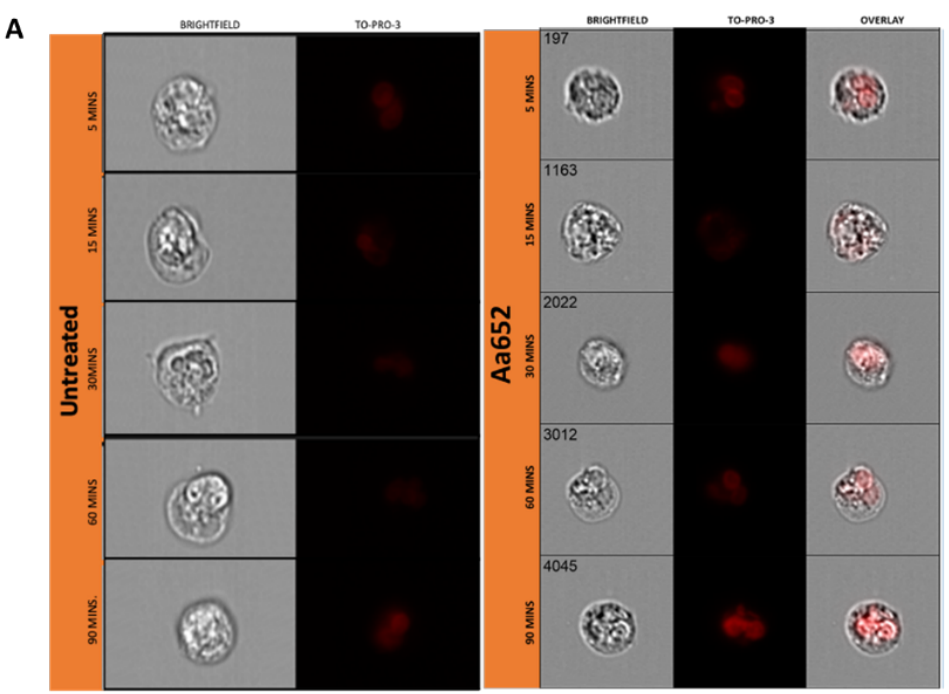
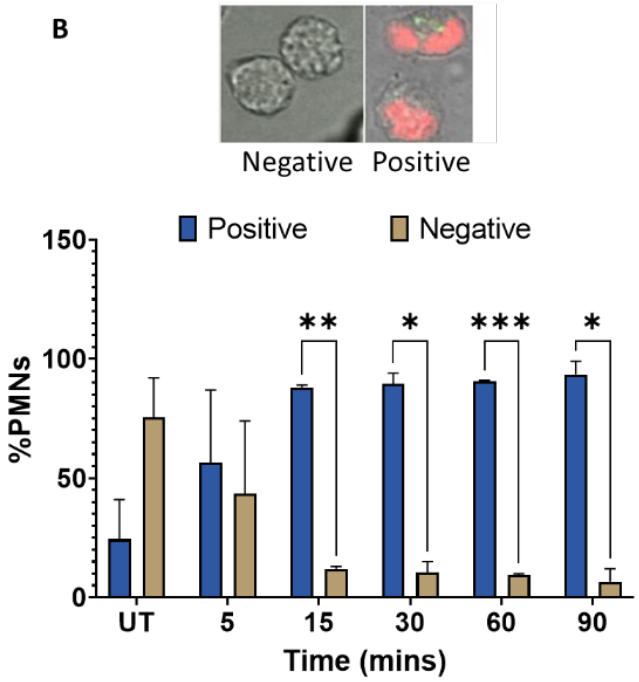


Figure 5.2. Human neutrophils membrane integrity is compromised when challenged with *A. actinomycetemcomitans* at MOI 50. Human neutrophils (3x10^6 cells/ml) were exposed to increasing MOIs of *Aa* (5-50) for 0, 15, 30, 60, 120 and 240 mins shaking in water bath at 37°C. Neutrophils cytotoxicity was determined by measuring released lactate hydrogenase (LDH) using a colorimetric assay and was presented as percentage of neutrophil cytotoxicity ± standard deviation (282) of 3 independent experiments. Statistical significance was determined with Two-way ANOVA with Tukey post-test **(A)**. The membrane of neutrophils was stained with wheat germ agglutinin (WGA) and membrane

integrity was determined by the amount of WGA that enter cell. CFSE was used to label bacteria **(B)**.


A
BRIGHTFIELD
TO-PRO-3
OVERLAY
Untreated
Aa652
5 MINS
15 MINS
30MINS
60 MINS
90 MINS.
197
1163
2022
3012
4045


B
Negative
Positive
Positive
Negative
%PMNs
150
100
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0
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UT
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90
Time (mins)

Figure 5.3. Human neutrophils membrane integrity is compromised by 15 mins of *A. actinomycetemcomitans* challenge. Human neutrophils ($4x10^6$ cells/ml) were challenged with *Aa* (MOI 50) for 5, 15, 30, 60 and 90 mins in suspension, shaking in a water bath at 37°C. The integrity of the membrane was evaluated by staining the cells with TO-PRO-3 stain (an impermeable DNA stain) and visualized by imaging flow cytometry **(A)**. PMNs were classified and quantified as positive or negative by the ability of the impermeable stain TO-PRO-3 to enter and stain the nucleus. Percentage of positive or negative neutrophils for TO-PRO-3 was plotted with ± SD of 3 independent experiments **(B)**. Statistical analysis was determined with Two-way ANOVA with Tukey post-test | Significance: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

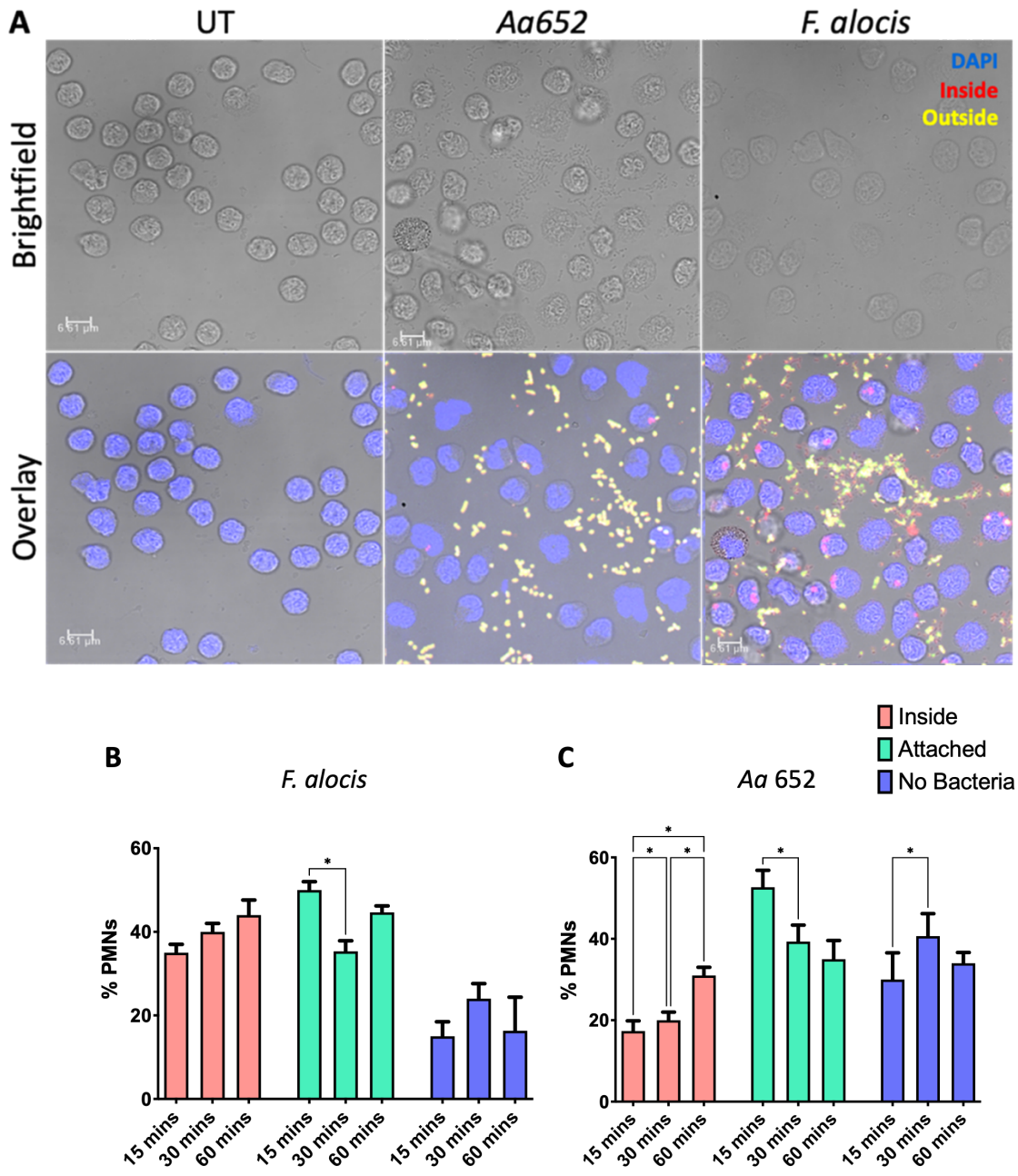


Figure 5.4. Human neutrophils can phagocytize *A. actinomycetemcomitans* at MOI 10. Human neutrophils (4x10^6 cells/ml) were challenged with *Aa* (MOI 10) or *Fa* (MOI 10) for 15, 30, 60 mins in suspension, shaking in a water bath at 37°C. Following, each condition was mounted and fixed into a coverslip and bacteria was differentially stained with a mouse polyclonal anti-*Aa* (1µg/ml) primary antibody. As secondary antibodies: goat anti-

mouse Alexa Fluor 488 (all *Aa*) and goat anti-mouse Alexa Fluor 647 (inside *Aa*). Example confocal images of differential staining **(A)**, a total 100 neutrophils per donor were counted and determined if *Fa* **(B)** and *Aa* **(C)** were internalized. Plotted are the percentage of neutrophils that internalized bacteria, have associated bacteria or no bacteria ± SD of 3 independent experiments. Statistical analysis was determined with Two-way ANOVA with Tukey post-test | Significance: * $p \leq 0.05$.

A
Brightfield
DAPI
Inside
Outside
UT
MOI 10
MOI 25
MOI 50
MOI 100
Aa652
JP2
JP2 ΔLtxA

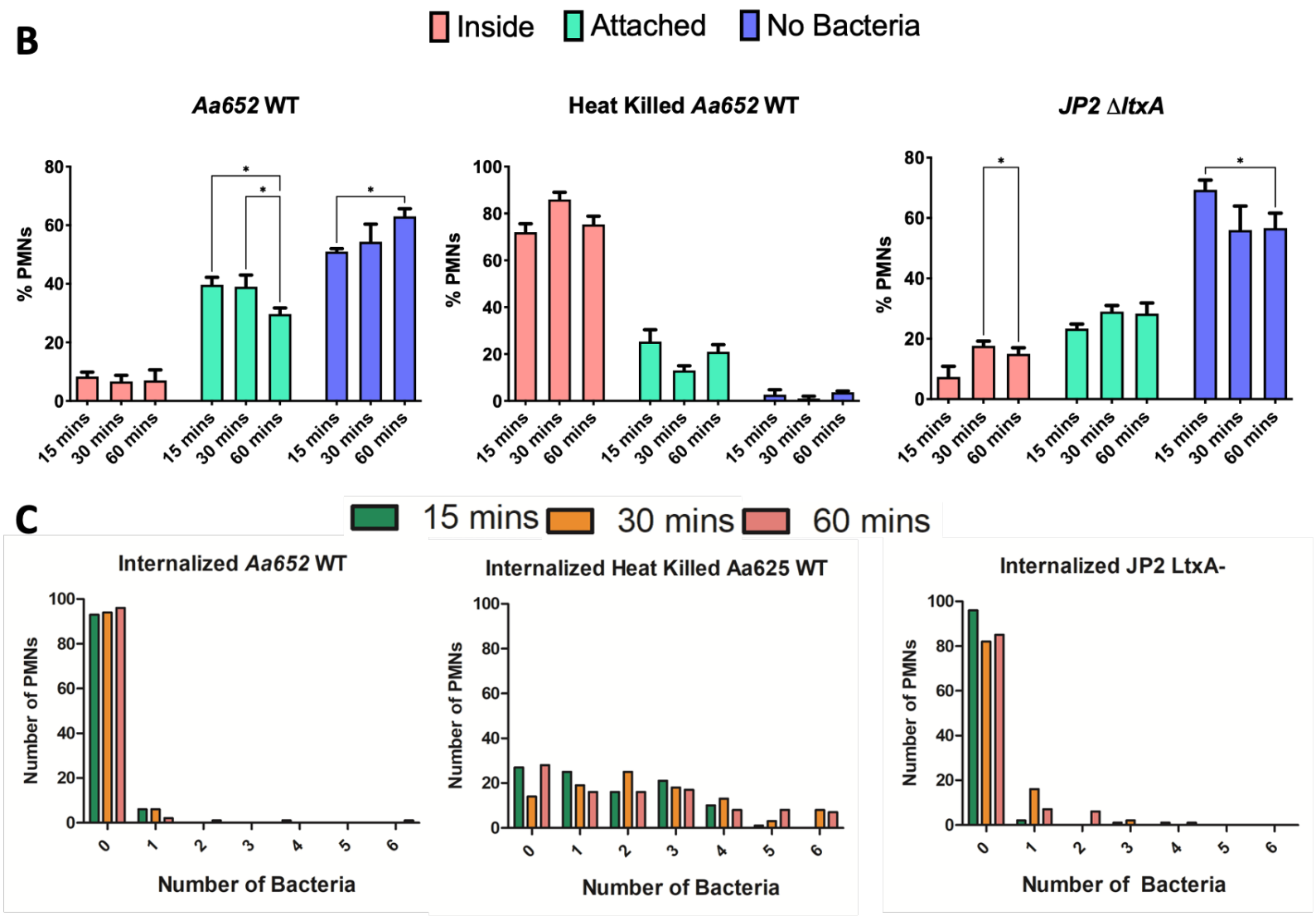
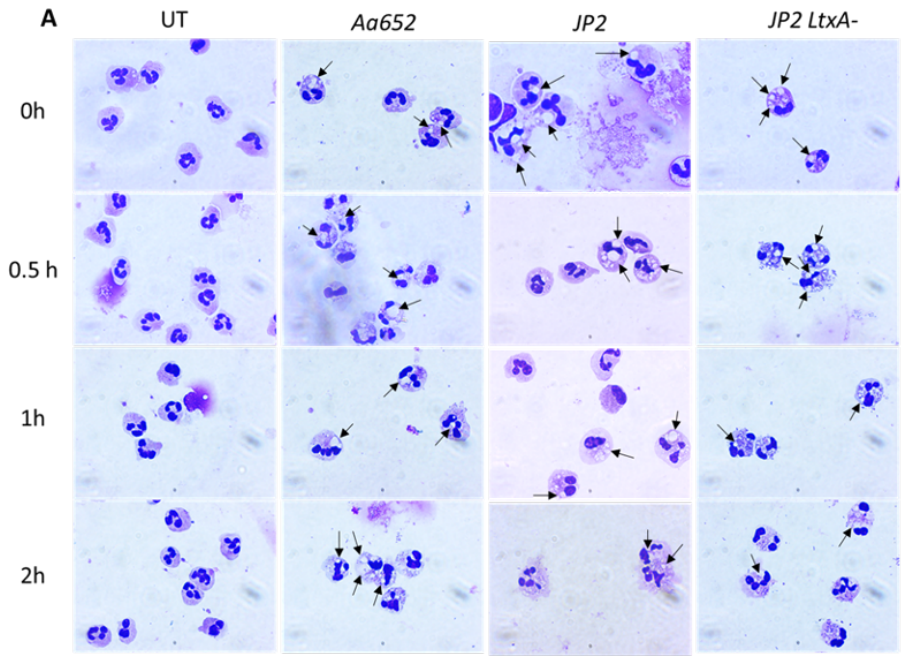
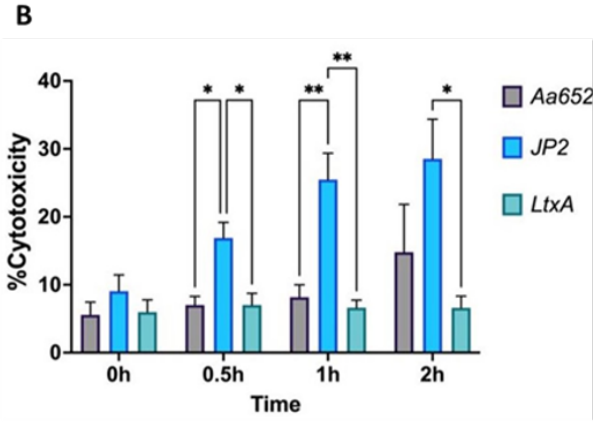

B
Inside
Attached
No Bacteria
Aa652 WT
Heat Killed Aa652 WT
JP2 ΔltxA
% PMNs
15 mins
30 mins
60 mins
C
15 mins
30 mins
60 mins
Internalized Aa652 WT
Internalized Heat Killed Aa625 WT
Internalized JP2 LtxA-
Number of PMNs
Number of Bacteria

Figure 5.5. *A. actinomycetemcomitans* resists internalization by human neutrophils at high MOIs. Human neutrophils ($4x10^6$ cells/ml) were challenged with *Aa* 652*,* HK *Aa* 652, JP2*,* JP2 *ΔLtxA* at increasing MOIs of 10, 25, 50 and 100*.* Representative images are shown of neutrophils challenged for 30 mins in suspension (A). Cells were challenged for 15, 30, 60 shaking in a water bath at 37°C. After washes, each condition was mounted and fixed into a coverslip and bacteria was differentially stained with a mouse polyclonal anti-*Aa* (1µg/ml) primary antibody. As secondary antibodies: goat anti-mouse Alexa Fluor 488 (all *Aa*) and goat anti-mouse Alexa Fluor 647 (inside *Aa*). Plotted are the percentage of neutrophils (B) that internalized bacteria, have associated bacteria or no bacteria ± SD of 3 independent experiments. Statistical analysis was determined with Two-way ANOVA with Tukey post-test | Significance: * $p \leq 0.05$. Number of internalized bacteria is shown for each condition (C).


A
UT
Aa652
JP2
JP2 LtxA-
0h
0.5 h
1h
2h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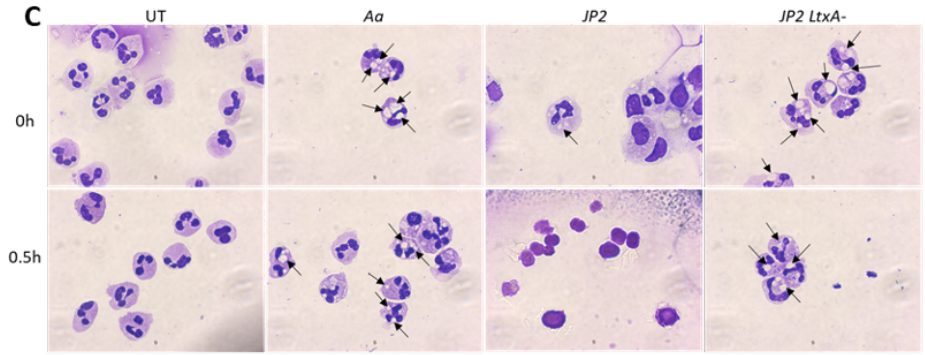
C
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Aa
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Figure 5.6. *A. actinomycetemcomitans* induces cytoplasmic vacuoles in human neutrophils regardless of LtxA expression with minimal cytotoxicity. Fresh Human neutrophils ($4x10^6$ cells/ml) were challenged with different *Aa* strains in suspension for up to 2h at 37°C shaking in a water bath. After challenge, a volume of 200 µl was collected at each time point and immediately taken to cytospin and Hema3 staining. Black arrows indicate cytoplasmic vacuoles. Cytospins images of neutrophils challenged with *Aa*, JP2 and JP2 *ΔLtxA at* MOI 10 (A). Cell supernatants were used to measure LDH using a colorimetric assay. Percentage of cytotoxicity of seven independent experiments is plotted (B). Cytospins images of neutrophils challenged with *Aa*, JP2 and JP2 *ΔLtxA at* MOI 25 (C). Mean cytotoxicity ± SD was plotted. Statistical analysis was determined with Two-way ANOVA with Tukey post-test. | N = 7 | Significance: * $p < 0.05$, ** $p < 0.01$.

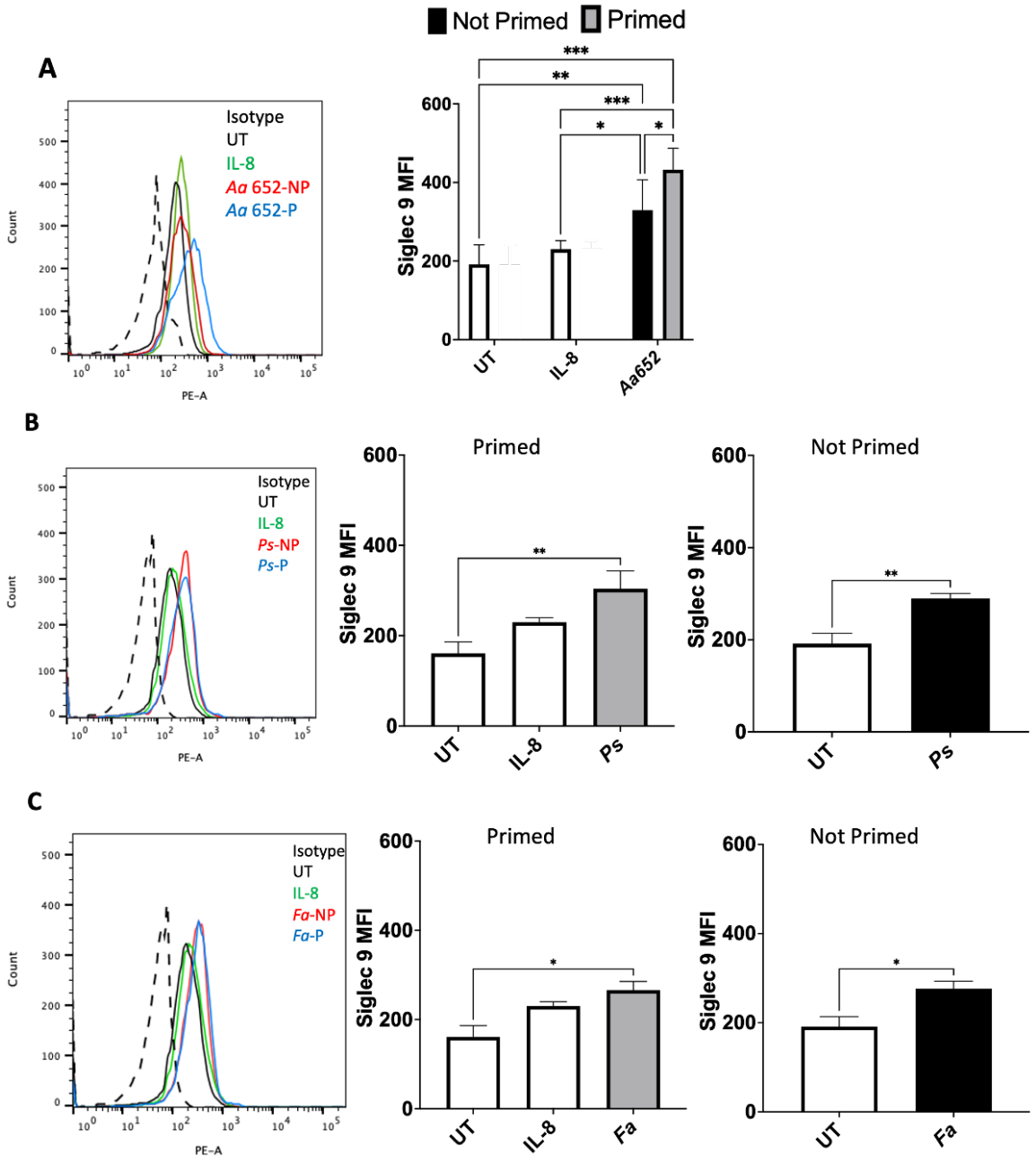

Figure 5.7. *A. actinomycetemcomitans* challenge results in increased Siglec-9 expression in human neutrophils. Human neutrophils (4x10^6 cells/ml) challenged with *Aa* at MOI 10 in suspension for 2h at 37°C shaking in a water bath. Primed neutrophils were incubated with IL-8 (10ng/ml) for 1h prior to bacterial challenge. Representative flow cytometry histogram profile (left panels) is provided for each set of conditions: *Aa* 652 (A), *Peptoanaerobacter*

stomatis (Ps) (B) and *Filifactor alocis (Fa)* (C). The MFI ± SD for Siglec-9 membrane expression is plotted. Statistical analysis was determined with Two-way ANOVA with Tukey post-test| N = 5 | Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

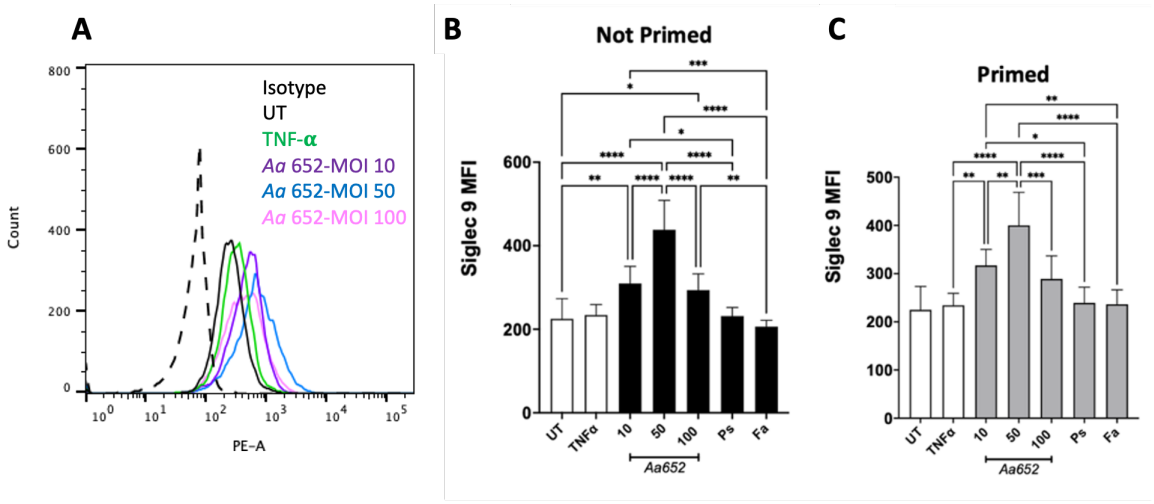

Figure 5.8. *A. actinomycetemcomitans* challenge enhances Siglec-9 expression in a dose dependent manner. Representative flow cytometry histogram profile **(A)**. Human neutrophils (4x10^6 cells/ml) challenged with *Aa* (MOI 10/50/100), *Ps* (MOI 10) and *Fa* (MOI 10) in suspension for up to 2h at 37°C shaking in a water bath **(B-C)**. Primed neutrophils were incubated with TNF-α (2ng/ml) for 15 min prior to bacterial challenge **(C)**. Statistical analysis was determined with Two-way ANOVA with Tukey post-test | N = 4 | Significance: ns = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

CHAPTER 6

SUMMARY AND FUTURE DIRECTIONS

Epinephrine interaction with *Aa* requires the expression of QseC periplasmic domain and FepA, but not YgiW

The QseBC TCS is associated with quorum sensing and is highly conserved in *Enterobacteriaceae* and *Pasteurellaceae* (89) but the signal recognized differs among species. In *Aa*, the *qseBC* operon encodes the genes for QseC, a sensor molecule, and QseB the response regulator (88). Previous findings made by our group identified catecholamines (norepinephrine and epinephrine) and iron as the signals sensed by QseC and that the periplasmic domain of QseC is required for *qseBC* operon expression (91). At this time the details of interaction between Cat-Fe and QseC had not been determined. **Recently, we published findings that demonstrated that QseC, in particular the QseC periplasmic domain (QseC-pr) is required for epinephrine interaction with *Aa* (210).** Moreover, there is the potential for iron or Cat-Fe to interact with QseC. The QseC sensor protein is structurally and functionally conserved in *Enterobacteriaceae* and *Pasteurellaceae*. In *E.coli,* QseC is not directly activated by iron but as a result of iron activating the PmrB sensor, which activates the PmrA regulator that ultimately binds QseB and induces the expression of the *qseBC* promoter (89). Although, *Aa* does not have the *pmrAB* genes, the sequence where QseB binds in the *qseBC* promoter is identical to that of PmrA binding site on the *pmrAB* promoter (89). The subgingival pocket where *Aa* is found is an

environment rich in catecholamines, for this reason *Aa* has evolved to take advantage of epinephrine/norepinephrine from the host to acquire ferrous/ferric iron and integrate this to the sensory functions of the PmrAB and QseBC TCS of *E.coli*. To determine if catecholamines and/or iron bind directly to QseC-pr, transformed *E.coli* BL21 cells that overexpress QseC-pr (pDJR1, with GST tag, Table 2.1) can be used to express QseC-pr by IPTG induction (see Chapter 2 for methods). The isolated protein can be used in equilibrium microdialysis experiments, where the protein of interest is added to one chamber and the ligand into another with a cellulose membrane (5kDA cut off) between them (see Chapter 2 for methods). This not only allows the determination of epinephrine or iron binding with QseC-pr, but the disassociation constant (K_d) can be measured. Finally, iron abundance in each chamber could be determined by ICP-MS, if a higher abundance is detected in the chamber containing QseC-pr, it will suggest interaction with iron. To confirm our results of the requirement of QseC-pr for epinephrine interaction with *Aa*, a similar approach can be used by replacing iron with epinephrine as the ligand. In the case that no interaction is determined with epinephrine or iron alone, Epi-Fe potential to bind QseC-pr can be investigated as well. Alternatively, a radioactively labeled epinephrine or iron could be used to assess binding to QseC. These experiments will allow to determine if Epi/Fe interact with QseC directly and if they interact as a complex or individually.

In both *Enterobacteriaceae* and *Pasteurellaceae*, the *qseBC* locus is associated with the *ygiW* gene, which encodes a putative periplasmic protein of the bacterial oligo-nucleotide/oligosaccharide-binding (OB)-fold (BOF) family (159, 160). **In this work we showed that epinephrine interacts with *Aa* independently of *ygiW* expression.** Members of the BOF family can act as scaffolds in ligand binding (160). Although little

characterization of BOF proteins exist, they have been linked to stress responses (161, 162). CusF, a BOF protein part of the CusCFBA copper-efflux system in *E. coli* was shown to bind copper and serve as a shuttle of copper, increasing the accessibility of copper in the periplasmic space (211). Preliminary observations by a previous lab member indicated that there was no induction of the *qseBC* operon in the presence of Cat-Fe in *Aa* when *ygiW* was not expressed (data not shown). To determine if YgiW plays a role in QseC activation, protein interaction of YgiW and QseC can be determined by pulldown assays. While we do not fully understand the role of YgiW in *Aa* at this time, is possible that YgiW in *Aa* shares similarities in metal binding with other BOF proteins involved in stress responses, but this requires further exploration. Future studies can determine if YgiW directly interacts with iron by utilizing transformed *E.coli* BL21 cells that overexpress YgiW (pDJR3, with HIS tag, Table 2.1) after IPTG induction. The equilibrium microdialysis approach described above can be applied to determine if YgiW binds to iron or epinephrine as a complex, or individually at the same time. Results will provide insight into the potential of YgiW serving as a shuttle of iron and/or Epi-Fe and activating QseC. If this is the case, it will suggest that QseC is indirectly activated by iron, similar to the PmrAB TCS in *E. coli.*

When *Aa* was grown in the presence of catecholamines and iron, the enterobactin operon which contains a gene for FepA, was the only iron acquisition system that was unchanged (91), suggesting that it may be the main transporter for Cat-Fe into the periplasm. **Using a *fepA* mutant strain of *Aa* we showed that interaction of epinephrine with *Aa* decreased in the absence of FepA.** However, because this interaction was not completely inhibited, FepA may not be the sole transport system for Cat-Fe. The importance of FepA in the induction of the *qseBC* operon and *Aa* growth can be determined

using a *fepA* mutant expressing the *lacZ* gene (pDJR29). Radioisotopes of epinephrine and iron can also be used to investigate binding to FepA. Finally, the role of FepA in virulence can be investigated using the oral gavage mouse model and the *Aa fepA* mutant strain and measure bone resorption.

In *E. coli*, FepA is an outer membrane protein that serves as a receptor for the ferric enterobactin siderophore (239, 240). The uptake of this siderophore into the bacterial cells is TonB-dependent (240, 241) and a region of FepA that lies outside the TonB box, is highly conserved among the *Pasteurellaceae* family (283). Expression of the FepA receptor which binds to catecholamines provides *Aa* the ability to acquire either ferrous or ferric iron and may provide *Aa* a competitive advantage against bacteria that do not have the ability to exploit these molecules. It is also possible that *Aa* may bind siderophores produced by other bacteria to acquire iron.

In healthy individuals, transferrin and lactoferrin in serum are only 30% saturated with iron, maintaining free iron levels at low levels that does not support bacterial growth. Catecholamines (i.e. norepinephrine) have a higher affinity for iron than transferrin or lactoferrin and can extract iron from these proteins, making iron readily available to bacteria (178).

We propose a model (Figure 6.1) where *Aa* uses epinephrine and iron from the environment as environmental cues to regulate gene expression that will facilitate adaptation to the anaerobic environment. *Aa* exploits epinephrine from the environment as a pseudosiderophore to sequester iron. Epinephrine and iron interact with FepA which helps transport iron into the periplasm in a TonB-dependent manner. In the periplasmic space, it is possible that iron is bound by YgiW, either in complex with the catecholamine

or independently. Given this, YgiW may serve as a shuttle of iron and/or epinephrine to activate QseC. Ultimately, iron is transported into the cell by an ABC transporter. Upon QseBC activation, iron uptake genes are downregulated except for ferritin, which is significantly upregulated by 10-fold (91). This suggests that *Aa* is in an iron replete environment, where high affinity uptake systems for iron are not necessary and their expression is downregulated to maintain iron homoeostasis. In contrast, ferritin expression is upregulated to safely sequester iron for storage, to prevent damage due to radical formation. Confirming this proposed model will provide a better understanding of the role that QseBC TCS signaling plays in *Aa* to acquire iron and may facilitate the development of therapies that will target critical steps to halt bacterial growth or the regulation of genes that favor bacterial survival in the anaerobic environment.

Human neutrophils store epinephrine in azurophilic granules and *A. actinomycetemcomitans* gains access to epinephrine by inducing degranulation

Marino *et al.* (224) detected catecholamines in the supernatant of human peripheral blood mononuclear cells but the report failed to determine if these catecholamines were released, and if so, what stimuli induced release. Based on work by others, it is known that there is a large infiltration of neutrophils at the subgingival pocket during periodontitis (27, 35, 231) and murine neutrophils have been shown to release and synthesize catecholamines when stimulated with LPS (166). **We expanded the current knowledge by demonstrating for the first time that human neutrophils store endogenous epinephrine in azurophilic granules, and that by inducing degranulation, *Aa* promotes the release of epinephrine.** Further, under anaerobic conditions **we showed that neutrophil-derived epinephrine can facilitate iron acquisition, promote *Aa***

growth and induce the *qseBC* operon. The use of host-derived catecholamines by *Aa* is also observed in *B. bronchiseptica*, *B. pertussis* and *E. coli*, which have been shown to use norepinephrine from the host to sequester iron from iron-loaded transferrin (137, 138, 154). It has been published that other Gram-negative bacteria, such as *Y. enterocolitica*, *P. aeruginosa* and *E. coli,* show a drastic increase in bacterial growth in the presence of epinephrine, norepinephrine, dopamine and dopa (176). The bacterial evolution to use host-derived catecholamines to adapt to environmental changes and persist serves as an example of convergent evolution.

As previously shown in our lab, this promotes the downregulation of genes involved in iron uptake and upregulates the expression of genes involved in anaerobic respiration and iron storage (91). Adding to the findings of the previous section, our *in vitro* results would suggest that in the subgingival pocket, human neutrophils may serve as an epinephrine source for *Aa* and provide an environment that may facilitate iron acquisition. To confirm if our *in vitro* findings that neutrophils serve as a source of epinephrine to *Aa* are recapitulated *in vivo*, we propose to use the experimental periodontitis model where mice are oral gavaged with *Aa*, followed by administration of a micro-dose (284) of TAT-fusion proteins, Syntanxin4 and SNAP23 (199), to selectively inhibit the release of granules in neutrophils. The inhibition of release of secretory vesicles and specific and azurophilic granules in murine neutrophils will be confirmed by measuring granule membrane markers by flow cytometry. Inhibition of release of gelatinase granules will be determined by measuring MMP9 release into supernatant. The levels of epinephrine in blood will be measured by ELISA. The impact that inhibition of degranulation in murine neutrophils has in *Aa* inducing bone loss will be measured. As controls untreated mice and

Aa-only infected mice will be used. This approach will allow to determine if neutrophils are the main source of epinephrine for *Aa*. There is not enough information of other bacteria that normally is found in co-infection with *Aa* to exploit neutrophil degranulation. However, the competitive advantage or benefit of neutrophils degranulation in other oral bacteria can be explored by co-infection of *Aa* with other bacteria that is normally found with *in vivo*, such as *Filifactor alocis*.

In our epinephrine release experiments, we observed the levels of epinephrine in cell lysates increased after 8h and 24h of bacterial challenge (Figure 4.2.C-D) suggesting *de novo* catecholamine synthesis by human neutrophils. **We showed that during *Aa* infection human neutrophils have significant levels of tyrosine hydroxylase**, the enzyme that catalyzes the rate limiting step of catecholamine synthesis (Figure 4. 5.A), expanding previous observations (234). However, **dopamine β-hydroxylase, which catalyzes dopamine into norepinephrine, decreased during *Aa* infection** (Figure 4.5.B). This result can be explained by the fact that not all dopamine will be made into noerpinephrine, therefore less demand for this enzyme. **We also demonstrated that the catecholamine inactivating enzymes catechol-o-methyltransferase and monoamine oxidase-A increased during *Aa* infection at 4h** (Figure 4.5.C-D), the same time point we see a peak in epinephrine release induced by *Aa* (Figure 4. 2.A). This suggests an increase requirement for catecholamine inactivation enzymes due to increased epinephrine release. In vivo, catecholamine levels are tightly controlled in different ways, such as by inactivation, at which state does not promote bacterial growth (154). It would be of interest to determine if the expression of these enzymes changes in neutrophils during *Aa* challenge. qRT-PCR at different time points during bacterial challenge will allow to

determine changes in gene transcription. These findings will provide information of the catecholamine turnover in neutrophils during *Aa* challenge.

We propose a model (Figure 6.2) where the encounter between neutrophils and *Aa* in the subgingival pocket promotes azurophilic granule exocytosis, and release of epinephrine. Consequently, epinephrine storage is depleted promoting catecholamine metabolism (210). In an environment where immune cells are releasing antimicrobials like lactoferrin and transferrin to starve bacteria by sequestering iron, and other bacteria release siderophores to acquire iron, *Aa* has the advantage of using epinephrine to acquire iron, promoting *Aa* growth and induction of the *qseBC* operon. Potential future therapies can target catecholamines for inactivation by complexing them with albumin or by sulfonation will render them useless to *Aa* to sequester iron (154) and prevent bacterial growth.

Effect of LtxA sublytic levels in neutrophil functions

The toxic effects of LtxA are highly dose dependent, as sublytic concentrations of the toxin not only induce increased calcium transport into cells but also activate neutrophils to promote oxidative burst and degranulation (273, 276). **In agreement with this, we demonstrated that challenge of human neutrophils with the minimally leukotoxic strain *Aa* 652 induces the mobilization of secretory vesicles, gelatinase granules, specific granules and azurophilic granules.** Johansson *et al.* (110) showed that purified leukotoxin A at high concentration induced release of granule contents from human neutrophils, and the authors observed increased expression of CD63 and CD66b along with the release of elastase and lactoferrin. However, these studies used high concentration of recombinant LtxA that most likely induces cell lysis, and the study did not differentiate or determine if the release of granules observed was due to true degranulation or cell lysis.

Both *Aa* 652 (minimally leukotoxic) and JP2 (highly leukotoxic) are found in periodontitis patients, therefore it is worth to determine if LtxA plays a role in inducing degranulation. For this, neutrophils can be challenged with JP2 *ΔltxA* strain and compared to JP2. Alternatively, neutrophils can be challenged with recombinant LtxA, to establish a direct role of LtxA in inducing degranulation. In addition, TEM and apoptosis markers could be used as well to determine if what we observed is due to degranulation or cell lysis. The *Aa* 652 strain can be used as an additional control to latrunculin and fMLF, given that we have establish that induces release of all granules in human neutrophils.

In contrast to the suggested LtxA dose dependent protective effect of *Aa* to resist internalization, it has also been argued that *Aa* resists neutrophil internalization and killing regardless of leukotoxin phenotype (129, 260). Supporting this, **we found that *Aa* 652 was internalized by neutrophils at low MOIs, but it resisted phagocytosis at higher population numbers (MOI 50).** Although this would argue that phagocytosis resistance by *Aa* 652 is due to more bacteria producing LtxA, we showed that *Aa* 652 challenge at MOI 50 extended the life span of neutrophils for 24h (Figure 4.4.C) with minimal cytotoxicity (Figure 5.3.B) compared to JP2 challenge at MOI 10 for 30 mins (Figure 5.6.B). Interestingly, the JP2 *ΔLtxA* resisted phagocytosis to a lesser extent than *Aa* 652, suggesting that **resistance to phagocytosis by neutrophils does not require LtxA**. On the other hand, JP2 was internalized by neutrophils only at low MOIs and as bacterial population numbers increased, we observed greater cell lysis. The self-aggregating phenotype and the expression of *tad* fimbriae (absent in lab stocks) has been shown to be important in resistance of neutrophil phagocytosis (129, 257). A potential scenario for *Aa* resistance of neutrophils internalization is a cooperative effect of LtxA and other virulence

factors. Other virulence factors that have been suggested to play a role in resistance of phagocytosis are complement inhibition by LPS, Omp29 and Omp100 (125, 127). Omp29 is known to bind C4-binding protein, inhibiting the activation of the classical and mannose-binding lectin (124) complement pathways (125). Omp100 captures the alternative complement pathway negative regulator, Factor H, and deposits it at the cell surface to inactivate C3b (127). In addition, to using unopsonized bacteria, all these genes are present in the strains we used, possibly explaining why *Aa* was able to resist internalization by neutrophils. To confirm this, *Aa* mutants for Omp29 and Omp100 can be tested for their ability to resist phagocytosis by human neutrophils.

Pore forming toxins, such as LtxA, are associated with the formation of vacuoles in cells due to exposure to bacterial or viral pathogens, a phenomenon known as cytoplasmic vacuolation (277, 278). **We showed that *Aa* induces cytoplasmic vacuolation in neutrophils, but unexpectedly, the JP2 *ΔltxA* induced cytoplasmic vacuolation similarly to *Aa* 652, at MOIs from 10 to 50. Therefore, *Aa* appears to induce cytoplasmic vacuolation in human neutrophils independently of LtxA.** Although, our results suggests that *Aa* induces cytoplasmic vacuolation independently of LtxA it does not eliminate a potential role of LtxA in inducing these vacuoles. Therefore, it is worth to confirm our observations by directly exposing human neutrophils to purified LtxA and evaluate the formation of cytoplasmic vacuoles. Further, to determine if there is significant difference in vacuoles formation numbers between *Aa* and JP2 *ΔLtxA*, quantification of cytoplasmic vacuolation can be done using transmission electron microscopy (TEM).

Finally, **we found that neutrophils were experiencing loss of membrane integrity, but our observations suggests that neutrophils can withstand the effect of LtxA at sublytic levels** and potentially employ membrane repair mechanisms to heal membrane injuries created by the toxin. This would be relevant in patients with periodontitis caused by *Aa* 652, where neutrophils will be exposed to sublytic levels of LtxA that can cause membrane damage and yet not cell lysis. However, this brings into question if it is energy favorable for the cell to repair such damage. The repair mechanism selected by the cell relies on the number and size of the pores, and cell type. Two potential membrane repair mechanisms that can be used by the cell are the ectocytosis method which it involves the externalization of microvesicles (267-270), and the endocytic method that consists of internalization of the membrane-pore containing portion that is targeted to the lysosome for degradation (271, 272). It will be of interest to determine what type of membrane repair mechanism is used by neutrophils after LtxA damage by *Aa*. In addition, identifying the type of vacuoles and their contents in neutrophils challenged with *Aa* 652 will provide information of the potential use of membrane repair mechanisms and if these are used, and it will allow to determine if the cytoplasmic vacuoles induced by *Aa* are associated with membrane repair processes. Further, identification of vacuole type and contents through TEM may provide information of what type of membrane repair system or cell death pathway is activated in the neutrophils in response to *Aa* 652.

***A. actinomycetemcomitans* increases Siglec-9 expression in human neutrophils**

Wu *et al.* (84) showed that murine neutrophils challenged with Gram-negative bacteria exhibit increased Siglec-9 expression in neutrophils, whereas Gram-positive bacteria suppress expression. **We expanded this finding by showing that an oral**

bacterium, *Aa,* enhances Siglec-9 expression at MOI of 10, 50, and 100 in human neutrophils. Further, we showed that pre-treatment of human neutrophils with IL-8 followed by *Aa* challenge resulted in a significant increase in Siglec-9 expression compared to *Aa* challenge alone.

Siglec-9 has been associated with the formation of cytoplasmic vacuoles (63). Activation of Siglec-9 in a pro-inflammatory environment diverts to a caspase-independent cell death, ROS production and vacuole formation (86). Additionally, Zeng *et al.* (83) demonstrated that Siglec-E (the Siglec-9 mouse homolog) is involved in modulating neutrophil functions, including induction of apoptosis both independent and dependent of caspase activation. To supplement our preliminary findings and determine if neutrophils are undergoing apoptosis or another cell death pathway the presence of cell death markers can be investigated, in addition to the requirement of caspase activation. To determine if Siglec-9 expression and cytoplasmic vacuolation are related events, Siglec-9 receptor can be blocked with Siglec-9 F(ab) fragments as previously done by von Gunten *et al.* (64) and the ability of *Aa* to induce cytoplasmic vacuolation can be determined. Because we have not directly determined if LtxA plays a role in cytoplasmic vacuolation it would be of interest to determine if LtxA plays a role in increasing Siglec-9 expression, potentially being the connection between Siglec-9 expression and cytoplasmic vacuolation. To test this, human neutrophils can be challenged with JP2 and JP2 *ΔLtxA* compared to *Aa* 652, and measure Siglec-9 expression by flow cytometry. In addition, Wu *et al.* (84) showed in mice that Gram-negative bacteria resist killing by enhancing Siglec-E expression, therefore it would be of interest to determine if blocking Siglec-9 has an effect on *Aa* clearance after internalization.

Sialic acid is the main ligand of Siglecs and is used by the host as a surveillance system to differentiate from self and non-self, and regulate unwanted immune responses (66-70). However bacteria has evolved mechanisms to exploit this by stealing sialic acid from host cells and using it as a disguise in order to suppress immune response (70). *Aa* has been shown to incorporate sialic acid to its LPS structure, like other *Pasteurellaceae* family members to dampen neutrophils inflammatory response (70, 71). To determine the requirement of *Aa* LPS for Siglec-9 activation, human neutrophils could be exposed to purified *Aa* LPS and evaluated for changes in Siglec-9 expression. This will allow us to determine if increased Siglec-9 expression in neutrophils is solely due to sialic acid on *Aa* LPS or an integrated effect of sialic acid binding and activation of Siglec-9 and TLR4 by *Aa* LPS.

Although sialic-acid is the main ligand of Siglec-9 (70), some Siglecs can bind other type of ligands, such as heat-shock proteins (HSP) (71). *Aa* is known to express several HSP and some have been suggested to serve as virulence factors. For example, in *Aa*, HSP60, has cross-reactivity with oxidized low-density lipoproteins (LDL) antibodies and is considered to have a role in the progression of atherosclerosis (285). If a role is determined for HSP60 in increasing Siglec-9 expression in neutrophils through binding, in addition to LPS, it will serve as novel advantage for *Aa* in the oral cavity by having more than one way to dampen neutrophil inflammatory response. Additionally, it will highlight the importance of modulating Siglec-9 expression for *Aa* survival. Given this blocking Siglec-9 activation can be explored further for therapy uses.

Summarizing the last two sections, presents a scenario (Figure 6.3) where at LtxA sublytic levels *Aa* induces degranulation of all neutrophil granules. *Aa* resists

internalization by neutrophils at higher MOIs independently of LtxA expression. Other virulence factors such as Omp29 and Omp100 may be responsible for phagocytosis resistance, or the resistance is due to a cooperative effect of such virulence factors and LtxA. *Aa* challenge of human neutrophils induces cytoplasmic vacuolation independently of LtxA and it can be due to vacuoles formed by endocytic membrane repair mechanism. Ultimately, as an effort to suppress the inflammatory response of neutrophils *Aa* increases Siglec-9 expression, that is further increased in IL-8 primed neutrophils.

In conclusion, *Aa* evades internalization by human neutrophils, and it induces degranulation to access epinephrine and sequester iron. This leads to QseBC activation and regulation of genes necessary for *Aa* to thrive and adapt to the anaerobic environment. *Aa* modulates neutrophils inflammatory response by increasing the expression of the inhibitory Siglec, Siglec-9. This way *Aa* modulates the inflammatory response of neutrophils to maintain and inflammatory environment that does not eliminate *Aa* but provides it with the necessary factors to persist.

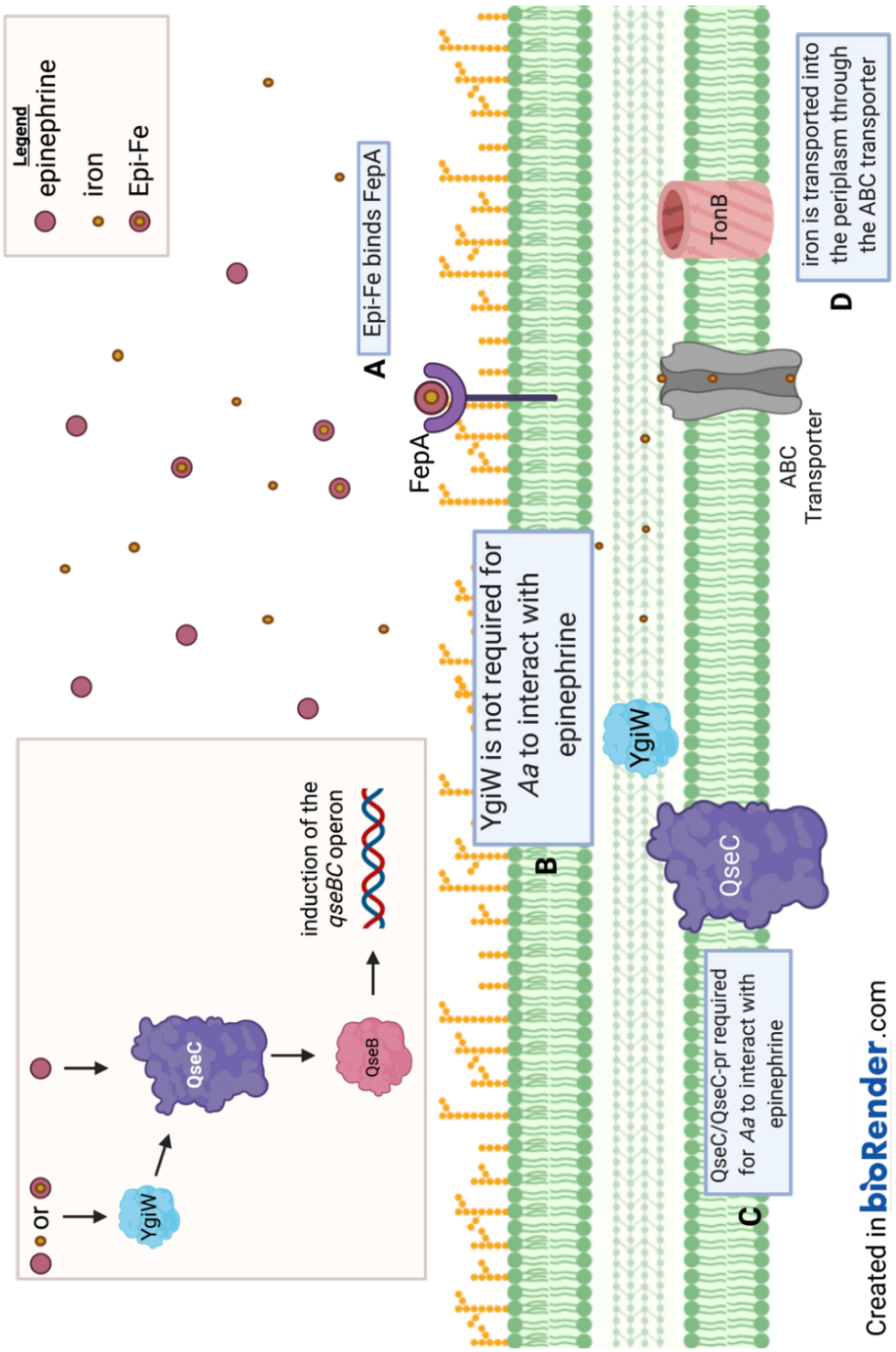

Figure 6.1 Epinephrine interaction with components of the QseBC TCS. We propose a model where epinephrine and iron bind FepA **(A)**. Both epinephrine and iron are transported into the periplasm where we found that epinephrin does not interact with

YgiW **(B)**, but binding of iron alone or of both epinephrine and iron to YgiW may be required (see insert). Then, YgiW serves as a shuttle of iron and epinephrine to activate QseC which interacts with epinephrine **(C)**, ultimately leading transport of iron to inside the cell by the ABC transporter **(D)** and to QseB activation and expression of the *qseBC* operon (see insert).

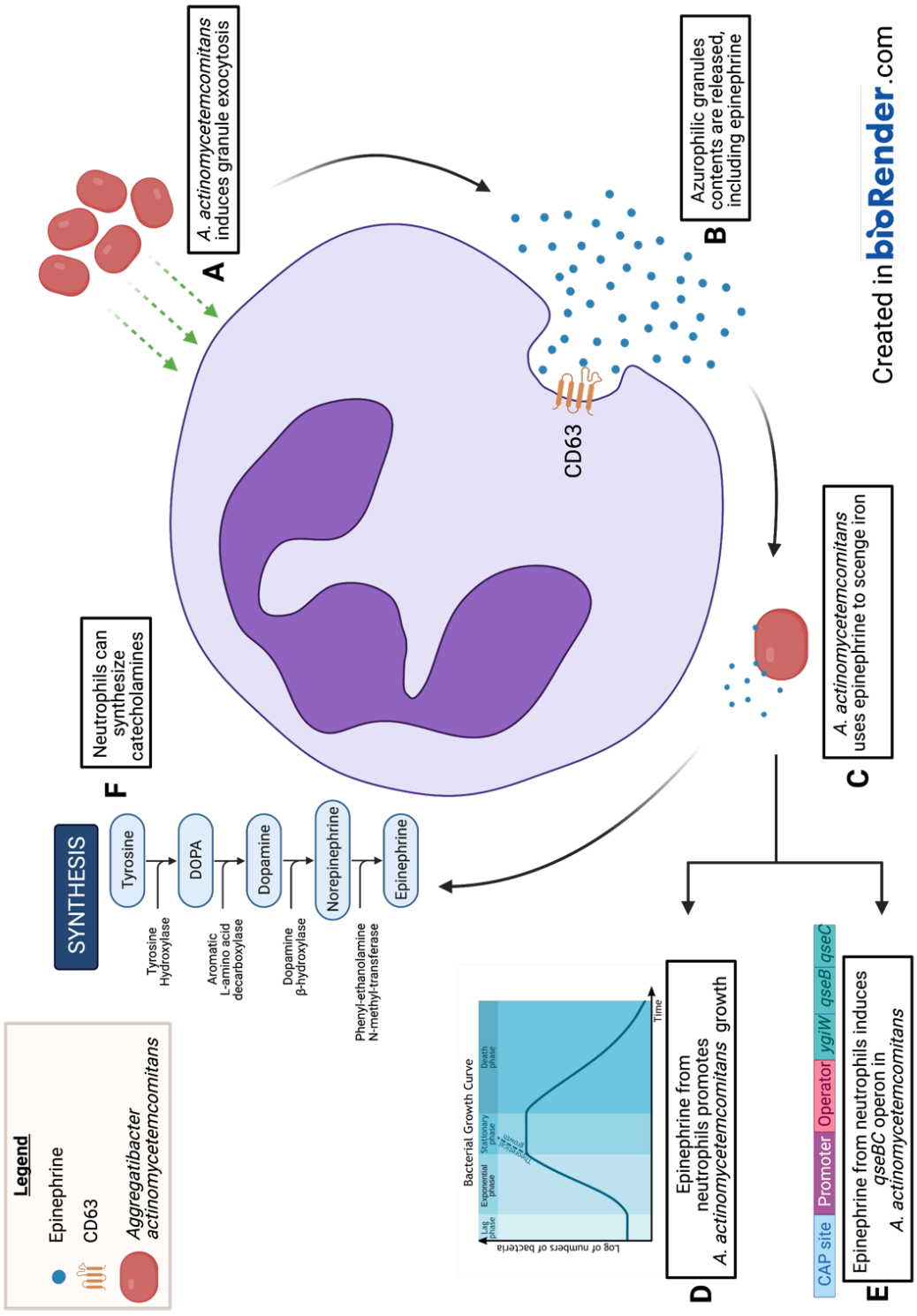

Figure 6.2 Model of how *A. actinomycetemcomitans* access epinephrine released by human neutrophils. We propose that *Aa* induces granule exocytosis **(A)** in human

neutrophils in order to access epinephrine which is store epinephrine in azurophilic granules **(B)**. Finally, *Aa* exploits neutrophil-derived epinephrine to scavenge for iron **(C)** promoting growth **(D)** and induction of the *qseBC* operon to regulate the expression of genes for priming anaerobic metabolism **(E)**. Consequently, epinephrine storage is depleted promoting catecholamine metabolism **(F)**.

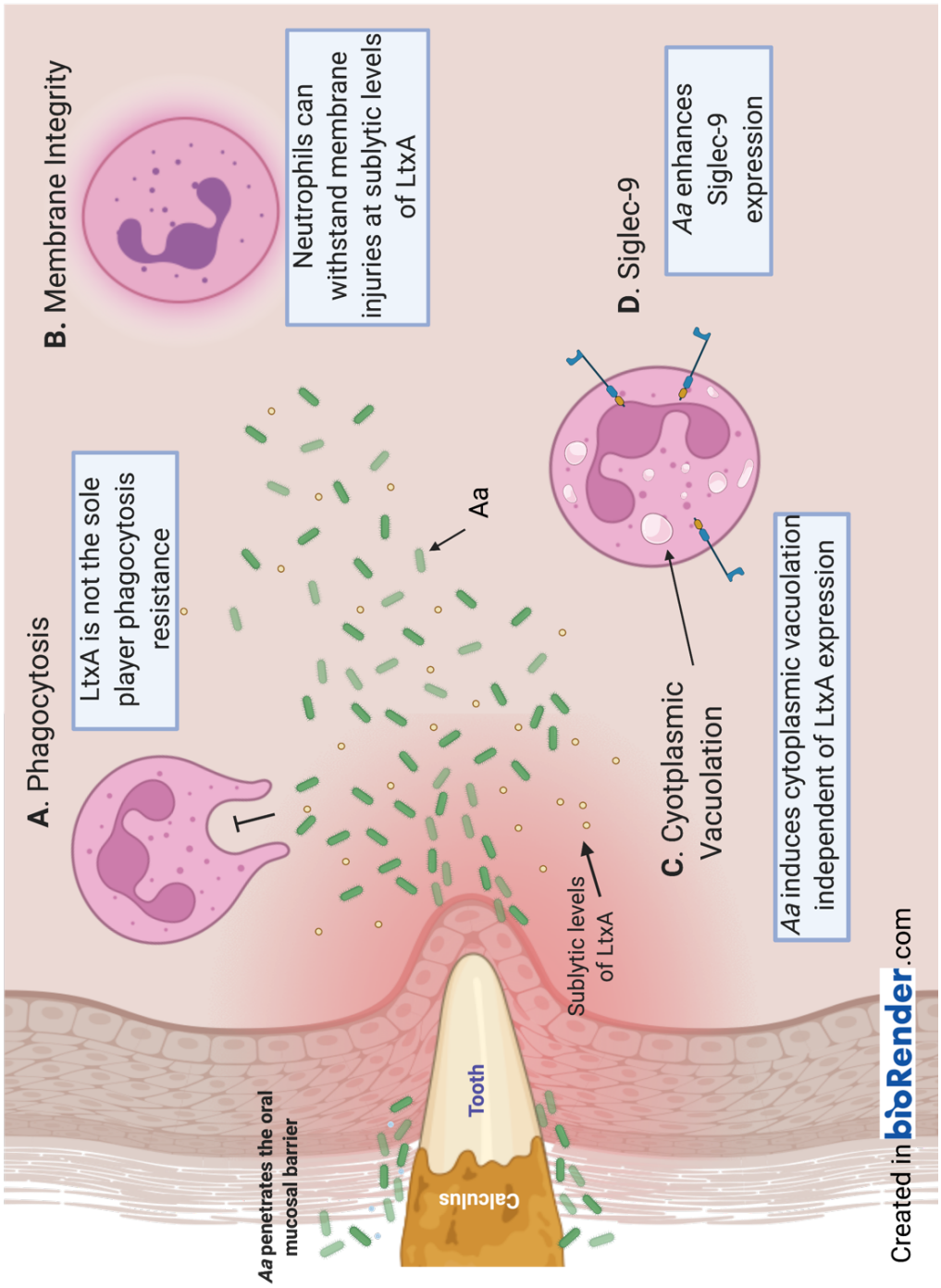

Figure 6.*3* Working model of sublytic effects of LtxA on neutrophils and induction of cytoplasmic vacuolation and increase of Siglec-9 by *A. actinomycetemcomitans* challenge.

We propose a scenario where at sublytic levels of LtxA, LtxA alone does not confer full protection to *Aa* from phagocytosis **(A)** by human neutrophils and can withstand

membrane injuries **(B)** repaired by membrane repair mechanisms. *Aa* challenge induces cytoplasmic vacuolation regardless of LtxA expression **(C)** and it also increases Siglec-9, that is further increased with IL-8 pre-treatment **(D)**.

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CURRICULUM VITAE

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Summary:

My experience in research spans a total of 17 years from undergraduate coursework, summer internships and training in graduate school. During these years I have taught college level classes, mentor high school and undergraduate students. I have enriched my scientific career by means of assisting conferences, training workshops, as a member of different organizations and going through the process of grant writing. Additionally, I am an active advocate and promoter of diversity and inclusion. My leadership, determination, and ability to train others will promote the increase of knowledge through the development of new ideas both in the lab and throughout the scientific community.

Education:

PhD Candidate, Microbiology & Immunology
University of Louisville, Louisville KY
Expected Graduation: May 2022

MS, Microbiology & Immunology
University of Louisville, Louisville KY
May 2016

MS, Biology
Illinois State University, Normal IL
May 2014

BS, Cellular and Molecular Biology
Minor in Mathematics
Universidad Ana G. Méndez, San Juan PR
May 2010

Mentoring Experience:

- Graduate Student Mentor as part of Cientifico Latino Graduate Student Engagement and Community (GSEC)
  Fall 2021 – Spring 2022

A team of two graduate students serve as mentors of up to four Latino first year graduate students in navigating their first year in graduate school. Students are at different programs around the country.

- Graduate Research Mentor
  May 2021 – July 2021
  University of Louisville, Dr. Silvia M. Uriarte Laboratory
  Zack K. Finnegan, Dental Student (D2), summer project title: "Expression of Leukotoxin A by *Aggregatibacter actinomycetemcomitans* partially contributes to inducing toxic vacuolation in human neutrophils"
- Undergraduate Research Mentor
  August 2012-December 2013
  Illinois State University, Dr. Laura A. Vogel Laboratory
  Jasmine Carr, undergraduate student, project title: "Characterization of B1 cells found in mouse peyer patches and peritoneal cavity"
  Katie Interial, undergraduate student, project title: "Determination of B1 cells ability to function as B2 cells"

Teaching Experience:

- Lecturer on Confocal Microcopy Technique, Fall 2020 & Fall 2021
  Microbiology & Immunology Methods Course for Graduate Students
  University of Louisville, Louisville KY
- Tutor in College Algebra, Pre-Calculus, Calculus I & II
  August 2017 – December 2017
  ClubZ Home Schooling and Tutoring Inc., Louisville, KY
- Teaching Assistant (T.A.)
  Spring 2013 & Spring 2014
  T.A. of Biotechnology Lab: Cells and Protein
  Illinois State University, Normal IL
- Lecturer and Head Teaching Assistant (T.A.)
  Fall 2012 & Fall 2013
  Lecturer and Head T.A. of Anatomy and Physiology Lab
  Illinois State University, Normal IL

Research Experience:

- Research mentor: Dr. Donald R. Demuth and Dr. Silvia M. Uriarte
  "Exploitation of the host immune response by Aggregatibacter actinomycetemcomitans mediated by QseBC", Doctoral Dissertation
  Department of Oral Immunology and Infectious Diseases,
  School of Dentistry, University of Louisville KY
  May 2015 – May 2022
- Research mentor: Dr. Laura A. Vogel
  "The effects of aging in B cell function", MS Thesis
  Department of Biological Sciences, Illinois State University, Normal IL
  January 2012 – May 2014

- Research mentor: Dr. Beatriz Zayas
  "Cytochrome C released from mitochondria after treatment with benzazolo quinolinium salts", Independent Research Course
  School of Environmental Affairs, Universidad Ana G. Méndez, San Juan PR
  January 2010 – May 2010
- Research mentor: Dr. Luis E. Vázquez-Quiñones
  "Proteases in the ectodomain shedding of EGFR", Undergraduate Research
  Department of Science & Technology, Universidad Ana G. Méndez, San Juan PR
  August 2008 – December 2009
- Research mentor: Dr. Cleber C. Ouverney
  "Validation of a fluorescent probe for detection of an oral bacterium in the environment", Summer Internship
  Department of Biology, San Jose State University, San Jose CA
  June 2009 – August 2009
- Research mentor: Dr. David T. Burke
  "Alternative mRNA Splicing of RAD 5 on mouse tissues", Summer Internship
  Department of Human Genetics, University of Michigan, Ann Arbor MI
  June 2008-August 2008
- Research mentor: Dr. Rob Knight
  "Compositional trends might improve RNA secondary structure prediction", Summer Internship
  Department of Chemistry and Biochemistry, University of Colorado at Boulder CO
  June 2007-August 2007

Awards /Honors:

Graduate School

- May 2021, School of Medicine Student Diversity Award, University of Louisville, Louisville, KY
- Spring 2021, K. Patricia Cross Future Leaders Award Finalist
- Fall 2017, Excellence in Research Award, Department of Microbiology & Immunology, University of Louisville, Louisville KY
- Fall 2016, Society for Advancement of Chicanos/Hispanics and Native Americans in Science (SACNAS) Conference 2016 Travel Award, Long Beach CA
- Summer 2014, Modeling Mucosal Immunity Summer School 2014 Travel Award, Virginia Polytechnic Institute and State University, Blacksburg VA
- Fall 2013, John H. Wallace Diversity Program Travel Award, Autumn Immunology Conference, Chicago IL
- Summer 2013, 5th Summer Institute in Statistics and Modeling in Infectious Diseases (SISMID) 2013 Tuition and Travel Award, University of Washington, Seattle WA
- Summer 2012, Modeling Immunity for Biodefense Tuition and Travel Award University of Rochester, Rochester NY

Undergraduate

- Spring 2010, Universidad Ana G. Méndez Honor Distinction, Universidad Ana G. Méndez, San Juan PR
- Fall 2009, Best Oral Presentation Award, AGMUS XX Undergraduate Research Symposium, San Juan PR
- Fall 2009, SACNAS Conference 2009 Travel Award, Dallas TX
- Spring 2007, Model Institutions for Excellence (MIE) Excellence List, Universidad Ana G. Méndez, San Juan PR
- Fall 2007, SACNAS Conference 2007 Travel Award, Kansa City KS
- Fall 2006, SACNAS Conference 2006 Travel Award, Tampa FL
- Fall 2005, MIE Excellence List, Universidad Ana G. Méndez, San Juan PR

Grants and Fellowships:

- Spring 2018 – Present, Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Fellowship (F31)
- Fall 2014 – Spring 2016, Integrated Programs in Biomedical Sciences (IPIBS) Fellowship

Leadership & Service:

- 2021-Present, Diversity, Equity, Accessibility, and Inclusion (DEAI) Committee Volunteer of the Federation of American Societies for Experimental Biology (FASEB), Reverse Mentoring Team
- 2021-Present, Junior Representative of the Diversity, Inclusion and Equity (33) Committee of the Society of Leukocyte Biology
- 2019 – 2021, Organization Assisting and Servicing International Students (OASIS), University of Louisville KY
- February 2021 (Talk), "Impostor Syndrome" as part of Women in Engineering Seminar at the University of Cincinnati, Ohio
- July 2020 (Talk), "Microbes: The Good & The Bad" as part of Louisville Science Pathways Summer Program, University of Louisville, Louisville KY
- February 2020 (Talk), "Microbes: The Good & The Bad" at Central High School, Louisville KY
- October 2019 (Talk), "Impostor Syndrome" at the Latino College Fair at Northern Kentucky University, Highland Heights, Kentucky
- 2018, Microbiology & Immunology Student Organization (MISO) Admissions Committee Student Representative, University of Louisville KY
- 2017-2019, Big Sister, Big Brothers & Big Sisters of America, Louisville, KY
- 2017 – 2021, President of the SACNAS student chapter, University of Louisville KY
- 2016, MISO Treasurer, University of Louisville, KY
- 2015, MISO Academic Student Representative, University of Louisville, KY

Memberships:

- 2018 – Present, Society of Leukocyte Biology
- January 2018 – December 2018, International Association of Dental Research

- January 2013 – December 2013, American Association of Immunology
- 2012 – Present, Phi Sigma Biological Society, Beta Lambda Chapter, Illinois State University, IL
- January 2007 – December 2007, American Society of Biochemistry and Molecular Biology
- 2006 – Present, Society for the Advancement of Chicanos, Latinos and Native Americans in Science (SACNAS)

Skills:

- Languages: Fully bilingual in Spanish and English; limited professional level Brazilian Portuguese
- Molecular Biology: PCR qPCR, RT-qPCR; electrophoresis; RNA isolation and purification; SDS-PAGE; ELISA; western blot; antibody production and purification; protein purification; cell culture; flow cytometry; confocal microscopy
- Microbiology: Sub-cloning; Fluorescent *In Situ* Hybridization (FISH); Bactericidal activity assays, gram stain, β-galactosidase assay; acid resistant; motility; biochemical tests; negative stain; capsule and spore stain; selective and differential; Transformation; phagocytosis assays, handling and usage of anaerobic bacteria and chamber
- Mouse Handling: adoptive transfer; tail vein injection; intraperitoneal injection; peritoneal lavage; oral gavage; heart puncture; eye bleeding; genotyping screening; organ dissections; euthanasia
- Data Analysis/Handling programs: Prism GraphPad, R, MATLAB, SAS, Python
- Programming: UNIX, C++, Java, Maple, Minitab and COBOL

Publications:

- **Ozuna, H.**, Uriarte, S. M. and Demuth, D. R. *The Hunger Games: Aggregatibacter actinomycetemcomitans exploits human neutrophils as an epinephrine source for survival*. Frontiers in Immunology (2021) 12:707096. doi: 10.3389/fimmu.2021.707096
    - Featured by Moselio (286) Schaechter in "All Small Things Considered" Blog from the American Society of Microbiology (ASM)
- Kunkel, G. H., Kunkel, C. J., **Ozuna, H.**, Miralda, I. and Tyagi, S. C. *TFAM overexpression reduces pathological cardiac remodeling*. Molecular and Cellular Biochemistry (2019) 454:139–152 https://doi.org/10.1007/s11010-018-3459-9
- Dinis, J.M., Barton, D.E., Ghadiri, J., Surendar, D., Reddy, K., Velasquez, F., Chaffe, C. L., Lee, M.C.W., Gavrilova, H., **Ozuna, H.**, Smits, S.A. and Ouverney, C. C. *In search of an uncultured human-associated TM7 bacterium in the environment.* Plos One (2011) 6(6): e21280. doi:10.1371/journal.pone.0021280

Presentations/Conferences Attended:

Graduate School

- Poster: "*Aggregatibacter actinomycetemcomitans* induces cytoplasmic vacuolation and modulates Siglec-9 expression in human neutrophils" March 30, 2022, Gordon Research Conference: Phagocytes, Oxnard, CA
- Poster: "*Aggregatibacter actinomycetemcomitans* induces cytoplasmic vacuolation and modulates Siglec-9 expression in human neutrophils" March 27, 2022, Gordon Research Seminar: Phagocytes, Oxnard, CA
- Seminar Talk: "*Aggregatibacter actinomycetemcomitans* exploits the immune response of human neutrophils in periodontitis", April 2021, Department of Biological Sciences Seminar Series, Northern Kentucky University, Highland Heights, Kentucky
- Poster: *"Induction of neutrophil granule exocytosis by the oral pathogen, Aggregatibacter actinomycetemcomitans, is independent of Leukotoxin A expression",* October 2018, Society of Leukocyte Biology Annual Meeting of the Society of Leukocyte Biology, Chandler, AZ
- Poster: *"Examining the interaction between Aggregatibacter actinomycetemcomitans and human PMNs",* March 2018*,* Annual Meeting of the International Association in Dental Research, Fort Lauderdale, FL
- Poster: "*Characterization of the Interaction of PMNs with Aggregatibacter actinomycetemcomitans",* October 2017, *Research*! Louisville, University of Louisville, Louisville, KY
- Flash Talk and Poster: *"Aged vs young B1 cell immune response in a SCID mouse model"*, November 2013, Autumn Immunology Conference, Downtown Marriott Hotel, Chicago IL
- Poster: *"Effects of aging on B cell function"*, June 2012, Modeling Immunity for Biodefense Symposium, University of Rochester, Rochester NY
- Poster: *"Effects of aging on B cell function"*, April 12, 2013, Phi Sigma Biological Society 14th Annual Research Symposium, Illinois State University, Normal IL
- Poster: *"Effects of aging on B cell function"*, April 5, 2013, Graduate School Research Symposium, Illinois State University, Normal IL

<u>Undergraduate</u>

- Talk: *"Validation of a fluorescent probe for detection of an oral bacterium in the environment",* March 13, 2010, The Puerto Rico Louis Stokes Alliance for Minority Participation (PRLSAMP) Conference 2010, Universidad de Puerto Rico, Mayaguez PR
- Poster: *"Validation of a fluorescent probe for detection of an oral bacterium in the environment",* October 17, 2009, SACNAS National Conference 2009, Sheraton Hotel and Convention Center, Dallas TX
- Talk: *"Validation of a fluorescent probe for detection of an oral bacterium in the environment"*, September 12, 2009**,** Model Institutions of Excellence Symposium 2009, Caribe Hilton Hotel, San Juan PR
- Talk: *"Validation of a fluorescent probe for detection of an oral bacterium in the environment"*, August 10, 2009**,** Research for Undergraduates in Molecular

Biology Applications (287) REU Program Final Presentation, San Jose State University, San Jose CA

- Talk: *"Validation of a fluorescent probe for detection of an oral bacterium in the environment"*, August 3, 2009**,** Bay Area REU Symposium 2009, California Academy of Sciences, San Francisco CA
- Poster: *"Alternative mRNA Splicing of RAD 5 on mouse tissues"*, March 14, 2009, The Puerto Rico Louis Stokes Alliance for Minority Participation (PRLSAMP) Conference 2009, Universidad de Puerto Rico, San Juan PR
- Poster: *"Alternative mRNA Splicing of RAD 5 on mouse tissues"*, September 13, 2008
  Model Institutions of Excellence Symposium 2008, Normandie Hotel, San Juan PR
- Poster: "*Alternative mRNA Splicing of RAD 5 on mouse tissues"*, July 31, 2008, SROP Final Presentation 2008, Rackham Building, Ann Arbor MI
- Poster: *"Alternative mRNA Splicing of RAD 5 on mouse tissues"*, July 26, 2008, CIC/SROP Conference 2008, Michigan State University, East Lansing MI
- Poster: *"Compositional Trends might improve RNA secondary structure",* October 13, 2007, SACNAS National Conference 2007, Kansas City Convention Center, Kansas City KS
- Poster: *"Compositional Trends might improve RNA secondary structure",* September 15, 2007, Model Institutions of Excellence Symposium 2007, Normandie Hotel, San Juan PR
- Poster: *"Compositional Trends might improve RNA secondary structure*", July 28, 2007, Leadership Alliance Conference 2007, Rocky Hill CT

References:

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