CELLULAR MECHANISMS OF *STAPHYLOCOCCUS AUREUS* α-HEMOLYSIN-MEDIATED ACTIVATION OF THE NLRP3 INFLAMMASOME

Ejiofor A.D. Ezekwe Jr

A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Curriculum of Pharmacology

Chapel Hill
2016

Approved by:

Joseph Duncan

Kendall Harden

Jenny Ting

Lee Graves

Stanley Lemon

ABSTRACT

Ejiofor A. D. Ezekwe Jr: Cellular Mechanisms of Staphylococcus aureus α-hemolysin-mediated Activation of the NLRP3 Inflammasome
(Under the direction of Joseph Duncan)

Background:

Staphylococcus aureus toxin, α-hemolysin, is secreted as a soluble monomer that forms a heptameric pore in the membranes of a range of host cell types. Hemolysin binds and activates A Disintergin and Metalloprotease 10 (ADAM10) and is a well-chronicled virulence factor in staphylococcal disease. ADAM10 activity is important for toxin-mediated pathology in a number of cell types. In host monocytes, α-hemolysin activates the nucleotide-binding domain and leucine-rich repeat containing gene family, pyrin domain containing 3 (NLRP3) inflammasome leading to production of the pro-inflammatory cytokines (IL-1β, IL-18) and pyroptotic cell death. Human airway epithelial cells express the components of the NLRP3 inflammasome but the involvement of this signaling pathway in the cellular response to α-hemolysin is unknown. We hypothesized that both ADAM10 and NLRP3 are involved in host cellular responses to α-hemolysin in both monocytic and respiratory epithelial cells.

Methods:

To elucidate the role of ADAM10 and its protease activity in α-hemolysin-mediated activation of the inflammasome, we used the immortalized monocyte cells line, THP1 and U937. Cells were treated with siRNA against or chemical inhibitors of ADAM10,

challenged with α-hemolysin, and NLRP3-inflammasome activation assessed by measuring secreted IL-1β, cell death, and activation of caspase-1.

To test for evidence of α-hemolysin-mediated inflammasome activation in respiratory epithelial cells, we used primary human tracheobronchial epithelial (hTBE) cells and measured their secretion of IL-1β in response to hemolysin challenge.

Results:

Loss of ADAM10 cell surface expression led to diminished α-hemolysin-mediated activation of the NLRP3 inflammasome and cell death. ADAM10 protease activity, however, was not required for NLRP3 activation in human monocytes. hTBEs secrete mature IL-1β in response to α-hemolysin treatment, suggesting a role for the inflammasome in their response to α-hemolysin.

Conclusions:

This work demonstrates that ADAM10's receptor and not its protease activity, is important for inflammasome activation by α-hemolysin in human monocytes. Preliminary evidence also suggests that the inflammasome may play a role in epithelial cell responses to α-hemolysin.

PREFACE

Chapter Two of this dissertation was previously published. Permission to include was provided by MDPI:

> Ezekwe, E. A., Weng, C. & Duncan, J. A. ADAM10 Cell Surface Expression but Not Activity Is Critical for Staphylococcus aureus α-Hemolysin-Mediated Activation of the NLRP3 Inflammasome in Human Monocytes. *Toxins (Basel)* **8,** (2016).

Chapter Three of this dissertation was conducted in collaboration with the Lab of Dr. Scott H. Randell with the assistance of Jeeho Kim. The Randell lab provided the human tracheobronchial epithelial for all experiments.

The outlined work was supported by the National Institutes of Health AI088255 and the Burroughs Wellcome Fund Career Award for Medical Scientists awarded to Joseph Duncan and the National Institutes of Health through the UNC Medical Scientist-Training Program (GM008719) and the Robert Watkins Fellowship from the American Society for Microbiology awarded to Ejiofor Ezekwe.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS:

ADAM10: A Disintegrin And Metalloprotease 10

Agr: accessory gene regulator

AIM2: absent in melanoma 2

ALI: air-liquid interface

ASC: apoptosis-associated speck-like protein containing a caspase recruitment domain

ATP: Adenosine triphosphate

CA-MRSA: Community Acquired Methicillin Resistant *Staphylococcus Aureus*

CAD: caspase-activated DNase

CARD: Caspase activation and recruitment domain

CGD: Chronic granulomatous disease

CLR: c-type lectins receptors

CNS: Central nervous system

DAMP: danger-associated molecular patterns

DNA: Deoxyribonucleic acid

DNase: deoxyribonuclease

dsDNA: double stranded deoxyribonucleic acid

Flu: Influenza

hAE: Human airway epithelium

Hla: α-hemolysin

HMGB1: High mobility group box 1

hTBE: human tracheobronchial epithelial cell

IL-1B: Interleukin-1-beta

K^+: Potassium

LDH: Lactose dehydrogenase

LPS: Lipopolysaccharide

LTA: Lipoteichoic acid

MRSA: Methicillin Resistant *Staphylococcus Aureus*

Nig: Nigericin

NLR: Nod-like receptor

NLRP3: Nucleotide-binding domain and Leucine-Rich repeat containing family Pyrin domain containing 3

PAMP: Pathogen-associated molecular patterns

PPR: Pathogen recognition receptors

RLR: Retinoic acid-inducible gene-I-like receptors

RNA: Ribonucleic acid

ROS: Reactive oxygen species

S. aureus: *Staphylococcus aureus*

siRNA: Short interfering ribonucleic acid

TLR: Toll-like receptor

CHAPTER ONE: Introduction

Innate Immunity and the NLRP3 inflammasome:

The innate immune system is one of the major functional barriers against infection. This system utilizes gene encoded pathogen recognition receptors (PRRs) to sense and respond to danger signals external and internal to the cell. PRRs are critical to the proper function of the innate immune system and are evolutionarily conserved across a wide array of organisms from *Drosophila* to human *[1-5].* Although immune cells of hematopoietic origin tend to express the widest array of PRR, most cell types express some PRRs allowing innate immune responses to be initiated at sites of invading microorganisms. These receptors respond to a broad array of stimuli, which have been termed danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). Some of the molecular pattern recognized by PRRs include lipids (e.g. lipopolysaccharide from gram-negative bacteria), proteins (e.g. porins from Neisseria species), bacterial cell wall components (e.g. peptidoglycan), nucleic acids (e.g. bacterial and viral DNA and RNA), and carbohydrates (e.g. Mannan from *candida albicans*) [2, 6]. PRR activation by DAMPS or PAMPS leads to a number of different cellular responses that have evolved to curtail pathogen invasion or replication including: production of cytokines that recruit cells of the immune system as well as induce programs of antimicrobial response, production of antimicrobial peptides,

priming of the adaptive immune system, production of reactive oxygen species, autophagy to contain pathogens and even programed cell death to contain infections and promote inflammation [2, 4, 6-11].

In mammals, there are many signaling proteins that fall under the umbrella of PRRs including Toll-like receptors (TLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), c-type lectins receptors (CLRs), and the Nod-like receptor (NLRs) proteins [11, 12]. The macromolecular structure known as the inflammasome is an effector mechanism common to several innate immune signaling pathways. The canonical inflammasome acts as platform for the activation of the cysteine proteinase, Caspase-1. Inflammasomes utilize a number of different PRR as sensors to initiate Caspase-1 activation. PRRs that can serve as the sensor components for the inflammasome include: AIM2 (absent in melanoma 2; a cytosolic dsDNA sensor) [13] and a number NLR family members including NLRP3 [4, 7, 14-16]. Caspase-1 activation leads to the processing, and release of the cytokines IL-1β and IL-18 [3, 4, 8, 17]. Inflammasome activation also leads to a form of inflammatory cell death termed pyroptosis [3, 18-20]. Pyroptosis is defined by being initiated by the pro-inflammatory caspases-1 and 11 leading to pore-formation in the cellular membrane and permeability to small molecular weight dyes. This leads to cell lysis due to the influx of water and ions and release of pro-inflammatory mediators such as HMGB1 (High mobility group box 1) [19-21]. Pyroptosis differs from apoptosis, a non-inflammatory pathway of programmed cell death, in a number of ways. Pyroptosis does not lead to the characteristic internucleosomal cleavage of DNA seen in apoptosis caused by activation of CAD (caspase-activated DNase). Apoptosis is also non-lytic, with intracellular

contents being contained within membrane bound apoptotic blebs, while pyroptosis is marked by lytic activity leading to cell swelling, rupture, and release of intracellular contents. Finally, the inflammatory caspases-1 and 11 cause pyroptosis, while apoptosis is executed by caspases-3 8, 9 and 10 [19, 20]. Downstream of caspase-1 activation is gasdermin D, a caspase-1 and caspase-11 substrate, which upon cleavage can induce cell death. Loss of gasdermin D greatly diminishes pyroptosis and the N-domain alone is enough to cause pyroptosis. The effector mechanisms leading to death downstream of gasdermin cleavage by caspase-1 are yet to be identified [22-24].

NLRP3 (Nucleotide-binding domain and Leucine-Rich repeat containing family Pyrin domain containing 3) was one of the first PRR sensors found to cause inflammasome formation. NLRP3 is a member of the NLR family of proteins, which are defined by a conserved three domain structure; a leucine-rich repeat domain, a nucleotide binding domain, and an N terminal effector domain [7, 14, 25]. The N terminal effector domain of NLRP3 is the pyrin domain. During inflammasome assembly, the pyrin domain of NLRP3 associates with the pyrin domain of the adapter molecule, ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain). The oligomerization of ASC with NLRP3 is thought to initiate a cascade of self-propagating ASC pyrin-ASC-pyrin associations leading to assembly of large macromolecular fibrils. Caspase-1 is recruited to this structure via CARD-CARD domain interactions between the C-terminal CARD domain of ASC and the amino terminal CARD domain of pro-Caspase-1. This super structure allows for pro-caspase-1 to cluster and brings caspase domains in proximity to allow for auto-cleavage and activate the protein [26-28].

While many NLRs are activated by specific stimuli, NLRP3 is activated by a broad range of DAMPs and PAMPS including extracellular ATP, alum, uric acid, and bacterial pore-forming toxins [17, 18, 29, 30]. In humans, NLRP3 mutations lead to the development of a group of autoinflammatory diseases termed cryopyrin-associated periodic fever syndromes including disease like Muckle-wells syndrome and familial cold autoinflammatory syndrome [31, 32]. These diseases have a spectrum of severity and are associated with periodic fevers, rashes, conjunctivitis, and arthralgias [32]. These diseases can often be treated with IL-1β blocking therapies. While it is not fully elucidated as to how these diverse sets of stimuli are encoded into NLRP3 activation, there are some general mechanisms that are thought to be important for NLRP3 inflammasome activation. These include potassium efflux, reactive oxygen species (ROS) based signaling and lysosomal destabilization [30, 33]. Potassium efflux has been strongly associated with inflammasome activity as increased extracellular K^+ or chemical inhibition of K^+ efflux have been shown to strongly inhibit NLRP3 activation in response to a broad range of activating agents including ATP and pore-forming toxin [28, 34, 35]. ROS has also been implicated in NLRP3 activation as use of ROS chemical scavengers and inhibition of NADPH oxidase activity have both been shown to block inflammasome activation in response to ATP and particulate matter including silica and asbestos [33, 36, 37]. Thioredoxin-interacting protein (TXNIP) has also been shown to bind the NLRP3 inflammasome in a ROS dependent manner in response to NLRP3 activating stimuli including uric acid crystals [38]. Interestingly, oxidized mitochondria DNA has also been shown to bind and activate the NLRP3 and inhibition of this process greatly diminishes inflammasome activation in response to ATP, the

pore-forming toxin nigericin, and alum [39]. Finally, there is strong evidence for the role of lysosomal destabilization in the activation of the NLRP3 inflammasome. It was discovered that lysosomal damage induced by NLRP3 activation factors such as uric acid crystals and silica leads to lysosomal disruption and leakage of lysosomal contents [40]. Chemical blockade of this process or treatment of cells with inhibitors of lysosomal proteases like cathepsin B can diminish inflammasome activation in response to those activating stimuli [30, 33]. Lysosomal destabilization has also been found to be an important mechanism for inflammasome activation in the context of bacterial infections as blockade of lysosomal acidification, phagocytosis or cathepsin B have all been shown to reduce NLRP3 activation in response to Group B streptococcal infection and *Streptococcus pneumonia* toxin pneumolysin [41, 42].

Bacteria often produce a number of virulence factors that use one or more of these pathways to activate the NLRP3 inflammasome [43]. *Staphylococcus aureus* is one such pathogen that is a major public health challenge and has multiple mechanisms by which it activates the inflammasome.

Staphylococcus aureus **and its role in human disease:**

Staphylococcus aureus is a gram-positive pathogen that is a major cause of morbidity and mortality in both hospital and community settings [44-46]. The bacterium is ubiquitous in our environment and a significant proportion of the population (about 20-30%) carry *S. aureus* primarily in their noses but the bacterium can colonize other parts of the body including the axillae, vagina, pharynx and skin [46-51]. Carriage of *S. aureus* is a risk factor for infection with the bacterium [48-51]. *S. aureus* is primarily

spread by skin to skin contact with a carrier or by contact with contaminated surfaces. *S. aureus* is capable of causing infection in normal healthy hosts but there a number of conditions that can lead to increased susceptibility to infection. This is particularly the case in conditions where the epithelial barrier is disrupted like traumatic injury, surgeries, or insertion of medical devices [45, 46, 52]. Additionally, patients with compromised granulocyte function through genetic conditions like CGD (chronic granulomatous disease) or through administration of leukoablative chemotherapy have increased risk of invasive *S. aureus* infection [53]. Finally, *S. aureus* has the capacity to form biofilms in invasive infection that can lead to prolonged and difficult to treat infections. Biofilms are most likely to be formed on non-biologic substances such as prosthetic joints and intravenous catheters [46, 54]. The bacterium is also capable of establishing niches in both the household and community settings by persisting on surfaces and being successfully transmitted from person to person, allowing for its wide dissemination from public places to the home and back again. This allows for continuous spread that may serve as a key sources for recurrent infections [55].

Due to the emergence of resistant strains like Methicillin Resistant *Staphylococcus aureus* (MRSA) the bacterium is becoming a serious public health challenge [44, 46, 56-58]. *S. aureus* was initially susceptible to penicillin but resistance to the drug resulted from acquisition of a betalactamase. This resistance soon became widespread. Oxacillin, methicillin and several other penicillin-derived drugs resistant to beta-lactamase were used effectively for many years [59]. Methicillin resistance resulted from acquisition of a PBP2A, a penicillin binding protein that does not bind methicillin or related penicillins and was first found a year after the introduction of methicillin in 1961

[46, 60]. Methicillin resistance was initially only found in *S. aureus* from hospital acquired strains that persisted in patients who were highly exposed to antibiotics. Then MRSA became widespread by the 1970s [60, 61]. Once mainly an issue in hospitalized patients, community-acquired MRSA (CA-MRSA) infections have been on the rise and often cause infections in previously healthy individuals [56-58]. This spread of antibiotic resistance has led to a need to better understand pathogenesis and develop novel therapeutic strategies

S. aureus is capable to causing serious infections in numerous tissue types including skin and soft tissues, bone, blood, and the lungs [45, 46, 62]. *S. aureus* skin infection is often characterized by the formation of abscesses but can lead to cellulitis, or in the most severe cases necrotizing fasciitis which can lead to tissue destruction and limb loss. In many communities across the United States MRSA is the most common cause of skin and soft tissue infections [63]. Treatment of S. aureus skin infections often includes lancing and drainage of abscesses in additional to treatment with oral antibiotics [56, 63]. Due to the prevalence of MRSA in skin infections beta-lactam antibiotics are no longer recommended for treatment of staphylococcal skin infections. So severe skin infections are often treated with more powerful antibiotics like parenteral vancomycin and linezolid [62, 64].

S. aureus is also the major causative agent in bone infection (osteomyelitis) and joint infections (infectious arthritis) [65, 66]. This can occur either through the spread of infection from surrounding tissue like skin and soft tissue or through the circulatory system due to *S. aureus* bacteremia [62]. The main risk factors for osteomyelitis include age, history of injection drug use, immunosuppression, or diabetes mellitus [52]. Often

staphylococcal osteomyelitis is difficult to treat requiring prolonged antibiotic courses of greater than 8 weeks to clear the infection [52]. If the infection affects artificial joints a debridement and implant retention procedure is used in conjunction with antibiotic treatment to save the implant [67, 68]. In many cases, S. aureus prosthetic joint infection is treated with prosthesis removal followed by prolonged antibiotic therapy. A new prosthetic joint replacement can be inserted after therapy [69].

Bacteremia is another major infection often caused by *S. aureus*. Bacteremia can lead to seeding of other sites, causing infections, particularly abscesses, in other parts of the body. Intravenous devices including catheters, pacemakers, defibrillators, and grafting materials used in revascularization procedures all pose serious risk for development of *S. aureus* bacteremia [45, 62]. Some other risk factors associated with *S. aureus* bacteremia are injection drug use due to inoculation of bacteria from contaminated skin, and hemodialysis because of the prolonged need for IV catheterization [44]. *S. aureus* bacteremia can have high rates of mortality ranging from 10-30% and mortality is often affected by the age and health status of the patient [70]. Patients often require as least two weeks of antibiotic treatment for uncomplicated infections and much longer treatment durations for patients with complicated disease like patients with ongoing disease of the heart valves [62, 71].

S. aureus is also an important causative agent of pneumonia in both the hospital and community settings, being among the top causative agents in hospital acquired infections [72-75]. Health-care associated *S. aureus* infection tends to affect elderly patients with multiple underlying diseases and often a history of ventilator use. This can lead to bacteremia, which is associated with poor outcomes [76, 77]. Community-

acquired pneumonia can often present as a superinfection after flu or other viral infections and lead to severe disease in previous healthy patients [78-80]. This was thought to be a major cause of deaths during the 1918 Flu pandemic [81]. A subset of patients with MRSA pneumonia can develop the life-threating complication of necrotizing pneumonia, which is associated with coughing up blood (hemoptysis), acute respiratory distress, pleural effusion and leukopenia. Additionally, strains that produce the Panton-Valentine toxin are associated with increased incidence of necrotizing pneumonia [81-84].

Staphylococcus aureus α-hemolysin: Structure, Function and Pathogenesis:

S. aureus has a number of virulence factors that are important for promoting its pathogenesis. These include exotoxins, immunomodulatory proteins, and protective factors that improve resistance to immune-mediated killing [45, 46]. *S. aureus* generates an array of exotoxins that damage or compromise host cell membranes. Many of these toxins are secreted as subunits that assemble into beta barrel pores in host cell membranes. Most *S. aureus* beta-barrel, pore-forming toxins are assembled from two different protein subunits and have been termed bicomponent leukotoxins [85]. However, the *S. aureus* toxin, alpha hemolysin (or alpha-toxin) forms beta-barrel pores from a single subunit, encoded by the *hla* gene [86]. These pore forming toxins are recruited to host target cells by high affinity toxin receptors on the host cell surface. The range of host cells affected by each toxin is dictated by the repertoire of toxin receptors. These toxin receptors tend to be host cell membrane proteins with tissue specific expression [85, 87, 88].

α-hemolysin (Hla) or alpha-toxin is one of the best-studied pore-forming toxins. Studies pointing to the hemolytic activity of *S. aureus* α-toxin have been on going for much of the last century. α-hemolysin's contributions to pathogenesis were first thoroughly explored after a vaccination incident in which several children died after exposure to a diphtheria toxin-anti toxin vaccination contaminated with *S. aureus* [86]. The authors described the hemolytic and dermatonecrotic activity of *S. aureus* supernatants upon injection into rabbits and postulated the existence of a single factor being responsible for the observed biological effects [89, 90]. Hla was later isolated and it was postulated the toxin mediated cellular damage by disrupting host cell membranes [91]. This conclusion was supported by the speed of cell lysis and leakage of low molecular weight markers including alpha-aminoisobutyric acid seen upon Hla intoxication of susceptible cells, and the presence of pore-like structure in the membrane of intoxicated cells [86, 92-94]. Not all cells are equally sensitive to Hla intoxication and early in vitro studies models of Hla intoxication used rabbit erythrocytes, which are highly sensitive to toxin treatment [95, 96]. Interestingly, human erythrocytes are comparatively more resistant to Hla intoxication while other human cell types including platelets and monocytes are highly sensitive [97, 98]. Hla production by the bacterium is regulated by the accessory gene regulator (*agr*) locus through the regulatory RNA molecule, RNA III that becomes active during the late-log and stationary phases of growth [99, 100]. This ties the highest production of Hla to the late-log/stationary phase of bacterial growth in culture. The Sae and Sar regulatory systems also play a role in Hla regulation. Sae regulates Hla transcription and Sar regulates Hla

production by enhancement of *agr* activity but can also promote Hla production in an *agr* independent fashion [101-103].

Hla is β-barrel pore-forming toxin that is secreted as a soluble monomer, which oligomerizes and forms a heptameric pore in the membranes of intoxicated cells [94, 104]. The assembled toxin has three distant structural motifs, the cap domain which is the externally facing portion of the toxin, the rim domain which interfaces with the external portion of the membrane, and the stem domain which is the structure that spans the width of the membrane [105]. The pores formed by these structures are approximately 1-3 nm in size and allow for the passage of molecules between 1 to 4 kDa in size [94]. There are a number of factors that have been shown to be important for Hla binding to host cell membranes. These include the presence of cholesterol as cholesterol depletion leads to decreased Hla binding and phosphocholine head group as depletion of the phosphocholine head groups with sphingomyelinase also greatly diminishes toxin binding [106]. Additionally, it was recently discovered that Hla binding to the cell surface metalloprotease ADAM10 (A Disintegrin and Metalloprotease 10) mediates the aforementioned differences in cell type sensitivity to the actions of the toxin [107]. There is a strong correlation between ADAM10 expression and susceptibility to Hla-mediated cytolysis. Loss of ADAM10 in cells blocks Hla's ability to bind and lyse cells, and ADAM10 is pulled down with Hla in intoxicated cells [107].

Hla is widely expressed in clinical isolates of *S. aureus* and is thought to be a critical virulence factor in human disease [86]. There are several studies in humans showing an association between anti-Hla antibody titers and protection from *S. aureus* infection [108-110]. One study in adults with high sepsis risk showed that higher

antibody titers to *S. aureus* exotoxins including α-hemolysin reduced their risk of disease [109]. In a second study completed in children, patients with invasive skin infection developed high anti-Hla titers and after a one-year follow-up, children that maintained high anti-Hla titers were protected from recurrent disease [108]. α-hemolysin is also a critical virulence factor in mouse models of disease [111-115]. In studies that compare α-hemolysin secreting *S. aureus* strains to mutant strains devoid of hemolysin secretion, numerous models show decreased pathogenesis. This decreased pathogenesis has been seen in models of *S. aureus* pneumonia [111-113], peritonitis [116, 117], skin infections [115, 118], corneal infections [119], endocarditis [120] and mastitis [121, 122]. In mouse models of *S. aureus* pneumonia, the loss of Hla production leads to stark reductions in the ability of the bacterium to cause lethal disease [111, 112]. In mouse models of *S. aureus* peritonitis, loss of Hla expression and active or passive immunization against Hla led to dramatically reduced lethality but not abscess formation cause by bacterial infection [116, 117]. In *S. aureus* skin infection loss of Hla expression or immunization of mice against Hla lead to reductions in dermonecrosis caused by *S. aureus* and reduction in the size of lesions at the site of infection [115, 118]. Corneal *S. aureus* infection in a rabbit leads to damage and inflammation marked by epithelial erosions some of which could be replicated by treatment of rabbits with Hla alone. Loss of Hla expression in *S. aureus* corneal infecting strains led to decreased corneal damage and the absence of epithelial erosions [119]. In a model of rabbit endocarditis, loss of Hla expression lead to decreased pathology marked by low levels of intravegetation staphylococcal densities as compared to parental strains with normal levels of Hla production [120]. In a mouse

model of *S. aureus* mastitis, loss of Hla expression led to decreased mortality, reduced invasion and lesser lesions than after infection with Wild-type strains [121]. All these studies illustrate the importance of Hla in reducing disease severity and mortality in animal models of infection.

An important component in α-hemolysin mediated pathogenesis is it ability to induce immune activation in leukocytes. It was first discovered in the late 1980s that Hla intoxication of human monocytes led to secretion of IL-1β [97]. Hla has now been shown to activate the NLRP3 inflammasome in human and murine monocytes and neutrophils leading to IL-1β secretion and NLRP3 dependent cell death [123-125]. This activation is dependent on K^+ efflux as high extracellular levels of K^+ diminish NLRP3 dependent IL-1β secretion and cell death [123, 125]. The consequences of Hla-mediated NLRP3 activation are complex and can have both positive and negative outcomes. In murine models of *S. aureus* pneumonia NLRP3 inflammasome activation is deleterious to the host [124]. Mice lacking NLRP3 showed decreased lung pathology, decreased IL-1β secretion in the lung and decreased mortality from pneumonia when treated with either Heated Killed Staphylococcus aureus + Hla or whole bacteria capable of secreting Hla. Additionally, loss of NLRP3 in this setting did not affect the bacterial burden or disease clearance in these mice [124]. Alternatively, in the skin diminished IL-1β signaling, which was shown to be NLRP3 and ASC dependent, leads to decreased bacterial clearance and increased lesion size [126]. More recent studies in mice have confirmed the importance of Hla activity on immune cells in mouse models of disease in determining disease outcome. Loss of the cell surface receptor for Hla, ADAM10 (A Disintegrin and Metalloprotease 10), in cells of the myeloid linage led to exacerbation of

skin infection but decreased severity of pneumonia caused by *S. aureus* [127]. Mice with loss of ADAM10 expression in cells of the myeloid lineage saw increased skin abscess size, dermonecrosis, and increased bacterial burden in their lesions. In a *S. aureus* pneumonia model, loss of ADAM10 expression in myeloid cells, reduced mortality caused by the bacterial infection. These finding were attributed to decreased inflammatory signaling as loss of ADAM10 led to defects in the ability of ADAM10$^{-/-}$ macrophages and neutrophils to produce IL-1β in response to treatment with Hla [127].

CHAPTER TWO: ADAM10 Cell Surface Expression but Not Activity is Critical for Staphylococcus aureus α-Hemolysin-Mediated Activation of the NLRP3 Inflammasome in Human Monocytes

2.1 Introduction:

Staphylococcus aureus is a gram-positive bacterium that is responsible for causing infections that lead to severe morbidity and mortality. *S. aureus* causes infections in a broad range of host tissues including the skin, vascular, and respiratory systems[45]. It is also a growing public health concern because of the emergence of antibiotic resistance including methicillin resistant strains that cause both hospital and community acquired infections [58, 82, 128].

Staphylococcus aureus produces an array of virulence factors that are important for the pathogenesis of infections caused by these bacteria. Among these virulence factors are several pore-forming toxins that attack host cells by permeabilizing their cell membranes. The pore-forming toxin, α-hemolysin (Hla) is one of the best studied of these factors and is critical for virulence in mouse models of infections caused by *S. aureus*[86, 111, 112, 115]. Hla is active against cells from a variety of tissues including respiratory epithelium, endothelium, immune cells, and keratinocytes [86]. This broad range of cellular targets stems from the nearly universal expression of the host cellular receptor for Hla, A Disintegrin and Metalloproteinase-10 or ADAM10 [107]. Additionally,

the level of ADAM10 expression on a given cell type dictates sensitivity to the toxin [107]. Genetic loss or chemical inhibition of ADAM10 protects cellular targets from Hla in tissue culture and mitigates Hla-induced pathology in mice [107, 127, 129-132]. Further, mice treated with ADAM10 inhibitors or with tissue specific knock out of ADAM10 exhibit resistance to *S. aureus* infection. In epithelial and endothelial cells, Hla's interaction with ADAM10 leads to the activation of ADAM10's metalloproteinase activity. This enhanced protease activity leads to the cleavage of cell surface adhesins, like E-cadherin, and disruption of cell-to-cell contacts [107, 131]. Consequently, it is believed that activation of ADAM10 by Hla is important for *S. aureus* ability to penetrate epithelial and endothelial barriers and thus cause invasive infection.

Hla is also a potent activator of the innate immune signaling protein, Nucleotide-binding domain and Leucine-Rich repeat containing family Pyrin domain containing 3 (NLRP3) inflammasome [123, 125]. The active NLRP3 inflammasome is a protein complex containing NLRP3 and the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) which is responsible for activation of the cysteine proteinase caspase-1. Active caspase-1 then goes on to proteolytically process the cytosolic, pro-inflammatory cytokines pro-IL-1β and pro-IL-18 into their active, secreted forms [133, 134]. In addition, NLRP3 activation leads to a program of necrotic cell death termed pyroptosis [134-136]. Mice with genetic deletion of *Nlrp3* have diminished inflammation in Hla-induced pneumonitis models and decreased severity of infection in a mouse model of Staphylococcal pneumonia [124]. Conversely in murine models of *S. aureus* skin infection IL-1β production is important for proper bacterial clearance [126, 127]. In this study, we sought to determine the role of Hla induced ADAM10 activation in

the NLRP3 inflammasome signaling pathway. We show that in human monocytes ADAM10 mediates NLRP3 activation and that the level of ADAM10 cell surface expression and not its protease activity, is important for NLRP3 activation.

Materials and Methods:

siRNA transfection of THP1 cells:

THP1 cells were purchased from ATCC (Manassas, VA, USA). THP-1 cells were maintained in RPMI 1640 media (Gibco, Thermo Fisher Scientific, Waltham, MA. USA) supplemented with 10% Fetal calf serum and Penicillin and Streptomycin as described in prior studies [123, 137]. Cells were transfected with the TransIT-TKO® transfection reagent from Mirus (Madison, WI, USA) per the manufacturers protocol (product #: MIR 2150). Cells were transfected with 50nM of pooled siRNA constructs in 6 well plates. siRNAs were obtained from the GE/Dharmacon (Lafayette, CO, USA) siGENOME library. A set of 4 siRNA was ordered for both ADAM10 (product #: D-004503-01-0002, D-004503-03-0002, D-004503-04-0002, D-004503-05-0002) and NLRP3 (product #:D-017367-01-0002, D-017367-02-0002, D-017367-03-0002, D-017367-04-0002) and pooled prior to transfection. Control siRNA used, include Lamin A/C (product #: D-001050-01-05), and a pool of non-targeting siRNA (Non-targeting siRNA 2, 4, and 5) (#: D-001210-02, D-001210-04, D-001210-05), were ordered from GE/Dharmacon (Lafayette, CO, USA) as well. Cells were incubated for 3 days after transfection prior to use in subsequent experiments.

Immunoblots analysis:

Westerns were performed as done by Craven et al.[123] Primary antibodies used were Adipogen Life Sciences (San Diego, CA, USA) anti NLRP3 monoclonal antibody (#:AG-20B-0014-C100) and ABCAM (Cambridge, MA, USA) anti-ADAM10 rabbit polyclonal (#: ab1997). Blots were imaged using a FluorChem E system from ProteinSimple (San Jose, CA, USA)

Treatment of Cells with inhibitors:

GI254023x was purchased from TOCRIS Bioscience (Bristol, UK) (product #: 3995) or Sigma (St. Louis, MO, USA) (product #: SML0789) and resuspended in DMSO to either 10 mM or 20 mM stocks. TAPI2 was purchased from Enzo Life sciences (Farmingdale, NY, USA) (product #: BML-PI135-0001) or Sigma (St. Louis, MO, USA) (product #: SML04020) and resuspended in pyrogene free water to 10mM. Cells were then treated with vehicle or inhibitor as indicated prior to challenge with inflammasome activating stimuli.

Treatment of cultured cells with inflammasome activators for cytokine studies:

THP-1 and U937 cells were suspended in RPMI 1640 media (Gibco, Thermo Fisher Scientific, Waltham, MA. USA) supplemented with 10% fetal calf serum and Penicillin and Streptomycin at $1x10^6$ cells/mL and plated in tissue culture treated 24 or 48 well plates. U937 cells were treated overnight with phorbol 12-myristate 13-acetate (PMA) (Sigma, St. Louis, MO, USA product #: P1585). When indicated, the NLRP3 inflammasome was primed in the cells by treatment with *E. coli* lipopolysaccharide (Invivogen, product #: tltl-3pelps) at a concentration of 100ng/ml for 3 hours. Recombinant α-hemolysin prepared as described by Craven et al. [123], α-hemolysin (30 µg/mL) for THP1 cells or (10 µg/mL) for U937 cells, Nigericin (50µM) (Sigma, St. Louis, MO, USA product #: P1585), or ATP (5 mM) was added at the indicated concentrations to induce NLRP3 inflammasome activation. After 1 hour, Cells and supernatants were collected by centrifugation at 17000 x g for 3 minutes. Cell culture supernatants were stored frozen at -80°C until assayed for cytokine content. LDH activity was measured in supernatants using the Cyto-tox ONE kit (Promega, Madison,

WI, USA, product #: G7891) read on a Perkin Elmer inspire plate reader. IL-1β was measured in cell culture supernatants using either Perkin Elmer Alphalisa (Product #: AL220C, Waltham, MA, USA) or BD bioscience (product #: 557953, San Jose, CA, USA) ELISA kits as per manufacturer protocols.

Treatment of cultured cells with inflammasome activator for cell death studies:

Cells were suspended in fresh media at $1x10^6$ cells/mL and plated in tissue culture treated 24 or 48 well plates. Recombinant α-hemolysin was prepared as described by Craven et al.[123] Cells were treated with α-hemolysin (30μg/ml) or Nigericin (50μM) at the indicated concentrations to induce NLRP3 inflammasome activation. After 1 hour, Cells and supernatants were collected by centrifugation at 17,000 x g for 3 minutes. Prior to the end of the experiment a control group of cells were lysed using 1% Triton X-100 as a lysis control. Cell culture supernatants were stored frozen at -80°C until assayed for LDH production.

Propidium Iodide studies:

Cells were suspended in fresh media at $1x10^6$ cells/mL and plated in tissue culture treated 48 well plates at 300 μL per well. Propidium Iodide was added to cells 5 minutes prior NLRP3 activating stimuli, α-hemolysin (30 μg/ml) or Nigericin (50 μM). Cells were incubate for 1 hour prior to cells being washed once with PBS and fixed using BD stabilizing fixative (product #: 338036, San Jose, CA, USA). Cells were then assayed by flow cytometry for Propidium iodide staining on a BD accuri C6 flow cytometer (San Jose, CA, USA).

Measurement of caspase-1 activity in treated cells:

Caspase-1 activity was measured using the FLICA® 660 caspase-1 assay kit,

far-red fluorescence (Product #: 9122) from ImmunoChemistry Technologies LLC (Bloomington, MN, USA). Cells were plated in a 48 well at 300 µL per well at $1x10^6$ cells/ml. The FLICA reagent was added and incubated with cells for 15 minutes prior to the addition of the indicated inflammasome activator, α-hemolysin (30 µg/ml) or Nigericin (50 µM). Cells were then incubated for an additional 30 minutes, transferred to 1.5 ml tubes, and washed twice with PBS. Cells were resuspended in 300 µl of PBS and 50 µL of supplied fixative. Accumulation of fluorescent caspase-1 inhibitor was assayed by flow cytometry on a BD accuri C6 flow cytometer (San Jose, CA, USA)

Measurement of ADAM10 cell surface immunofluorescence staining protocol:

siRNA transfected cells were resuspended in 2% BSA in 1x PBS with 0.1% sodium azide at a concentration of $1x10^7$ cells/ml. One-hundred microliters of cells were added into sterile tubes with 5ul of either PE mouse IgG1, κ Isotype control Ab from Biolegend (San Diego, CA, USA) (product #: 400113) or PE anti-human CD156c (ADAM10) from Biolegend(product #: 352703). Cells were incubated with antibodies for 15-20 minutes in the dark at 4°C. Cells were then twice washed with 2mL of buffer and spun down at 350 G for 5 minutes. Cells were resuspended in 500 µl of buffer prior to being analyzed by flow cytometry on a BD accuri C6 flow cytometer (San Diego, CA, USA).

In the inhibitor time course assays cells were resuspended in fresh RPMI media at a concentration of $1x10^6$ cells/ml. Cells were plated at 300 µl per well in a 48 well sterile tissue culture treated plate. Inhibitors and vehicle controls were added at the indicated times after which cells were washed with 500ul of cell staining buffer from Biolegend® (San Diego, CA, USA) (product #: 420201) and spun down at 2500 RPM for

3minutes. Cells were resuspended in 100 µl of cell staining buffer prior to the addition of 5 µl of either PE mouse IgG1, κ Isotype control Ab from Biolegend (product #: 400113, San Diego, CA, USA)) or PE anti-human CD156c (ADAM10) from Biolegend (product #: 352703, San Diego, CA, USA)). The plate was incubated at 4°C for 15-20 minutes in the dark. After which the cells were washed twice with 500 µl of cell staining buffer, spun down at 2500 RPM for 3 minutes and resuspended in a final volume of 300ul prior to being analyzed by flow cytometry on a BD accuri C6 flow cytometer.

Measurement of metalloprotease activity in cells treated with TAPI2:

Metalloprotease activity was measured using the Mca-P-L-A-Q-A-V-Dpa-R-S-S-S-R-NH_2 Fluorogenic Peptide Substrate III from R&D systems. (Catalog #: ES003, Minneapolis, MN, USA) THP1 cells were resuspended at a concentration of 1 $x10^6$ cells/ml in 25 mM Tris buffer at a pH 8.0 as per manufacturers protocol. The assay was run in a 96 well plate in a 100 µL reaction with a final concentration of 10 µM of substrate per well. Reads were done every 2 minutes and TAPI2 100 µM or vehicle were added after 12 minutes and reads continued for 30 minutes.

Purified ADAM10 activity assay:

This assay was conducted using the SensoLyte® 520 ADAM10 Activity Assay from ANASPEC (Fremont, CA, USA) as per manufacturers protocol. (Catalog #: 72226) GI254023X was used a final concentration of 20 µM and TAPI2 100 µM. Reads were taken every five minutes for a total of 60 minutes.

2. Results:

ADAM10 expression is required for α-hemolysin induced cell death in human monocyte-derived cells

Previous work has shown ADAM10 to be important for the activity of α-hemolysin (Hla) towards a variety of host cell types [107, 130, 131]. Loss of expression of ADAM10 using either siRNA in immortalized human epithelial cells or tissue specific genetic knock-out in mouse epithelial cells blocks Hla induced cell death [107]. Lung epithelium specific knock out of the ADAM10 gene protects mice from pulmonary injury induced by Hla inhalation or live *S. aureus* instillation [129]. Targeted deletion of ADAM10 in mouse myeloid cells also protects them from Hla induced death in a murine pneumonia model [127]. We sought to confirm that ADAM10 expression is required for human monocytic cell responsiveness to Hla. Monocytic THP1 cells were transfected with siRNA directed against ADAM10 (both individual siRNAs and pooled siRNA) and after three days cell surface expression was characterized by flow cytometry. We were able to achieve significant reductions in detectable cell surface expression of ADAM10 as compared to our non-targeting siRNA controls (Figure 2.1B-D). Immunoblot analysis also showed reductions of total ADAM10 (Figure 2.1E). Because it has previously been shown that NLRP3 expression is required for Hla-induced cell death in monocytes, siRNA directed to NLRP3 were used as a positive control. While siRNA directed to NLRP3 reduced NLRP3 expression levels, it had no effect on ADAM10 levels measured by flow cytometry or immunoblot (Figure 2.1C-E). siRNA transfected THP1 cells were then treated with Hla and cytolysis was subsequently assessed by measuring release of

cytosolic LDH into the culture supernatant. Depletion of NLRP3 or ADAM10 by siRNA significantly reduced Hla-induced LDH release as compared with controls. (Figure 2.1F) To demonstrate the effect of ADAM10 depletion was specific to Hla-induced cell death and not generally suppressive of NLRP3 activation, siRNA transfected cells were also treated with nigericin, a pore forming toxin known to activate NLRP3, and assessed for cell death [138]. As expected, NLRP3 depletion blocked nigericin-induced death while ADAM10 depletion had no effect. (Figure 2.1G)

ADAM10 expression is required for Hla-mediated NLRP3 inflammasome activation in monocytes.

Hla-induces inflammatory cell death by activating the NLRP3 inflammasome in monocytes [123]. Activation of the NLRP3 inflammasome leads to activation of the cysteine proteinase caspase-1 and subsequent processing and secretion of the cytokines IL-1β and IL-18 [133]. To determine whether ADAM10 is required for Hla-mediated NLRP3 activation, THP1 cells transfected with individual siRNA directed against ADAM10 as well as pooled siRNA targeting ADAM10 and NLRP3 were challenged with α-hemolysin and IL-1β secretion by the cells as well as activation of caspase-1 were assessed. Cells depleted of ADAM10 expression using 4 different siRNA against ADAM10 all demonstrated a marked reduction in IL-1β secretion when compared to non-targeting control siRNA. (Figure 2.2A) Caspase-1 activation was assessed by accumulation of a fluorescent inhibitor of caspase-1 (caspase-1 FLICA) after toxin administration. Knockdown of ADAM10 or NLRP3 by pooled siRNA transfection of THP1 cells significantly decreased caspase-1 activation in response to

Hla (Figure 2.2B & 2.2C). Cells depleted of ADAM10 or NLRP3 expression by transfection of pooled siRNA exhibited markedly reduced release of IL-1β in response to Hla exposure. (Figure 2.2D) To test whether ADAM10 depletion effected NLRP3 activation in general or only in response to Hla, siRNA-transfected cells were treated with nigericin or ATP, which activates NLRP3 through activation of the P2X7 purinergic receptor [139]. ADAM10 knockdown did not affect secretion of IL-1β after treatment with either ATP or nigericin. (Figure 2.2D) In contrast to the reductions in Hla-induced IL-1β secretion observed after transfection with NLRP3 or ADAM10 directed siRNA, secretion of MIP-1α, a cytokine that is not dependent on caspase-1 proteolysis for secretion, was not reduced at all in these cells relative to the control siRNA transfected cells. (Figure 2.2E) Thus, ADAM10 expression is critical for NLRP3 activation by Hla but not by other NLRP3 activating stimuli.

The protease activity of ADAM10 is not required for Hla-mediated activation of NLRP3-induced cell death.

Chemical inhibition of ADAM10 using GI254023X, a specific inhibitor of ADAM10 reduces Hla-mediated cytotoxicity and cleavage of extracellular E-cadherin in epithelial and endothelial cells [129, 131, 140]. To determine whether the protease activity of ADAM10 was required for Hla-induced NLRP3 activation, we treated cells with TAPI2, a non-specific metalloprotease inhibitor or GI254023X. THP1 cells were treated with inhibitor for 15-30 minutes prior to challenge with Hla. Short-term treatment of THP1 cells (15-30 min) with these inhibitors did not reduce Hla-induced cytotoxicity (Figure 3A&B). Inoshima *et al.* demonstrated that treatment of epithelial cells with GI254023X

led to diminished ADAM10-mediated E-cadherin cleavage within minutes of addition of the inhibitor [10]. To ensure short-term treatment with ADAM10 inhibitors could suppress protease activity in the 30 min time-frame of our experiments, we tested the effect of the inhibitors on measurable protease activity. We found that TAPI2 and GI254023X were able to immediately impact the rate of peptide substrate cleavage by purified ADAM10 (Figure 2.3C). Further, total metalloproteinase activity in intact THP1 cells was immediately diminished by the addition of TAPI2 (Figure 2.3D). Combined these results demonstrate that the proteinase activity of ADAM10 is not required α-hemolysin-induced cell death.

Inhibitors of ADAM10 protease activity reduce Hla-mediated activation of the NLRP3 inflammasome through down regulation of surface ADAM10 levels.

In contrast to the findings with short-term inhibition of ADAM10, overnight treatment with GI254023X diminished Hla-mediated but not nigericin-induced cytotoxicity in THP1 cells. (Figure 2.4A). Overnight treatment with TAPI2, also caused a downward trend in Hla-induced death, though this did not meet statistical significance. (Figure 2.4B) Overnight treatment with TAPI2 or GI254023X inhibited Hla-induced IL-1β secretion but not nigericin-induced IL-1β secretion in THP1 cells while 30 minute inhibitor treatment had no effect on IL-1β secretion (Figure 2.4C-F) because inhibition of ADAM10 protease activity did not immediately diminish Hla-induced NLRP3 inflammasome activation, we tested whether prolonged inhibitor treatment altered NLRP3 expression levels using immunoblot analysis. Levels of NLRP3 were not altered by treatment of THP1 cells with GI254023X for 20 hours (Figure 2.5A), consistent with

the continued response of these cells to nigericin (Figure 2.4D&F). We then sought to determine whether chemical inhibition altered the quantity of surface expressed ADAM10. Treatment of THP1 cells with either inhibitor for 30 minutes had no significant effect on cell surface expression of ADAM10 detectable by flow cytometry. After 20 hours, treated THP1 exhibited diminished detectable surface ADAM10. (Figure 2.5A, B & C) To better understand the time dependent difference in the response of THP1 cells treated with these inhibitors, we assessed the ADAM10 cell surface expression over time during treatment with GI254023X using flow cytometry. Cell surface expression of ADAM10 decreased over time when compared to vehicle treated cells. As expected, the loss of ADAM10 expression was accompanied by similar reductions in Hla-induced IL-1β secretion at the corresponding timepoints in THP1 cells. (Figure 2.5 D & E) To ensure the effects of ADAM10 inhibition on ADAM10 cell surface expression and Hla-induced IL-1β was not limited to THP1 cells, we tested whether prolonged GI254023X exposure in U937 cells had an effect on ADAM10 levels and IL-1β secretion. U937 cells treated with GI254023X replicated the effects observed with THP1 cells. (Figure 2.6)

Discussion:

ADAM10 is important for α-hemolysin binding to target cells [107]. In addition, Hla binding leads to increased ADAM10 proteolytic activity in keratinocytes, endothelial cells, and epithelial cells [107, 129-131]. This increased activity leads to disruption in cell-to-cell contacts through cleavage of E-cadherin and plays a key role in *S. aureus* pathogenesis. In addition to causing disruption of cell-to-cell contacts, Hla is known to induce potent pro-inflammatory signals in myeloid cells, including the production of IL-1β and induction of programmed necrotic cell death [123]. These pro-inflammatory actions of Hla require host cell NLRP3 inflammasome activity [123]. Targeted deletion of ADAM10 in myeloid cells results in diminished lung levels of IL-1β in a mouse model of *S. aureus* pneumonia [13]. In a *S. aureus* sepsis model, elimination of ADAM10 from platelets and myeloid lineages resulted in diminished IL-1β observed in liver homogenates, decreased lung and liver pathology, and decreased mortality in mice [132]. We have now shown that ADAM10 is required for Hla to activate the NLRP3 inflammasome in human monocytes (Figure 2.2). Our data suggest that the diminished tissue levels of IL-1β observed in these mouse models lacking ADAM10 are the direct result of diminished inflammasome activation in myeloid cells from the infected animals. Interestingly, loss of ADAM10 has also been implicated in lung epithelial injury from other bacterial pore-forming toxins, like pneumolysin from *Streptococcus pneumonia* [129]. We have demonstrated that myeloid cell ADAM10 is not required for NLRP3 activation by nigericin, a second pore-forming toxin. The difference in ADAM10 requirement for cellular injury between epithelial cells and monocytic cells in response

to pore-forming toxins other than Hla may have to do with role of ADAM10 proteolytic activation in those cell types.

In mice, ADAM10 inhibitors prevent the loss epithelial barrier integrity that is typically induced by *S. aureus* Hla during infections of the skin and lung [129, 130]. The enhancement of ADAM10 proteolytic activity by Hla is clearly tied to that aspect of infection pathogenesis, which is likely important for the bacteria to establish invasive infection of the tissues the toxin is acting on. However, in published studies of *S. aureus* infection in mice lacking ADAM10 in myeloid lineage cells, the role of ADAM10 proteolytic activity, as opposed to high affinity Hla-binding remains unknown. Our data now demonstrate that ADAM10 proteolytic activity is not required for host inflammasome activation in isolated immune cells. Because inhibitors of ADAM10 proteolytic activity ultimately result in reduced levels of myeloid cell surface expression of ADAM10, these inhibitors can still reduce Hla-induced inflammasome activation. These data suggest that in myeloid cells ADAM10-facilitated targeting of Hla to the cell is sufficient for NLRP3 inflammasome activation, which leads to both IL-1β secretion and cell death. The exact mechanism by which these inhibitors lower ADAM10 cell surface expression remains to be explored. ADAM some family members are known to undergo autoproteolysis, it is possible that processing is important for maturation and/or proper trafficking of ADAM10 [141]. Although we have not demonstrated that pharmacologic ADAM10 inhibition reduces cell surface ADAM10 expression on other cell types, our findings open the possibility that ADAM10 inhibitors may improve outcomes in murine *S. aureus* infection models through multiple mechanisms including both reduction in ADAM10-mediated adherence factor cleavage and reduction of Hla-

receptor on multiple cell types. Further, the mechanisms by which Hla activates NLRP3 remain to be elucidated but are also still potentially attractive therapeutic targets for adjunctive therapy to antibiotic therapy in severe *S. aureus* infections.

In mouse models, the consequences of the loss of Hla activity towards myeloid cells differs based on the location of infection. The loss of ADAM10 in the immune cell compartment or global loss of NLRP3 was beneficial to the host in a mouse pneumonia model of *S. aureus* infection, reducing mortality compared to mice with intact ADAM10 or NLRP3 [127]. Loss of ADAM10 from the myeloid lineage is deleterious to the host in the skin infection model of mouse staphylococcal disease by leading to increased lesion size and bacterial burden [127]. Prior studies by our group and others have tied Hla-induced inflammasome activation to worsened host outcomes in *S. aureus* infections [124]. Genetic deletion of NLRP3 improved clinical parameters in mouse pneumonia without effecting pathogen burden, suggesting that blunting the inflammasome mediated inflammatory response to *S. aureus* could be beneficial even after infection is established [124]. This combination of findings suggested that inhibition of ADAM10 could be an attractive mechanism to reduce deleterious effects of robust inflammatory response during severe *S. aureus* infection. Because the effects of Hla on myeloid derived human cells is likely redundant to several other pore-forming, immune cell-restricted toxins, known as leukotoxins, made by *S. aureus,* the role of Hla activity on immune cells in human infection is unknown. Like Hla, these leukotoxins also activate the NLRP3 inflammasome [137, 142]. Thus, during *S. aureus* infection, the host inflammasome will be activated in immune cells regardless of the interaction between ADAM10 and Hla. The role of the NLRP3 inflammasome in mediating Hla effects on

non-immune cells remains another open question in the field. Several studies have shown Hla antibody titers are important predictors of disease outcome in patients with *S. aureus* infections [143, 144]. Gaining a better understanding of how Hla specifically contributes to human disease by targeting leukocytes and other cell types will be an important step in the development of novel, specific therapies for Staphylococcal disease.

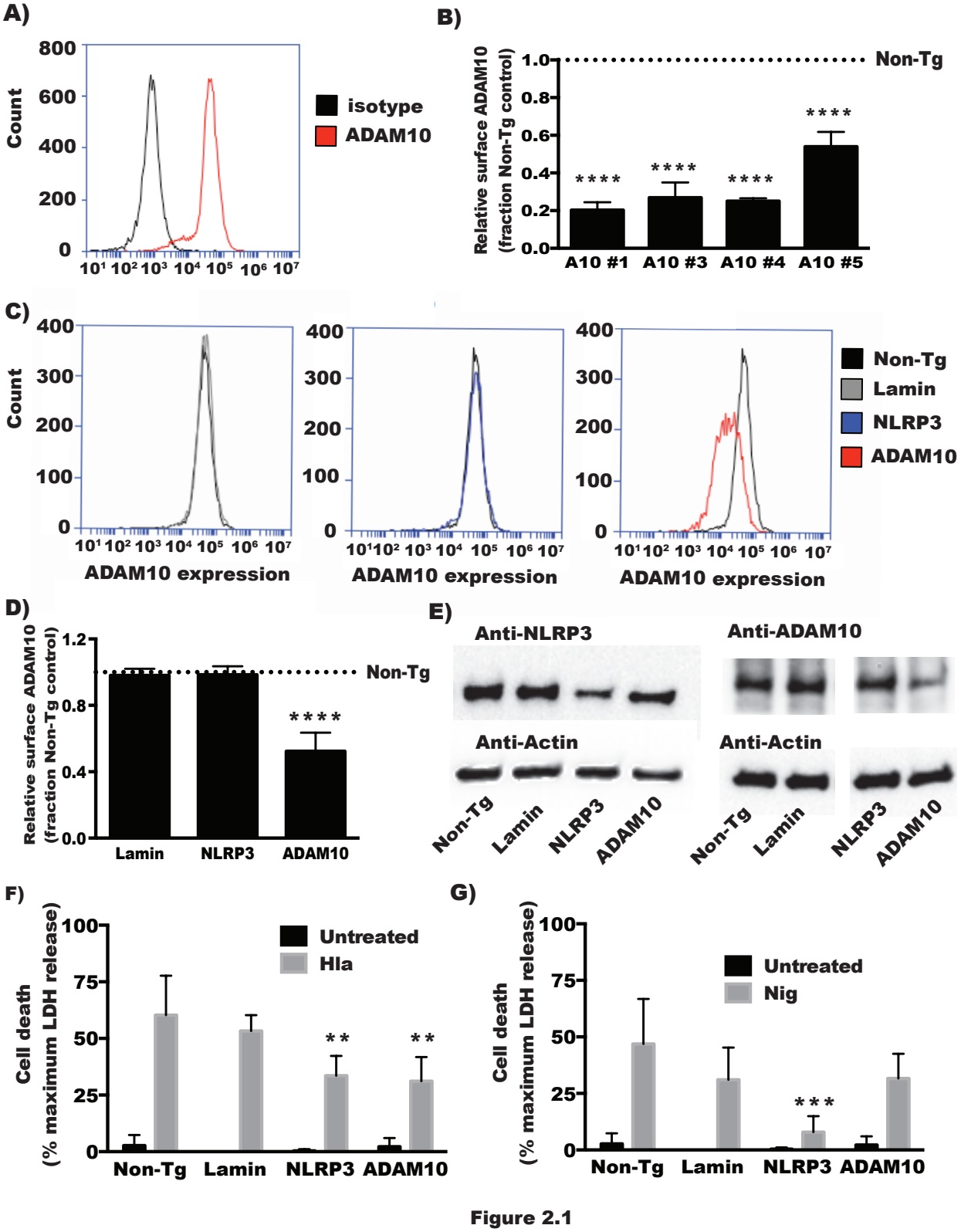
A)
Count
800
600
400
200
0
10^1 10^2 10^3 10^4 10^5 10^6 10^7
isotype
ADAM10
B)
Relative surface ADAM10
(fraction Non-Tg control)
1.0
0.8
0.6
0.4
0.2
0.0
Non-Tg
****
A10 #1
A10 #3
A10 #4
A10 #5
C)
Count
400
300
200
100
0
ADAM10 expression
Non-Tg
Lamin
NLRP3
ADAM10
D)
Relative surface ADAM10
(fraction Non-Tg control)
1.2
0.8
0.4
0.0
Non-Tg
****
Lamin
NLRP3
ADAM10
E)
Anti-NLRP3
Anti-ADAM10
Anti-Actin
Anti-Actin
Non-Tg
Lamin
NLRP3
ADAM10
F)
Cell death
(% maximum LDH release)
100
75
50
25
0
Untreated
Hla
**
Non-Tg
Lamin
NLRP3
ADAM10
G)
Cell death
(% maximum LDH release)
Untreated
Nig
***

Figure 2.1

Figure 2.1: (A) Histogram showing staining with ADAM10-directed antibody compared to isotype control measured by flow cytometry. (B-D) THP1 cells were transfected with the indicated siRNA three days prior to assessing ADAM10 cell surface expression via flow cytometry, as detailed in the experimental methods section and demonstrated in A. (B) The relative surface expressed ADAM10 mean fluorescence intensity of THP1 cells transfected with 4 different siRNA targeting ADAM10 as compared to the non-targeting control siRNA transfected cells. (C) Histogram showing fluorescence intensity of ADAM10 staining between cells transfected with non-targeting siRNA and siRNA pools targeting Lamin, NLRP3, and ADAM10. (D) The relative surface expressed ADAM10 mean fluorescence intensity of siRNA pool transfected THP1 cells as compared to the non-targeting control. For both B and D, n=3. (E) Whole cell lysates from cells transfected with the indicated siRNA pools were analyzed by immunoblot analysis with antibodies for ADAM10, NLRP3, and Actin as a loading control. (F&G) siRNA-transfected THP1 cells were treated with α-hemolysin (Hla) (30 μg/mL) or Nigericin (Nig) (50 μM) for 1 hour. Culture supernatants from untreated and toxin-treated cells were assayed for LDH production as compared to a detergent-lysis control (n=3). For B&D, **** indicates statistically significant difference from Non-Tg transfected cells ($p \leq .0001$) determined by one-way ANOVA with Dunnett's multiple comparisons testing. For F&G, ** and *** indicates statistically significant difference from Non-Tg transfected cells ($p \leq .01$ and $p \leq .001$ respectively) determined by two-way ANOVA testing with Dunnett's multiple comparison testing.

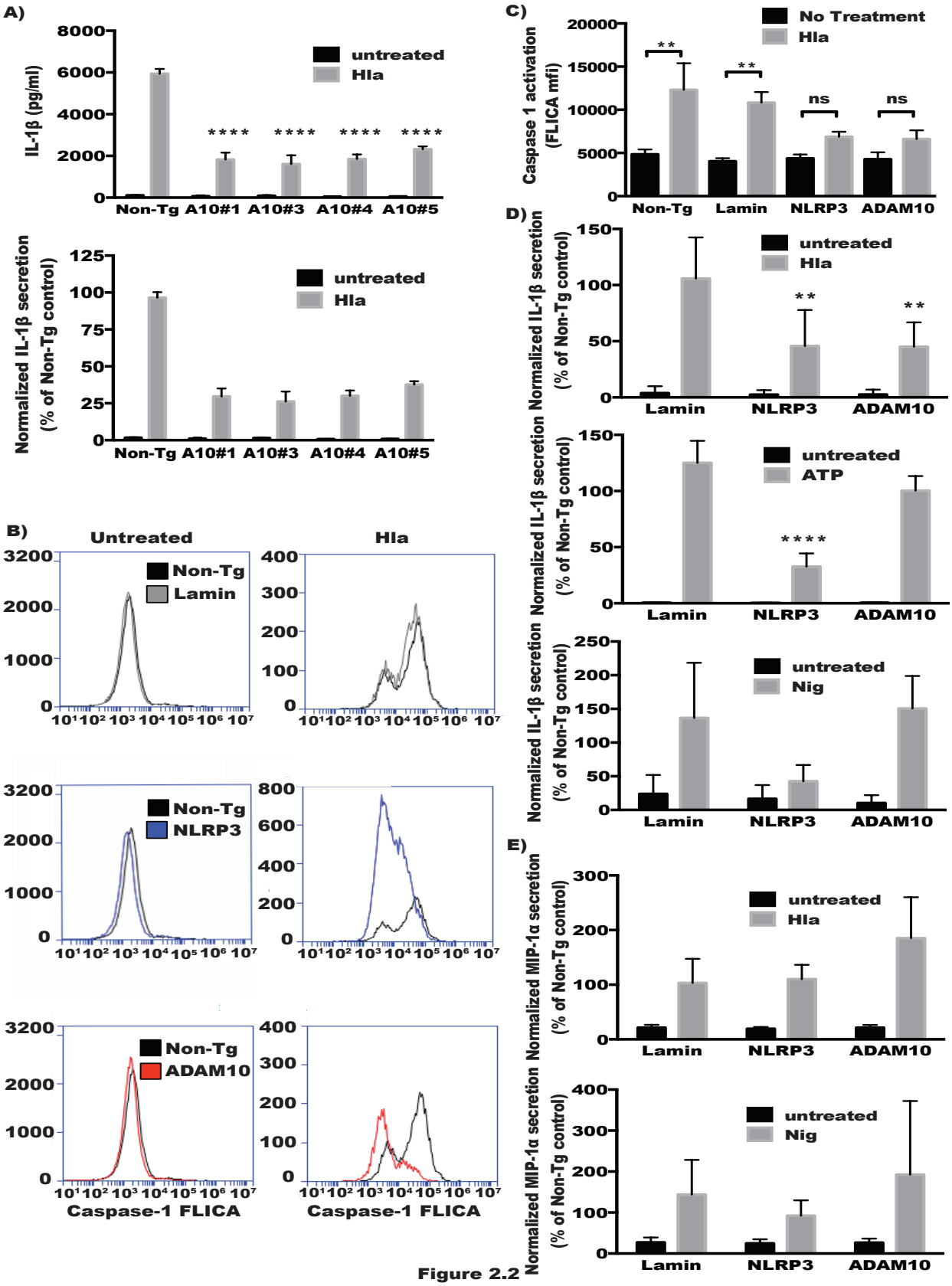
A)
IL-1β (pg/ml)
untreated
Hla
Non-Tg A10#1 A10#3 A10#4 A10#5
Normalized IL-1β secretion (% of Non-Tg control)
B)
Untreated
Hla
Non-Tg
Lamin
NLRP3
ADAM10
Caspase-1 FLICA
C)
Caspase 1 activation (FLICA mfi)
No Treatment
Hla
Non-Tg Lamin NLRP3 ADAM10
D)
Normalized IL-1β secretion (% of Non-Tg control)
ATP
Nig
E)
Normalized MIP-1α secretion (% of Non-Tg control)
Lamin NLRP3 ADAM10

Figure 2.2

Figure 2.2: THP1 cells were treated with the indicated siRNA three days prior to assessment, as in Fig 2.1. siRNA-transfected THP1 cells were treated with LPS (100ng/mL) for 3 hours before the addition of an NLRP3 simulating agent for 1 hour. Cell culture supernatants were then assayed for cytokine production. For some analysis the relative secretion of cytokine as compared with toxin-treated, non-targeting siRNA-transfected control cells is plotted. A) Absolute (top) and relative (bottom) IL-1β secretion in hemolysin treated THP1 cells transfected with individual ADAM10 siRNAs (n=3). (B) THP1 cells transfected with the indicated siRNA pool were treated with Caspase-1 FLICA reagent prior to the addition of α-hemolysin (30 μg/ml) for 30 minutes. Cells are then washed, fixed and assayed by flow cytometry. Representative histograms showing caspase-1 FLICA reagent based fluorescence in resting (left) and α-hemolysin-treated (right) cells previously transfected with the indicated siRNA. (C) Caspase-1 FLICA activation mean fluorescence intensity in cells treated with hemolysin (30 μg/mL) (n=3). (D) Relative IL-1β secretion as compared with non-targeting control in cells treated with α-hemolysin (30 μg/mL) (n=7) top, ATP (5 mM) (n=4) middle, and nigericin (50 μM) (n=3) bottom. (E) Relative MIP1-α secretion as compared with non-targeting control in cells treated with hemolysin (30 μg/ml) (n=3) or nigericin (50 μM) (n=3). For (A & D) ** & **** indicates statistically significant difference from Non-Tg ($p \leq .01$ and $p \leq .0001$, respectively) determined by one-way ANOVA with Dunnett's multiple comparisons testing. For (C) ** Indicates statistically significant difference between no treatment and Hla treatment ($p \leq .01$) determined by one-way ANOVA with Sidak's multiple comparison testing.

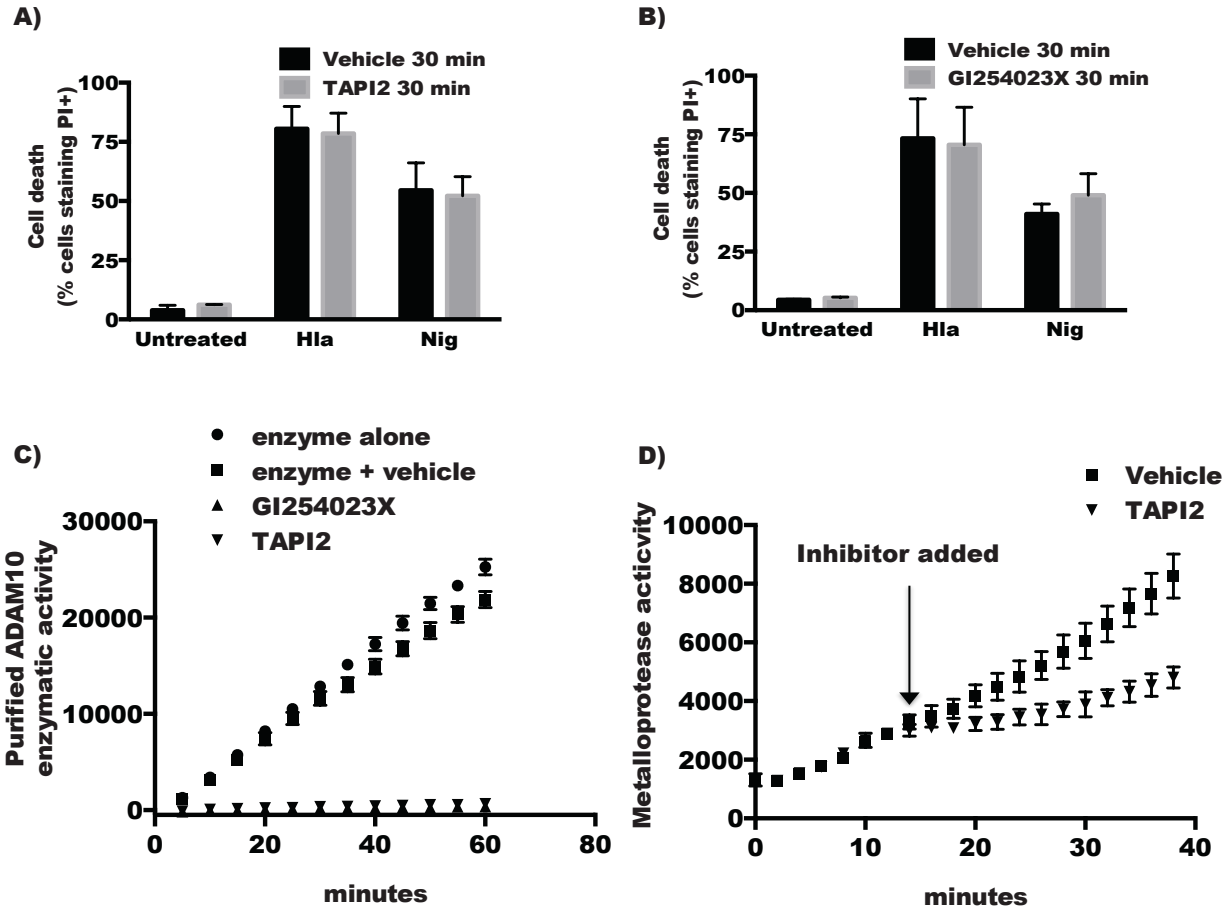
A)
Vehicle 30 min
TAPI2 30 min
Cell death
(% cells staining PI+)
100
75
50
25
0
Untreated
Hla
Nig
B)
Vehicle 30 min
GI254023X 30 min
Cell death
(% cells staining PI+)
100
75
50
25
0
Untreated
Hla
Nig
C)
enzyme alone
enzyme + vehicle
GI254023X
TAPI2
Purified ADAM10
enzymatic activity
30000
20000
10000
0
0
20
40
60
80
minutes
D)
Vehicle
TAPI2
Inhibitor added
Metalloprotease acticvity
10000
8000
6000
4000
2000
0
0
10
20
30
40
minutes

Figure 2.3

Figure 2.3: (A&B) THP1 cells were incubated with either GI254023x (20 μM) or TAPI2 (100 μM) inhibitors or inhibitor vehicle (DMSO for GI254023X or H_2O for TAPI2) for 30 minutes, propidium iodide (PI) was then added to the cells followed by treatment with either nothing (untreated, n=3), α-hemolysin (30 μg/mL) (Hla, n=3), or nigericin (50 μM) (Nig, n=3) for 1 hour. Cell death was assessed by measuring cells that stained positive for PI using flow cytometry. (C) Purified recombinant ADAM10 protein was mixed with vehicle (square), 20μM GI254023X (upward triangle) or 100μM TAPI2 (downward triangle) and immediately assayed for ADAM10 protease activity as described in the Materials and Methods for 60 minutes with measurements taken every five minutes. (D) Metalloprotease activity was measured in THP1 cells by incubating intact cells with a fluorogenic peptide substrate and measuring fluorescent intensity every 2 min. After 10 min, the metalloproteinase inhibitor TAPI2 (100μM) or vehicle was added to the reaction and fluorescence intensity was measured an additional 30 min.

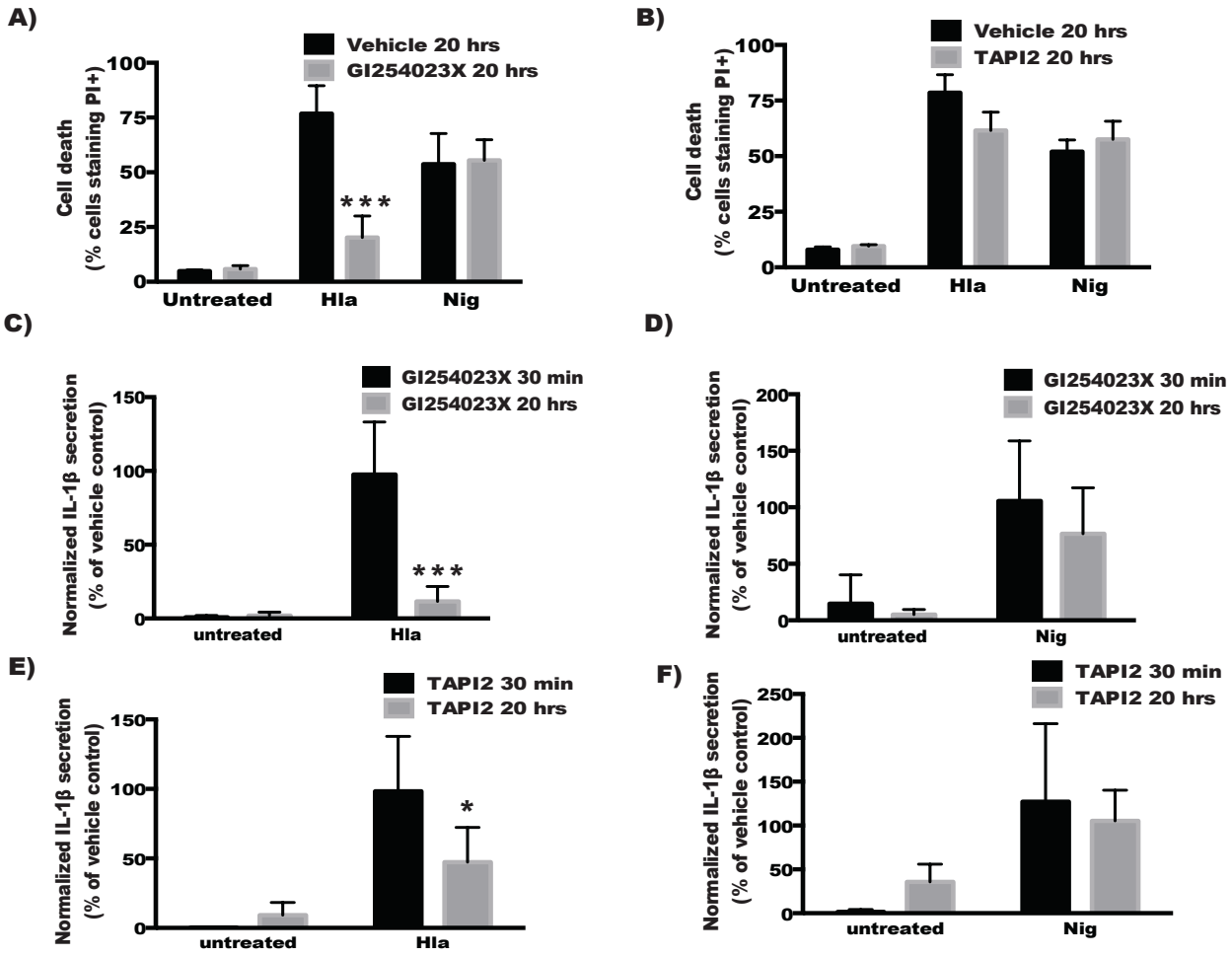
A)
Vehicle 20 hrs
GI254023X 20 hrs
Cell death
(% cells staining PI+)
100
75
50
25
0
***
Untreated
Hla
Nig
B)
Vehicle 20 hrs
TAPI2 20 hrs
Cell death
(% cells staining PI+)
100
75
50
25
0
Untreated
Hla
Nig
C)
GI254023X 30 min
GI254023X 20 hrs
Normalized IL-1β secretion
(% of vehicle control)
150
100
50
0
***
untreated
Hla
D)
GI254023X 30 min
GI254023X 20 hrs
Normalized IL-1β secretion
(% of vehicle control)
200
150
100
50
0
untreated
Nig
E)
TAPI2 30 min
TAPI2 20 hrs
Normalized IL-1β secretion
(% of vehicle control)
150
100
50
0
*
untreated
Hla
F)
TAPI2 30 min
TAPI2 20 hrs
Normalized IL-1β secretion
(% of vehicle control)
250
200
150
100
50
0
untreated
Nig

Figure 2.4

Figure 2.4: (A&B) THP1 cells were incubated with either GI254023x (20 μM) or TAPI2 (100 μM) inhibitors or inhibitor vehicle (DMSO for GI254023X or H_20 for TAPI2) for 20 hours, propidium iodide (PI) was then added to the cells followed by treatment with either nothing (untreated, n=3), α-hemolysin (Hla, n=3), or nigericin (Nig, n=3) for 1 hour. (C-F) THP1 cells were incubated with the indicated inhibitors either overnight (20 hours) or for 30 minutes. Cells were subsequently incubated with LPS (100 ng/mL) for 3 hours followed by no addition (untreated, n=3), α-hemolysin (30 μg/mL) (Hla, n=3), or nigericin (50 μM) (Nig, n=3) for one hour. Cell-culture supernatants were then collected and assayed for IL-1β. Results are reported as either relative secretion to secretion from vehicle exposed cells subsequently treated with the indicated toxin (Hla or Nig). * & *** indicates statistically significant difference from vehicle treated cells intoxicated with Hla ($p \leq .05$ or $p \leq .001$, respectively) determined by one-way ANOVA with Dunnett's multiple comparisons testing.

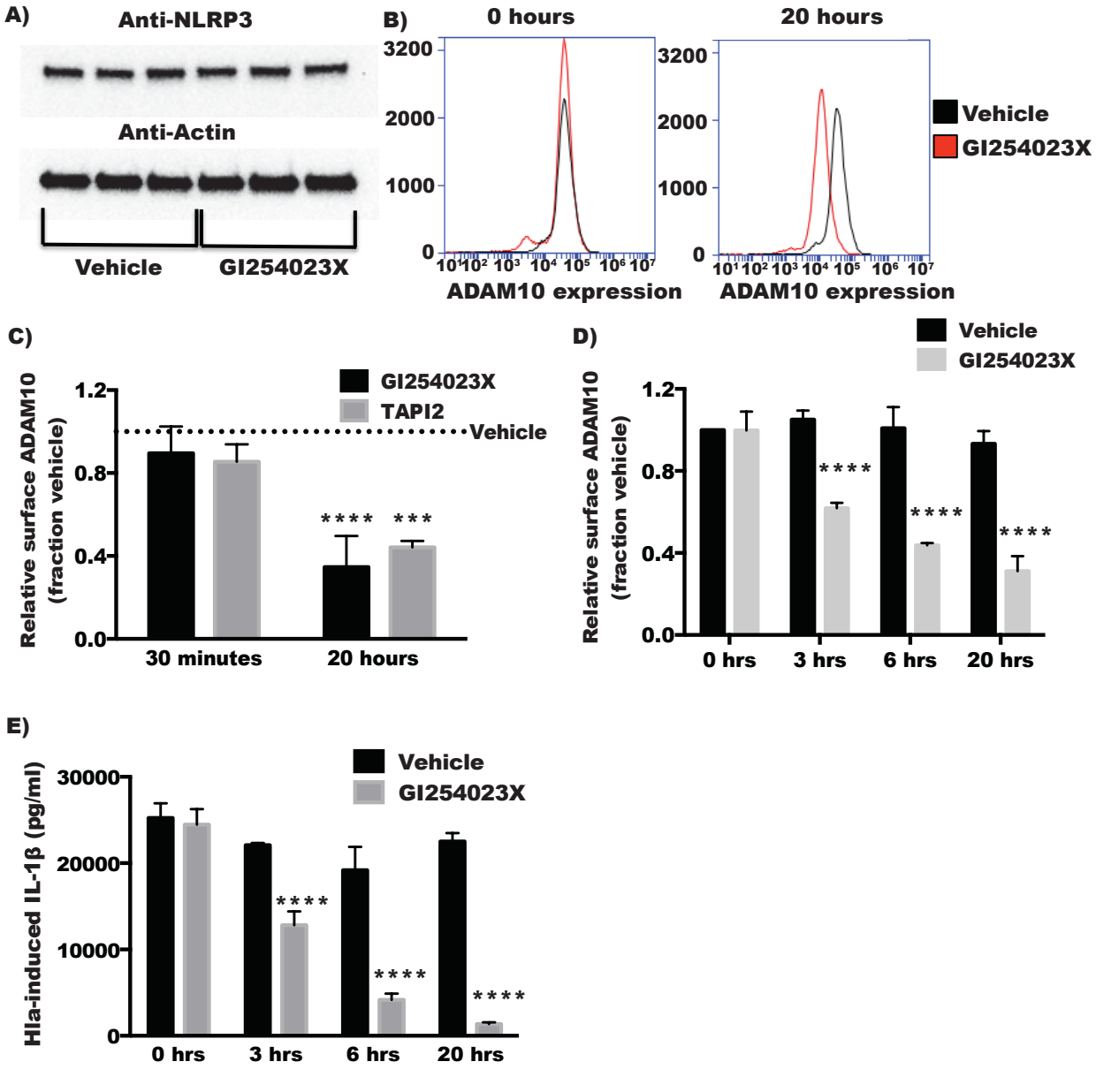
A)
Anti-NLRP3
Anti-Actin
Vehicle
GI254023X
B)
0 hours
20 hours
3200
2000
1000
0
ADAM10 expression
Vehicle
GI254023X
C)
Relative surface ADAM10 (fraction vehicle)
GI254023X
TAPI2
Vehicle
1.2
0.8
0.4
0.0
****
***
30 minutes
20 hours
D)
Vehicle
GI254023X
Relative surface ADAM10 (fraction vehicle)
****
****
****
0 hrs
3 hrs
6 hrs
20 hrs
E)
Hla-induced IL-1β (pg/ml)
Vehicle
GI254023X
30000
20000
10000
0
****
****
****
0 hrs
3 hrs
6 hrs
20 hrs

Figure 2.5

Figure 2.5: (A) Cells were treated with either vehicle (DMSO) or GI254023X (20 μM) for 20 hours and cell lysates were analyzed by immunoblot for NLRP3 and Actin (loading control) as indicated. Lysates from three separate vehicle treated cell populations and three separate GI254023X-treated cell populations were tested. (B) Representative histograms showing the cell surface staining in vehicle- or ADAM10 inhibitor-treated THP1 cells at either 0 or 20 hours of inhibitor treatment. (C) THP1 cells were treated with GI254023X (20 μM), TAPI2 (100 μM), or vehicle overnight or 15-30 minutes before flow cytometric analysis of ADAM10 cell surface expression. Each bar represents the mean of the mean fluorescent intensity of cell surface staining from multiple experiments (n=3) (D-F) THP1 cells were treated with GI254023X (20 μM) or DMSO for 0, 3, 6 hours, or overnight before flow cytometric analysis of ADAM10 cell surface expression or treatment with α-hemolysin (30 μg/mL) was carried out as above. (n=3) For (C), *** and **** Indicates statistically significant reduction when compared to vehicle-treated controls ($p \leq .001$ and $p \leq 0.0001$, respectively) determined by one-way ANOVA with Dunnett's multiple comparisons testing. For (D&E), **** Indicates statistically significant reduction when compared to vehicle treated control at the same time point ($p \leq .0001$) determined by two-way ANOVA with Sadik's multiple comparisons testing.

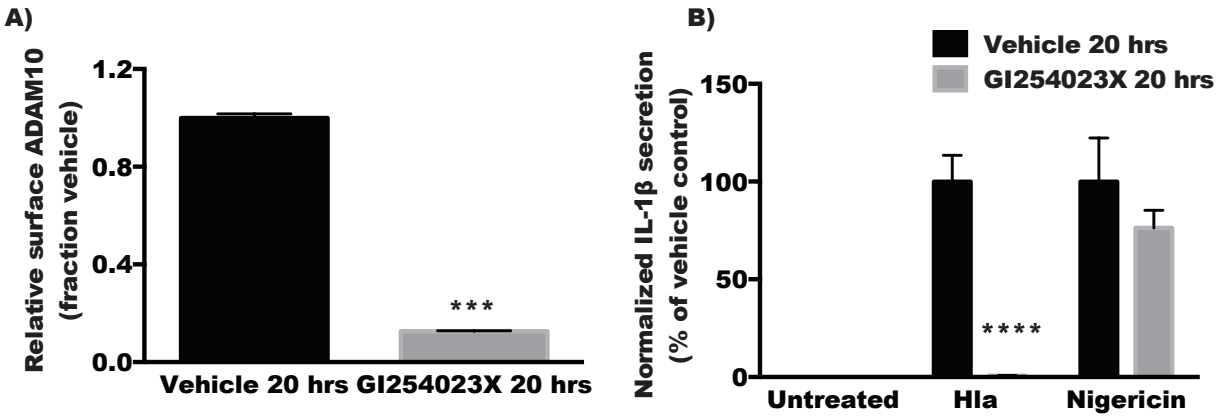
A)
Relative surface ADAM10
(fraction vehicle)
1.2
0.8
0.4
0.0
***
Vehicle 20 hrs
GI254023X 20 hrs
B)
Vehicle 20 hrs
GI254023X 20 hrs
Normalized IL-1β secretion
(% of vehicle control)
150
100
50
0
****
Untreated
Hla
Nigericin

Figure 2.6

Figure 2.6:(A) U937 cells were treated with GI254023X (20 μM) or vehicle for 20 hours and ADAM10 cell surface expression measured. (B) PMA-differentiated U937 cells were treated with GI254023X (20 μM) or vehicle for 20 hours prior to treatment with α-hemolysin (10 μg/mL). For (A) *** Indicates statistically significant reduction when compared to vehicle treatment (p≤ .0001) determined by paired T-test. For (B) **** Indicates statistically significant when compared to vehicle treated control cells (p≤ .0001) determined by one-way ANOVA with Sadik's multiple comparisons testing.

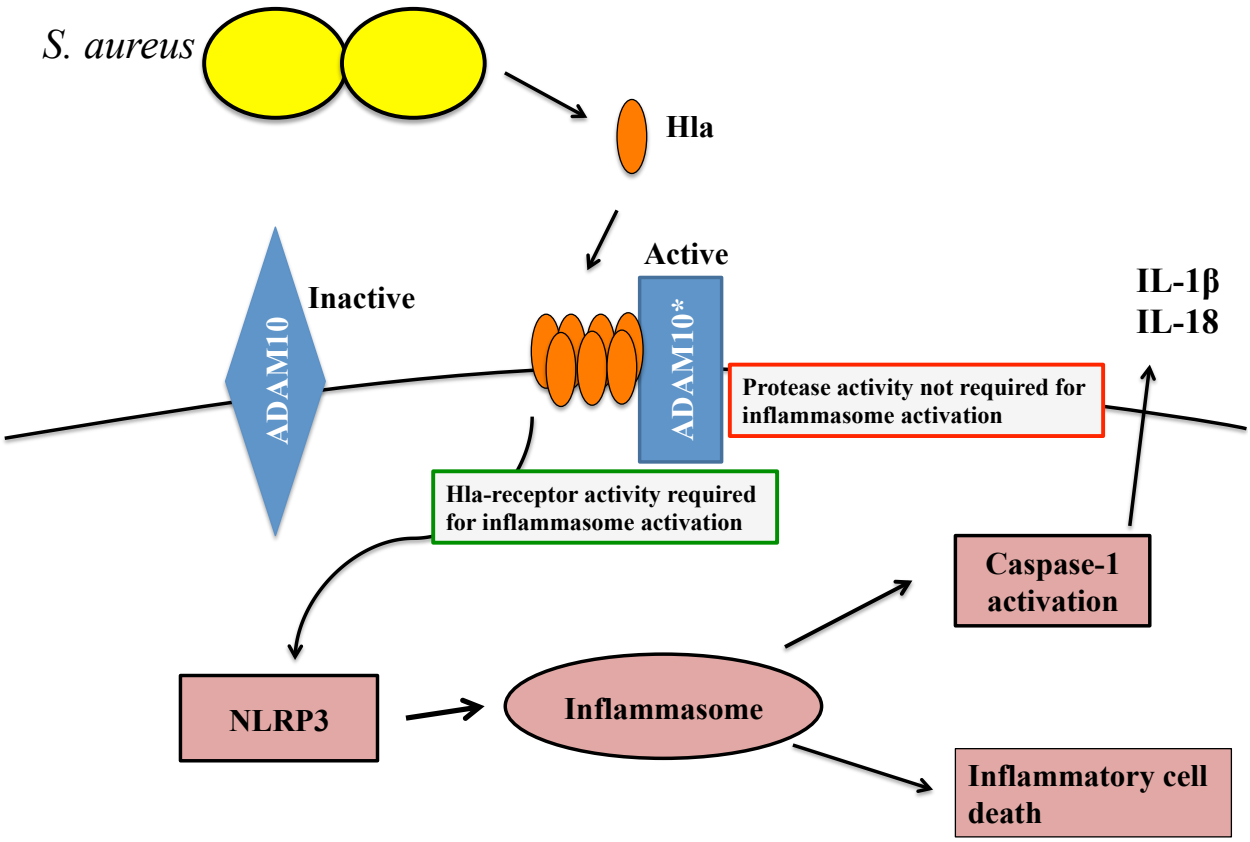

Fig 2.7 Graphical Abstract

CHAPTER THREE: α-hemolysin-mediated activation of the NLRP3 Inflammasome in Primary Human Airway Epithelial cells

Introduction:

Staphylococcus aureus can cause serious respiratory tract infection including infection of the airways (bronchitis or bronchiolitis) and the alveoli (pneumonia). *S. aureus* can cause necrotizing infections of the lungs characterized by severe localized inflammation, leading to high morbidity and mortality [82, 84]. The high mortality of these conditions in the face of antibiotic therapy demonstrates a need to understand the pathogenesis of these infections in order to develop therapies that prevent the development of infection (vaccination) or that can mitigate the severity of established infections. The bacterium is known to produce several virulence factors including α-hemolysin (Hla), which are important for pulmonary pathogenesis [45, 46, 57]. Unlike many S. aureus pore forming toxins, Hla can act on both epithelial cells and immune cells in the lung [86]. Hla is a soluble monomeric protein that once secreted by the bacterium forms a heptameric pore in a broad range of host cell membranes [94, 104]. Additionally, Hla is a critical virulence factor in murine models of staphylococcal infection and has being implicated in human disease [111, 112, 115, 131, 143, 144]. A Disintegrin and Metalloprotease 10 (ADAM10) acts as a cellular receptor for Hla [107]. Knockdown of ADAM10 protects immortalized human epithelial cell cultures from Hla-induced death [107]. Genetic deletion of ADAM10 in the lung epithelium using a Cre

recombinase driven by the surfactant protein C promoter or chemical inhibition of the protein, mitigates Hla-induced pathology and makes animals resistant to lethal pneumonia in mouse models of disease [129]. Additionally, in epithelial and endothelial cells, Hla has been shown to increase the protease activity of ADAM10 leading to cleavage of cellular adherence factors like E-cadherin [107, 130].

Hla also potently activates the host signaling complex known as the Nucleotide-binding domain and Leucine-Rich repeat containing family Pyrin domain containing 3 (NLRP3) inflammasome in monocytes [123, 124]. The NLRP3 inflammasome is an important innate immune signaling complex that is part of a large collection of host signaling moieties that are responsible for sensing and responding to microbial threats. The NLRP3 inflammasome, once activated, forms a protein complex comprised of NLRP3, apoptosis-associated speck-like protein (ASC), and pro-caspase-1. The inflammasome converts pro-caspase-1 into the proteolytically active caspase-1 enzyme that cleaves the precursors of IL-1β and IL-18 into their active forms. Additionally, inflammasome activation induces a pro-inflammatory program of cell death characterized by the morphologic features of necrosis [134-136]. In a murine model of Hla intoxication during S. aureus infection genetic loss of NLRP3 was seen to be beneficial for both survival and immune-mediated damaged by the toxin [124].

Primary airway epithelial cells also express a functional NLRP3 inflammasome that can be activated by particulate matter and Influenza A virus infection [145, 146]. Primary airway epithelial cells can be prepared from nasal turbinate, trachea and bronchi from human donor airways. The tissue is dissected and dissociated using proteases. Cells are then scraped from the epithelial surface and plated directly onto

porous support for the production of ALI (Air-liquid interface) cultures or are plated on collagen-coated tissue cultures plates using Bronchial epithelial growth medium (BEGM) or ALI growth media or ALI cell as defined here by Fulcher et al [147]. Cells grown on tissue culture treated plates assume a squamous, poorly-differentiated phenotype while ALI cultures assume a fully polarized architecture reminiscent of the normal intact airway [147]. ALI cells differentiate into both secretory and ciliated cells, make and move mucus by ciliary action, and are capable of being infected by airway specific pathogens like RSV (Respiratory Syncytial Virus) [147-150]. In work conducted by Hirota et al., these primary airway epithelial cells were found to express the inflammasome components NLRP3, caspase-1, and IL-1β by both In situ staining of cross sections of human bronchus and immunoblot analysis of primary human airway epithelial (hAE) cells. Primary hAE cells also secrete IL-1β and show evidence of caspase-1 cleavage in response to urban particulate matter treatment [145]. Hirota and colleagues were able to reverse particulate matter induced IL-1beta secretion by treating the hAE cells with siRNA against NLRP3. Allen et al. also showed strong induction of IL-1β secretion in primary hAE grown in ALI cultures in response to influenza infection [146].

In this chapter, we have begun to address the hypothesis that NLRP3 inflammasome signaling is activated by *S. aureus* α-hemolysin in respiratory epithelium and that this signaling is an important factor in inflammatory signaling in the respiratory tract.

Methods and Materials:

Treatment of cultured cells with α-hemolysin for cytokine studies:

Human primary airway epithelial were harvested and grown as review here [147, 151]. Prior to treatment, hAE cells grown in plastic tissue culture treated plates were washed and media changed. Cells were then treated with 1 μg/ml of lipoteichoic acid for 24 hours after which media was again collected and fresh media added. Cells were then treated with Hla (100 μg/ml) and supernatants collected after either 4 or 24 hours of intoxication and all supernatants were collected by centrifugation at 13000 RPM for 3 minutes. Cell culture supernatants were stored frozen at -80°C until assayed for cytokine production. IL-1β was measured using BD bioscience (product #: 557953, San Jose, CA, USA) ELISA kits as per manufacturer protocols. Recombinant α-hemolysin was prepared as described by Craven et al [123].

For cells grown in the Air-liquid interface format, one day prior to that start of treatment cells are washed and fed fresh media. The next day samples were collected from both the apical and basolateral sides of the culture. The cells were then washed and fresh media is added. Cells were subsequently challenged apically with 20% pseudomonas aeruginosa culture filtrates in media for 24 hours prior to challenge with Hla. Samples were collected the next day from both the apical and basolateral sides of the culture, cells were washed and fresh media added to cultures. Cells were then challenged apically with 50 μl of media containing the 50 μg/ml of Hla for 24 hours. Supernatant were again collected both apically and basolaterally and all supernatants were centrifuged at 13000 RPM for 3 minutes. Supernatants were stored frozen at -80°C until assayed for cytokine production. IL-1β was measured using BD bioscience

(product #: 557953, San Jose, CA, USA) ELISA kits as per manufacturer protocols. We used cells grown for 3 days at ALI and fully differentiated ALI cultures grown for over 21 days (used on day 35).

Treatment of cultured cells with α-hemolysin for cell death studies:

Human primary airway epithelial were harvested and grown as detailed in this review [147, 151]. Prior to treatment hAE cells grown on plastic were washed and media changed. Cells were then treated with 1 µg/ml of lipoteichoic acid for 24 hours after which media was again collected and fresh media added. Cells were then treated with Hla (100 µg/ml) and supernatants collected after either 4 or 24 hours of intoxication. Cells and supernatants were collected by centrifugation at 13000 RPM for 3 minutes. Prior to the end of the experiment a control group of cells were lysed using 1% Triton X-100 as a lysis control. Cell culture supernatants were stored frozen at -80°C until assayed for LDH production. LDH was measured using the CytoTox-ONE Homogeneous Membrane Integrity Assay from Promega (product #: 557953, Madison, WI, USA) as per manufacture protocols.

Caspase-1 activity was measured using the FAM-FLICA® caspase-1 assay kit, far-red fluorescence (Product #:97) from ImmunoChemistry Technologies LLC (Bloomington, MN, USA). hAE cells were plated on glass chamber slips and allowed to grow overnight. The FLICA reagent was added and incubated with cells for 5 minutes prior to the addition of α-hemolysin for one hour. Cells were washed once using 1x FAM-FLICA® caspase-1 assay kit wash buffer after which 1 µg/ml Hoeschst 3334L from caspase-1 kit in 500uL total volume was added to each slip. Cells were further stained

for 10 minutes before being washed twice with wash buffer. Cells were resuspended in 1ml of PBS containing FAM-FLICA® caspase-1 assay kit fixative diluted 1:10. Accumulation of fluorescent caspase-1 inhibitor was assayed by microcopy using a EVOS Cell imaging system by ThermoFisher (Waltham, MA. USA).

Results:

Primary human airway epithelial cells grown on collagen treated tissue culture plates show makers of inflammasome activation in response to treatment with α-hemolysin.

To begin to test whether the NLRP3 inflammasome plays a role in airway epithelial responses to S. aureus hemolysin, we tested whether primary epithelial cells died, activated caspase-1 and produced IL-1β in response to Hla treatment. Primary human airway epithelial cells were grown to confluence in tissue culture treated plates prior to challenge with Hla. 24 hours prior to challenge with Hla, we treated cells with lipoteichoic acid to promote the production of pro-IL-1β via TRL2 stimulation and NFκB signaling. Cells were then challenged with Hla and supernatants collected at either 4 or 24 hours for measurement of cytokine production and cell death via LDH release. Cells treated with Hla showed higher secretion of IL-1β and cell death than untreated cells especially at 24 hours after toxin addition (Figure 3.1 A&B). Next to test whether treatment of hAE with Hla leads to detectable levels of caspase-1 activation, we stained cells with Caspase-1 FLICA, which irreversibly binds to active caspase-1 enzyme. Upon binding the FLICA reagent fluoresces and we are able to wash out unbound reagent and detect caspase-1 activation via fluorescence microscopy. Cells were pretreated with FLICA reagent and then challenged with Hla for one hour. We then washed, fixed, and imaged the cells. In Hla-treated cells we were able to detect a strong fluorescence signal indicative of caspase-1 activation in the Hla treated population. (Figure 3.1C)

Primary human airway epithelial cells grown at air liquid interface produce IL-1β secretion in response to treatment with α-hemolysin.

hAE cells grown at grown with an Air-liquid interface (ALI) provide an excellent model for studying the cells of the human airway as they mimic the morphology and variety of cell types seen in the human airway [147]. To determine if these cells were able to secrete IL-1β in response to challenge with Hla, we used two sets of ALI cultures. One culture that has been grown for at least 21 days which is considered full maturity and another ALI culture grown for 3 three day prior to treatment. The three-day culture mimics a less mature and more pro-inflammatory airway as marked by higher levels of baseline IL-8 production. Cells were treated with *Pseudomonas aeruginosa* culture filtrate 24 hours prior to Hla challenge to promote immune activation because previous work has shown the Pseudomonas culture filtrates to promote Toll-like receptor signaling in ALI cultures[152]. Cells were then challenged with Hla and after 24 hours supernatants collected to assay for cytokine production. Hla treatment simulated IL-1β production from both sets of cultures with the Day 3 culture secreting higher levels of cytokine. (Figure 3.2)

Discussion:

hAE cells are known to express NLRP3 inflammasome components. hAE cells are also capable of secreting IL-1β in response to challenge with Influenza A or particulate matter. We now have evidence that α-hemolysin treatment also leads to secretion of IL-1β in hAE cultures grown on collagen treated tissue culture plates and at ALI. Treatment with Hla also leads to the activation of caspase-1 as measured by FAM FLICA. These findings point to a possible role for the NLRP3 inflammasome in mediating Hla-induced pathogenesis in these cells. We currently know the importance of ADAM10 and its activity in mediating Hla effects in epithelial cells but NLRP3's role has yet to be thoroughly investigated. NLRP3 activation and secretion of IL-1β, a powerful inflammatory cytokine, from these cell types could have important implications in mediating early inflammatory events in the course of S. aureus infection. These cells are the first to be encountered by the bacterium during infection and their ability to secrete inflammatory cytokines could be critical in the early immune response to the bacterium.

In our model, day 3 ALI culture intoxication with Hla led to higher levels of IL-1β secretion and presumably inflammasome activation. If indeed the day 3 cultures represent more a proinflammatory cellular environment than their day 21 counterparts, this may provide evidence as to why the injured inflamed lung is more susceptible to bacteria infections that lead to severe disease. This can be observed keenly in the case of influenza and *Staphylococcus aureus* superinfection. In both human disease and animal models, influenza immediately followed by a *S. aureus* infection leads to more severe disease than either infection alone [153-155]. Our lab has previously shown that

in the setting of S. aureus pneumonia, NLRP3 activation is harmful and leads to increase mortality in mouse model of disease [124] and influenza infection simulates NLRP3 and release of IL-1β from these cells [143], so this hyper-activation of the inflammasome could further worsen disease outcomes.

As this project is in its early stages, there are several lines of investigation I would like to follow up in the future. Using siRNA and/or viral RNAi delivery, I would like to knockdown key inflammasome components such as NLRP3, ASC, and caspase-1 to determine their roles in mediating the IL-1β secretion and cell death, we observe in hAE cultures. I would also like to determine using a super-infection model with influenza, what role NLRP3 activation in the airway epithelium may have in the increased pathology seen in this setting [80, 154, 155].

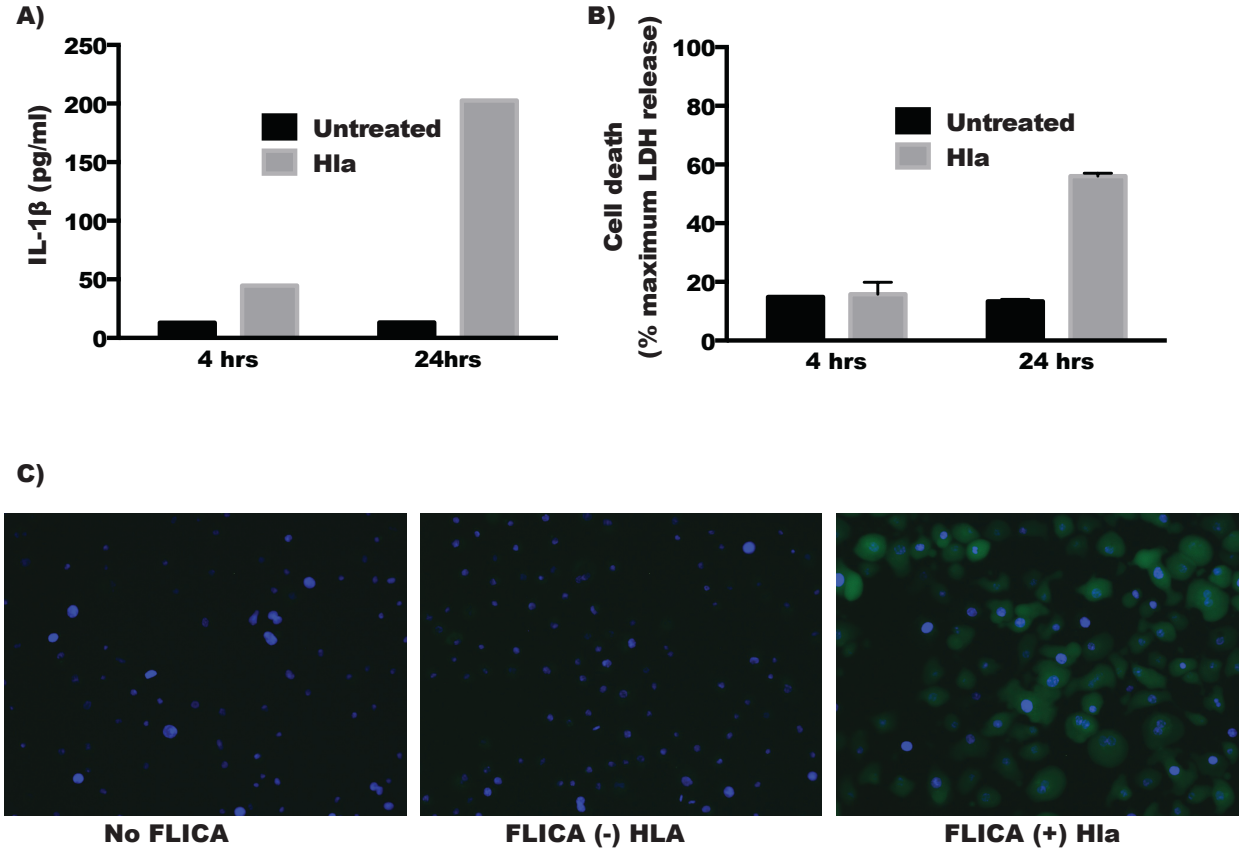
A)
IL-1β (pg/ml)
250
200
150
100
50
0
Untreated
Hla
4 hrs
24hrs
B)
Cell death
(% maximum LDH release)
100
80
60
40
20
0
Untreated
Hla
4 hrs
24 hrs
C)
No FLICA
FLICA (-) HLA
FLICA (+) Hla

Figure 3.1

Figure 3.1: A&B) Primary human airway epithelial cells were challenged with 1 μg/ml of lipoteichoic acid for 24 hours after which cell were treated with 100 μg/ml of Hla. Supernatants were collected at either 4 or 24 hours after toxin treatment and assayed for IL-1β production and LDH release. C) Primary human airway epithelial cells were grown on glass chamber slide and treated with Caspase-1 FLICA, washed and challenged with 100ug/ml of Hla for one hour. Cell were then washed and the nuclei counterstained before imaging by Fluorescence microscopy.

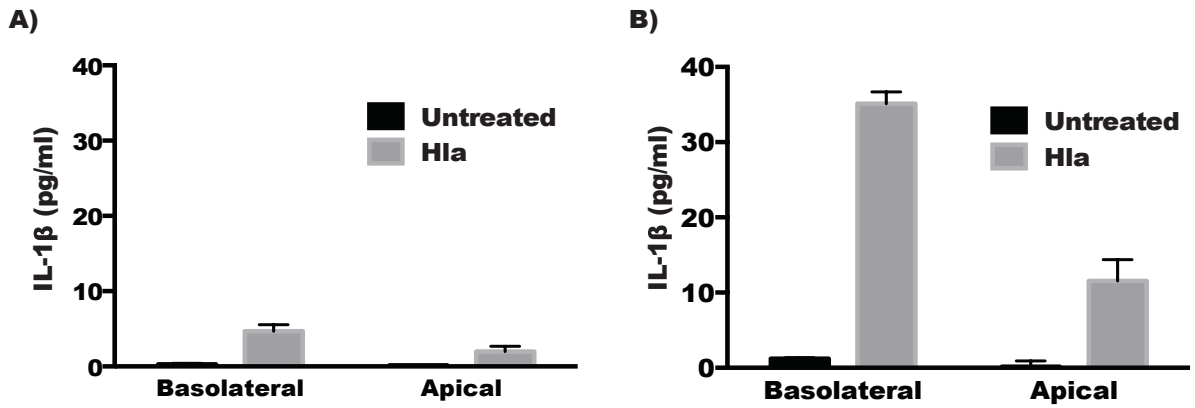

Figure 3.2

Figure 3.2: Primary epithelial cells grown at air-liquid interface for either 3 or 35 days. The cells were washed and then challenged with 20% *Pseudomonas aeruginosa* culture filtrate in media for 24 hours prior to Hla challenge. Cell were challenge with 50 μg/ml of Hla apical and after 24 hours samples were collected from both the apical and basolateral sides of the cultures and assayed for IL-1β production.

CHAPTER FOUR: Significance and Future directions

In the outlined studies, we explored the mechanisms by which the ADAM10-NLRP3 inflammasome signaling axis is required for S. aureus-mediated pathogenesis in monocytes and primary human epithelial cells. In chapter Two, we explored the role of ADAM10 in Hla-mediated activation of the NLRP3 inflammasome in human monocytes. ADAM10 is the cell surface receptor for Hla and loss of ADAM10 diminishes the ability of Hla to bind and intoxicate target cells [86, 107, 111, 127, 129-132]. Hla binding to ADAM10 also elicits increased protease activity in both epithelial [107, 129, 130] and endothelial cells [131]. This enhanced ADAM10 protease activity leads to disruption of cell-to cell contact and presumably break down of the epithelial/endothelial barrier. Interestingly, in models of S. aureus pneumonia and skin infection, specific ADAM10 knockout in cells of myeloid origin recapitulated the effects seen in previous NLRP3 knockout mouse models [124, 126, 127]. But the specific role of ADAM10 and its protease activity in NLRP3 inflammasome activity had not been explored. We have demonstrated that ADAM10's ability to serve as a cell surface receptor for hemolysin and not its protease activity is important for facilitating Hla-mediated NLRP3 inflammasome activation. siRNA knockdown and chemical blockade of the ADAM10 leads to diminished α-hemolysin-mediated inflammasome activation. This was due to the reduction in ADAM10 cell surface expression caused by both RNAi treatment and

prolonged inhibitor exposure. This latter finding is especially novel as treatment with ADAM10 chemical inhibitors not only inhibited protease activity but also decreased ADAM10 cell surface expression in a time dependent fashion, which to my knowledge has not been previously reported.

There are a number of questions that arise from this work that would warrant further study. First, I would like to determine the mechanisms behind the inhibitor-mediated suppression of ADAM10 cell surface expression. There is a possibility that ADAM10 proteolysis is being blocked leading to improper trafficking and maturation of the protein [141]. Another question that arises is whether inhibitor-mediated suppression of ADAM10 cell surface expression is specific to cells of myeloid origin or is a general mechanism by which long-term inhibitor treatment affects all cell types that express ADAM10. This has implications because in animal models where the ADAM10 inhibitor GI254023X was shown to reduce mortality from S. aureus infection, it was presumed that inhibitor either blocked Hla binding or that the blockade of ADAM10 protease function was what conferred the better outcomes [129-131]. Our works points to the suppression of ADAM10 cell surface expression as the mechanism by which this inhibitor reduces Hla-mediated pathology by the bacterium in-vivo. Finally, the exact mechanism by which Hla activates the NLRP3 inflammasome down stream of ADAM10 has yet to be determined. We know from prior work in the lab that high extracellular K+ can diminish Hla-mediated inflammasome activation but the deeper mechanism behind this have yet to be explored. One possible mechanism downstream of this that could bridge the gap is ROS and the generation of oxidized mitochondrial DNA. There is evidence that oxidized mitochondrial DNA may play an important role as a downstream

activation step for NLRP3 activation as it has been shown to be important for both ATP and nigericin-mediated NLRP3 inflammasome activation in macrophages [39].

These studies help to clarify the role of ADAM10 in hemolysin-mediated pathogenesis in monocytes, illustrating cell-type specific differences in Hla-ADAM10-mediated pathology. Pointing to a promising future for ADAM10 inhibitors as potential treatments to not only protect patient's epithelial/endothelial cell barrier integrity but to also reduce hemolysin-mediated immune activation in the context of S. aureus infection.

In Chapter Three, we sought to determine the role of NLRP3 signaling in mediating α-hemolysin-induced pathogenesis in epithelial cells. These cells are the first line of defense in maintaining airway integrity in S. aureus infections and often directly encounter pathogens. Primary human epithelial cells express inflammasome components and secrete IL-1β in response to particulate matter [145] and influenza infection[146]. Epithelial cells express ADAM10 and are sensitive to Hla intoxication leading to disruption of cell-to-cell contacts and cell death [107, 129] in cultured cells and animal models of disease. While the role of ADAM10 in hemolysin-mediated pathogenesis is well elucidated in epithelial cells, the functions of NLRP3 in mediating hemolysin pathogenesis in epithelial cells are unknown. Using a primary human airway epithelial cell model we sought to shed some light on the role of NLRP3 in this process. We were able to show strong evidence of NLRP3 activation in this model with hAE cells grown both with traditional cell culture methods on collagen coated plates and at air-liquid interface. With ALI cultures being the better model of well-differentiated airway epithelium with both secretory and ciliated cells types present [147, 151]. Both cells grown at air-liquid interface and on traditional cell culture plates secreted IL-1β in

response to hemolysin intoxication and we were able to detect caspase-1 activation via caspase-1 FLICA staining in response to hemolysin treatment. These studies point to inflammasome activation being present in hemolysin treated hAE and a possible role for this activation in mediating hla-induced pathogenesis.

This project is still in its early stages and there is much left to pursue. We would like to determine whether the IL-1β is being generated in a NLRP3 dependent or if some other inflammasome may be responsible. We would like to begin by knocking down a number of inflammasome components including NLRP3, ASC and caspase-1 in hAE cells to determine what role if any they play in this process. This can be readily done with cells grown on collagen plates, which are more readily transfected. We also know from both animal models of infection and in studies of human patients that influenza followed by bacterial super-infection leads to worse outcomes and severe disease [154, 155]. In addition, we know that influenza infection can stimulate IL-1β production in an NLRP3 dependent manner in these cell types [146]. So in order to follow up on this finding we would like to develop an influenza/S. aureus super infection model to determine if NLRP3 or more broadly inflammasome activation has a more profound effect on influenza infected cells in the context of hemolysin intoxication. The studies outlined in this thesis help to delineate the role of NLRP3 in S. aureus α-hemolysin-mediated immune activation in both monocytes and epithelial cells. This is an important step in furthering our understanding of the complex interactions of α-hemolysin and by extension Staphylococcus aureus with different cell types.

Gaining a better understanding of the cellular dynamics involved in *S. aureus* pathogenesis gives us the best chance to develop new therapeutics to combat this deadly disease as antibiotic resistance continues to spread.

REFERENCES:

1. Trdá, L., et al., *Perception of pathogenic or beneficial bacteria and their evasion of host immunity: pattern recognition receptors in the frontline.* Frontiers in plant science, 2015. **6**: p. 219.

2. Kumar, H., T. Kawai, and S. Akira, *Pathogen recognition in the innate immune response.* The Biochemical journal, 2009. **420**(1): p. 1-16.

3. Davis, B.K., H. Wen, and J. Ting, *The Inflammasome NLRs in Immunity, Inflammation, and Associated Diseases.* Annual Review of Immunology, 2011. **29**(1): p. 707-735.

4. Martinon, F., A. Mayor, and J. Tschopp, *The inflammasomes: guardians of the body.* Annual review of immunology, 2009. **27**: p. 229-265.

5. Hoffmann, J.A., et al., *Phylogenetic perspectives in innate immunity.* Science (New York, N.Y.), 1999. **284**(5418): p. 1313-1318.

6. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen Recognition and Innate Immunity.* Cell, 2006. **124**(4): p. 783-801.

7. Kersse, K., et al., *NOD-like receptors and the innate immune system: coping with danger, damage and death.* Cytokine & growth factor reviews. **22**(5-6): p. 257-276.

8. Martinon, F., K. Burns, and J. Tschopp, *The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta.* Molecular cell, 2002. **10**(2): p. 417-426.

9. Kawai, T. and S. Akira, *The roles of TLRs, RLRs and NLRs in pathogen recognition.* International immunology, 2009. **21**(4): p. 317-337.

10. Takeuchi, O. and S. Akira, *Innate immunity to virus infection.* Immunological reviews, 2009. **227**(1): p. 75-86.

11. Kumar, H., T. Kawai, and S. Akira, *Pathogen recognition by the innate immune system.* International reviews of immunology, 2011. **30**(1): p. 16-34.

12. Takeuchi, O. and S. Akira, *Pattern recognition receptors and inflammation.* Cell, 2010.

13. Bürckstümmer, T., et al., *An orthogonal proteomic-genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome.* Nature …, 2009.

14. Dagenais, M., J. Dupaul-Chicoine, and M. Saleh, *Function of NOD-like receptors in immunity and disease.* Current opinion in investigational drugs (London, England : 2000), 2010. **11**(11): p. 1246-1255.

15. Schroder, K. and J. Tschopp, *The inflammasomes.* Cell, 2010. **140**(6): p. 821-832.

16. Bauernfeind, F., et al., *Inflammasomes: current understanding and open questions.* Cellular and molecular life sciences : CMLS, 2011. **68**(5): p. 765-783.

17. Lamkanfi, M. and Vishva M. Dixit, *Mechanisms and Functions of Inflammasomes.* Cell, 2014. **157**(5): p. 1013-1022.

18. Lamkanfi, M., *Emerging inflammasome effector mechanisms.* Nature reviews. Immunology, 2011. **11**(3): p. 213-220.

19. Bergsbaken, T., S.L. Fink, and B.T. Cookson, *Pyroptosis: host cell death and inflammation.* Nature Reviews Microbiology, 2009.

20. Jorgensen, I. and E.A. Miao, *Pyroptotic cell death defends against intracellular pathogens.* Immunological reviews, 2015. **265**(1): p. 130-142.

21. Fink, S.L. and B.T. Cookson, *Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages.* Cellular microbiology, 2006.

22. He, W.-t.T., et al., *Gasdermin D is an executor of pyroptosis and required for interleukin-1β secretion.* Cell research, 2015. **25**(12): p. 1285-1298.

23. Shi, J., et al., *Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death.* Nature, 2015. **526**(7575): p. 660-665.

24. Vince, J.E. and J. Silke, *The intersection of cell death and inflammasome activation.* Cellular and Molecular Life Sciences, 2016.

25. Ting, J., et al., *The NLR gene family: a standard nomenclature.* Immunity, 2008. **28**(3): p. 285-287.

26. Lu, A., et al., *Unified polymerization mechanism for the assembly of ASC-dependent inflammasomes.* Cell, 2014. **156**(6): p. 1193-1206.

27. Cai, X., et al., *Prion-like polymerization underlies signal transduction in antiviral immune defense and inflammasome activation.* Cell, 2014. **156**(6): p. 1207-1222.

28. Jo, E.-K.K., et al., *Molecular mechanisms regulating NLRP3 inflammasome activation.* Cellular & molecular immunology, 2015.

29. Vanaja, S.K., V.A. Rathinam, and K.A. Fitzgerald, *Mechanisms of inflammasome activation: recent advances and novel insights.* Trends in cell biology, 2015. **25**(5): p. 308-315.

30. Jo, E.K., et al., *Molecular mechanisms regulating NLRP3 inflammasome activation.* Cell Mol Immunol, 2015.

31. Hoffman, H.M., et al., *Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome.* Nature genetics, 2001. **29**(3): p. 301-305.

32. Gross, O., et al., *The inflammasome: an integrated view.* Immunological reviews, 2011. **243**(1): p. 136-151.

33. Jin, C. and R. Flavell, *Molecular mechanism of NLRP3 inflammasome activation.* Journal of clinical immunology, 2010. **30**(5): p. 628-631.

34. Pétrilli, V., et al., *Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration.* Cell death and differentiation, 2007. **14**(9): p. 1583-1589.

35. Arlehamn, C., et al., *The role of potassium in inflammasome activation by bacteria.* The Journal of biological chemistry, 2010. **285**(14): p. 10508-10518.

36. Cassel, S.L., et al., *The Nalp3 inflammasome is essential for the development of silicosis.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(26): p. 9035-9040.

37. Dostert, C., et al., *Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica.* Science (New York, N.Y.), 2008. **320**(5876): p. 674-677.

38. Zhou, R., et al., *Thioredoxin-interacting protein links oxidative stress to inflammasome activation.* Nature Immunology, 2009. **11**(2): p. 136-140.

39. Shimada, K., et al., *Oxidized Mitochondrial DNA Activates the NLRP3 Inflammasome during Apoptosis.* Immunity, 2012. **36**(3): p. 401-414.

40. Hornung, V., et al., *Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization.* Nature immunology, 2008. **9**(8): p. 847-856.

41. Gupta, R., et al., *RNA and β-hemolysin of group B Streptococcus induce interleukin-1β (IL-1β) by activating NLRP3 inflammasomes in mouse macrophages.* Journal of Biological …, 2014.

42. Hoegen, T., et al., *The NLRP3 inflammasome contributes to brain injury in pneumococcal meningitis and is activated through ATP-dependent lysosomal cathepsin B release.* Journal of immunology (Baltimore, Md. : 1950), 2011. **187**(10): p. 5440-5451.

43. Greaney, A.J., S.H. Leppla, and M. Moayeri, *Bacterial exotoxins and the inflammasome.* Frontiers in Immunology, 2015. **6**.

44. Klevens, R.M., et al., *Invasive methicillin-resistant Staphylococcus aureus infections in the United States.* Jama, 2007.

45. Franklin, D.L., *Staphylococcus aureus Infections.* New England Journal of Medicine, 1998. **339**(8): p. 520-532.

46. Gordon, R.J. and F.D. Lowy, *Pathogenesis of methicillin-resistant Staphylococcus aureus infection.* Clinical infectious diseases, 2008.

47. Williams, R.E., et al., *Nasal staphylococci and sepsis in hospital patients.* Br Med J, 1959. **2**(5153): p. 658-62.

48. von Eiff, C., et al., *Nasal carriage as a source of Staphylococcus aureus bacteremia. Study Group.* N Engl J Med, 2001. **344**(1): p. 11-6.

49. Wertheim, H.F., et al., *The role of nasal carriage in Staphylococcus aureus infections.* Lancet Infect Dis, 2005. **5**(12): p. 751-62.

50. Kluytmans, J., A. van Belkum, and H. Verbrugh, *Nasal carriage of Staphylococcus aureus: epidemiology, underlying mechanisms, and associated risks.* Clin Microbiol Rev, 1997. **10**(3): p. 505-20.

51. Perl, T.M. and J.E. Golub, *New approaches to reduce Staphylococcus aureus nosocomial infection rates: treating S. aureus nasal carriage.* Ann Pharmacother, 1998. **32**(1): p. S7-16.

52. Tong, S.Y., et al., *Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management.* Clinical microbiology reviews, 2015. **28**(3): p. 603-661.

53. Holland, S.M.S.M., *Chronic granulomatous disease.* Hematology/oncology clinics of North America. **27**(1): p. 89-99.

54. Donlan, R.M. and J.W. Costerton, *Biofilms: survival mechanisms of clinically relevant microorganisms.* Clinical microbiology reviews, 2002.

55. Knox, J., A.C. Uhlemann, and F.D. Lowy, *Staphylococcus aureus infections: transmission within households and the community.* Trends Microbiol, 2015. **23**(7): p. 437-44.

56. Lee, M.C., et al., *Management and outcome of children with skin and soft tissue abscesses caused by community-acquired methicillin-resistant Staphylococcus aureus.* Pediatr Infect Dis J, 2004. **23**(2): p. 123-7.

57. Diep, B. and M. Otto, *The role of virulence determinants in community-associated MRSA pathogenesis.* Trends in microbiology, 2008. **16**(8): p. 361-369.

58. Young, L.M. and C.S. Price, *Community-acquired methicillin-resistant Staphylococcus aureus emerging as an important cause of necrotizing fasciitis.* Surg Infect (Larchmt), 2008. **9**(4): p. 469-74.

59. Chambers, H., *Community-associated MRSA--resistance and virulence converge.* The New England journal of medicine, 2005. **352**(14): p. 1485-1487.

60. Hiramatsu, K.K., *The emergence and evolution of methicillin-resistant Staphylococcus aureus.* Trends in microbiology (Regular ed.). **9**(10): p. 486-493.

61. Hiramatsu, K., *Molecular Evolution of MRSA.* Microbiology and Immunology, 1995. **39**(8): p. 531-543.

62. Tong, S.Y., et al., *Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management.* Clin Microbiol Rev, 2015. **28**(3): p. 603-61.

63. Daum, R.S.R.S., *Clinical practice. Skin and soft-tissue infections caused by methicillin-resistant Staphylococcus aureus.* The New England journal of medicine. **357**(4): p. 380-390.

64. Stevens, D.L., et al., *Practice guidelines for the diagnosis and management of skin and soft tissue infections: 2014 update by the infectious diseases society of America.* Clin Infect Dis, 2014. **59**(2): p. 147-59.

65. Sheehy, S.H., et al., *The microbiology of chronic osteomyelitis: Prevalence of resistance to common empirical anti-microbial regimens.* Journal of Infection, 2010. **60**(5): p. 338-343.

66. Julia Chelsom, C.O.S., *Vertebral Osteomyelitis at a Norwegian University Hospital 1987-97: Clinical Features, Laboratory Findings and Outcome.* Scandinavian Journal of Infectious Diseases, 1998. **30**(2): p. 147-151.

67. Cobo, J., et al., *Early prosthetic joint infection: outcomes with debridement and implant retention followed by antibiotic therapy.* Clinical Microbiology and Infection, 2011. **17**(11): p. 1632-1637.

68. Peel, T.N., et al., *Outcome of Debridement and Retention in Prosthetic Joint Infections by Methicillin-Resistant Staphylococci, with Special Reference to Rifampin and Fusidic Acid Combination Therapy.* Antimicrobial Agents and Chemotherapy, 2013. **57**(1): p. 350-355.

69. Bejon, P., et al., *Two-stage revision for prosthetic joint infection: predictors of outcome and the role of reimplantation microbiology.* Journal of Antimicrobial Chemotherapy, 2010. **65**(3): p. 569-575.

70. van Hal, S.J., et al., *Predictors of Mortality in Staphylococcus aureus Bacteremia.* Clinical Microbiology Reviews, 2012. **25**(2): p. 362-386.

71. Chong, Y.P., et al., *Treatment Duration for Uncomplicated Staphylococcus aureus Bacteremia To Prevent Relapse: Analysis of a Prospective Observational Cohort Study.* Antimicrobial Agents and Chemotherapy, 2013. **57**(3): p. 1150-1156.

72. Kollef, M.H., et al., *Epidemiology and Outcomes of Health-care–Associated Pneumonia: Results From a Large US Database of Culture-Positive Pneumonia.* Chest, 2005. **128**(6): p. 3854-3862.

73. *Guidelines for the Management of Adults with Hospital-acquired, Ventilator-associated, and Healthcare-associated Pneumonia.* American Journal of Respiratory and Critical Care Medicine, 2005. **171**(4): p. 388-416.

74. Wunderink, R.G., *How Important is Methicillin-Resistant Staphylococcus aureus as a Cause of Community-Acquired Pneumonia and What is Best Antimicrobial Therapy?* Infectious Disease Clinics of North America, 2013. **27**(1): p. 177-188.

75. Moran, G.J., et al., *Prevalence of Methicillin-Resistant Staphylococcus aureus as an Etiology of Community-Acquired Pneumonia.* Clinical Infectious Diseases, 2012. **54**(8): p. 1126-1133.

76. Huang, S.S., et al., *Methicillin-Resistant Staphylococcus aureus Infection and Hospitalization in High-Risk Patients in the Year following Detection.* PLoS ONE, 2011. **6**(9).

77. DeRyke, C.A., et al., *Epidemiology, Treatment, and Outcomes of Nosocomial Bacteremic Staphylococcus aureus Pneumonia.* Chest, 2005. **128**(3): p. 1414-1422.

78. Kallen, A.J., et al., *Staphylococcus aureus Community-Acquired Pneumonia During the 2006 to 2007 Influenza Season.* Annals of Emergency Medicine, 2009. **53**(3): p. 358-365.

79. Robertson, L., *IMPORTANCE OF STAPHYLOCOCCUS AUREUS IN PNEUMONIA IN THE 1957 EPIDEMIC OF INFLUENZA A.* The Lancet (British edition). **272**(7040): p. 233-236.

80. Finelli, L., et al., *Influenza-Associated Pediatric Mortality in the United States: Increase of Staphylococcus aureus Coinfection.* Pediatrics, 2008. **122**(4): p. 805-811.

81. Kallen, A.J., et al., *Staphylococcus aureus community-onset pneumonia in patients admitted to children's hospitals during autumn and winter of 2006–2007.* Epidemiology and Infection, 2010. **138**(05): p. 666.

82. Francis, J., et al., *Severe community-onset pneumonia in healthy adults caused by methicillin-resistant Staphylococcus aureus carrying the Panton-Valentine leukocidin genes.* Clinical infectious diseases : an official publication of the Infectious Diseases Society of America, 2005. **40**(1): p. 100-107.

83. Francis, J.S., et al., *Severe Community-Onset Pneumonia in Healthy Adults Caused by Methicillin-Resistant Staphylococcus aureus Carrying the Panton-Valentine Leukocidin Genes.* Clinical Infectious Diseases, 2005. **40**(1): p. 100-107.

84. Gillet, Y., et al., *Factors Predicting Mortality in Necrotizing Community-Acquired Pneumonia Caused by Staphylococcus aureus Containing Panton-Valentine Leukocidin.* Clinical Infectious Diseases, 2007. **45**(3): p. 315-321.

85. Alonzo, F. and V.J. Torres, *The bicomponent pore-forming leucocidins of Staphylococcus aureus.* Microbiology and molecular biology reviews : MMBR, 2014. **78**(2): p. 199-230.

86. Berube, B. and J. Wardenburg, *Staphylococcus aureus α-Toxin: Nearly a Century of Intrigue.* Toxins, 2013. **5**.

87. Dumont, A.L. and V.J. Torres, *Cell targeting by the Staphylococcus aureus pore-forming toxins: it's not just about lipids.* Trends in microbiology, 2013. **22**(1): p. 21-27.

88. Yoong, P. and V.J. Torres, *The effects of Staphylococcus aureus leukotoxins on the host: cell lysis and beyond.* Current Opinion in Microbiology, 2013. **16**(1).

89. Burnet, F.M., *The production of staphylococcal toxin.* The Journal of Pathology and Bacteriology, 1930. **33**(1): p. 1-16.

90. Burnet, F.M., *The exotoxins ofStaphylococcus pyogenes aureus.* The Journal of Pathology and Bacteriology, 1929. **32**(4): p. 717-734.

91. Bernheimer, A.W. and L.L. Schwartz, *Isolation and Composition of Staphylococcal Alpha Toxin.* Journal of General Microbiology, 1963. **30**(3): p. 455-468.

92. Thelestam, M., R. Möllby, and T. Wadström, *Effects of staphylococcal alpha-, beta-, delta-, and gamma-hemolysins on human diploid fibroblasts and HeLa cells: evaluation of a new quantitative as say for measuring cell damage.* Infection and immunity, 1973. **8**(6): p. 938-946.

93. Thelestam, M. and R. Möllby, *Sensitive assay for detection of toxin-induced damage to the cytoplasmic membrane of human diploid fibroblasts.* Infection and immunity, 1975. **12**(2): p. 225-232.

94. Füssle, R., et al., *On the mechanism of membrane damage by Staphylococcus aureus alpha-toxin.* The Journal of Cell Biology, 1981. **91**(1): p. 83-94.

95. Cassidy, P. and S. Harshman, *Studies on the binding of staphylococcal 125I-labeled α-toxin to rabbit erythrocytes.* Biochemistry, 1976. **15**(11): p. 2348-2355.

96. Hildebrand, A., M. Pohl, and S. Bhakdi, *Staphylococcus aureus alpha-toxin. Dual mechanism of binding to target cells.* The Journal of biological chemistry, 1991. **266**(26): p. 17195-17200.

97. Bhakdi, S., et al., *Release of interleukin-1 beta associated with potent cytocidal action of staphylococcal alpha-toxin on human monocytes.* Infection and immunity, 1989. **57**(11): p. 3512-3519.

98. Bhakdi, S., et al., *Staphylococcal alpha toxin promotes blood coagulation via attack on human platelets.* The Journal of experimental medicine, 1988. **168**(2): p. 527-542.

99. Peng, H.L., et al., *Cloning, characterization, and sequencing of an accessory gene regulator (agr) in Staphylococcus aureus.* Journal of bacteriology, 1988. **170**(9): p. 4365-4372.

100. Novick, R.P., et al., *Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule.* The EMBO journal, 1993. **12**(10): p. 3967-3975.

101. Cheung, A.L., J.M. Koomey, and C.A. Butler, *Regulation of exoprotein expression in Staphylococcus aureus by a locus (sar) distinct from agr.* Proceedings of the National Academy of Sciences, 1992.

102. Xiong, Y.Q., et al., *Regulation of Staphylococcus aureus α-Toxin Gene (hla) Expression by agr, sarA, and sae In Vitro and in Experimental Infective Endocarditis.* The Journal of Infectious Diseases, 2006. **194**(9): p. 1267-1275.

103. Giraudo, T.A., L.A. Cheung, and R. Nagel, *The sae locus of Staphylococcus aureus controls exoprotein synthesis at the transcriptional level.* Archives of Microbiology. **168**(1): p. 53-58.

104. Gouaux, J.E., et al., *Subunit stoichiometry of staphylococcal alpha-hemolysin in crystals and on membranes: a heptameric transmembrane pore.* Proceedings of the National Academy of Sciences, 1994. **91**(26): p. 12828-12831.

105. Song, L.L., *Structure of staphylococcal alpha-hemolysin, a heptameric transmembrane pore.* Science (New York, N.Y.). **274**(5294): p. 1859.

106. Valeva, A., et al., *Evidence that clustered phosphocholine head groups serve as sites for binding and assembly of an oligomeric protein pore.* J Biol Chem, 2006. **281**(36): p. 26014-21.

107. Wilke, G. and J. Bubeck Wardenburg, *Role of a disintegrin and metalloprotease 10 in Staphylococcus aureus alpha-hemolysin-mediated cellular injury.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(30): p. 13473-13478.

108. Fritz, S.A., et al., *A serologic correlate of protective immunity against community-onset Staphylococcus aureus infection.* Clin Infect Dis, 2013. **56**(11): p. 1554-61.

109. Adhikari, R.P., et al., *Lower antibody levels to Staphylococcus aureus exotoxins are associated with sepsis in hospitalized adults with invasive S. aureus infections.* J Infect Dis, 2012. **206**(6): p. 915-23.

110. Kolata, J., et al., *Distinctive patterns in the human antibody response to Staphylococcus aureus bacteremia in carriers and non-carriers.* Proteomics, 2011. **11**(19): p. 3914-27.

111. Bubeck Wardenburg, J., et al., *Poring over pores: alpha-hemolysin and Panton-Valentine leukocidin in Staphylococcus aureus pneumonia.* Nature medicine, 2007. **13**(12): p. 1405-1406.

112. Bubeck Wardenburg, J., R. Patel, and O. Schneewind, *Surface proteins and exotoxins are required for the pathogenesis of Staphylococcus aureus pneumonia.* Infection and immunity, 2007. **75**(2): p. 1040-1044.

113. Bubeck Wardenburg, J. and O. Schneewind, *Vaccine protection against Staphylococcus aureus pneumonia.* The Journal of experimental medicine, 2008. **205**(2): p. 287-294.

114. Ragle, B. and J. Bubeck Wardenburg, *Anti-alpha-hemolysin monoclonal antibodies mediate protection against Staphylococcus aureus pneumonia.* Infection and immunity, 2009. **77**(7): p. 2712-2718.

115. Kennedy, A.D., et al., *Targeting of alpha-hemolysin by active or passive immunization decreases severity of USA300 skin infection in a mouse model.* The Journal of infectious diseases, 2010. **202**(7): p. 1050-1058.

116. Rauch, S., et al., *Abscess Formation and Alpha-Hemolysin Induced Toxicity in a Mouse Model of Staphylococcus aureus Peritoneal Infection.* Infection and Immunity, 2012. **80**(10): p. 3721-3732.

117. Menzies, B.E. and D.S. Kernodle, *Passive immunization with antiserum to a nontoxic alpha-toxin mutant from Staphylococcus aureus is protective in a murine model.* Infection and immunity, 1996. **64**(5): p. 1839-1841.

118. Patel, A.H., et al., *Virulence of protein A-deficient and alpha-toxin-deficient mutants of Staphylococcus aureus isolated by allele replacement.* Infection and immunity, 1987. **55**(12): p. 3103-3110.

119. O'Callaghan, R.J., et al., *Specific roles of alpha-toxin and beta-toxin during Staphylococcus aureus corneal infection.* Infection and immunity, 1997. **65**(5): p. 1571-1578.

120. Bayer, A.S., et al., *Hyperproduction of alpha-toxin by Staphylococcus aureus results in paradoxically reduced virulence in experimental endocarditis: a host defense role for platelet microbicidal proteins.* Infection and immunity, 1997. **65**(11): p. 4652-4660.

121. Bramley, A.J., et al., *Roles of alpha-toxin and beta-toxin in virulence of Staphylococcus aureus for the mouse mammary gland.* Infection and immunity, 1989. **57**(8): p. 2489-2494.

122. Jonsson, P., et al., *Virulence of Staphylococcus aureus in a mouse mastitis model: studies of alpha hemolysin, coagulase, and protein A as possible virulence determinants with protoplast fusion and gene cloning.* Infection and immunity, 1985. **49**(3): p. 765-769.

123. Craven, R., et al., *Staphylococcus aureus alpha-hemolysin activates the NLRP3-inflammasome in human and mouse monocytic cells.* PloS one, 2009. **4**(10).

124. Kebaier, C., et al., *Staphylococcus aureus α-hemolysin mediates virulence in a murine model of severe pneumonia through activation of the NLRP3 inflammasome.* The Journal of infectious diseases, 2012. **205**(5): p. 807-817.

125. Muñoz-Planillo, R., et al., *A critical role for hemolysins and bacterial lipoproteins in Staphylococcus aureus-induced activation of the Nlrp3 inflammasome.* Journal of immunology (Baltimore, Md. : 1950), 2009. **183**(6): p. 3942-3948.

126. John, S.C., et al., *Neutrophil-derived IL-1β Is Sufficient for Abscess Formation in Immunity against Staphylococcus aureus in Mice.* PLoS Pathogens, 2012. **8**(11).

127. Becker, R.E., et al., *Tissue-Specific Patterning of Host Innate Immune Responses by S taphylococcus aureus α-Toxin.* Journal of innate immunity, 2014.

128. Miller, L., et al., *Necrotizing fasciitis caused by community-associated methicillin-resistant Staphylococcus aureus in Los Angeles.* The New England journal of medicine, 2005. **352**(14): p. 1445-1453.

129. Inoshima, I., et al., *A Staphylococcus aureus pore-forming toxin subverts the activity of ADAM10 to cause lethal infection in mice.* Nature medicine, 2011. **17**(10): p. 1310-1314.

130. Inoshima, N., Y. Wang, and J. Bubeck Wardenburg, *Genetic Requirement for ADAM10 in Severe Staphylococcus aureus Skin Infection.* The Journal of investigative dermatology, 2012. **132**(5): p. 1513-1516.

131. Powers, M., et al., *ADAM10 Mediates Vascular Injury Induced by Staphylococcus aureus α-Hemolysin.* The Journal of infectious diseases, 2012.

132. Powers, Michael E., et al., *Synergistic Action of Staphylococcus aureus α-Toxin on Platelets and Myeloid Lineage Cells Contributes to Lethal Sepsis.* Cell Host & Microbe, 2015. **17**(6): p. 775-787.

133. Agostini, L., et al., *NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder.* Immunity, 2004. **20**(3): p. 319-325.

134. Willingham, S., et al., *Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/cryopyrin/NLRP3 and ASC.* Cell host & microbe, 2007. **2**(3): p. 147-159.

135. Willingham, S., et al., *NLRP3 (NALP3, Cryopyrin) facilitates in vivo caspase-1 activation, necrosis, and HMGB1 release via inflammasome-dependent and -independent pathways.* Journal of immunology (Baltimore, Md. : 1950), 2009. **183**(3): p. 2008-2015.

136. Anand, P., R. Malireddi, and T.-D. Kanneganti, *Role of the nlrp3 inflammasome in microbial infection.* Frontiers in microbiology, 2011. **2**: p. 12.

137. Melehani, J.H., et al., *Staphylococcus aureus Leukocidin A/B (LukAB) Kills Human Monocytes via Host NLRP3 and ASC when Extracellular, but Not Intracellular.* PLoS pathogens, 2015. **11**(6).

138. Perregaux, D. and C.A. Gabel, *Interleukin-1 beta maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity.* The Journal of biological chemistry, 1994. **269**(21): p. 15195-15203.

139. Ferrari, D., et al., *The P2X7 receptor: a key player in IL-1 processing and release.* Journal of immunology (Baltimore, Md. : 1950), 2006. **176**(7): p. 3877-3883.

140. Ludwig, A., et al., *Metalloproteinase inhibitors for the disintegrin-like metalloproteinases ADAM10 and ADAM17 that differentially block constitutive and*

phorbol ester-inducible shedding of cell surface molecules. Combinatorial chemistry & high throughput screening, 2005. **8**(2): p. 161-171.

141. Murphy, G., *Regulation of the proteolytic disintegrin metalloproteinases, the 'Sheddases'.* Seminars in cell & developmental biology, 2009. **20**(2): p. 138-145.

142. Perret, M., et al., *Cross-talk between Staphylococcus aureus leukocidins-intoxicated macrophages and lung epithelial cells triggers chemokine secretion in an inflammasome-dependent manner.* Cellular microbiology, 2012.

143. Adhikari, R.P., et al., *Lower Antibody Levels to Staphylococcus aureus Exotoxins Are Associated With Sepsis in Hospitalized Adults With Invasive S. aureus Infections.* Journal of Infectious Diseases, 2012. **206**(6): p. 915-923.

144. Fritz, S.A., et al., *A Serologic Correlate of Protective Immunity Against Community-Onset Staphylococcus aureus Infection.* Clinical Infectious Diseases, 2013. **56**(11): p. 1554-1561.

145. Hirota, J., et al., *The airway epithelium nucleotide-binding domain and leucine-rich repeat protein 3 inflammasome is activated by urban particulate matter.* The Journal of allergy and clinical immunology, 2012. **129**(4): p. 1116-1125.

146. Allen, I., et al., *The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA.* Immunity, 2009. **30**(4): p. 556-565.

147. Fulcher, M.L., et al., *Well-Differentiated Human Airway Epithelial Cell Cultures*, in *Human Cell Culture Protocols*, J. Picot, Editor. 2005, Humana Press: Totowa, NJ. p. 183-206.

148. Matsui, H., et al., *Coordinated clearance of periciliary liquid and mucus from airway surfaces.* The Journal of clinical investigation, 1998. **102**(6): p. 1125-1131.

149. Zhang, L., et al., *Respiratory syncytial virus infection of human airway epithelial cells is polarized, specific to ciliated cells, and without obvious cytopathology.* Journal of virology, 2002. **76**(11): p. 5654-5666.

150. Bernacki, S.H., et al., *Mucin gene expression during differentiation of human airway epithelia in vitro. Muc4 and muc5b are strongly induced.* American journal of respiratory cell and molecular biology, 1999. **20**(4): p. 595-604.

151. Randell, S.H., et al., *Primary epithelial cell models for cystic fibrosis research.* Methods Mol Biol, 2011. **742**: p. 285-310.

152. Wu, Q., et al., *Airway epithelial cell tolerance to Pseudomonas aeruginosa.* Respiratory research, 2005. **6**: p. 26.

153. T, H. and W. A, *Ménage à trois of bacterial and viral pulmonary pathogens delivers coup de grace to the lung.* Clinical & Experimental Immunology, 2004.

154. Rothberg, M.B., S.D. Haessler, and R.B. Brown, *Complications of viral influenza.* Am J Med, 2008. **121**(4): p. 258-64.

155. Chertow, D.S., et al., *Influenza A and methicillin-resistant Staphylococcus aureus co-infection in rhesus macaques – A model of severe pneumonia.* Antiviral Research, 2016. **129**: p. 120-129.