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Factors Contributing to Pathogenesis of *Pseudomonas aeruginosa*

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Ami Joy Hughes

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ABSTRACT

Pseudomonas aeruginosa is a Gram-negative pathogen that frequently causes severe nosocomial infections through expression of virulence factors, evasion of immune clearance and resistance to therapeutic antimicrobial agents. These factors have led the US Centers for Disease Control and Prevention (CDC) and Infectious Disease Society of America (IDSA) to identify *P. aeruginosa* as a severe threat to public health. Thus, understanding *P. aeruginosa* pathogenesis is of interest and concern to the medical and scientific communities. In the current study, we examined factors that contribute to pathogenesis, including the host immune response and antimicrobial resistance. Using mice deficient in components of the inflammasome, an important component of the host innate immune defense to infection, we demonstrated that the inflammasome adaptor protein apoptosis speck-like protein containing a CARD (ASC) is associated with severe pre-lethal illness in a mouse model of pneumonia. In the absence of ASC, infected mice exhibited improved survival and bacterial clearance from the lungs compared to mice lacking the inflammasome effector protein caspase-1/11 and C57BL/6 (B6) mice. Interestingly, we found that ASC is required for activation of caspase-3 in addition to caspase-1. Furthermore, activation of ASC-dependent caspase-3 activity was dependent on *P. aeruginosa* possessing a functional type III secretion system. Collectively, these findings may provide an alternate strategy for *P. aeruginosa* to promote severe infection *in vivo* by activating caspase-3 through ASC, independent of caspase-1 activation. Next, we utilized a pre-existing library of *P. aeruginosa* isolates to analyze relationships between antimicrobial resistance and bacterial virulence using statistical models. We found that strains with higher bacterial virulence had a lower odds ratio of exhibiting an antibiotic-resistant or MDR phenotype by logistic regression. Interestingly, *P. aeruginosa* strains that secrete the T3SS effector protein ExoU had higher odds ratio of antibiotic resistance. We also found a correlation between resistance to a greater number of antimicrobial classes and lower virulence scores by linear

regression. Collectively, these models demonstrate an inverse relationship between antimicrobial resistance and bacterial virulence. In summary, we describe how *P. aeruginosa* exacerbates the ASC-mediated immune response to promote severe illness in a mouse model of pneumonia and how strains of increased virulence are more likely to be antibiotic susceptible or exhibit resistance to fewer antimicrobial classes. These results further our understanding of the various strategies that *P. aeruginosa* employs to overcome clearance mechanisms in promoting disease.

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CHAPTER 1: **Introduction**

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium that causes nosocomial infections such as pneumonia, urinary tract infections (UTIs), bloodstream infections (BSIs) and wound infections [1]. The US Centers for Disease Control and Prevention (CDC) estimates that *P. aeruginosa* causes 51,000 infections in hospitalized patients annually [2]. The prevalence of *P. aeruginosa* nosocomial infections, severity of infection outcomes in people and emergence of antimicrobial resistance led the CDC to list multidrug-resistant (MDR) *Pseudomonas* as a "serious" threat to public health in 2013 [2]. Thus, understanding *P. aeruginosa* pathogenesis is of interest to the scientific and medical communities.

Manifestation of bacterial infections can be thought of in terms of 3 factors: 1) virulence of the pathogen, 2) susceptibility of the host and 3) effectiveness of treatment (Figure 1). Virulence refers to the ability of the pathogen to cause illness. Host susceptibility refers to the ability of a host to recognize a pathogen and mount, or fail to mount, a successful immune response to that pathogen. Ability to treat infections refers to availability and appropriate use of efficacious therapeutic antimicrobial agents. Disease manifests when a pathogen is able to establish infection and the host immune system fails to clear the pathogen. Even if the host defenses fail and bacteria are able to cause disease, effective antimicrobial agents may ameliorate disease by killing the pathogen (bactericidal agents) or attenuating bacterial growth (bacteriostatic agents) [2]. However, pathogens like *P. aeruginosa* have evolved to become resistant to many antimicrobial agents. In this way, infections are a balance of these three factors: virulence of the bacterium, host immune defenses and therapeutic antimicrobial agents (Figure 1). The current work examines factors that contribute to *P. aeruginosa* pathogenesis related to the host

immune response and to *P. aeruginosa* antimicrobial resistance. Specifically, we examine activation of the host inflammasome during acute *P. aeruginosa* pneumonia. We also examine the prevalence of antibiotic resistance among *P. aeruginosa* clinical isolates and the impact of antibiotic resistance on bacterial virulence.

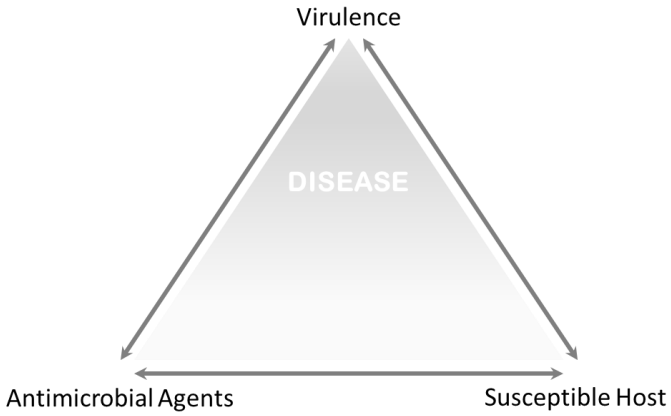


Figure 1 – **Factors influencing *P. aeruginosa* pathogenesis**. *P. aeruginosa* infections are a balance of bacterial virulence, the host immune response and therapeutic antimicrobial agents in determining whether a pathogen is successful in causing illness or if illness is ameliorated by the host defenses and/or treatment.

P. AERUGINOSA TYPE III SECRETION SYSTEM

P. aeruginosa has developed an arsenal of virulence determinants that allow its persistence and spread within a host. Extensive research has been conducted regarding virulence determinants of *P. aeruginosa* [1]. Notably, *P. aeruginosa* has a large genome of 5.5-7 Mbp [3] that includes both core genome (highly conserved genes shared by all or nearly all *P. aeruginosa* strains) and accessory genome (variable genes found in some *P. aeruginosa* strains but absent in others) [4]. Virulence determinants may be encoded in core and accessory genetic elements [4]. Some of the many virulence determinants of *P. aeruginosa* include flagellum, pili, lipopolysaccharide (LPS), pyoverdine that scavenges iron, the

type III secretion system (T3SS) and secreted effectors ExoU, ExoS, ExoT and ExoY [1, 4]. The current work focuses on the T3SS.

The T3SS is a multi-protein complex that spans the inner membrane, periplasm and outer membrane of the bacterium, creating a needle-like apparatus on the cell surface [5, 6]. The basal structure of the needle apparatus includes the lipoprotein PscJ [6, 7]. The PscI protein forms the inner rod complex of the assembly [8] and PscC forms the outer membrane channel protein [6]. PscF oligomerizes to form the needle complex [6]. Upon contact with mammalian cells, the tip of the needle-like apparatus forms a pore through the membrane of the target cell. This pore, referred to as the translocon, is made of a complex of PopD, PopB and PcrV proteins [5, 6]. The bacterium is able to secrete effectors from the bacterial cytoplasm directly into the host cytosol through the channel created by the T3SS. Disruption of any of the proteins required for formation of the needle-like apparatus (including PscI, PscF, PscC or PscJ) renders the T3SS non-functional [7-10].

As mentioned previously, there are four major effector proteins secreted by the T3SS: ExoU, ExoS, ExoT and ExoY. While nearly all *P. aeruginosa* strains encode the genes of the secretion apparatus and translocon [11], the genes encoding the effector proteins are not present in all strains. Just like the genes of the translocon, nearly all strains encode the *exoT* gene, which shares over 80% sequence identity with the *exoS* gene [11]. However, just over 70% of *P. aeruginosa* strains encode the *exoS* gene [11]. ExoT and ExoS are bi-functional proteins that have ADP-ribosyltransferase (ADPRT) activity and a GTPase activating protein (GAP) domain [6]. Once secreted into eukaryotic cells, ExoS targets Rho and Rac leading to cell rounding [6]. The *exoY* gene is present in approximately 86% of strains [11], and the ExoY protein has adenylyl cyclase activity [6]. Conversely, the *exoU* gene is found in a minority of

strains, with less than 30% of clinical isolates encoding the ExoU protein [6, 11]. Furthermore, the *exoS* and *exoU* genes are nearly mutually exclusive with the majority of strains encoding one or the other but very few strains encoding both [6, 11]. The ExoU protein has phospholipase A_2 activity that cleaves phospholipids in the cell membrane of eukaryotic cells leading to rapid cell lysis [6, 12]. In this way, the T3SS effectors have specific protein functions that promote infection by disrupting cellular processes upon translocation into the cytosol of mammalian cells.

HOST INFLAMMASOME-MEDIATED IMMUNE RESPONSE

Mammals have evolved a multi-layered defense system to prevent infection and rapidly kill microbes. The inflammasome is a multimeric protein complex that is an important component of the innate immune response to many endogenous and exogenous danger signals [13, 14]. It is activated by a broad spectrum of microbial pathogens including *P. aeruginosa*, *Salmonella enterica, Shigella flexneri, Bacillus anthracis, Listeria monocytogenes* and *Legionella pneumophila.* Our understanding of how *P. aeruginosa* interacts with the inflammasome is rapidly evolving.

While some studies have shown that inflammasome activation by *P. aeruginosa* is beneficial to the host [8, 15, 16], increasing evidence suggests that this bacterium can also cause an excessive and pathologic inflammasome-mediated response that promotes bacterial persistence [17-20]. *P. aeruginosa* has developed mechanisms to subvert inflammasome signaling, turning an important host defensive shield into a bacterial offensive weapon. A better understanding of these processes and their roles in pathogenesis is vital and may identify targets for therapeutic intervention. Indeed, recent studies have shown that treatment of *P. aeruginosa* infections with macrolides may be beneficial by reducing inflammasome activation [21]. Novel interventions are critically needed to combat *P. aeruginosa*

infections, which are increasingly difficult to treat with conventional antibiotics [22, 23].

Overview of Inflammasome Assembly and Activation

During infection, the inflammasome functions as a sensor that recognizes the presence of pathogenic bacteria and initiates a proinflammatory response. The current model of inflammasome assembly proposes that a pattern recognition receptor (PRR) in the cytosol of a host immune cell senses a pathogen associated molecular pattern (PAMP) and triggers an activation cascade [24, 25]. At the core of the active inflammasome is a NOD-like receptor (NLR). NLR proteins include three structural domains with a pyrin or caspase recruitment domain (CARD) at the amino terminus, a central nucleotide oligomerization domain (NOD) and a leucine rich repeat (LRR) at the carboxy terminus. Through these domains, NLRs directly or indirectly bind additional proteins to form the inflammasome complex. While many inflammasomes are known to be activated during bacterial infection [14], the NRLC4 inflammasome has been best studied in the context of *P. aeruginosa* [8, 26].

Briefly, the first step in inflammasome activation occurs when the cell becomes primed by sensing the presence of bacteria through surface-exposed Toll-like receptors (TLRs). This sets in motion a signaling cascade that causes expression of immature forms of proinflammatory cytokines. The NLRC4 inflammasome itself becomes activated upon recognition of microbial PAMPs in the cytosol such as *P. aeruginosa* flagellin and components of the type III secretion apparatus. PAMP recognition is mediated by NLR apoptosis-inhibitory proteins (NAIPs). NAIPs then bind to NLRC4 proteins, which in turn form a complex with the inactive form of the cysteine protease pro-caspase-1. This interaction may utilize the adapter molecule apoptosis-associated speck-like protein containing CARD (ASC). Once assembled, the regulatory pro-domain of caspase-1 is autoproteolytically cleaved. Mature caspase-1 cleaves the

regulatory pro-domains from cytokines interleukin-1β (IL-1β) and IL-18. These mature cytokines are then secreted through a poorly characterized mechanism. This process is dependent on a decrease in intracellular K^+ [27]. Cytokine release initiates a broader innate immune response involving recruitment of phagocytes to the site of infection [28]. Another consequence of NLRC4 activation is death of the host cell by pyroptosis, a proinflammatory form of regulated cell death [29, 30]. Each of these processes contributes to the host immune response to *P. aeruginosa* infection. Inflammasome activation is tightly regulated and endogenous factors can dampen this activation under specific circumstances.

In summary, microbial PAMPs are sensed in the cytosol by the inflammasome. This initiates a robust immune response to clear the infecting bacteria. The processes of the inflammasome response can be divided into the following steps: 1) priming, 2) ligand recognition, 3) functions of effector pathways and 4) activity regulation (Figure 2). Our understanding of each of these steps in the context of *P. aeruginosa* infection is discussed in the following sections.

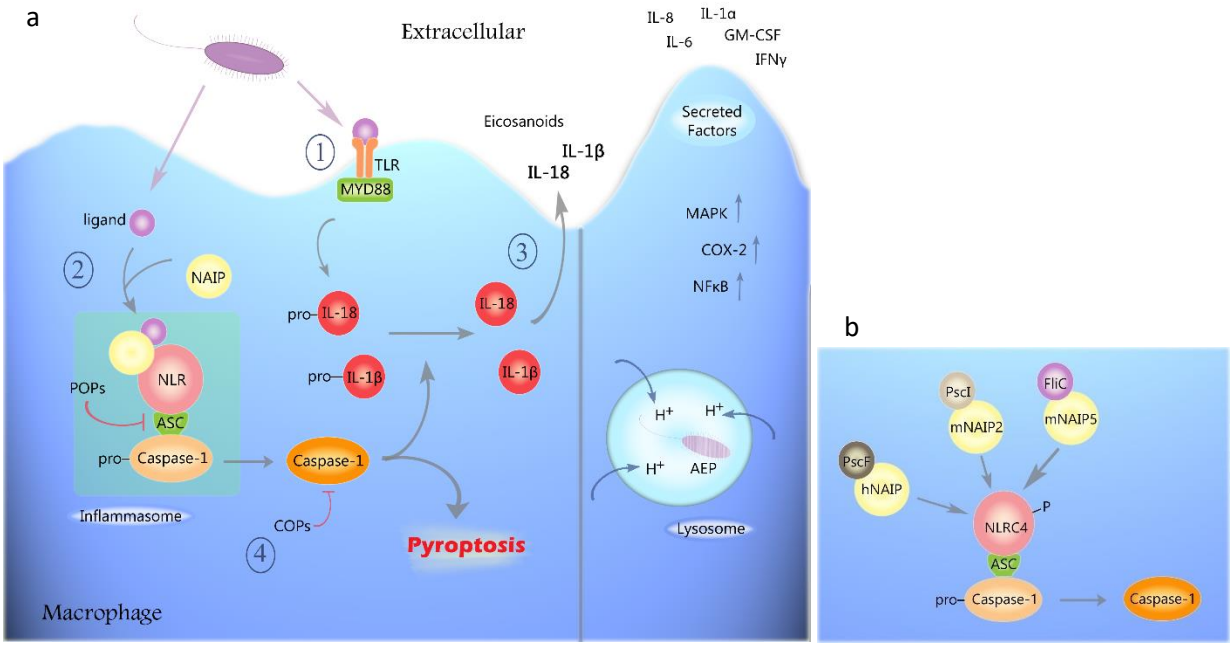


Figure 2 – Overview of inflammasome activation cascade by *P. aeruginosa*. (Figure adapted from [31]) a) Inflammasome activation can be divided into four broad steps: *1* Priming – Inflammasome assembly is initiatied by recognition of a PAMP at the cell membrane that primes transcription of immature cytokines. *2* Ligand recognition – PAMP recognition in the cytosol by NAIPs results in oligomerization of the inflammasome complex and subsequent autoproteolysis of caspase-1. *3* Caspase-1 effector functions – caspase-1 initiated downstream effector pathways including processing of proinflammatory cytokines, production of eicosanoids and cell death by pyroptosis. *4* Regulation of the inflammasome response – Inflammasome assembly is regulated by POPs and COPs. Implications of effector functions during *P. aeruginosa* will be discussed in Chapter 2. b) Specificity of NAIPs for ligands and transduction of ligand recognition into assembly of the NLR and pro-caspase-1.

Inflammasome Priming

Inflammasome activation occurs in a multi-step process that requires two independent signaling events. The first signal activates a toll-like receptor (TLR) on the cell surface that in turn activates a signaling cascade culminating in transcription of specific genes to yield immature cytokines [32, 33]. TLR engagement, however, is not sufficient to induce caspase-1 autoproteolysis or secretion of the mature forms of IL-1β or IL-18 cytokines [16, 32, 33]. The second signal is ligand recognition (discussed in the next section), which activates an NLR in the cell cytoplasm. NLR activation by itself cannot promote the

synthesis of pro-IL-1β or pro-IL-18 but is required for caspase-1-dependent cytokine processing and release [8, 16, 32, 33]. Thus, TLR engagement primes macrophages for inflammasome activation by producing pools of immature cytokines, which are subsequently processed upon stimulation of intracellular NLRs.

The processes of priming and activation may be linked in certain contexts such that a separate priming step may not be observable or required. For example, in the context of infection, a cell is assaulted with multiple bacterial PAMPs (LPS, flagellin, lipoproteins, etc.) at the same time, and priming may occur concomitantly with inflammasome ligand recognition. Pre-stimulation to induce immature cytokine transcription may not be required *in vivo* or during infection of macrophages *in vitro* with live bacteria [15]. Additionally, there is evidence that pro-IL-18 is constitutively expressed in human peripheral blood mononuclear cells (PMBCs). However, the second NLR signal is still required for maturation and secretion of IL-18 [32]. This suggests that the model for IL-18 maturation varies slightly from the two-signal activation of inflammasomes discussed previously. A similar variation of the two-signal activation model occurs in the gut and allows the host to discriminate between non-pathogenic and pathogenic bacteria. In the context of the gut, resident intestinal macrophages are anergic to TLR stimulation by LPS, CpG DNA and lipoproteins from constant engagement by commensal bacteria [34]. Whereas intestinal macrophages failed to produce TNF or IL-6 in response to TLR stimulation, these cells constitutively produced immature pro-IL-1β. As a result, they produced mature capase-1 and IL-1β when exposed to pathogenic bacteria but not commensal bacteria [34]. This response was dependent on NLRC4, demonstrating a role for the inflammasome in the immune response to pathogenic bacteria in the gut [34]. In this way, the host appropriately regulates inflammasome activation through the two-signal model such that the inflammasome is activated when pathogenic bacteria are detected but fails

to mount an immune response against normal gut flora.

Inflammasome Ligand Recognition

Ligand recognition is the paramount step to signal assembly of the inflammasome complex. Three *P. aeruginosa* PAMPs are known to activate the inflammasome. They are 1) flagellin, the major component of flagella [8, 9], 2) the T3SS [9, 26] and 3) pilin, the major component of type IV pili [35]. While the specific inflammasome activated by type IV pilin has yet to be identified, both flagellin and the T3SS activate the NLRC4 inflammasome.

Activation of the NLRC4 inflammasome requires sensing of the ligands in the cytosol of the target cell. Yet how the ligands, particularly flagellin, gain access to the cytosol has long been a perplexing question. Early studies examining the activation of the inflammasome noted a dependence of flagellin recognition on functional T3SS [7, 27, 36, 37]. This was initially interpreted as T3SS-dependent delivery of flagellin into the cytosol. Indeed, studies with *S. enterica* established a direct link between secretion of flagellin and functional T3SS [38]. However, it has since been shown that flagellin and T3SS are distinct activators of the inflammasome [24-27]. Consistent with this finding, non-flagellated strains of *P. aeruginosa* are capable of activating the inflammasome [7, 15, 16, 27]. Thus, the manner by which these ligands access the cytosol for detection by the inflammasome remains unclear, but it is now understood that flagellin and T3SS independently activate the inflammasome.

Previously, the nature of ligand recognition (specific or non-specific, direct or indirect) by NLRC4 was the source of some debate. It had been known for some time that murine NAIP5 played an important role in flagellin recognition by NLRC4 [39]. In the absence of NAIP5, *L. pneumophila* no longer activated the

NLRC4 inflammasome [39]. During *P. aeruginosa* infection, however, inflammasome-dependent cell death [39] and cytokine processing [26] were not abrogated in NAIP5-deficient murine macrophages. Unlike *L. pneumophila* that does not have a T3SS, early studies of flagellin-dependent activation of the inflammasome during *P. aeruginosa* infection were likely confounded by T3SS-dependent activation of the inflammasome. The specific nature of ligand recognition was examined using a reconstituted inflammasome expression system in non-immune cells. In this way, it was demonstrated that flagellin is directly recognized by murine NAIP5 [24, 25]. *L. pneumophila* flagellin interacted with NAIP6 in addition to NAIP5. Components of the T3SS apparatus are also recognized by different NAIPs. It was discovered that PscI is recognized by murine NAIP2 [24, 25]. Similarly, NAIP1 recognizes the T3SS needle proteins of bacteria such as *S. enterica,* enterohemorrhagic *Escherichia coli, S. flexneri and Burkholderia* spp. [40]. It remains unclear whether this is true for PscF of *P. aeruginosa.* Upon ligand recognition, the NAIP proteins subsequently oligomerize with NLRC4 in the inflammasome complex [24, 25, 40]. Incorporating this information into the current model of NLRC4 activation suggests that ligand recognition is mediated by distinct NAIPs that directly bind specific ligands and in turn interact with NLRC4 to initiate assembly of the inflammasome complex.

An unanticipated aspect of flagellin-mediated recognition of *P. aeruginosa* is that motility, rather than the mere presence of flagella, is associated with increased inflammasome activation. The *motAB* and/or *motCD* genes are required for flagellar rotation and bacterial motility but do not affect flagellar architecture. Previous studies demonstrated that disruption of these genes, which rendered *P. aeruginosa* strains non-motile, conferred resistance to phagocytosis [41, 42]. Non-motile strains also failed to fully induce inflammasome-dependent maturation of pro-caspase-1 [43]. Similar observations were made with *S. enterica;* during infection, non-motile strains elicited reduced levels of IL-1β secretion

and pyroptosis-like cytotoxicity compared to motile strains [37]. Collectively, the data suggest that flagellar motility, not just specific ligand recognition, plays a role in activating the inflammasome, although the mechanisms by which this occurs remain unclear.

Specific recognition of NAIP/NLRC4-ligands is dependent on critical residues in the carboxy termini of these ligands. NAIP5-dependent recognition requires leucine residues in the C-terminus of flagellin [9, 25, 39]. Interestingly, this region of flagellin is distinct from the region necessary for TLR5 signaling [39], indicating that the NLRC4 and TLR5 pathways rely upon detection of different PAMPs within the same protein. Alignment of the *S. enterica* T3SS inner rod protein PrgJ and flagellin revealed sequence similarity in the C-termini at these critical residues [9]. Likewise, these residues are conserved between PrgJ and the homologous *P. aeruginosa* T3SS inner rod protein PscI. This region of PscI is required for T3SS-dependent activation of NLRC4 in murine macrophages [9]. Although T3SS ligands and FliC are recognized by different NAIPs in murine macrophages, this homology may be significant in human macrophages that only have a single NAIP [25]. While PscI is necessary for inflammasome recognition, mutations in genes encoding other *P. aeruginosa* T3SS apparatus proteins also abolish inflammasome activation. These include genes encoding the outer membrane channel protein PscC [8], the basal structure component PscJ [7] and the translocon proteins PcrV, PopB and PopD [7, 27]. In the absence of PscC or PscJ, *P. aeruginosa* fails to assemble a functional T3SS apparatus. In contrast, null mutants of PcrV, PopB or PopD assemble a functional T3SS apparatus but fail to inject effector proteins into host cells because they cannot form the channel in the host cell plasma membrane through which these effectors are transported. Presumably, these T3SS mutants do not elicit an inflammasome response because they fail to deliver PscI (or possibly PscF) to the host cytosol. Collectively, these findings demonstrate that NLRC4 ligand recognition is sequence specific and distinct from other known receptor-

mediated signaling pathways.

Most of the current knowledge regarding inflammasome ligand recognition is based on studies in murine cells. Key differences, however, have been identified using human macrophages. Whereas mice produce multiple NAIP proteins, humans have only a single variant [25, 40]. Intriguingly, human NAIP (hNAIP) failed to recognize flagellin under the conditions tested. Furthermore, hNAIP recognizes the T3SS needle protein PscF [25]. Although a direct interaction between PscI and hNAIP has not been shown, there is evidence that PscI can also act as a ligand for hNAIP recognition and activation of NLRC4 in human THP1 cells [44]. As with murine NAIP2/5 and their ligands, human NAIP recognition of the needle protein was dependent on amino acids in the C terminus [25], although the required amino acids are distinct from those recognized in the rod protein [9]. Further studies are needed to fully characterize the differences between human and murine NLRC4 inflammasomes and to clarify the role of flagellin in human inflammasome activation. Even though human and murine NAIPs differ, the central theme of ligand recognition appears to be consistent in both cell types. Ligand recognition is mediated by NAIP(s), which interact with NLRC4 to initiate assembly of the inflammasome complex.

Although much is known about inflammasome activation by flagellin and the T3SS, the specifics of how type IV pili are recognized by inflammasomes remains unclear. Type IV pili are long protein polymers of pilin that mediate twitching motility and adhesion [45]. Type IV pilin was shown to activate the inflammasome by liposomal transfection of bone marrow derived macrophages (BMDMs). Delivery of pilin to these cells resulted in cleavage of caspase-1 and secretion of mature IL-1β [35]. Intriguingly, IL-1β release was abrogated in BMDMs from caspase-$1^{-/-}$ mice but not $NLPR3^{-/-}$, $NLRC4^{-/-}$ or $ASC^{-/-}$ macrophages [35]. Additionally, $NLRC4^{-/-}$, $ASC^{-/-}$ and caspase-$1^{-/-}$ BMDMs were only marginally protected

from cell death by pyroptosis following transfection [35]. Thus, the activity of inflammasomes containing NLRP3, NLRC4, or ASC cannot completely explain the activation of caspase-1 upon exposure to type IV pilin.

Inflammasome Effector Functions

The canonical effector of the NLRC4 inflammasome is caspase-1. Upon maturation, caspase-1 promotes the immune response to bacterial infection by inducing multiple pathways. Caspase-1-dependent release of mature IL-1β and IL-18 is a characteristic outcome of inflammasome activation and is responsible for a number of downstream inflammatory events. Recently, the production of eicosanoids, a class of lipid mediators, was shown to also play a role in the inflammasome-mediated immune response. Finally, inflammasome activation induces the pyroptotic cell death pathway. Each of these effector pathways contributes to resolution of bacterial infection in the appropriate context.

Interleukin 1 family cytokines IL-1β and IL-18 are translated as the inactive precursors pro-IL1β and pro-IL-18, respectively, that require cleavage by caspase-1. Cleavage of the regulatory pro-domain results in release of active cytokines. Mature IL-1β and IL-18 are then recognized by cell surface receptors and induce the NF-κB or MAPK signaling pathways, respectively, to produce IL-6, IL-8 and IL-1α [46]. IL-1β release has pleiotropic effects that together promote a successful immune response. In the context of *P. aeruginosa* infection, Descamps and colleagues have shown that TLR5 engagement results in secretion of IL-1β, which is required for acidification of the phagosome after bacterial uptake by macrophages. Subsequent activation of the lysosomal cysteine protease asparagine endopeptidase (AEP) is necessary for bacterial killing. These authors speculated that inflammasome activation mediated the production of IL-1β that they observed [16]. Of note, inflammasome-dependent

processes are not the only manner by which mature IL-1β is secreted. Cleavage of pro-IL-1β by the serine protease elastase of neutrophils also occurs [47]. IL-18 stimulates T lymphocytes to produce IFNγ and bolsters the neutrophil response by promoting production of GM-CSF [28]. In this way, assembly of the inflammasome complex results in a cascade of events that ultimately produces a robust cytokine response and activates multiple signaling pathways.

The inflammasome and inflammasome-dependent cytokines also converge on the eicosanoid pathway. Macrophages produce eicosanoids, a family of biologically active lipids, through the action of cyclooxygenase (COX) and lipoxygenase (LOX). COX-dependent eicosanoids include prostaglandin D_2, prostaglandin E_2 (PGE2), prostaglandin I_2 (PGI2) and thromboxane A_2 (TXA2). They modulate the function of immune cells and result in a fever response [48]. Leukotrienes, such as the neutrophil chemoattractant leukotriene B_4 (LTB4), are LOX-dependent [49]. It has been known for some time that eicosanoid signaling and IL-1β signaling are linked [48, 49]. Likewise, IL-18 also promotes the eicosanoid response indirectly through IL-1α production, although this response is slower than the IL-1β response [46]. The NLRC4 inflammasome stimulated the rapid and robust production of both LOX-dependent and COX-dependent eicosanoids in response to stimulation with flagellin [50]. This “eicosanoid storm” was dependent on caspase-1 but independent of IL-1β or IL-18, indicating an alternate target of caspase-1. Interestingly, activation of either NLRC4 or NLRP1b inflammasomes resulted in eicosanoid release. Thus, the eicosanoid response may be universally upregulated in a caspase-1 dependent manner and not specific to the activation of any one inflammasome. The rapid induction of the eicosanoid response was associated with the enhanced severity of infection, both *in vitro* following stimulation with purified flagellin and *in vivo* following challenge with *Bacillus anthracis* [50]. Whether inflammasome-mediated eicosanoid production plays a protective role under other conditions requires further investigation. This

study was conducted with the *L. pneumophila* flagellin, and it remains to be demonstrated whether homologous *P. aeruginosa* flagellin causes a similar phenomenon. It will be interesting to learn whether eicosanoid release is clinically relevant to the inflammasome-mediated response to *P. aeruginosa* infection.

As stated above, cell death by pyroptosis is another effector function of caspase-1 and inflammasome activation. Pyroptosis is marked by membrane permeabilization and release of intracellular components [27, 51]. It is inherently inflammatory by virtue of IL-1β and IL-18 release associated with caspase-1 activation [52]. However, while cytokine release requires activation of caspase-1 by cleavage of the regulatory pro-domain, cell death does not require caspase-1 activation [53]. An alternate model of inflammasome assembly suggests that NLRC4 can directly interact with pro-caspase-1 by virtue of its N-terminal CARD domain [53], obviating the need for ASC. Pro-caspase-1 and NLRC4 were sufficient to induce cell death. This may explain certain observations that cell death after *P. aeruginosa* intoxication was dependent on NLRC4 but independent of ASC [8] or caspase-1 catalytic activity [54]. Mechanisms of programmed cell death, including pyroptosis, will be discussed in more detail in future sections.

While there is an established understanding of the role for caspase-1 in pyroptosis and cytokine maturation, the next steps in the inflammasome pathway that lead to cell death and cytokine release have been poorly understood. Recent studies have identified gasdermin D as another target of caspase-1 that may provide clarity to these unknown events in inflammasome activation [55]. Gasdermins have affinity for phospholipids in eukaryotic cell membranes. Upon cleavage by caspase-1 or caspase-11, gasdermin D forms a pore in the cell membrane. Gasdermin D membrane pores may provide channels for release of IL-β and IL-18 from the cytosol into the extracellular milieu [55]. However, accumulation

of gasdermin D pores in the membrane results in rapid cell lysis due to compromised membrane integrity [55]. In this way, caspase-1-dependent cleavage of gasdermin D may provide clarity to the downstream processes of caspase-1 effector functions: providing a means for release of mature cytokines and a mechanism for caspase-1 dependent cell death.

Regulation of the Inflammasome Response

Appropriate regulation is necessary for inflammasomes to maintain productive control of infections without causing off-target disease pathologies [14]. Several regulatory strategies have been discussed thus far, such as the two-signal activation model and production of inactive pro-caspase-1, pro-IL1β and pro-IL-18 that require post-translational processing for release of mature proteins. Cytokine levels are also regulated by Toll IL-1R 8 (TIR8), which reduces levels of IL-1β and IL-18 [56, 57] and prevents excessive tissue damage. These mechanisms modulate the inflammasome response to stimuli.

There is mounting evidence that the inflammasome is also regulated at the level of complex formation. Assembly of the NLRC4 inflammasome complex requires phosphorylation of NLRC4 itself. Murine NLRC4 is phosphorylated by kinase PKCδ at serine residue 533 [58]. In the absence of S533 phosphorylation, NLRC4 was unable to oligomerize with ASC or caspase-1 [58]. Furthermore, phosphorylation was required for unimpaired cleavage of pro-caspase-1, pro-IL-1β and pro-IL-18 after infection with *S. enterica* [58]. While NLRC4 phosphorylation has not been studied in the context of *P. aeruginosa* infection, it will be interesting to examine whether the hyper-inflammatory response observed during certain *P. aeruginosa* infections [20] can be attributed to bacterial interference with cellular processes that would otherwise attenuate inflammasome activation.

Immunomodulatory proteins also regulate inflammasome complex formation by competing for ASC or pro-caspase-1 binding. CARD-only proteins (COPs) and pyrin-only proteins (POPs) are small proteins that sequester inflammasome components, preventing association with the complex. COPs sequester caspase-1 from the inflammasome complex through interaction with the CARD domain of caspase-1 [59]. POPs interact with ASC, preventing association with NLRs [60-62] and thereby inhibiting downstream events associated with the inflammasome cascade. Interestingly, POP1 prevents NF-κB signaling but enhances caspase-1 cleavage of pro-IL-1β [60]. In contrast, POP2 disrupts both NF-κB signaling and caspase-1 activity [61, 62]. Although five COPs and two POPs have been identified in humans, they are lacking in mice, preventing mechanistic studies of these proteins in regulation of inflammasome activity during mouse models of infection [60]. Additionally, POPs have not been implicated in regulation of NLRC4. Thus, inflammasome activation is regulated by a complex network of protein interactions, but the implications of these mechanisms to infections by *P. aeruginosa* are unknown.

Non-Canonical Inflammasome Pathway

Caspase-1-deficient mice used in this study and others were found to be doubly deficient in caspase-1 and caspase-11 [63]. Therefore, the contribution of caspase-11 to inflammasome activation cannot be excluded and may have unintentionally confounded prior conclusions. Recent studies have demonstrated a role for caspase-11 in cleavage of pro-caspase-1 and subsequent maturation of pro-IL-1β and pro-IL-18 [63]. Additionally, caspase-11 resulted in secretion of IL-1α and cell death by pyroptosis, independent of its interaction with caspase-1. Thus, caspase-11 intersects with the NLRC4 inflammasome activation cascade at the level of pro-caspase-1. These results indicate a previously unappreciated role for caspase-11 in inflammasome activation. Additional studies have demonstrated

that caspase-11 synergizes with the NLRP3 inflammasome through TLR4 engagement by LPS of Gram-negative bacteria like *E. coli* and *Citrobacter rodentium.* Caspase-11 may also directly bind LPS in the cytosol [64]. However, these functions of caspase-11 were not observed in response to all inflammasome stimuli. Notably, infection of BMDMs with *E. coli* resulted in caspase-11-dependent IL-1β release but not stimulation with ATP or *P. aeruginosa* [63]. Regardless, the contribution of caspase-11 to the host response against *P. aeruginosa* must be explored further to understand the unique role of each inflammasome component in the context of various infection conditions.

General P. aeruginosa Strategies to Counter the Inflammasome-Mediated Immune Response

Inflammasomes have certainly evolved to protect mammals from microbes. Therefore, it is not surprising that the inflammasome functions to clear *P. aeruginosa* in certain infection models [8, 15, 43]. It is also anticipated, however, that successful pathogens have evolved countermeasures to subvert or even co-opt inflammasomes. In support of the last possibility, inflammasome activation is associated with improved bacterial persistence and worse infection outcomes in some model systems. In these infections, the bacteria exploit the inflammasome response in such a way that it is ineffective in resolving the infection and detrimental to the host by inducing pathological inflammation [17-20]. *P. aeruginosa* uses a multi-faceted approach to counter the inflammasome immune response, including the dampening of caspase-1 effector function, the down-regulation of inflammasome ligands and the exploitation of inflammasome-induced inflammation.

T3SS Effectors Dampen Inflammasome Activation

The T3SS is a major virulence determinant that injects effector proteins through a needle-like apparatus into the cytosol of the host cell in a manner that is dependent on cell-cell contact. The importance of

the T3SS in pathogenesis is underscored by the evolution of inflammasomes to detect components of the T3SS secretion apparatus. While components of the T3SS apparatus are activators of the NLRC4 inflammasome, the effector proteins ExoU and ExoS are capable of blocking the activity of the inflammasome.

ExoU has phospholipase A_2 activity that requires ubiquitin and phosphatidylinositol 4,5-bisphosphate (PIP2) as coactivators [12, 65]. ExoU translocation results in rapid lysis of the target cell but also dampens the inflammasome. Caspase-1 maturation was inhibited during infection of murine macrophages with ExoU-secreting *P. aeruginosa* [15]. Inhibition of caspase-1 was dependent on the phospholipase activity of ExoU, although the mechanism by which this occurred is unknown [15]. Cleavage of pro-casapase-1, pro-IL-1-β and pro-IL-18 occurred when cells were infected with isogenic strains containing a disrupted *exoU* gene [7, 15]. ExoU secretion caused rapid lysis of target cells, but cell death was still observed when the *exoU* gene was disrupted. This latter form of cell death was presumably pyroptotic as it was dependent on NLRC4 and was associated with the release of LDH from the cell, indicating membrane damage [15]. In this manner, ExoU has the dual functions of causing lysis of target cells and inhibition of caspase-1 maturation. Furthermore, in a mouse model of peritonitis, mice were able to clear ExoU-deficient bacteria in an NLRC4-dependent manner [15]. Thus, ExoU dampening of the inflammasome response appears to be biologically relevant to bacterial persistence.

Like ExoU, ExoS also suppresses the inflammasome by inhibiting maturation of caspase-1 [51]. The ADPRT domain of ExoS was required for inhibition of caspase-1 [51]. ExoS causes an apoptotic-like form of cell death related to caspase-3 activation [51]. In the absence of ExoS, infection resulted in pyroptosis characteristic of inflammasome activation [51]. ExoT also shifted cell death towards apoptosis in a

manner that was dependent on its ADPRT activity [66]. Thus, ExoS (and perhaps ExoT) dampens the inflammasome response through inhibition of caspase-1 maturation and shifts the cell death phenotype to a mechanism that does not propagate the immune response. Similar inhibition of the inflammasome occurred with a related T3SS effector of *Yersinia enterolitica,* YopE [67, 68]. However, unlike ExoS, YopE-dependent caspase-inhibition required the protein's GAP activity [68]. This difference may be attributed to the fact that the effectors are similar but not identical. YopE has GAP activity but no ADPRT activity, and the protein is significantly smaller than ExoS [69]. Despite the two proteins sharing similar helical structure in their GAP domains, the amino acid sequences of YopE and ExoS only share 22% identity [69]. Furthermore, YopE is specific to eukaryotic proteins RhoA and Rac1 [69], while ExoS targets Ras for ADP-ribosylation to cause cell rounding and apoptosis [68, 70]. In spite of their structural differences and different cellular targets, expression of ExoS in *Yersinia pseudotuberculosis* can complement a YopE deletion [70]. Thus, both YopE and ExoS translocation into eukaryotic cells results in an alteration of the cell death phenotype with a reduction in cell death by pyroptosis even though inhibition is achieved by different functional activities [51, 68].

Several questions remain regarding the role of ExoS in dampening the inflammasome response. An independent study that examined the roles of ExoS, ExoT and ExoY in inflammasome activation did not observe differences in caspase-1 maturation, IL-1β release, or cell death attributed to these effectors [8]. Each effector was studied in the absence of the others so the results were not confounded by redundancy. In this study, BALB/c mice, which are known to be more resistant to *P. aeruginosa* infection, were used [71]. Indeed, although the bacteria elicited an NLRC4-dependent inflammasome response during acute infection *in vivo,* the infection was ultimately cleared regardless of inflammasome activation [8]. In contrast, inflammasome inhibition by ExoS was observed using DBA2 mice [51], which

are more susceptible to *P. aeruginosa* infection [71]. In these latter experiments, the outcome of infection was not reported, although a similar model of acute pneumonia was used [51]. Therefore, the apparent discrepancy between these reported observations may be due to experimental differences. Further studies are required to clarify the role of ExoS in inflammasome inhibition and the consequence for infection outcomes.

Evasion of Inflammasome Activation by Loss of PAMPs

The ability to circumvent host defensive barriers distinguishes successful pathogens from other microbes. *Pseudomonas aeruginosa* is no exception. While dampening the immune response is important during acute infection, bacteria must also neutralize host defense systems during chronic infection. Characterization of isolates from cystic fibrosis (CF) patients chronically infected with *P. aeruginosa* demonstrated that these isolates exhibited marked decreases in functional T3SS [72] and production of flagella and pili [73, 74]. Whereas 90% of *P. aeruginosa* environmental isolates were capable of type III secretion, only 49% of isolates cultured from newly infected children with CF were secretion-positive [72]. This percentage was further reduced to only 4% of isolates from chronically infected adults, demonstrating a clear loss of functional type III secretion following longer periods of infection [72]. Similarly, *P. aeruginosa* isolates from chronically-infected CF patients exhibited a loss of pili and flagella [73, 74]. The frequency of non-flagellated strains during chronic CF infection was particularly high compared to isolates from the environment or acute infections [73, 74]. Despite the loss of motility, non-flagellated strains were associated with worse clinical outcomes in CF patients [74]. The reasons why type III secretion, flagella and pili are lost during chronic infections are unclear but may involve the inability of patients to clear *P. aeruginosa* in the absence of robust inflammasome activation.

Several observations support a model whereby loss of T3SS favors *P. aeruginosa* persistence in chronic respiratory infections. Pulmonary infection of mice with a T3SS-negative strain caused reduced cytokine responses and neutrophil recruitment relative to a T3SS-positive strain [7]. Additionally, T3SS-negative bacteria were not cleared from the lungs as quickly as isogenic strains that assembled the T3SS apparatus. Finally, caspase-1 played an integral role in the differential response to T3SS-competent versus T3SS-null strains [7]. Thus, the ability of *P. aeruginosa* to persist during chronic infections may be at least partially attributable to evasion of the inflammasome-mediated immune response by loss of functional T3SSs.

The absence of flagella during chronic infection may also allow *P. aeruginosa* bacteria to evade immune detection. *P. aeruginosa* possesses a single polar flagellum that is required for swimming motility, colonization and full virulence during infection. Flagella mediate bacterial penetration across the epithelial barrier and binding to the basolateral cell surface [75, 76]. However, rotating flagella also engage TLR5, causing release of chemokine and cytokine signals [41, 76]. TLR5 and MYD88 signaling were essential for bacterial clearance in certain *in vivo* infection models [16]. In contrast, non-flagellated isogenic strains of *P. aeruginosa* failed to stimulate TLR5/MYD88 signaling or secrete IL-1β, indicating the absence of inflammasome activation [16, 41]. As a consequence, non-flagellated bacteria persisted in higher numbers than flagellated bacteria [16, 20]. Therefore, loss of flagella may represent a means to avoid inflammasome activation and promote bacterial persistence during chronic infection.

Infection-Induced Pathologic Inflammation

In contrast to evading and dampening the inflammasome response, *P. aeruginosa* also has the ability to amplify and take advantage of inflammasome-induced inflammation in certain contexts. Mounting

evidence suggests that *P. aeruginosa* induces pathological inflammation and persists better under these conditions [17-20]. Contrary to studies demonstrating that IL-1β is necessary for bacterial clearance [16], other reports suggest that this cytokine can also cause excessive recruitment of neutrophils [19] and induce tissue damage [17, 19] under certain conditions. Similar tissue damage has been observed with IL-18 [18]. Thus, inflammasome activation by *P. aeruginosa* has the potential to adversely impact outcomes.

Flagella have been implicated in the pathological inflammation observed in certain infection models. The production of flagella was associated with inflammasome activation and increased tissue damage in a mouse model of acute pneumonia [20]. The magnitude of tissue damage was dramatically reduced by depletion of alveolar macrophages or chemical inhibition of caspase-1 by YVAD [20]. Interestingly, NLRC4$^{-/-}$ mice were better able to clear flagellated *P. aeruginosa* than their wild-type counterparts. Thus, *P. aeruginosa* is able to thrive in the midst of flagellin-mediated inflammasome activation.

In summary, *P. aeruginosa* has developed a number of mechanisms to hinder or confound the inflammasome-mediated immune response. At first glance, some of these mechanisms appear to be contradictory and result in both enhancement and inhibition of an inflammatory response. Data supporting a definitive explanation for these differences are not currently available. It is conceivable, however, that different mechanisms are employed by different *P. aeruginosa* strains or in different infection contexts. For example, ExoU- and ExoS-mediated dampening of the inflammasome may occur during the first hours of infection when bacteria are few and vulnerable to sterilizing effects of inflammation [7, 15, 51]. After an acute infection has been established and large numbers of bacteria are present, bacteria may switch to a strategy of inducing an overly exuberant immune response [17-

20]. *P. aeruginosa* may exploit off target tissue damage from pathologic inflammation by a number of mechanisms. For example, tissue damage may promote dissemination. During chronic infections, which require bacteria to persist for months and years, *P. aeruginosa* may adopt a stealth strategy by eliminating some PAMPs all together [7, 16, 20]. Collectively, these strategies represent a multipronged approach by *P. aeruginosa* to escape the inflammasome-mediated immune response and promote infection.

Inflammasome Activation During Specific Types of Infection

P. aeruginosa infection has been evaluated using a number of infection models at different body sites, including the peritoneal cavity, the eye and the lung. To be successful in such diverse body sites, it must overcome several host defenses, such as epithelial barriers containing tight junctions between cells to block bacterial invasion, the mucociliary transport system of the airways, the antimicrobial properties of tears in the eye and the innate immune response (reviewed in [77]). During infection, cytokines and chemokines are released from macrophages and epithelial cells to initiate the immune response. In addition to the inflammasome response, other cytokines such as IL-6, IL-8 and TNF-α are also secreted during *P. aeruginosa* infection [77]. Neutrophils are recruited to the site of infection to promote bacterial clearance through phagocytosis and production of defensins, elastases and reactive oxygen species [77]. Thus, inflammasome activation is one part of a robust and sophisticated host defense system against bacterial infection. Perhaps not surprisingly, the consequences of *P. aeruginosa* inflammasome interactions are themselves complex and appear to vary in different models of infection. In some models, inflammasome activation is required for bacterial clearance [8, 15, 43] while other models indicate that such activation leads to worse infection outcomes [17-20]. Whether inflammasome activation is protective or detrimental to the host may be related to the route of

infection, the duration of infection, the magnitude of bacterial load, or other experimental differences between the various infection models employed.

Peritonitis is an infection of the abdominal cavity that is a common complication of ascites, peritoneal dialysis, or rupture of the intestines [78-80]. Although many different bacteria can cause peritonitis, infection by *P. aeruginosa* is especially severe [78, 80]. A mouse model of peritonitis is convenient because bacteria are easily injected directly into the peritoneum. Inflammasome activation is essential for clearance of *P. aeruginosa* during peritonitis [15, 43]. Infection of peritoneal macrophages *in vitro* with *P. aeruginosa* caused increased caspase-1-dependent release of mature IL-1β [15, 43] and increased cell death [15]. Inflammasome activation was dependent on recognition of either the T3SS apparatus or flagellin [15, 43]. *P. aeruginosa* modulated the magnitude of inflammasome activation by translocation of ExoU into macrophages [15] or by loss of flagellin and/or flagellar motility [43]. These results were confirmed *in vivo* as well. Although host survival outcomes were not explicitly reported, inflammasome-competent mice were better able to clear bacteria after intraperitoneal challenge with *P. aeruginosa* [15, 43].

Keratitis is an infection of the cornea that can lead to blindness. *P. aeruginosa* is the most common pathogen cultured from contact lens wearers with keratitis [81]. The ability to persist in biofilms on the lens surface undoubtedly contributes to the frequency with which *P. aeruginosa* causes keratitis, but epidemiological evidence also supports a role for specific virulence factors. For example, strains that secrete the T3SS effector ExoU are especially common in *P. aeruginosa* contact lens keratitis [81]. The pathology of keratitis is associated with robust inflammation that results in opacification of the cornea, damage to the epithelium and stroma, as well as corneal perforation [19, 47, 82-84]. Inflammation plays

a prominent role in the pathogenesis of *P. aeruginosa* keratitis. Proinflammatory cytokines are produced from multiple pathways during these infections. IL-1β is produced from macrophages following TLR/MYD88-dependent signaling [84], which presumably feeds into the inflammasome-dependent pathway for priming of pro-IL-1β and pro-IL-18 and subsequent maturation by caspase-1 [19]. Mature IL-1β is also produced from neutrophils through cleavage by neutrophil elastase [47]. IL-1β from the TLR/MYD88 pathway or the neutrophil elastase pathway is necessary for restriction of bacterial numbers at early time points during infection (generally, less than 48 hours) but also results in corneal opacification [47, 84]. Conversely, prolonged high levels of IL-1β are associated with worse outcomes over longer infection courses (generally, over several days) [82]. Bacterial loads and clinical scores for corneal damage were improved in caspase-1 deficient mice despite decreased neutrophil recruitment, suggesting a role for the inflammasome in causing pathological inflammation [19]. Similarly, chemical inhibition of caspase-1 at 18 hours post-infection also resulted in improved clinical scores and reduced corneal perforation [19]. Of therapeutic relevance, these outcomes were further improved by the addition of antibiotics [19]. Thus, persistent inflammasome activation during *P. aeruginosa* keratitis results in worse infection outcomes that can be rescued by inhibition of caspase-1-dependent release of proinflammatory cytokines.

In a National Healthcare Safety Network study, *P. aeruginosa* was the fifth most common cause of overall nosocomial infections and the second most common cause of ventilator-associated pneumonia during the period from 2009 – 2010 [85]. Multiple murine infection models of acute pneumonia, including intranasal and intratracheal inoculation models, have been used to investigate the pathology associated with *P. aeruginosa* pneumonia. Whether inflammasome activation is beneficial or detrimental to the host during acute pneumonia caused by *P. aeruginosa* remains controversial. Some

studies have reported that inflammasome activation was required for more rapid clearance of *P. aeruginosa*; bacteria infecting mice deficient in components of the inflammasome cascade persisted in greater numbers during infection [7, 8]. In contrast, other studies that employed the same infection models reported detrimental outcomes associated with inflammasome activation. Inflammasome activation was associated with increased bacterial persistence [17, 18, 20, 57] and increased tissue damage [17, 18, 20]. Bacterial clearance was improved and tissue damage was reduced when levels of IL-1β or IL-18 were inhibited [17, 18, 57]. Furthermore, a recent study found that infection outcomes were improved following depletion of alveolar macrophages or chemical inhibition of caspase-1 [20]. Thus, studies differ as to whether inflammasome activation is beneficial or detrimental for the host in acute pneumonia.

Variations in experimental design may explain the differing conclusions regarding the consequences of inflammasome activation during acute pneumonia. In particular, different strains of *P. aeruginosa* were used in the various studies. Whereas some studies used strain PAK, which secretes the T3SS effector ExoS [8, 20], others used the strain PA103, which secretes ExoU and is non-flagellated [7, 17, 18]. Although similar routes of infection were used, the size of the bacterial inoculum differed greatly between studies. Higher doses of bacteria resulted in early mortality, preventing measurements at later time points. This precludes comparisons of longer infections between studies using lower and higher inocula. Furthermore, the protective or detrimental role of the inflammasome may differ between mild and severe infections. Collectively, experimental differences regarding bacterial strain, inoculum size and duration of infection may have influenced the conclusions of each study. Understanding the implications of inflammasome activation during acute pneumonia, therefore, must be carefully considered within the context of the experimental design.

Mechanisms of Programmed Cell Death

Pyroptosis is a form of programmed cell death that is intrinsically different from apoptosis [52]. Apoptosis is dependent on caspase-3, caspase-6 and caspase-8 and is marked by release of membrane blebs that are phagocytosed by neighboring cells [30, 52]. In contrast, pyroptosis is dependent on caspase-1 and characteristically leads to the formation of membrane pores that result in cell swelling and lysis from osmotic stress [30]. Whereas apoptosis tends to be accompanied by minimal or no inflammation, pyroptosis is a proinflammatory process by virtue of IL-1β and IL-18 release.

As mentioned above, apoptosis is dependent on various caspases that initiate a process of programmed cell death and phagocytic uptake of the dying cell by neighboring cells. Although there are multiple signaling pathways and caspases involved, caspases are not redundant and are activated in response to specific intrinsic or extrinsic stimuli [86]. Like caspase-1, all caspases have multiple effector domains that facilitate interactions with receptors and other caspases. Caspases related to apoptosis are generally classified into two groups – initiator caspases and executioner caspases [86]. Initiator caspases like caspase-2, caspase-8 and caspase-9 are composed of a regulator pro-domain, a large subunit and a small subunit. Initiator caspases are activated in response to stimuli like release of cytochrome C from mitochondrion, release of nuclear components following DNA damage, or ligation of first apoptosis signal receptor (Fas) on the cell surfaces [86]. Activation of initiator caspases occurs through homotypic dimerization of the caspase and recruitment of executioner caspases. Executioner caspases include caspase-3, caspase-7 and caspase-6 [86]. Executioner caspases are composed of a large and small subunit which are proteolytically cleaved upon assembly with the initiator complex. The executioner caspases are then active to proteolytically cleave cellular targets leading to phenotypic

changes associated with cell death like cell rounding, membrane bleb formation and release of apoptotic bodies [86]. In this way, caspases associated with apoptosis respond to stimuli to initiate assembly of multi-protein complexes with executioner caspases for activation of a programmed cell death pathway.

Although we tend to think of apoptosis and pyroptosis as distinct mechanisms of programmed cell death, there is increasing evidence that there is cross-talk between these pathways. For example, there is evidence that NLRP3 and ASC are required for caspase-8-dependent apoptosis in murine tubular epithelial cells [87]. This would represent a heterotypic interaction between ASC and caspase-8 as ASC has pyrin and CARD domains while caspase-8 has two death effector domains (DEDs) [64]. Yet, Chung and colleagues show a clear association of NLRP3, ASC and caspase-8 in speck-like formations at the mitochondria of cells treated with apoptosis inducers [87]. Another study of Shiga toxin treated human THP1 cells showed a dependence of caspase-3 on ASC [88]. How programmed cell death pathways may be activated during *P. aeruginosa* infection or how they may influence ASC and caspase-1-dependent inflammasome activation is unknown.

A recent report indicates that programmed cell death pathways induced by PAMPs may be more varied than previously thought. Lage and colleagues showed that purified flagellin from *S. enterica* was able to induce macrophage cell death even in the absence of ASC, NLRC4, or caspase-1 [89]. Furthermore, *in vitro* infection with live bacteria resulted in increased cell death that was correlated with reduced bacterial persistence [89]. Collectively, this suggests that flagellin is activating a cell death mechanism that is independent of the NLRC4 inflammasome pathway but that is required to restrict *Salmonella* during infection. Further investigation revealed that lysosomal proteases cathepsin B and cathepsin D

played a role in this form of cell death. Interestingly, inhibition of cathepsin-B reduced flagellin-dependent cell death but was also associated with decreased IL-1β [89]. This suggests that the caspase-1-dependent inflammasome cell death pathway and the cathepsin B-dependent cell death pathway may be related. Additional studies are required to determine whether flagellin activates the cathepsin B cell death pathway in the context of *P. aeruginosa* infection in a similar manner to *S. enterica*.

At first glance, a signaling cascade that leads to the death of an important cell of the immune system (macrophages) would appear to favor bacterial pathogens rather than the host. But studies using *Salmonella enterica, L. pneumophila,* and *B. thailandensis* have demonstrated that caspase-1-dependent pyroptosis is an important mechanism for eradicating intracellular bacteria by exposing them to neutrophils in the extracellular environment [29]. Whether the same process is important for *P. aeruginosa*, which is primarily an extracellular pathogen but does access the intracellular compartment to a small degree [90], is unclear. Furthermore, lysosomes fuse with the plasma membrane during pyroptosis, releasing antimicrobial factors into the extracellular milieu [30]. In this way, pyroptotic cell death actively contributes to the inflammasome response to microbial infections by exposing intracellular bacteria to the extracellular environment and expelling intracellular antimicrobial components into the extracellular milieu.

THERAPEUTIC ANTIMICROBIAL AGENTS

One aspect that is often overlooked in mouse models of infection is the ability to treat infections with antimicrobial agents. Whereas antimicrobial therapies are typically withheld in scientific models of disease to evaluate determinants of bacterial virulence, nosocomial infections are always treated with antimicrobial agents. There are currently 8 classes of antimicrobials effective for treatment of *P.*

aeruginosa infections: aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, penicillin+β-lactamase inhibitor combination drugs, monobactams, phosphonic acids and polymyxins [91]. Yet, antibiotics are not a cure-all for *P. aeruginosa* infections due to increasing antibiotic resistance [2]. Between 13% - 20% of *P. aeruginosa* hospital-acquired infections are caused by multidrug-resistant (MDR) strains [2, 92, 93] while 6% are caused by extensively drug-resistant (XDR) strains [92]. *P. aeruginosa* strains resistant to all clinically relevant antibiotics, or pan drug-resistant (PDR) strains, have also been reported [2, 92]. Not surprisingly, antibiotic resistance affects infection outcomes in humans. Patients with MDR *P. aeruginosa* infections have a higher probability of mortality, longer hospitalizations and increased medical costs [2, 93, 94]. However, it is unclear if poor infection outcomes reported in MDR infections are due to a paucity of treatment options or other factors such as the baseline illness of the patient, the host immune response or virulence of the infecting strain.

Mechanisms of Antibiotic Resistance in P. aeruginosa

P. aeruginosa employs multiple mechanisms to achieve antimicrobial resistance [95-97]. Some mechanisms are effective against multiple antimicrobial classes. For example, drug efflux pumps of the resistance-nodulation-division (RND) family are often intrinsically multi drug-resistant because of the broad substrate capacity of these systems. RND drug efflux pumps are multi-protein complexes that include an outer membrane protein, an inner membrane protein and a membrane fusion protein that form a channel through the cytoplasm to the extracellular milieu [96]. Some examples of RND drug efflux pumps include the MexAB-OprM system that confers resistance to fluoroquinolones, cephalosporins, β-lactams, carbapenems and monobactam [96, 98], MexCD-OprJ that confers resistance to quinolones, cephalosporins and erythromycin, and MexEF-OprN that confers resistance to chloramphenicol and quinolones [98]. Antimicrobial resistance mechanisms like the β-lactamase

enzyme AmpC confer resistance to multiple antimicrobial classes but have more narrow specificity than the efflux pump described above. Increased production of AmpC results in resistance to antibiotics that contain a β-lactam ring such as cephalosporins and penicillins [96]. All *P. aeruginosa* strains produce some background levels of AmpC [96]. Other mechanisms are specific to one class of antibiotics. An example of a class-specific resistance mechanism includes antimicrobial modifying enzymes like aminoglycoside acetyltransferases (AACs), aminoglycoside phosphoryltransferases (APHs) and aminoglycoside nucleotidyltransferases (ANTs) that inactivate aminoglycosides through the addition of a functional group to the drug compound [97]. OprD-mediated resistance is another well-studied class-specific antimicrobial resistance mechanism [96]. Carbapenems diffuse into the cell through the OprD porin in addition to basic amino acids and peptides that are the normal substrates for this porin [96, 99-101]. Several studies have shown that insertions into or deletions of the *oprD* gene result in increased resistance to carbapenems [96, 99-101]. Thus, *P. aeruginosa* antimicrobial resistance is a multi-factorial process that can involve multiple mechanisms.

How a strain acquires antibiotic resistance is another question of importance. Certain genes such as *ampC* are chromosomally encoded and are inherited vertically during bacterial growth [96]. Other genes such as those encoding aminoglycoside modifying enzymes may be on plasmids or in mobile elements [96, 97]. There is evidence that strains evolve to develop antimicrobial resistance in the presence of antibiotic stress, as well. In laboratory experiments where *P. aeruginosa* strain PAO1 was grown in the presence of carbapenems or fluoroquinolones and passaged in broth culture for 7 days, the bacteria acquired genetic transitions, transversions and frameshifts [102]. Some of these genetic mutations resulted in enhanced antimicrobial resistance compared to strains passaged in the absence of antibiotic stress [102]. Furthermore, the rate of genetic mutations was significantly greater in strains

grown under antibiotic stress compared to strains grown in the absence of antibiotics [102]. These results demonstrate that *P. aeruginosa* has the ability to acquire or develop antibiotic resistance in the presence of antimicrobial stress. This process may be clinically relevant to the use of antimicrobial agents to treat *P. aeruginosa* infections. Independent studies found an association between prescribing patterns of antibiotics for *P. aeruginosa* infections and development of resistance to those agents. Kallel and colleagues evaluated nearly 600 *P. aeruginosa* isolates collected from a medical surgical intensive care unit over a 5-year period and compared antibiotic resistance of the strains with antimicrobial agents used to treat the infections [103]. They found a temporal relationship between *P. aeruginosa* resistance to fluoroquinolones and carbapenems with prior use of the antibiotic agents [103]. Similarly, Carmelli and colleagues found that use of carbapenems, fluoroquinolones and penicillin were associated with increased risk of emergence of resistance in subsequent infections [104]. Collectively, these studies provide evidence that the use of certain antimicrobial agents to treat *P. aeruginosa* infections may in fact be a double-edged sword that is capable of treating susceptible infections or promoting development of antibiotic resistance.

Fitness Cost of Antibiotic Resistance

There is increasing evidence in the scientific literature that antibiotic resistance comes with a fitness cost to bacteria. Melnyk and colleagues conducted a meta-analysis of studies examining fitness of bacteria with chromosomal mutations that resulted in antibiotic resistance [105]. They found that the overall consensus in published studies is that antibiotic resistance is associated with a fitness cost. By their methods, a fitness ratio of 1 indicated no difference between fitness of antibiotic resistant strains compared to antibiotic susceptible strains and a ratio less than 1 was indicative of reduced fitness for antimicrobial resistant strains. They found antibiotic resistant strains had a fitness ratio of 0.88

compared to susceptible ancestral strains [105]. However, they also found significant variation in the fitness cost of antimicrobial resistance in different bacterial species where certain species (such as *Mycobacterium tuberculosis,* for example) had a greater magnitude of fitness cost and other species (like *E. coli, Enterococcus faecium and Borellia burgdorferi*) exhibited a minor fitness cost or no cost of antibiotic resistance [105]. Notably, this meta-analysis did not include studies of *P. aeruginosa* so it is unclear how the authors conclusions may be applied to this particular Gram-negative bacterium.

Limited studies have been conducted examining fitness of antimicrobial resistant *P. aeruginosa*. One study examining growth rates and competition between antibiotic susceptible and antibiotic resistant strains of *P. aeruginosa* found that the *in vitro* growth rate of resistant strains was somewhat slower than susceptible strains. Antibiotic resistant strains where out-competed in *in vitro* competition assays with susceptible strains [106]. Notable, this study used strains that were antibiotic resistant by over-expression of the MexAB efflux pump due to an in-frame deletion of the gene encoding the repressor MexR or strains that had in-frame deletions of the *oprD* gene [106]. They found that there was a synergistic effect of multiple drug resistance mechanisms such that mutants of both MexR and OprD were further attenuated compared to single mutants of either system [106]. Another study of antibiotic resistant *P. aeruginosa* mutants that over-produce the MexAB-OprM efflux pump found that these strains exhibited reduced persistence in water, a common environmental niche for *P. aeruginosa* exposure [107]. Interestingly, persistence of the MexAB-OprM over-producing mutants on solid surfaces was also attenuated but biofilm formation was unchanged between the mutants and the parental strains of *P. aeruginosa* [107]. Yet, the observations were not replicated in other antibiotic resistant strains that over-produce the MexCD-OprJ efflux pump, although these mutants did ultimately exhibit a viability decrease over the course of several days [107]. Still others have found that over-

production of the MexEF-OprN efflux pump was associated with defects in production of cell signaling autoinducer [98]. Collectively, these studies provide evidence that *P. aeruginosa* strains with mutations leading to antimicrobial resistant phenotypes may also have reduced fitness in bacterial competition, bacterial persistence in the environment and impaired cell-to-cell signaling. However, not all mutations or antimicrobial resistance mechanisms result in the same fitness defects. Furthermore, these studies utilized isogenic mutants of *P. aeruginosa* that resulted in a resistance phenotype rather than strains that developed or acquired resistance naturally [98, 106, 107]. This is notable as strains that develop antimicrobial resistance naturally may also acquire or develop compensatory mutations to mitigate the cost of antibiotic resistance during natural selection [105]. Further studies of bacterial fitness of strains that naturally-acquired antimicrobial resistance genes or mutations that result in antimicrobial resistant phenotypes are required.

Contrary to the evidence above, some antimicrobial resistance mechanisms are associated with increased fitness *in vivo*. Using a transposon mutant library, Skurnik and colleagues found that mutants of the *oprD* gene, which confers resistance to carbapenems, were better able to colonize the gut of mice [100]. Furthermore, *oprD* mutants were capable of disseminating from the gut to the spleen of neutropenic mice [100]. The fact that *oprD* mutants exhibited improved bacterial dissemination to the spleen suggested that these strains may be better able to cause invasive illness than carbapenem-susceptible strains. Importantly, the authors found their observations to be consistent for carbapenem-resistant clinical isolates as well as *oprD* mutants in their transposon library [100]. Although the authors demonstrate that carbapenem resistant strains had a fitness advantage over susceptible strains in host colonization, it remains to be seen if the ability to colonize a host is predictive of the ability to cause clinical disease or enhance disease severity.

Antibiotic Resistance and Virulence

In humans, infections due to antimicrobial resistant strains are often associated with severe outcomes including worse morbidity and increased risk of mortality [2, 93, 94]. Whether this is due to the virulence of the strain causing more severe illness or inappropriate antibiotic therapy for treatment of antimicrobial resistant infections is unclear [108]. In one study of over 100 clinical isolates of *P. aeruginosa* bloodstream infections (BSIs), the authors found that over 80% of patients received appropriate antibiotic therapy [109]. Yet, the crude in-hospital mortality rate for patients in this study was 36%, suggesting that factors other than appropriate antibiotic treatment contribute to severe infection outcomes [109]. Therefore, it is important to understand how antimicrobial resistance may be affecting virulence of *P. aeruginosa* and the ability of the bacterium to cause severe infections.

There are several outstanding questions regarding the relationship between antibiotic resistant *P. aeruginosa* and bacterial virulence. One study of *P. aeruginosa* clinical isolates found an association between ExoU and the MDR phenotype [110]. Given that ExoU is a potent virulence determinant [6], one might hypothesize that MDR strains would be more virulent than antibiotic susceptible strains. On the contrary, over-production of the efflux pumps MexCD-OprJ or MexEF-OprN is associated with decreased transcription of T3SS genes [111]. This suggests that virulence may be down-regulated upon expression of antimicrobial resistance mechanisms. Few studies have been conducted to examine these questions experimentally. In their study of bacterial fitness, Sanchez and colleagues found that MexAB-OprM overproducing strains were avirulent in a *C. elegans* infection model where the isogenic antibiotic susceptible strain resulted in killing of the nematodes [107]. Conversely, MexAB-OprM overproduction was not found to have an effect on inhibition of the amoeba *Dictyostelium* compared to isogenic

parental strains [112]. In the *Dictyostelium* model, strains overproducing MexEF-OprN were more permissive to amoeba growth than antibiotic susceptible strains [112]. Furthermore, the MexEF-OprN overproducing strain was avirulent in a rat model of pneumonia, and virulence was restored upon complementation of MexE regulation [112]. Collectively, these studies suggest that antimicrobial resistance due to overproduction of RND efflux pumps is associated with reduced bacterial virulence although there is disagreement in the outcome related to expression of specific antimicrobial resistance mechanisms. In a mouse model of disease, Abdelraouf and colleagues found that MDR-*P. aeruginosa* mutants of the laboratory strain PAO1 exhibited reduced bacterial burden in the lungs and improved host survival compared to mice infected with the antibiotic susceptible parental PAO1 strain [106]. However, they did not see a difference in survival of mice infected with the parental PAO1 strain and an MDR clinical isolate of *P. aeruginosa* [106]. In this way, an MDR clinical isolate failed to replicate their findings using laboratory modified antibiotic resistant strains. The authors make no effort to identify the resistance mechanisms expressed by the clinical isolate. Therefore, it is possible that the clinical isolate expressed different antimicrobial resistance mechanisms than their laboratory modified strains, confounding their conclusions. Furthermore, the authors do not exclude the possibility that the reduced virulence phenotype in mice may be related to the growth defect of the antimicrobial resistant strains *in vitro* [106]. Additional studies are required to understand the capacity for antimicrobial resistant *P. aeruginosa* isolates to cause infection.

SUMMARY

The ability of a pathogen to cause severe illness is a multi-factorial issue that is determined by bacterial virulence, the host immune response and ability to treat infection with antimicrobial therapeutic agents. *P. aeruginosa* expresses multiple virulence determinants to promote infection, namely the T3SS that is

the subject of the current study. The inflammasome is an integral component of the host innate immune response against microbial infections. Multiple virulence determinants of *P. aeruginosa* are known to activate the inflammasome, including flagellin, components of the T3SS and type IV pilin. Upon sensing these microbial PAMPs, the inflammasome initiates an activation cascade resulting in autoproteolytic cleavage of pro-caspase-1 and subsequent maturation of proinflammatory cytokines IL-1β and IL-18, production of eicosanoids and initiation of a pyroptotic cell death cascade. These events have evolved to facilitate the host immune response to *P. aeruginosa* and aid in bacterial clearance. However, *P. aeruginosa* has developed several strategies to circumvent the inflammasome response. Maturation of caspase-1 can be inhibited by the T3SS effectors ExoU or ExoS, effectively dampening the activity of the inflammasome. During chronic infection, flagellin, T3SS proteins and pili may not be produced, allowing bacteria to evade immune detection. Conversely, inflammasome activation can be exploited to induce pathologic inflammation that is ineffective at clearing the bacteria. Ultimately, whether inflammasome activation is beneficial for the host to promote clearance or to the bacterium to promote persistence may be related to the context of the infection and to the virulence determinants expressed by the particular strain of *P. aeruginosa*.

Even if the host immune response fails to clear infection, antimicrobial therapies are used in human infections to promote resolution of clinical illness. Several different classes of antimicrobial agents have been developed including aminoglycosides, fluoroquinolones, cephalosporins, carbapenems, monobactam and penicillin + β-lactamase inhibition combination therapies. As evidence of the robustness of *P. aeruginosa* pathogenesis, the bacterium has acquired or evolved resistance mechanisms to antimicrobial agents. *P. aeruginosa* antimicrobial resistance mechanisms include overproduction of RND drug efflux pumps, production of β-lactamases, expression of antimicrobial

modifying enzymes and acquisition of genetic mutations that reduce membrane permeability to antimicrobial agents. Although *P. aeruginosa* has multiple strategies to confer antibiotic resistance to the strain, antimicrobial resistance may also come with a cost to bacterial fitness and/or virulence. In this way, antimicrobial resistance mechanisms may provide an advantage to strains in the presence of antimicrobial stress compared to antimicrobial susceptible strains, but it is unknown if resistant strains are as capable of persisting in environmental niches or establishing severe illness in the absence of antimicrobial stress. Thus, the interplay between the bacterium, the host inflammasome-mediated immune response and the efficacy of antimicrobial agents is a complex balance of factors that ultimately determines clearance of the bacteria or manifests in disease (Figure 1).

In the following chapters, we will examine factors that contribute to *P. aeruginosa* pathogenesis related to the host inflammasome-mediated immune response and antimicrobial resistance. We will demonstrate that *P. aeruginosa* activates the caspase-1-dependent inflammasome and caspase-3/7-dependent apoptosis during acute pneumonia. Activation of these pathways and progression of severe illness in mice are dependent on functional T3SS. Furthermore, the inflammasome adaptor protein ASC is required for caspase-3/7 activity and may contribute to severe infection outcomes independent of caspase-1. We will also demonstrate that antimicrobial resistance is prevalent among *P. aeruginosa* clinical isolates. Using statistical modeling, we reveal that there is an inverse relationship between antimicrobial resistance and bacterial virulence such that ABR and MDR strains require higher bacterial inocula to cause illness in mice. We also show that these conclusions are generally consistent with trends of antimicrobial resistance and bacterial virulence of the Gram-negative pathogen *Klebsiella pneumoniae*. Taken together, these statistical models suggest that the inverse relationship between antimicrobial resistance and bacterial virulence may be more broadly applicable to other Gram-negative

bacteria. Collectively, the findings of this study further elucidate the strategies that *P. aeruginosa* employs to exacerbate the host immune response through the detrimental role of ASC in severe infection outcomes in mice and demonstrate a possible virulence cost of antimicrobial resistance.

CHAPTER 2: ***P. aeruginosa*** **activation of the inflammasome is detrimental to infection**

OVERVIEW

There is mounting evidence that inflammasome activation by *P. aeruginosa* is associated with increased bacterial persistence and host tissue damage [17-20]. Mice lacking NLRC4 or treated with chemical inhibitors to block capsase-1, IL-1β and IL-18 demonstrated improved bacterial clearance and reduced infection-related tissue damage [17, 18, 20]. Yet, others have shown that neutrophils secrete IL-1β independent of caspase-1 [47] or the inflammasome adaptor protein ASC [113] in response to *P. aeruginosa* infection. Taken together, this evidence highlights the ongoing questions regarding the role of the canonical caspase-1 dependent inflammasome in cell death, IL-β release and infection outcomes during *P. aeruginosa* infection.

Additionally, PSE9 secretes the T3SS effector ExoS (Appendix 1.5), which is known to activate caspase-3 to cause apoptosis [51, 114]. ExoS shifts the immune response towards a caspase-3/7-mediated mechanism of programmed cell death by apoptosis [51] and prevents pyroptosis-like cell death to dampen IL-1β release [51]. Hence, the T3SS promotes infection through the effectors that it secretes, stimulates the host inflammasome-mediated immune response and modulates inflammasome activity through the effector protein ExoS. Yet, the mechanisms of caspase-3/7 activation in the context of *P. aeruginosa* inflammasome activation is poorly understood.

In the current chapter, we utilize a highly virulent clinical isolate of *P. aeruginosa,* PSE9, to evaluate the implications of inflammasome activation in a mouse model of pneumonia and characterize inflammasome activation by *P. aeruginosa in vitro*. We demonstrate that the inflammasome adaptor

protein ASC is contributing to severe infection outcomes via a caspase-1-independent mechanism. Furthermore, we demonstrate that caspase-3/7 is activated during PSE9 infection and that caspase-3/7 activation is dependent on ASC. Functional T3SS is sufficient to activate caspase-3/7 in the absence of ExoS.

RESULTS

P. aeruginosa promotes infection through ASC

To examine the implications of inflammasome activation *in vivo*, we infected wild-type C57BL/6 mice (B6), ASC-deficient mice ($ASC^{-/-}$) and caspase-1/11-deficient mice ($C1/11^{-/-}$) with a highly virulent $ExoS^{+}$ clinical strain of *P. aeruginosa,* PSE9 [115]. We hypothesized that $ASC^{-/-}$ and caspase-$1/11^{-/-}$ mice would be equally susceptible to infection because ASC is upstream of caspase-1 in inflammasome assembly. However, ASC is not required for all caspase-1 effector functions [53]. Therefore, we compared survival and bacterial clearance from B6, $ASC^{-/-}$ and $C1/11^{-/-}$ mice to parse out the consequences of inflammasome activation *in vivo*. Under these infection conditions, PSE9 was highly lethal to B6 mice. An inoculum of 3.5×10^5 CFU caused nearly all mice to develop pre-lethal illness within 96 hours (Figure 3a and b) and bacteria persisted in the lungs up to 30 hours post-infection (hpi) (Figure 2c, Appendix 1.1). B6 mice infected with a PSE9 strain lacking a T3SS needle due to a disruption of the *pscF* gene (PSE9Δ*pscF*) survived the infection time course (Figure 3a). PSE9Δ*pscF* numbers in the lungs were approximately equivalent to those of PSE9 at 30 minutes post-infection but were reduced as early as 18 hpi (Figure 3c and d). These results indicate that the T3SS contributes significantly to the virulence of PSE9. Pre-lethal illness of $ASC^{-/-}$ mice infected with PSE9 was delayed by approximately 18 hours compared to B6 control mice, and overall survival of $ASC^{-/-}$ mice was 46% (Figure 3b). Consistent with these findings, 80% of $ASC^{-/-}$ mice exhibited a reduction in bacterial numbers in their lungs by 30 hpi

Figure 3c and d). These data demonstrate that ASC is detrimental to infection outcomes, as mice lacking the inflammasome adaptor protein had improved survival and reduced bacterial burden in the lungs compared to B6 control mice. Interestingly, C1/11$^{-/-}$ mice were equally susceptible to infection as B6 control mice (Figure 3b). Although the median bacterial burden in the lungs of C1/11$^{-/-}$ mice was reduced over 1 log compared to B6 mice at 30 hpi, the difference was not statistically significant (Figure 3c and d). Therefore, caspase-1/11 did not make a significant contribution to poor infection outcomes during pneumonia caused by PSE9 because the absence of caspase-1/11 did not improve survival or significantly reduce bacterial clearance.

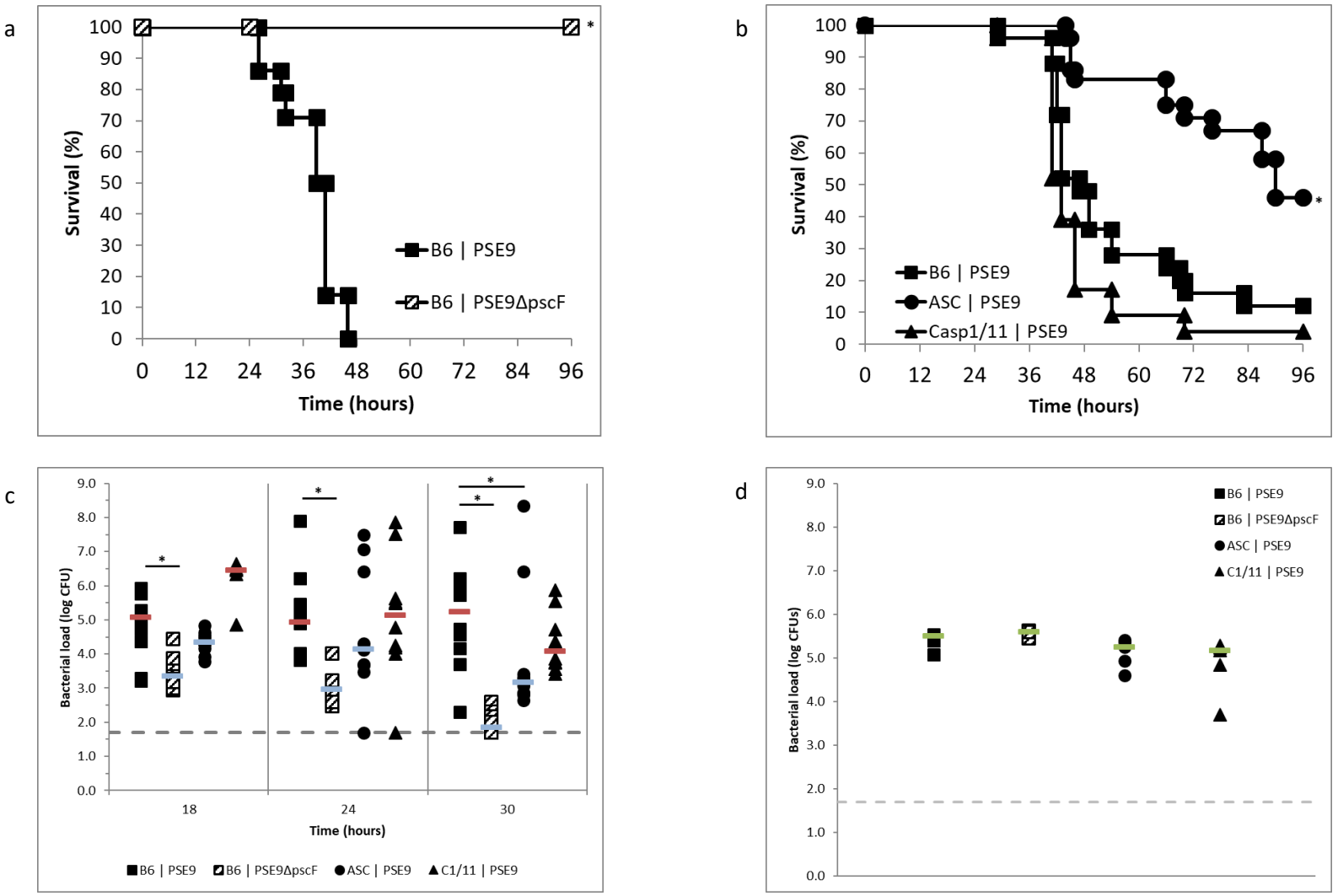


Figure 3 – ASC$^{-/-}$ mice exhibit improved survival during PSE9 pneumonia. a) Survival of B6 mice infected with PSE9 (n=14) or PSE9Δ*pscF* (n=15). b) Survival of B6 (n=25), ASC$^{-/-}$ (n=24) or casp1/11$^{-/-}$ (n=23) mice infected with PSE9. c) Bacterial CFU recovered from the lungs of infected mice at indicated times (n=10 mice per group) or d) 30 minutes post-infection (n=5 mice per group). The target inoculum was 3.5 x 10^5 CFU. Each symbol represents a mouse, bars represent group median. Data are combined from two or three independent experiments. (*p<0.05)

We next investigated possible mechanisms by which ASC could contribute to severe infection outcomes in mice infected with PSE9. ASC is required for processing IL-18 and IL-1β cytokines that contribute to a pathological immune response to *P. aeruginosa* [17-20, 53]. However, the fact that caspase-1/11$^{-/-}$ mice did not have improved outcomes suggested that the ASC-induced survival benefit was not due to inflammasome-dependent cytokines IL-18 or IL-1β. To verify this *in vivo,* IL-18$^{-/-}$ mice were infected with PSE9. We observed that IL-18$^{-/-}$ mice succumbed to infection within 48 hours and exhibited no significant survival improvement compared to B6 mice (Figure 4). Therefore, loss of IL-18 did not attenuate infection as was observed with ASC. Taken together, these results suggest that PSE9 is activating an ASC-dependent immune response that is detrimental to outcomes but independent of caspase-1 and IL-18.

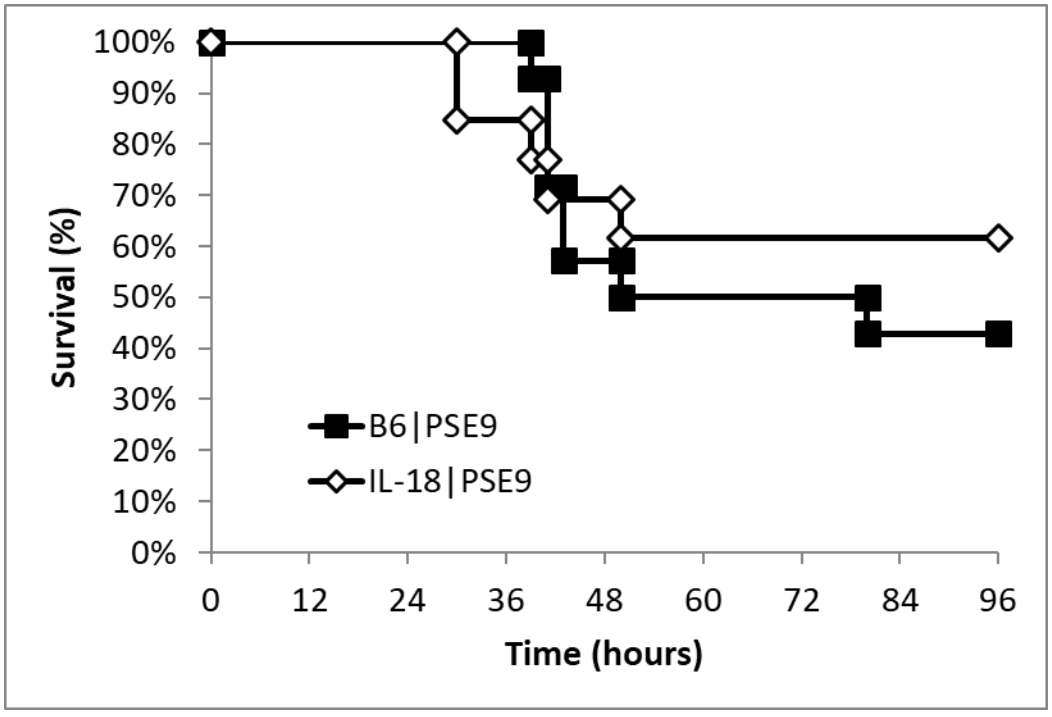


Figure 4 – IL-18$^{-/-}$ are equally susceptible to PSE9 as B6 mice in an acute pneumonia model. Data are combined from two independent experiments (B6 n=14 mice, IL-18$^{-/-}$ n=13 mice).

P. aeruginosa engages the canonical inflammasome pathway in vitro

Given the uncoupling of ASC from caspase-1 *in vivo*, we next assessed the nature of inflammasome activation *in vitro* to determine whether PSE9 engaged the canonical caspase-1 pathway in a novel way. Cell death and cytokine release were assessed *in vitro* with bone marrow-derived macrophages (BMDMs) from B6 mice. Infection of B6 BMDMs with the parental strain PSE9 for 2.5 hours resulted in 60% cell death (Figure 5a). Infection with PSE9Δ*pscF* demonstrated that the T3SS was required for a significant portion of this cell death (Figure 5a). T3SS-dependent killing could be due to direct toxicity of injected effector proteins or to the host pyroptotic cell death response. To distinguish between these two possibilities, we infected BMDMs from mice lacking components of the inflammasome pathway. BMDMs from mice deficient in IL-18 were susceptible to cell killing upon infection with PSE9 at similar levels to B6 BMDMs (Figure 5c and d). Cell death was reduced by 50% in $ASC^{-/-}$ BMDMs (Figure 5b and d) infected with PSE9 relative to B6 BMDMs and was nearly abrogated in $C1/11^{-/-}$ BMDMs (Figure 5c and d). These results indicate that PSE9 killing of macrophages occurs through a caspase-1/11-mediated process that is partially dependent upon ASC. These findings are consistent with prior reports of *P. aeruginosa* activation of the inflammasome pathway to cause pyroptosis [7, 8, 20, 25].

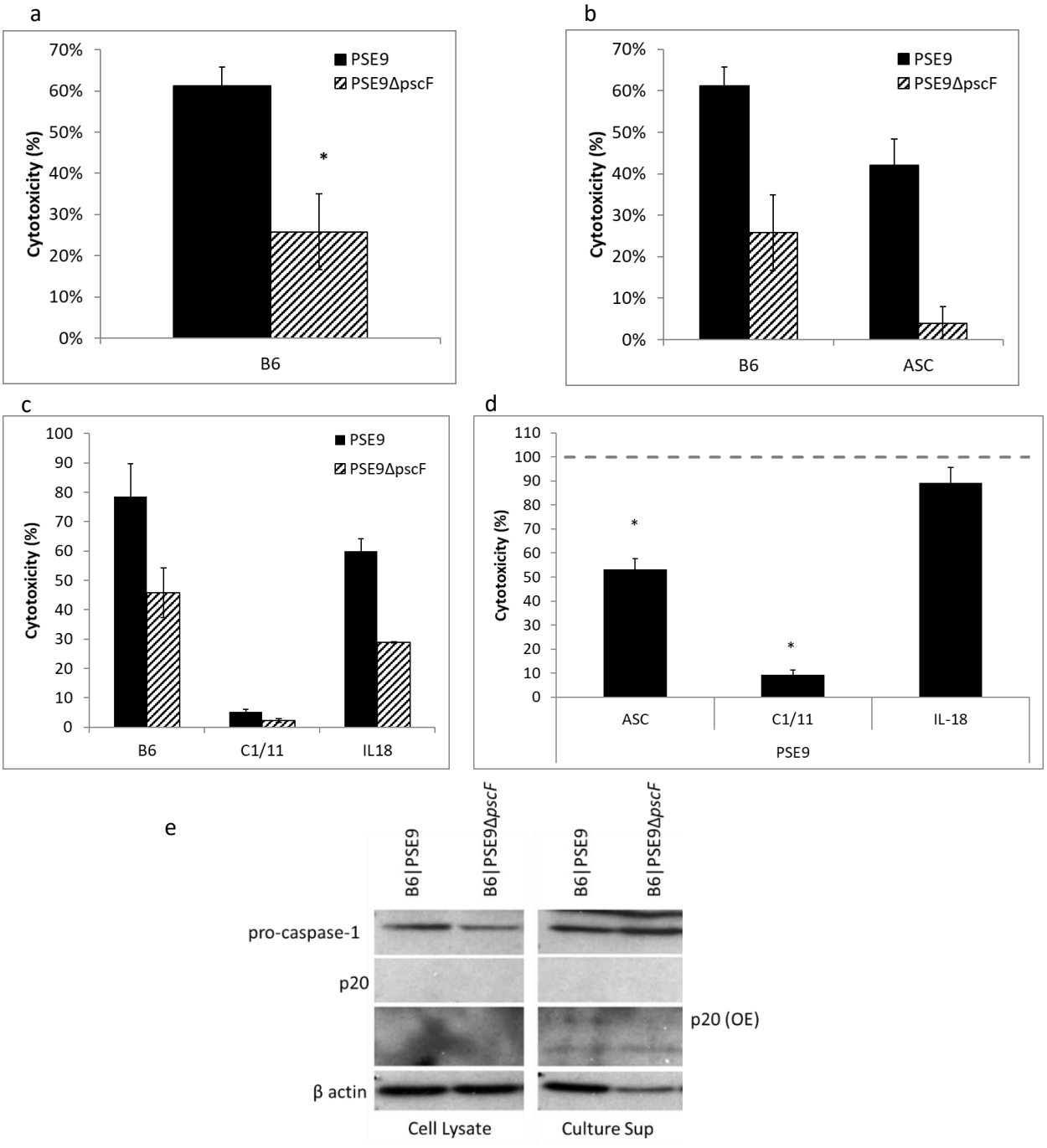


Figure 5 – The T3SS and the inflammasome contribute to cell death of BMDMs *in vitro.* a) Cytoxicity of B6 BMDMs infected with PSE9 or PSE9Δ*pscF.* b) Cytotoxicity of ASC$^{-/-}$ BMDMs, c) casp1/11$^{-/-}$ or IL-18$^{-/-}$ BMDMs infected with PSE9. Cytotoxicity measured by LDH release and normalized to 100% cell lysis. d) Cytotoxicity of indicated BMDMs normalized to B6 BMDMs infected with PSE9 from panels b & c. e) Caspase-1 immunoblot from cell lysates and culture supernatants of BMDMs infected with *P. aeruginosa*. B-actin was used as a loading control. OE = over exposed for faint band detection, p20 = 20kDa processed portion of caspase-1.

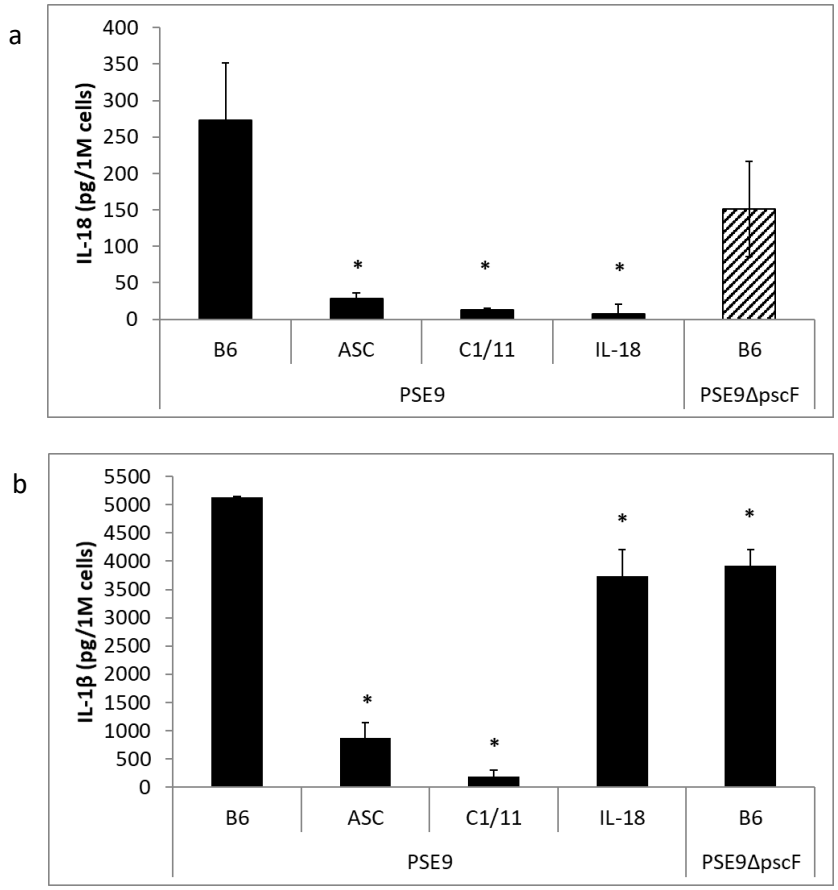


Figure 6 – Cytokine release from infected BMDMs *in vitro.* a) IL-18 release from indicated cell types. b) IL-1β release from indicated cell types. Bars represent the mean of experimental replicates. Error bars are standard deviation. Data are representative of two or three independent experiments. (* $p < 0.05$)

Another indication of canonical inflammasome activation is cleavage of the regulatory pro-domain from caspase-1. We looked for evidence of caspase-1 activation following PSE9 infection of B6 BMDMs by immunoblot and detected caspase-1 cleavage product in culture supernatants (Figure 5e). Furthermore, both IL-18 and IL-1β were detectable in culture supernatants (Figure 6a and b, respectively). IL-18 and IL-1β were still detectable albeit significantly reduced in PSE9Δ*pscF* infected B6 BMDMs compared to PSE9, demonstrating that type III secretion is required for release of some of the cytokines. IL-18 and IL-1β release were significantly attenuated in PSE9 infected-BMDMs lacking ASC relative to B6 (Figure 6a and b, respectively). Cytokine release was nearly completely eliminated in caspase-1/11$^{-/-}$ macrophages (Figure 6a and b). Taken together, these results are consistent with the current model of the canonical inflammasome activation in which PSE9 infection leads to caspase-1 activation and cytokine release in a process that requires ASC (Broz, von Moltke et al. 2010). While type III secretion is a robust ligand of inflammasome activation, evidenced by the enhancement of pyroptosis and cytokine release when T3SS is intact, other components of PSE9 may also play a role. With regard to the current study, these results further suggest that the unexpected role of ASC *in vivo* (Figure 3b) cannot be explained by aberrant activation of the canonical inflammasome or caspase-1 effector functions in macrophages because our *in vitro* results are consistent with the published literature.

Caspase-11 pathway is not required for PSE9-mediated cell death or cytokine release

The caspase-1$^{-/-}$ mice used in this study also harbor a mutation in the gene encoding caspase-11 [63]. Caspase-11 functions as a non-canonical inflammasome and indirectly contributes to IL-1β release through IL-1α autocrine signaling and modulation of caspase-1 [63]. Previous studies indicated that caspase-11 is not activated by *P. aeruginosa* [20, 63]. Nevertheless, we evaluated activation of the non-canonical inflammasome to ensure that our previous results were not confounded by activity of

caspase-11. We hypothesized that if the non-canonical inflammasome were activated downstream of ASC, cell death and cytokine release from caspase-11$^{-/-}$ BMDMs would replicate results from our ASC$^{-/-}$ BMDMs.

Caspase-11 is activated through TLR4 engagement by Gram-negative bacteria in conjunction with NF-κB mediated transcription and subsequent translation of NLRP3 [64, 116]. Therefore, BMDMs were isolated from mice with genetic disruptions of the genes encoding NLRP3 and caspase-11 to observe cell death and cytokine release in the absence of the non-canonical inflammasome. Unlike the ASC$^{-/-}$ and caspase-1/11$^{-/-}$ BMDMs described above, both caspase-11$^{-/-}$ (Figure 7a and c) and NLRP3$^{-/-}$ BMDMs (Figure 7b and c) exhibited cell death at similar levels to B6 BMDMs upon PSE9 infection. Furthermore, both genotypes were competent to release IL-18 and IL-1β (Figure 7d and e, respectively). In fact, both caspase-11$^{-/-}$ and NLRP3$^{-/-}$ BMDMs released more of these cytokines relative to B6 controls (Figure 7d and e). The current findings suggest that caspase-11 and NLRP3 may be involved in dampening inflammatory cytokines IL-18 and IL-1β upon detection of *P. aeruginosa* because BMDMs deficient in either component exhibit enhanced cytokine release (Figure 7d and e). Regardless, it is clear that ASC is not acting through caspase-11 and NLRP3 as neither NLRP3$^{-/-}$ nor caspase-11$^{-/-}$ BMDMs replicated the results of our ASC$^{-/-}$ BMDMs reported above (Figure 5b and d). Therefore, we conclude that the non-canonical inflammasome is not activated downstream of ASC by PSE9 and does not account for the *in vivo* role of ASC.

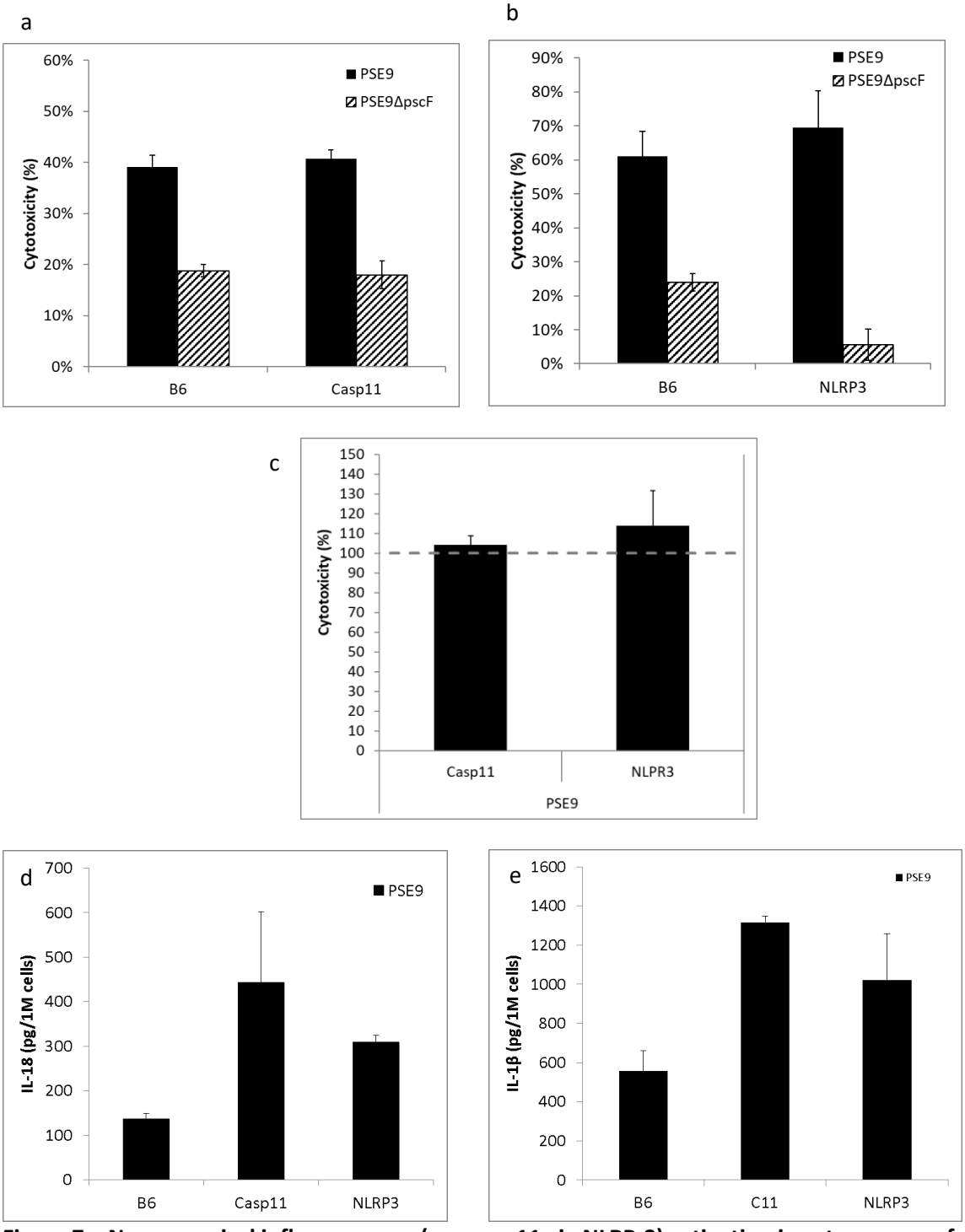


Figure 7 – Non-canonical inflammasome (caspase-11 via NLRP-3) activation is not necessary for PSE9 mediated cell death. a) Cytotoxicity of casp11$^{-/-}$ or b) NLRP3$^{-/-}$ BMDMs infected with PSE9 measured by LDH release and normalized to 100% cell lysis. c) Cytotoxicity of indicated BMDMs normalized to B6 controls from panels a & b. d) IL-18 release from indicated cells infected with PSE9. e) IL-1β release from indicated cells infected with PSE9. Bars represent the mean of experimental replicates. Error bars are standard deviation. Data are representative of two or three independent experiments. (*p<0.05)

Caspase-3 is activated in parallel to caspase-1 during PSE9 infection

To this point, we have demonstrated that the canonical caspase-1-dependent inflammasome is activated in the context of PSE9 infection (Figure 5) but that elimination of this pathway does not ameliorate lethality of infection *in vivo* (Figure 3b). Yet, elimination of ASC does ameliorate lethality of PSE9 infection *in vivo* (Figure 3b), suggesting that ASC may be worsening infection through a caspase-1-independent pathway. One possibility is that PSE9 engagement of ASC has consequences to caspases in addition to caspase-1. Heterotypic interactions of ASC with caspase-8 have been reported [87], and PSE9 secretes the T3SS effector ExoS (Figure 10), which activates caspase-3 to cause apoptosis [51]. We evaluated PSE9-mediated activation of other caspases by immunoblot analysis. The active fragments of caspase-9, caspase-12, caspase-8 and caspase-3/7 were all detected in uninfected cells, likely due to programmed cell death mechanisms intrinsic to the cellular lifecycle (Figure 8a). Although we were unable to detect the cleavage products of caspase-9 or caspase-12 in PSE9 infected BMDMs (Figure 8a) the active fragments of both caspase-8 and caspase-3/7 were present in PSE9 infected cells (Figure 8a). It was unclear whether either of these caspases were activated in response to PSE9 or whether their detection was again due to background levels of programmed cell death.

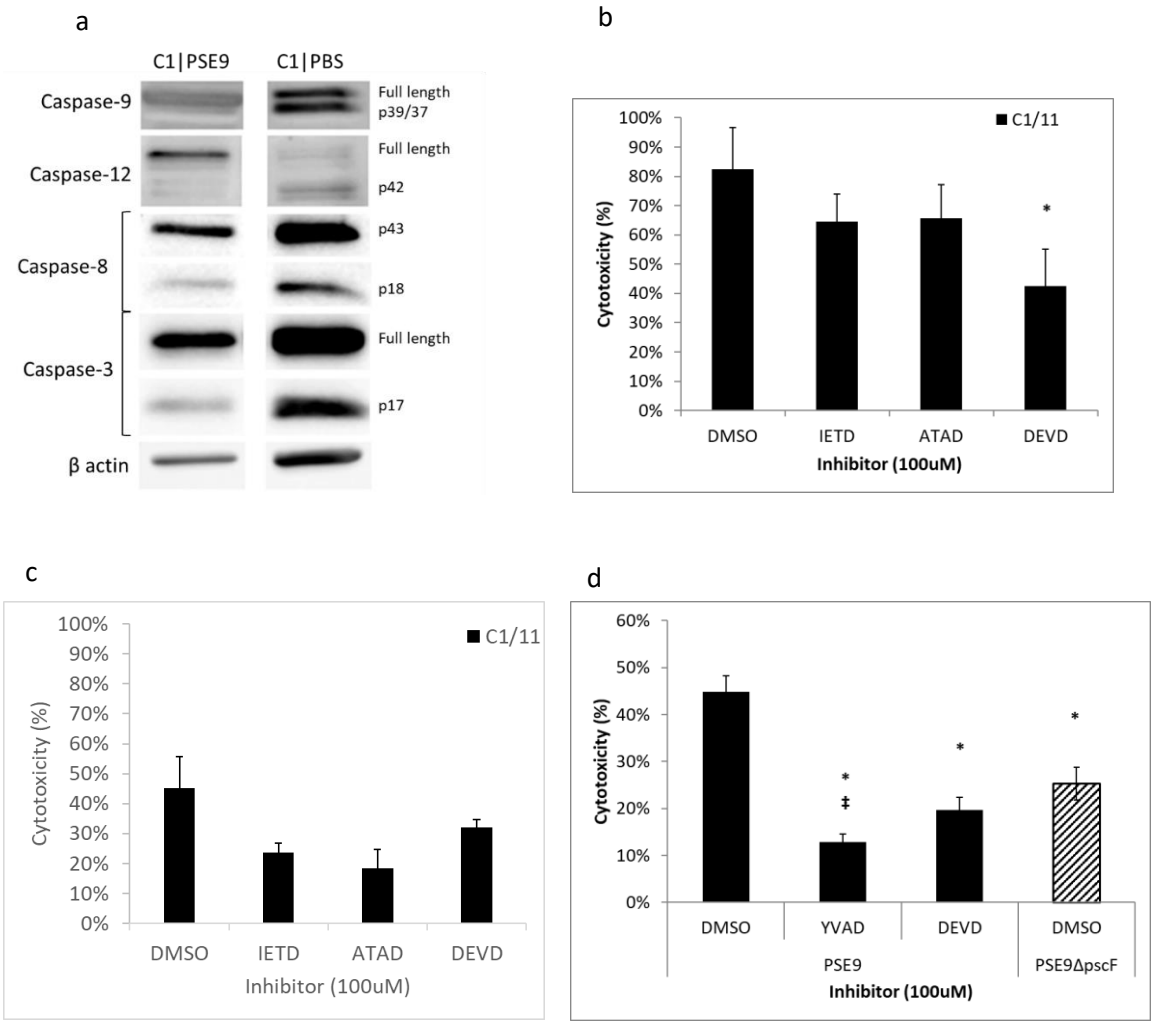


Figure 8 – Caspase-3 contributes to cell death during PSE9 infection. a) Immunoblot of cell lysates for indicated caspases in casp1/11$^{-/-}$ BMDMs infected with PSE9 or mock infected with PBS, as indicated. b) Cytotoxicity following treatment of casp1/11$^{-/-}$ BMDMs with PSE9 for 4 hours with caspase inhibitors. c) Cytotoxicity following treatment of casp1/11$^{-/-}$ BMDMs with caspase inhibitors and staurosporine. d) Cytotoxicity following treatment of B6 BMDMs with caspase inhibitors and infection with indicated strains for 2.5 hours. Cytotoxicity is measured by LDH release and normalized to 100% cell lysisBars represent experimental mean and error bars represent standard deviation. Data representative of two or three independent experiments. (*$p < 0.05$ relative to bar 1, ‡ $p > 0.05$ relative to bar 4)

Our previous *in vitro* assays did not detect substantial cell death independent of caspase-1/11 (Figure 5c) but we reasoned that pyroptosis could be masking cell death caused by caspases other than caspase-1. To determine whether caspase-8 or caspase-3/7 contributed to cell death during PSE9 infection, we infected caspase-1/11$^{-/-}$ BMDMs with PSE9 and measured cell death by LDH release at 4 hours (rather than 2.5 hours as used in Figure 5). Indeed, at this later time point caspase-1/11$^{-/-}$ BMDMs were killed by PSE9 (approximately 80% cell death, Figure 8b). To address whether other caspases played a role in this killing, we included inhibitors of caspase-12 (z-ATAD), caspase-8 (z-IETD) or caspase-3/7 (z-DEVD) in the media during co-incubation of BMDMs with PSE9. Although caspase-8 and caspase-12 inhibitors reduced cell death by one half in response to apoptosis induction by staurosporine (Figure 8c), cell death in response to PSE9 infection was only marginally reduced with these inhibitors (Figure 8b), and the differences were not statistically significant. However, addition of the caspase-3/7 inhibitor z-DEVD significantly reduced cell death in response to both staurosporine and PSE9 infection (Figure 8b and c, respectively). This result suggested that caspase-3/7 is activated in response to PSE9. Given the difference in timing between these experiments and our previous assays (Figure 4), we repeated this experiment with B6 BMDMs using a 2.5 hour infection to determine whether caspase-3/7 inhibition reduced cell death at earlier time points and in the presence of caspase-1. PSE9 infection of B6 BMDMs treated with z-YVAD to inhibit caspase-1 predictably reduced cell death (Figure 8d). Furthermore, z-DEVD treatment also significantly reduced cell death of PSE9-infected B6 BMDMs (Figure 8d). These results suggest that caspase-3/7 contributes to cell death during PSE9 infection even when caspase-1 is present and active.

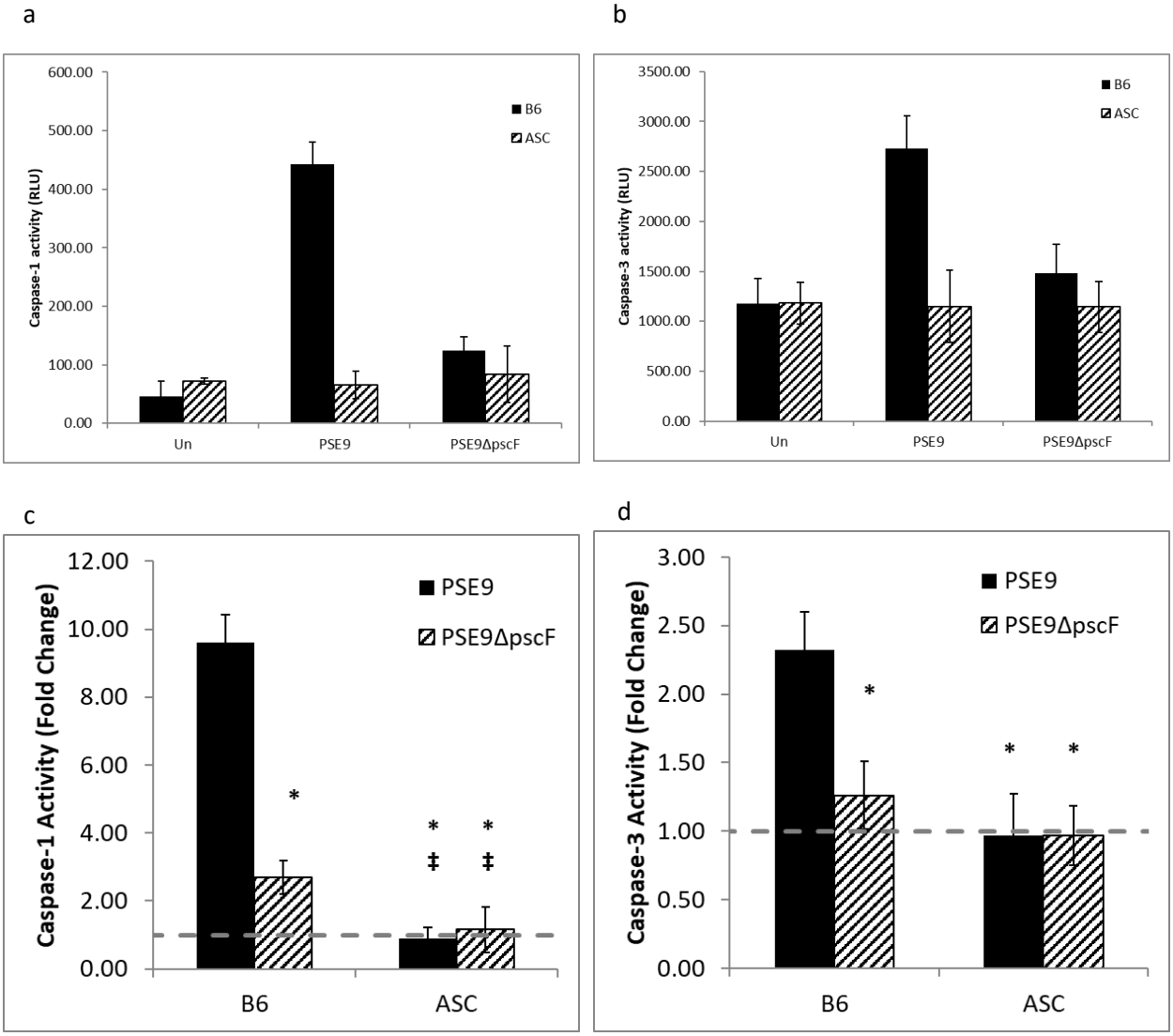


Figure 9 – Caspase-1 and caspase-3 are active in parallel *in vitro.* a) Caspase-1 activity in B6 and ASC$^{-/-}$ BMDMs infected with indicated strains. b) Caspase-3 activity in B6 and ASC$^{-/-}$ BMDMs infected with indicated strains. c) Caspase-1 activity relative to uninfected cells from panel a. d) Caspase-3 activity relative to uninfected cells from panel b. show fold-increase in activation relative to uninfected cells. Bars represent experimental mean. Error bars represent standard deviation. Data representative of independent experiments. (*$p < 0.05$ relative to bar 1, ‡ $p > 0.05$ relative to bar 2)

Caspase-3 activity is reduced in the absence of ASC

Consistent with the reports of others [51], we have demonstrated that the caspase-3/7 pathway is active during *P. aeruginosa* infection *in vitro*. However, it is unclear whether ASC contributes to the caspase-3/7 cell death pathway. To address this question, we utilized a bioluminescent reporter assay to measure caspase activity *in vitro.* First, we assessed activity of caspase-1 using this technology (Figure 9a and c). Whereas B6 BMDMs infected with PSE9 demonstrated a 10-fold increase in caspase-1 activity relative to uninfected macrophages, cells infected with PSE9Δ*pscF* exhibited a much smaller 2.5-fold increase in caspase-1 activity (Figure 9a and c). Moreover, caspase-1 activity was not detected above background levels in BMDMs lacking ASC (Figure 9a and c). Notably, this assay detects cleaved caspase-1, which has been shown to require ASC [53]. Therefore, these results are consistent with our findings and with previous studies showing that caspase-1 activation is ASC-dependent.

We next turned to the question of caspase-3 activity in the absence of ASC. We anticipated that caspase-3 activity as measured by the bioluminescent reporter would be reduced in the absence of ASC if the adaptor protein interacts with the apoptotic pathway upstream of caspase-3. Overall, we did not observe the same magnitude of caspase-3 activation (Figure 9b and d) as caspase-1 activation (Figure 9c). Yet, caspase-3 activity was enhanced 2.5-fold in B6 BMDMs intoxicated with PSE9 but was absent in $ASC^{-/-}$ macrophages (Figure 9b and d). Based on these findings, we conclude that caspase-3 activation is dependent on the presence of ASC. Furthermore, this interaction demonstrates an alternate role for ASC during *P. aeruginosa* infection.

Functional type III secretion is required for caspase activation

Although ExoS is secreted in a T3SS-dependent manner, this effector protein and the needle apparatus have different effects on inflammasome activation and cell death during infection [7, 9, 24, 25, 51]. We sought to determine whether our observed ASC-dependent caspase-3/7 activation was due to the T3SS apparatus or the ExoS effector. To do so, we utilized a panel of previously characterized T3SS mutants in a *P. aeruginosa* strain PA99 background: PA99S, which secretes only ExoS; PA99null, which produces a functional TSS apparatus but does not secrete any effectors; and PA99Δ*pscJ,* which lacks a functional T3SS (Figure 10) [10]. First, we assessed these mutants *in vitro* by infecting B6 BMDMs and ASC-deficient BMDMs and measuring cell death by LDH release (Figure 11a). B6 BMDMs were susceptible to cell death when infected with PA99S and PA99null (Figure 11a). However, cell death was abrogated in cells infected with PA99Δ*pscJ,* consistent with our previous findings that a functional T3SS is required for cell death. Furthermore, cell death was significantly reduced, but not eliminated, in $ASC^{-/-}$ BMDMs infected with PA99S and PA99null (Figure 11a). These results are consistent with our conclusion that an ASC-dependent pathway or pathways are contributing to cell death of macrophages during *P. aeruginosa* infection.

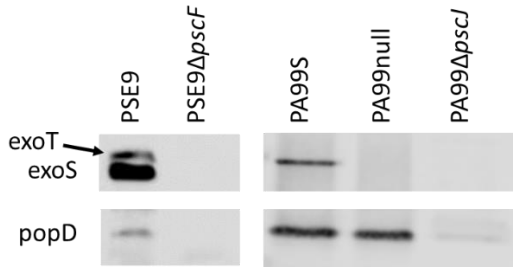


Figure 10 – Immunoblot analysis of bacterial culture supernatants for T3SS proteins. Bands representing effectors (ExoS and ExoT) and component of T3S needle apparatus (PopD) are indicated.

We reasoned that if ExoS were responsible for the observed activation of caspase-3/7 (Figure 8d and 9d), then caspase-3/7 activity would be similarly abrogated in cells infected with PA99null and PA99Δ*pscJ* since both mutants fail to secrete ExoS. As observed in the PSE9 background, caspase-1 and caspase-3/7 were both activated by PA99S in B6 BMDMs, but enhanced caspase activation was lost in macrophages infected with PA99Δ*pscJ* (Figure 11b and c). When evaluating the role of the T3SS in the absence of ExoS (i.e. the PA99null strain), we observed substantial activation of both caspase-1 and caspase-3/7 (Figure 11b and c, respectively). Activation of both caspases was less than that observed with the PA99S strain, although the reduction of caspase-3/7 was not statistically significant (Figure 11c). These results demonstrate that the presence of the T3SS apparatus in the absence of effectors is sufficient to stimulate activation of caspase-1 and caspase-3/7. Furthermore, both caspase-1 and caspase-3/7 activation were lost in $ASC^{-/-}$ BMDMs in all conditions tested (Figure 9a-d, Figure 11b and c). These results demonstrate that *P. aeruginosa* T3SS activates both pyroptotic and apoptotic mechanisms of programmed cell death and that both pathways are dependent on the adaptor protein ASC.

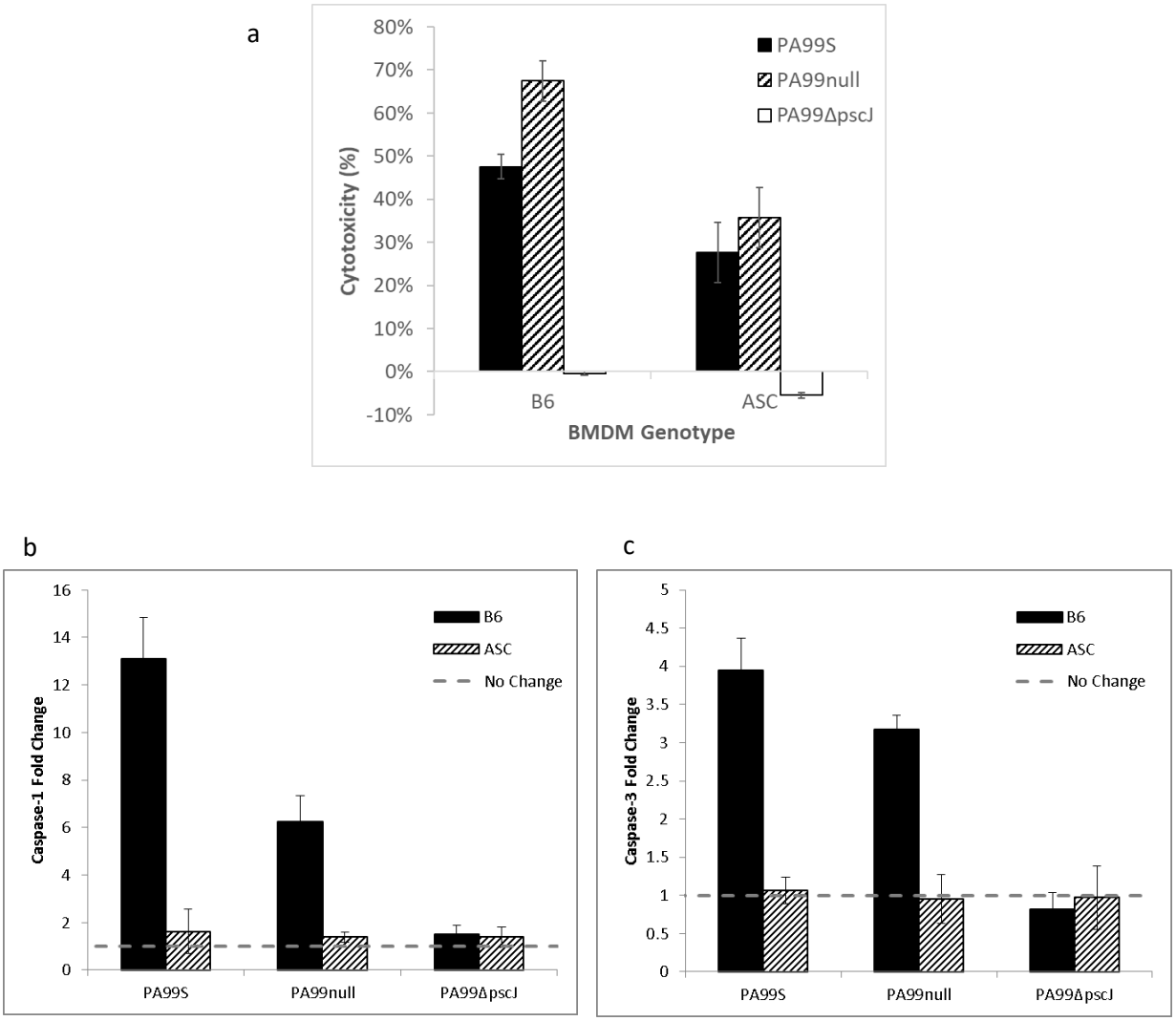


Figure 11 – T3S apparatus contributes to caspase activation. a) Cytotoxicity measured by LDH release of B6 BMDMs intoxicated with PA99 and normalized to 100% cell lysis. b) Caspase-1 activity in B6 and ASC BMDMs after intoxication with PA99. c) Caspase-3 activity in B6 and ASC BMDMs after intoxication with PA99. Data in b) and c) are normalized to uninfected cells. Bars represent experimental mean. Error bars represent standard deviation. Data representative of independent experimental replicates. (*$p < 0.05$)

DISCUSSION

In the current chapter, we sought to evaluate the relationship between *P. aeruginosa* and the host inflammasome-mediated immune response during acute pneumonia. Inflammasome activation by *P. aeruginosa* has been described in the literature by others [8, 20, 26, 51]. We described a novel role for the inflammasome adaptor protein ASC in promoting severe infection outcomes *in vivo*. Using a mouse model of acute pneumonia, we showed that mice lacking ASC have improved survival compared to B6 mice and that 80% of these mice cleared bacteria from their lungs. This result demonstrates that ASC is detrimental to infection outcomes because bacterial clearance and host survival are improved in the absence of ASC. Because caspase-1 is downstream of ASC in the inflammasome activation cascade, we hypothesized that caspase-1/11$^{-/-}$ mice would exhibit similar survival outcomes to ASC$^{-/-}$ mice. In fact, we observed that caspase-1/11$^{-/-}$ mice exhibited less than 10% survival, similar to B6 mice, suggesting that the role of ASC *in vivo* may be independent of its role in the caspase-1 inflammasome complex.

There is evidence in the literature that ASC plays differential roles in caspase-1-dependent inflammasome functions in that ASC is required for cytokine processing but is not required for cell death by pyroptosis [53]. Whereas disruption of caspase-1/11 affected both cell death and cytokine processing, disruption of ASC only disrupted cytokine processing. We hypothesized that survival of infected IL-18$^{-/-}$ mice would replicate that of our ASC$^{-/-}$ mice if a deficiency in cytokine processing explained our *in vivo* results. However, PSE9 infected IL-18$^{-/-}$ mice were equally susceptible to infection as B6 mice. Taken together, these results confirmed that the poor survival outcome of ASC mice is unrelated to the downstream caspase-1-dependent inflammasome pathway.

Possible explanations for our *in vivo* results included the following: aberrant activation of the inflammasome pathway downstream of ASC, relevance to infection of a caspase-11 non-canonical pathway that is also lacking in caspase-1/11$^{-/-}$ mice, or an inflammasome-independent role of ASC. We evaluated these possible mechanisms for ASC *in vitro*. First, we described caspase-1-dependent inflammasome activation by measuring cell death and cytokine release in PSE9-infected BMDMs to determine whether the inflammasome was aberrantly activated. We found that PSE9 infection of BMDMs resulted in caspase-1-dependent cell death and release of IL-18 and IL-1β indicative of inflammasome activation. While cell death was partially reduced in the absence of ASC, cytokine release was dependent on ASC. These results are consistent with the published literature and the current understanding of *P. aeruginosa* activation of the inflammasome [8, 26, 53]. Thus, our *in vitro* results suggest that aberrant inflammasome activation was not the explanation for our *in vivo* results.

We next evaluated the role of the non-canonical caspase-11 pathway to determine whether the deficiency of caspase-11 in caspase-1/11$^{-/-}$ mice was confounding our analysis. We also evaluated BMDMs lacking NLRP3, as this receptor has been implicated in the non-canonical caspase-11 pathway [64, 116]. We hypothesized that if ASC was engaging with the caspase-11 pathway, PSE9 infected caspase-11$^{-/-}$ and NLRP3$^{-/-}$ BMDMs would exhibit similar cell death and cytokine release to ASC$^{-/-}$ BMDMs. Whereas ASC$^{-/-}$ BMDMs had reduced cell death and cytokine release upon PSE9 infection, neither cell death nor cytokine release were reduced in caspase-11$^{-/-}$ or NLRP3$^{-/-}$ BMDMs. Like others who evaluated *P. aeruginosa* activation of caspase-11 before us, we did not find a contribution of caspase-11 in inflammasome activation [20, 63].

Collectively, these results confirm that the canonical caspase-1-dependent inflammasome pathway is activated in response to PSE9 infection in a predictable and consistent way. But, these results did not explain the caspase-1/11-independent role of ASC we observed *in vivo.* This led us to the conclusion that clinical isolates of *P. aeruginosa* engage an alternate ASC pathway that is detrimental to infection outcomes independent of the caspase-1 inflammasome.

To this point, we identified a novel role for ASC in *P. aeruginosa* infection outcomes that suggested ASC was engaging an alternate caspase-pathway. To unmask interactions between ASC and alternate caspases, we utilized an *in vitro* cell infection in the absence of caspase1/11. This allowed us to detect active caspases associated with apoptotic mechanisms of cell death without loss of signal in cell lysates due to membrane damage from caspase-1-mediated pyroptosis [52]. Heterotypic interactions of ASC with caspase-8 and caspase-12 have been reported by others [87]. Yet, neither caspase-8 nor caspase-12 were observed to contribute to cell death during *P. aeruginosa* infection of BMDMs. However, we did observe caspase-3 activation during PSE9 infection *in vitro*. We also observed a loss of caspase-3 activation in the absence of ASC, indicating that ASC is required for caspase-3 activation during PSE9 infection. Lee, *et al.* also described a reduction in caspase-3 activity in the absence of ASC when human monocyte-like THP1 cells were stimulated with Shiga toxin [88]. Whereas the authors found caspase-3 to be dependent on NLRP3 in addition to ASC [88], we did not find evidence of NLRP3 contribution to cell death or cytokine release in BMDMs infected with *P. aeruginosa*. The report of Lee, *et al.* supports our finding of a relationship between ASC and caspase-3. This suggests that the mechanisms by which ASC participates in caspase-3 activation during bacterial infection are complex.

Caspase-3 has been shown to play a role in *P. aeruginosa* pathogenesis [51, 114]. Specifically, the ADPRT activity of ExoS has been shown to be required for induction of caspase-3 activity and cell death by apoptosis in infected HeLa cells [114]. The work of Galle *et al.* was the first to demonstrate that ExoS promotes a caspase-3-dependent cell death pathway in the context of caspase-1 activation [51]. But, the authors postulated that ExoS modulates inflammasome activation by shifting away from caspase-1 activation towards caspase-3 activation and the mechanism of caspase-3 activation in this context remained unclear. The implications of dual cell death pathways through caspase-1 and caspase-3 on host infection outcomes and bacterial pathogenesis were poorly understood. We found that functional T3SS is sufficient to activate caspase-3 even in the absence of ExoS. Furthermore, we found that T3SS activation of caspase-3 is dependent on ASC and activation occurs in parallel to ASC-dependent caspase-1 activation.

CHAPTER 3: ***P. aeruginosa*** **antibiotic resistance is inversely correlated with virulence**

OVERVIEW

In addition to the host immune defenses, antimicrobial therapies ameliorate bacterial infections. However, emergence of *P. aeruginosa* antimicrobial resistance alters efficacy of antibiotic treatments. There are reports that infections due to MDR-*P. aeruginosa* are associated with increased morbidity and mortality in hospital patients [2, 93, 108]. Yet, it is unclear if severe infection outcomes are due to a lack of effective treatment options for MDR-infections or due to virulence of the infecting strain. Laboratory studies demonstrate that antimicrobial resistance may come with a fitness cost to the bacteria [98, 105-107]. Furthermore, it is unknown if fitness defects related to antimicrobial resistance manifest as attenuated virulence during infection. Limited studies have evaluated the relationship between antimicrobial resistance and bacterial virulence. Studies examining antimicrobial resistance due to overproduction of the drug efflux pump MexAB-OprM reported differing results. Using a *C. elegans* model of *P. aeruginosa* infection, Sanchez and colleagues reported attenuated virulence of MexAB-OprM overproducing strains[107]. Conversely, Cosson and colleagues did not find an attenuation related to this drug efflux pump using a *Dictyolstelium* inhibition model [98]. In examining carbapenem resistance related to disruptions of the *oprD* gene, Skurnik and colleagues found that antibiotic resistant strains exhibited improved gut colonization and dissemination to the spleen in neutropenic mice [100]. Thus, there is a lack of consensus in the literature regarding the effect of antimicrobial resistance on bacterial virulence.

In the current chapter, we use a clinical library of *P. aeruginosa* isolates examine the relationship between antimicrobial resistance and bacterial virulence from previous experiments in a mouse model

of infection. Using statistical modeling, we demonstrate that there is an inverse relationship between antimicrobial resistance and bacterial virulence.

RESULTS

Antibiotic resistance of P. aeruginosa strains

We utilized a library of previously described *P. aeruginosa* clinical isolates to examine prevalence of antimicrobial resistance [109]. In total, 89 *P. aeruginosa* isolates were tested for resistance to at least one therapeutic agent in each of the following classes: aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, monobactams and combination penicillin/β-lacatamase inhibitor drugs (Table 1). Minimum-inhibitory concentration (MIC) for the agents tested was initially tested at the time of diagnosis by the Clinical Microbiology Laboratory at Northwestern Memorial Hospital (NMH) by the Vitek2 or Etest (for Aztreonam). MICs were collected from the medical record, where available. In cases where MIC data was incomplete or missing, samples were retested in the Clinical Microbiology Laboratory using the same methods (Table 2). Fifty-four percent of the strains (n = 48) were found to be antibiotic resistant (Figure 12a). The antibiotic resistance phenotypes of the strains were defined according to the following definitions: strains resistant to at least one antibiotic in all 6 drug class tested were called extensively-drug resistant (XDR), strains resistant to at least one antibiotic in 3 to 5 drug classes were called multi-drug resistant (MDR) [91]. The term antibiotic resistant (ABR) is used in reference to strains that are resistant to at least one antibiotic in any of the antimicrobial classes tested [91]. We found that 7% of the isolates in this population were XDR, 27% were MDR and 20% were ABR (Figure 12a). Resistance to fluoroquinolones, cephalosporins and monobactams were most commonly observed in our strain population (Figure 12b). These findings are consistent with published reports regarding the prevalence of MDR and XDR strains among clinical isolates of *P. aeruginosa* [2, 92, 93].

Table 1 – Antibiotics and antimicrobial classes tested for *P. aeruginosa* and *K. pneumoniae.* Every strain was tested for at least one antibiotic in each drug class indicated.

Drug Class	P. aeruginosa	K. pneumoniae
Aminoglycosides	Gentamicin, Tobramycin, Amikacin	Gentamicin, Tobramycin, Amikacin
Carbapenems	Meropenem, Imipenem	Meropenem, Imipenem, Ertapenem
Cephalosporins	Ceftazidime, Cefepime	Ceftriaxone, Cefepime
Fluoroquinolones	Ciprofloxacin, Levoquin	Ciprofloxacin
Monobactam	Aztreonam	Aztreonam
Penicillin + βlactamase Combo	Piperacillin/Tazobactam	Piperacillin/Tazobactam, Ampicillin/ Sulbactam
Folate Inhibitor	-	Trimethoprim/Sulfamethoxalone

Table 2 – Summary of *P. aeruginosa* strains with MICs in indicated ranges for each antimicrobial class. Number indicates frequency of strains with MICs in Susceptible, Intermediate or Resistant ranges according to CLSI breakpoints for each antimicrobial class.

	Susceptible	Intermediate	Resistant
Fluoroquinolones	52	2	35
Aminoglycosides	64	9	16
Carbapenems	69	11	9
Monobactam	53	15	21
Cephalosporin	73	5	11
Piperacillin + βlactamas combo	63	14	12

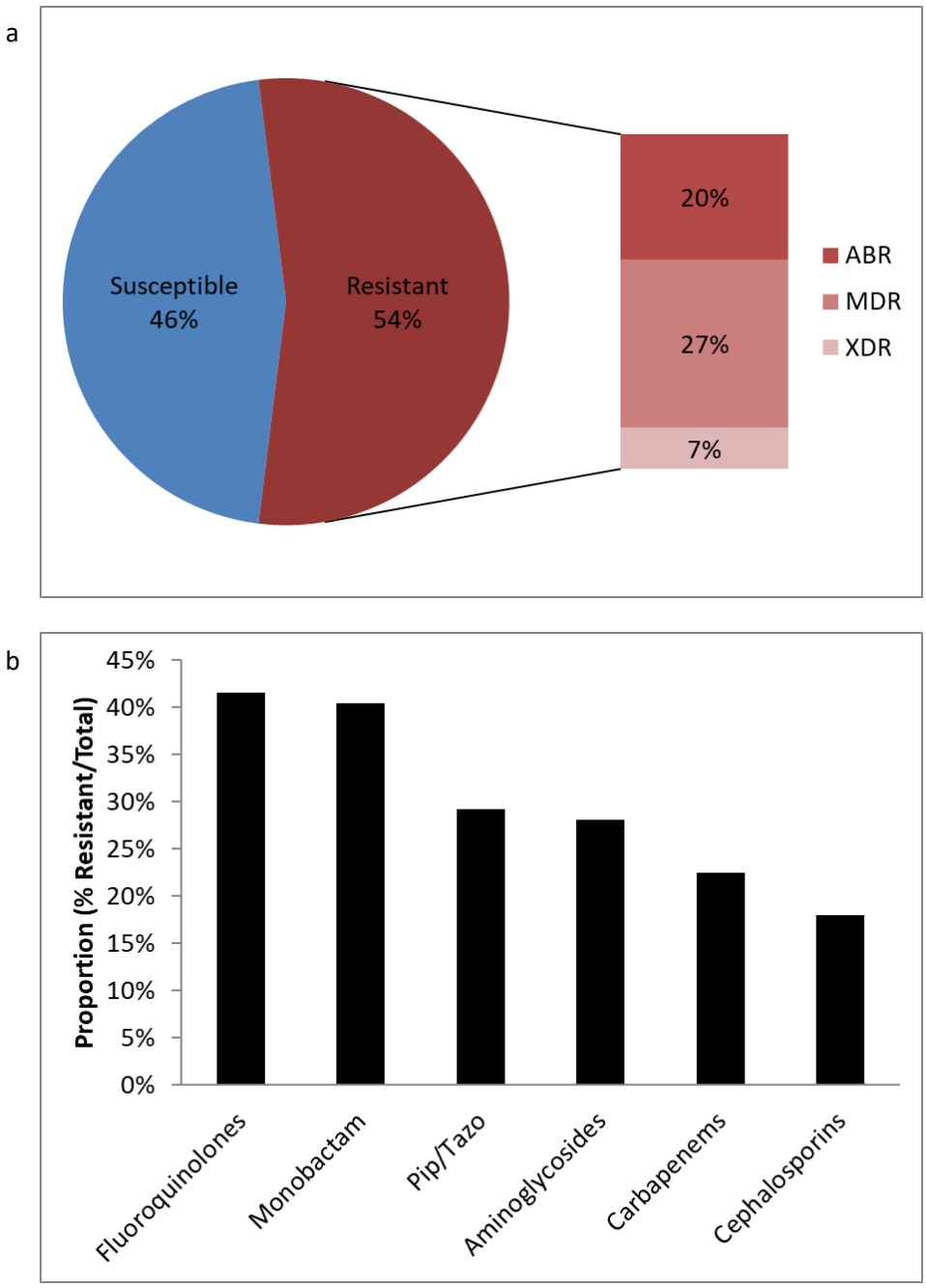


Figure 12 – Summary of antibiotic resistance phenotypes of *P. aeruginosa* bloodstream isolates. a) Percent of strains that are antibiotic susceptible, ABR (dark red), MDR (pink) or XDR (light pink). b) Percent of strains with resistance to at least one therapeutic agent in indicated classes. (n = 89)

In examining prevalence of drug resistance among MDR strains, certain combinations of antibiotic classes were more common (Figure 13). The vast majority of MDR strains were resistant to fluoroquinolones (n = 23/24, 95.8%, Figure 13). The combinations of fluoroquinolone resistance + piperacillin/tazobactam resistance + monobactam resistance was observed in 75% of MDR strains (n = 18/24, Figure 13a – c). Interestingly, resistance to aminoglycosides and/or carbapenems were more common among strains with increasing antibiotic resistance. Nearly 82% (n = 9/11) of strains resistant to 5 drug classes were resistant to aminoglycosides (Figure 13c). That proportion is reduced to 60% (n = 3/5) and 50% (n = 4/8) for strains resistant to 4 classes (Figure 13b) and 3 classes (Figure 13a), respectively. Similarly, nearly 91% (n = 10/11) of strains resistant to 5 drug classes were resistant to carbapenems (Figure 13c) while only 40% of strains resistant to 4 drug classes (Figure 13b) and 12.5% (n = 1/8) of strains resistant to 3 drug classes (Figure 13a) were resistant to carbapenems. In this way, antibiotic resistance and the MDR phenotype were common among the strains in our population.

a

3 Classes			*N*
Fluoroquinolones	Monobactam	Aminoglycosides	4
		Carbapenems	1
		Piperacillin/Tazobactam	1
	Piperacillin/Tazobactam	Cephalosporin	1
Monobactam	Piperacillin/Tazobactam	Cephalosporin	1

b

4 Classes				*N*
Fluoroquinolones	Piperacillin/Tazobactam	Monobactam	Aminoglycosides	2
			Carbapenems	1
			Cephalosporins	1
		Aminoglycosides	Carbapenems	1

c

5 Classes					*N*
Fluoroquinolones	Piperacillin/Tazobactam	Carbapenems	Monobactam	Aminoglycosides	5
				Cephalosporins	2
			Aminoglycosides	Cephalosporins	3

Figure 13 – Combinations of resistance to antimicrobial classes in MDR *P. aeruginosa.* a) Strains resistant to 3 antimicrobial classes, b) 4 antimicrobial classes, and c) 5 antimicrobial classes. Rows of each table represent indicated number of strains with the same pattern of antimicrobial resistance. Columns indicate antimicrobial class. Depth of blocks in each column represent antimicrobial classes shared between multiple rows.

Relative virulence of P. aeruginosa isolates

The bacterial strains used in this study were previously challenged in a mouse model of bacteremia for other studies (unpublished data). We collected data from that study to create relative virulence scores for each strain and conduct a post-hoc analysis of antimicrobial resistance related to virulence. Specifically, strains were scored based on the bacterial dose that was required for greater than 50% but less than 100% pre-lethal infection in the mouse model of disease. Based on these values, bacteria received virulence scores from 1 to 5, with 1 being the least virulent strains (requiring the highest bacterial dose to induce disease) and 5 being the most virulent strains (requiring the lowest bacterial dose to cause severe illness). Figure 14a depicts the distribution of strains across virulence score groups. Overall, few strains were categorized as highly virulent receiving a score of 5 (n = 3/89) or low virulence receiving a score of 1 (n = 6/39, Figure 14a). The majority of strains exhibited a moderate virulence phenotype receiving a score of 3 (n = 33, Figure 14a). Using this method, we were able to categorically define the ability of *P. aeruginosa* strains to establish severe illness as a measure for bacterial virulence.

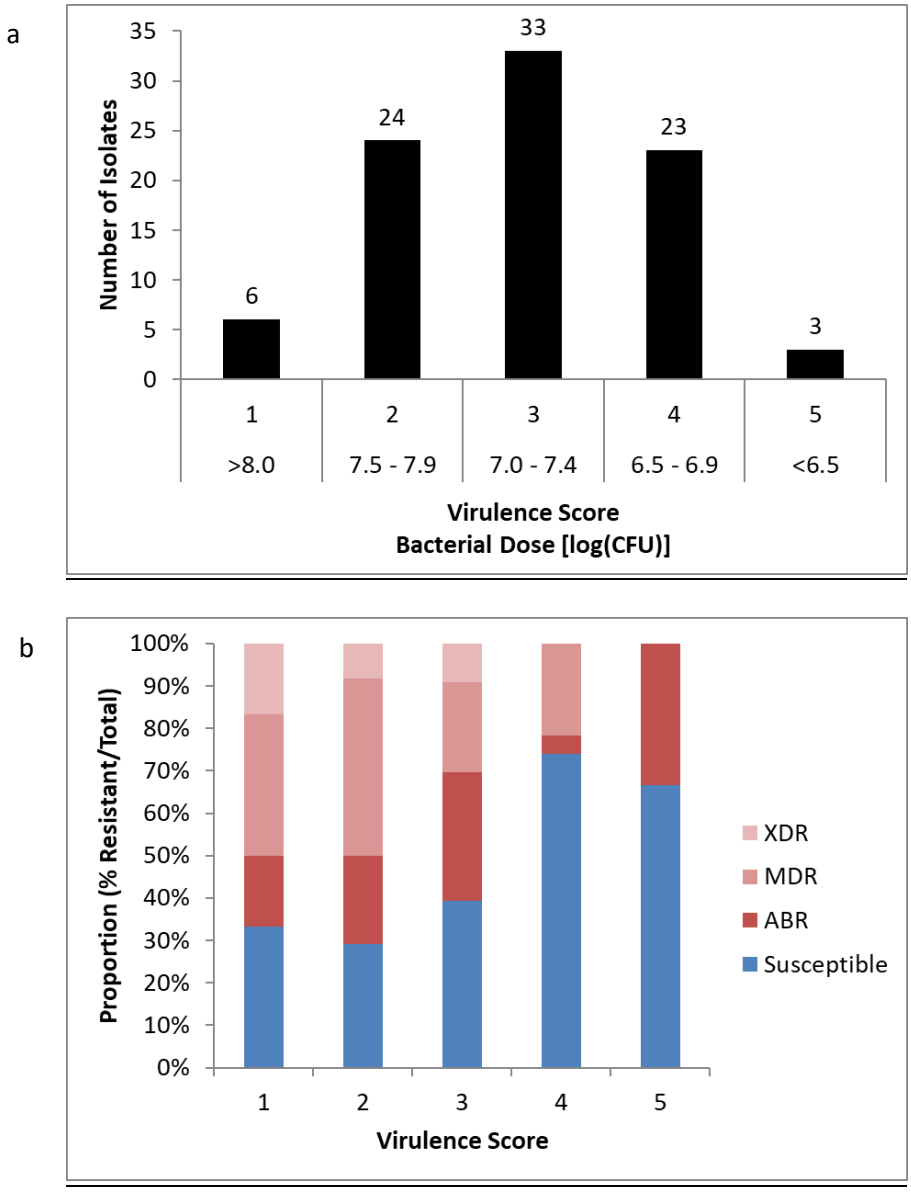


Figure 14 – Distribution of *P. aeruginosa* antimicrobial resistance phenotypes by strain virulence (n = 89). a) Histogram of strains by virulence score where 1 represents the least virulent group and 5 represents the most virulent group. b) Proportion of antibiotic susceptible (blue), ABR (dark red), MDR (pink) and XDR (light pink) strains stratified by virulence score.

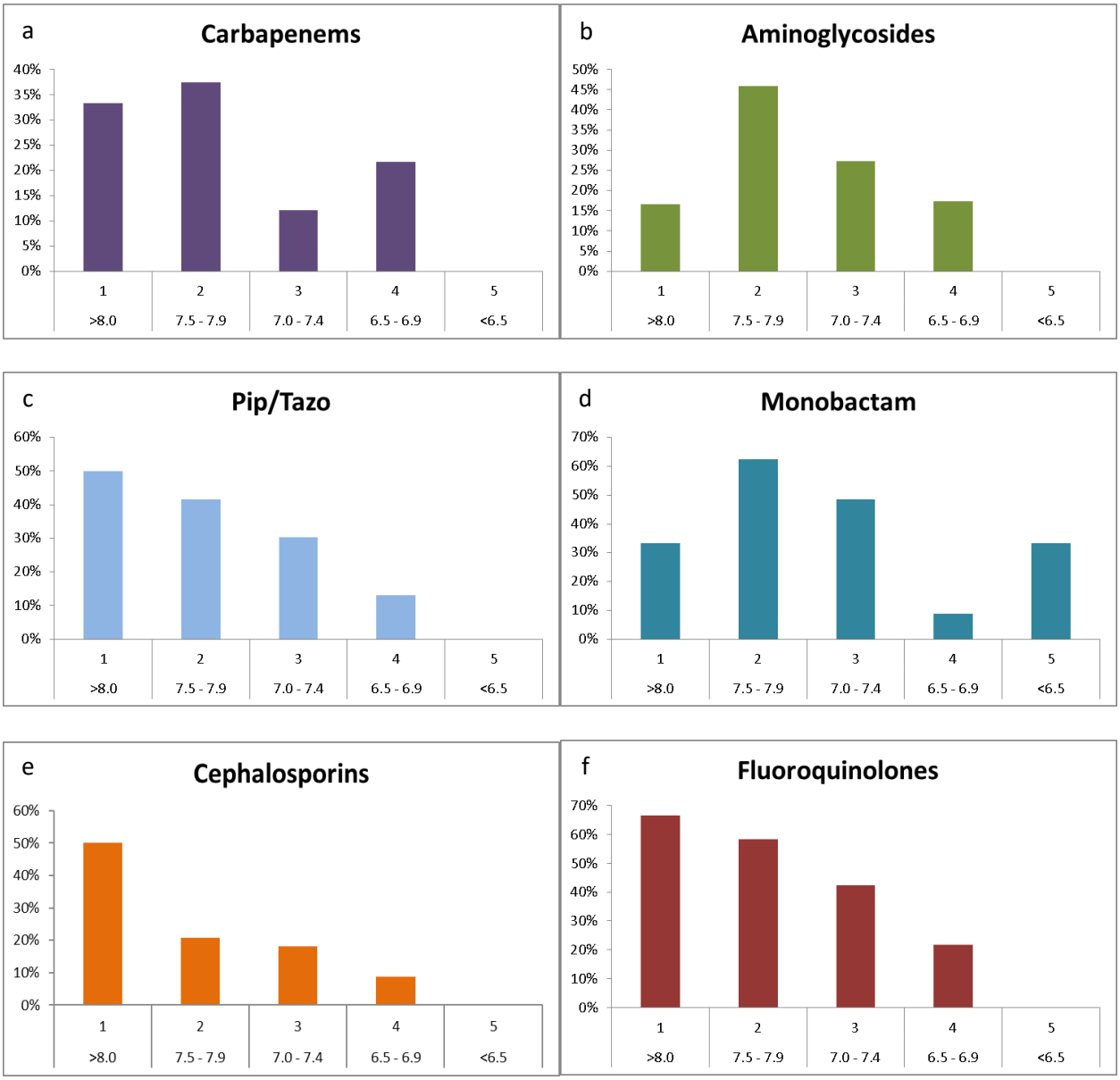


Figure 15 – Percent of *P. aeruginosa* strains resistant to at least on therapeutic agent in indicated antimicrobial class stratified by virulence rank. a) Carbapenem resistance stratified by virulence rank. b) Aminoglycoside resistance stratified by virulence rank. c) Piperacillin/tazobactam resistance stratified by virulence rank. d) Monobactam resistance stratified by virulence rank. e) Cephalosporin resistance stratified by antibiotic resistance. f) Fluoroquinolone resistance stratified by virulence rank.

Antibiotic resistance is inversely correlated with virulence in P. aeruginosa

The primary goal of the current study was to observe whether there is a relationship between antibiotic resistance and *P. aeruginosa* virulence. In this regard, we stratified strains by their resistance phenotype and virulence score. We observed that a greater proportion of the more virulent strains (virulence scores of 4 or 5) were antibiotic sensitive or resistant to less than 3 drug classes (ABR, Figure 14b). Conversely, a greater proportion of lower virulent strains with a virulence score of 1 or 2 had MDR or XDR phenotypes (Figure 14b). We used logistic regression analysis to calculate the odds ratio of a strain exhibiting a resistance phenotype based on increasing virulence score (Table 3). In this analysis, an odds ratio greater than one indicates that strains of increased virulence have higher odds of exhibiting an antibiotic resistance phenotype than strains of lower virulence. An odds ratio less than one indicates that strains of increased virulence have lower odds of exhibiting an antibiotic resistance phenotype than strains of lower virulence. The first model used an ABR variable that included all strains resistant to at least 1 antimicrobial agent (inclusive of MDR and XDR phenotypes). The odds ratio for antibiotic resistance was 0.49 (95% confidence interval [CI] 0.30-0.81), indicating that increased virulence is associated with lower odds of having an antibiotic resistant phenotype (Table 3 and Figure 16a). In the second model, we examined increasing antibiotic resistance phenotypes utilizing a variable that included all strains resistant to at least 3 antimicrobial classes (MDR/XDR). Similarly, we found that increased virulence was associated with lower odds of a strain having an MDR phenotype (OR 0.54, 0.33-0.89 95% CI, Table 3 and Figure 16b). Finally, we examined extensive antibiotic resistance using the XDR phenotype as a variable. Although the trend of ABR and MDR/XDR relationships was also consistent with the XDR phenotype, this logistic regression model was not statistically significant, likely due to the fact that only a small proportion of our strains exhibited the XDR phenotype (Table 3 and Figure 16c).

Table 3 – Logistic regression models of association between *P. aeruginosa* antibiotic resistance and virulence score. *Indicates statistically significant models

Univariate		
	OR	95% CI
ABR	0.49	0.30-0.81*
MDR/XDR	0.54	0.33-0.89*
XDR	0.49	0.2-1.24
Multivariate		
	OR	95% CI
ABR	0.39	0.22-0.69*
ExoU	6.51	1.33-31.8*
ExoS	2.21	0.55-8.94
MDR/XDR	0.41	0.23-0.74*
ExoU	6.63	1.36-32.39*
ExoS	2.52	0.59-10.87
XDR	0.43	0.15-1.24
ExoU	2.16	0.17-28.0
ExoS	1.62	0.15-17.09

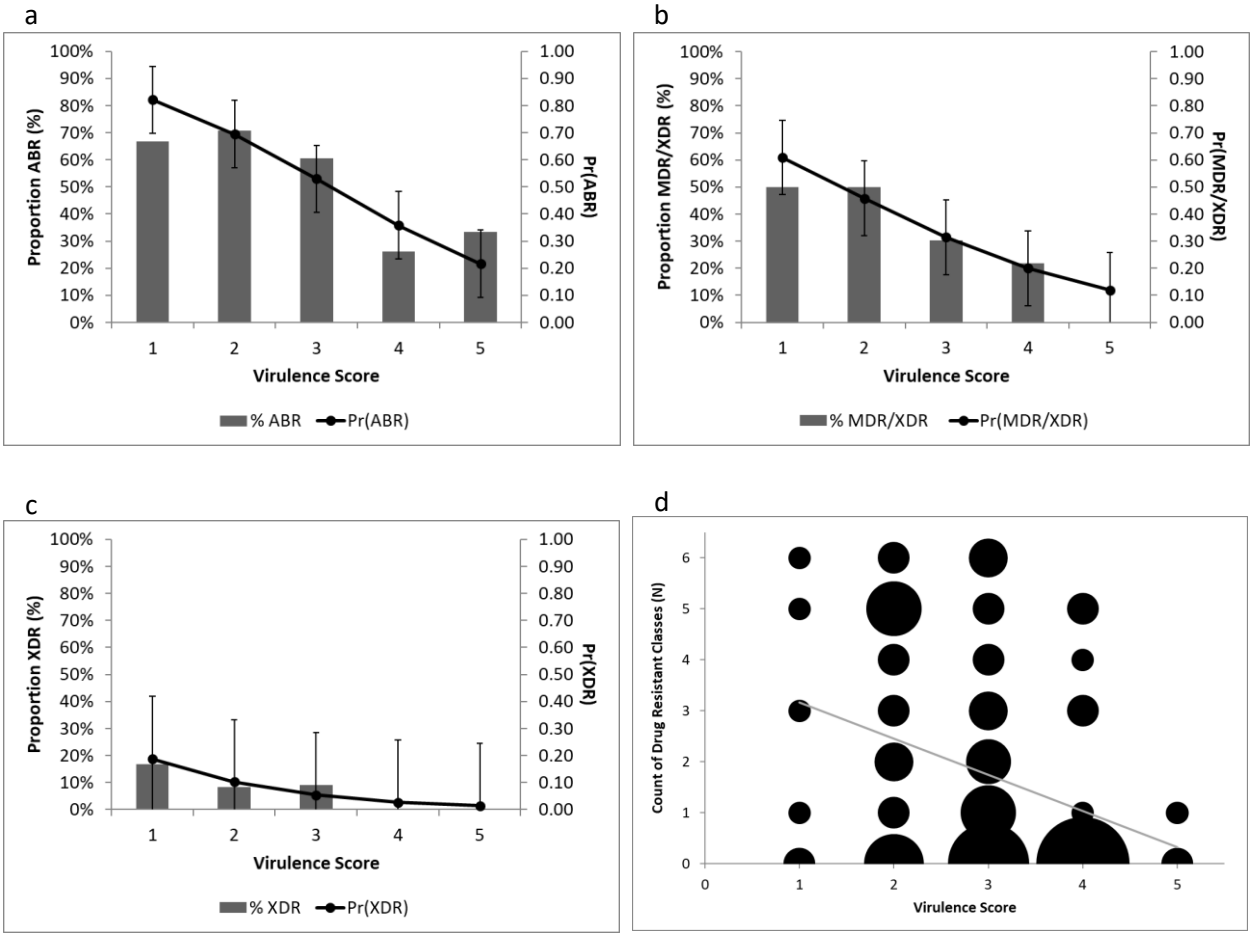


Figure 16 – Regression models of *P. aeruginosa* antibiotic resistance and virulence score. Percent of strains that are resistant to a) at least 1 class of antibiotics (ABR), b) 3 or more classes of antibiotics (MDR/XDR) in each virulence group, or c) at least one antibiotic in all classes tested (XDR). Bars represent percent of strains resistant to the specified minimum number of drug classes (left axis). Circles represent the calculated probability of antibiotic resistance from regression models by virulence score (right axis). d) Number of drug classes a strain is resistant to versus virulence score where each circle represents a strain and the area of each circle is weighted for the number of strains it represents. Line represents the linear regression model fit to the data represented.

The above analysis relied on inclusive categorical grouping by antibiotic resistance. However, the definition for MDR includes strains exhibiting a range of resistance to 3, 4 and 5 classes of antibiotics. Therefore, we repeated the analysis by evaluating the number of antibiotic classes that each strain was resistant to versus its virulence score. These data are represented in Figure 16c where increasing weight is given to points that represent a greater number of strains. All of our most virulent strains with a virulence score of 5 were resistant to either 0 or 1 antibiotic classes. Conversely, lower virulent strains with a virulence score of 1 or 2 were generally resistant to a greater number of antibiotic drug classes. This trend was statistically significant by linear regression (β coefficient = -0.71, p = 0.002, Figure 16c). This suggests that virulence score is reduced by 0.71 points for each additional antibiotic class that a strain is resistant to. Taken together, our statistical models demonstrate that there is an inverse relationship between antibiotic resistance and bacterial virulence such that bacterial virulence is associated with lower odds of antibiotic resistance.

Effector protein ExoU is associated with increased antibiotic resistance

Others have reported that the virulence factor ExoU is associated with increased antibiotic resistance [110]. *P. aeruginosa* uses the T3SS to inject effectors like ExoU, ExoS, ExoT and ExoY directly into target cells [6]. However, not all strains express functional T3SS [110, 115] and less than 30% of clinical isolates encode the *exoU* gene [11]. The strains used in this study were assessed for the presence of functional T3SS and secretion of ExoU and ExoS effector proteins by immunoblot from culture supernatants *in vitro* for other ongoing studies in the lab. Data were collected and summarized for statistical analysis in Table 8. Functional T3SS was detected in detected in 76 of 89 isolates (85%). Twenty-seven isolates secreted the virulence factor ExoU and 48 secreted ExoS. One strain secreted both ExoU and ExoS. We then added variables for $ExoU^+$ and $ExoS^+$ secretion to our regression model to observe whether the presence

of virulence factors affects the relationship between antibiotic resistance and virulence (Table 3). Indeed, there was a statistically significant relationship between antibiotic resistance phenotypes and the ExoU^{+} variable in the multiple regression model (Table 3). Strains that secreted ExoU had higher odds of exhibiting ABR and MDR/XDR phenotypes than strains that did not secrete ExoU with the same virulence score (OR = 6.51 and OR = 6.63, respectively, Table 3). However, the odds ratios for the ABR and MDR models relative to virulence score were still less than one at 0.39 (0.22-0.69 95% CI) and 0.41 (0.23-0.74 95% CI), respectively (Table 3). The T3SS effector ExoS was not associated with increased odds of antibiotic resistance in our study or others (Table 3, [110, 117]). In this revised statistical model, strains with higher virulence scores have lower odds of being antibiotic resistant. At the same time, strains that are antibiotic resistant within each virulence group have higher odds of secreting ExoU. These findings suggest that while ExoU is correlated with increased antibiotic resistance (consistent with the published literature [110]) overall bacterial virulence of *P. aeruginosa* is inversely correlated with antibiotic resistance.

DISCUSSION

In the current section, we examined the relationship between antimicrobial resistance and bacterial virulence using a library of 89 clinical isolates of *P. aeruginosa* and previously generated virulence data. We found that MDR and XDR resistance phenotypes were represented in our strain population. Using a measure of bacterial virulence from a mouse model of disease, we demonstrated that antimicrobial resistance was inversely correlated with virulence. More virulent strains that required a lower bacterial dose to cause severe illness in mice were susceptible to antibiotics or resistant to few antimicrobial classes. In this way, we found a statistically significant inverse relationship between antimicrobial resistance and bacterial virulence of *P. aeruginosa*.

In examining patterns of antibiotic resistance, we observed that the combination of fluoroquinolone resistance + piperacillin/tazobactam resistance + monobactam resistance was most common among MDR-*P. aeruginosa* isolates. If antibiotic resistance to different antimicrobial classes were random, we would not expect to observe patterns in combinations of resistance classes. Others have reported relationships between prescribing patterns for antibiotic agents in treatment of infection and emergence of antibiotic resistance in clinical isolates [103, 104]. The patterns observed here may indicate a preference towards prescribing fluoroquinolones more frequently. Treatment of *P. aeruginosa* infections with aminoglycosides and carbapenems may not have been as common at this institution as resistance to these drug classes emerged in strains with increased resistance to 4, 5 or 6 antimicrobial classes. If resistance to multiple antimicrobial classes was achieved by the same mechanism, we would expect predictable combinations in antimicrobial resistance such as cephalosporin resistance + piperacillin/tazobactam resistance + monobactam resistance related to production of an extended spectrum β-lactamase [96]. Yet, we did not observe covariance of these three antimicrobial classes (Figure 13a-c). We cannot exclude the possibility that a universal resistance mechanism such as overproduction of MexAB-OprM may explain our observations regarding combinations of resistance to antimicrobial classes. These possibilities must be explored in future studies.

Others have reported a correlation between the T3SS effector protein ExoU and antimicrobial resistance [110]. Conversely, transcription of the T3SS apparatus was found to be reduced in *P. aeruginosa* PAO1 mutants that overexpress the drug efflux pumps MexCD-OprJ or MexEF-OprN [111]. In the current study, we also found increased odds ratio for the ExoU$^+$ variable and antimicrobial resistance or MDR

phenotype. However, the overall statistical model of bacterial virulence that included this variable had a low odds ratio for antimicrobial resistance. Collectively, our results suggest that antimicrobial resistance is inversely correlated with bacterial virulence but that antimicrobial resistant strains have higher odds of secreting ExoU by T3SS.

Our findings of a virulence cost related to antimicrobial resistance are consistent with the reports of others [106, 107, 112]. However, the studies in the published literature relied on genetically modification of *P. aeruginosa* strains in the laboratory to examine virulence cost of specific antimicrobial resistance mechanisms. In doing so, experimental methods such as differences in infection models between *Caenorhabditis elegans* and the amoeba *Dictysotelium* may have contributed to discrepant findings, particularly related to the role of MexAB-OprM overproduction in virulence attenuation [107, 112]. In the current study, we were able to conduct a population-level analysis of antimicrobial resistance utilizing a library of clinical isolates. The strains used in our analysis acquired antimicrobial resistance in the context of clinical infection rather than by laboratory manipulation. One may expect to observe no virulence cost to antimicrobial resistance that emerges in the context of clinical infection as these strains were capable of causing disease in people [109]. However, the statistical models of the current study demonstrate an inverse relationship between antimicrobial resistance and virulence when comparing antimicrobial resistant *P. aeruginosa* isolates to a population of *P. aeruginosa* isolates.

CHAPTER 4: ***K. pneumoniae* antibiotic resistance is inversely correlated with virulence**

OVERVIEW

Having identified an inverse relationship between antimicrobial resistance and bacterial virulence in a population of *P. aeruginosa* clinical isolates in the previous section, we next sought to evaluate this relationship in other Gram-negative bacteria. *Klebsiella pneumoniae* is another Gram-negative bacterium that is a common cause of nosocomial infections [2]. Like *Pseudomonas*, *Klebsiella* is of concern due to increasing antimicrobial resistance and limited treatment options [2]. Others have reported reduced virulence of antimicrobial resistant *K. pneumoniae* in a *Galleria mellonella* model of infection [118]. The authors found that *K. pneumoniae* strains producing a carbapenemase exhibited 50% less lethality of the *Galleria* compared to carpabenemase-negative isolates [118]. However, it is unclear if these conclusions would be the same in mouse models of illness where the host has a complex immune response combating infection. Based on the evidence in the published literature and our previous findings, we sought to evaluate the relationship between antimicrobial resistance of *K. pneumoniae* and bacterial virulence to determine if our statistical approach was relevant for Gram-negative bacteria other than *P. aeruginosa*.

RESULTS

Antimicrobial resistance of K. pneumoniae

A library of *K. pneumoniae* clinical isolates was collected for other studies ongoing in the laboratory. Strains were collected Northwestern Memorial Hospital and various other institutions (see methods for a detailed summary). We utilized this library to examine the prevalence of antimicrobial resistance. MICs for antimicrobial agents were reported at the time of infection diagnosis in the Clinical

Microbiology Laboratory using the Vitek2 or appropriate test method. Where MIC data was missing or incomplete, strains were retested for antibiotic resistance using the same methods (Table 4). In total, 43 isolates were assessed for resistance to at least one therapeutic agent in each of the following classes according to CLSI breakpoints [119]: aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, monobactam, penicillin+β-lactamase inhibitor combination drugs and a folate inhibitor (Table 1). The cephalosporin cefazolin has 2 different MIC breakpoints for UTIs or bloodstream infections [119]. We utilized the bloodstream breakpoint as this infection is more relevant to the current study than UTIs. We found that 100% of the strains in this population were resistant to cefazolin (Table 9). This was surprising because intrinsic resistance to cefazolin (a bactericidal agent that binds to penicillin binding protein) has not been reported for *K. pneumoniae,* to our knowledge. Given that cefazolin did not appear to be a clinically relevant antimicrobial agent for *K. pneumoniae* outside of UTIs*,* we excluded cefazolin from further analysis to prevent biasing our conclusions. We found that the majority of isolates in this population were antibiotic resistant with 28% of strains exhibiting an MDR phenotype and 35% exhibiting an XDR phenotype (Figure 17a).

Table 4 – Summary of *K. pneumoniae* strains with MICs in indicated ranges for each antimicrobial class. Number indicates frequency of strains with MICs in Susceptible, Intermediate or Resistant ranges according to CLSI breakpoints for each antimicrobial class.

	Susceptible	Intermediate	Resistant
Cephalosporins	16	0	27
Aminoglycosides	21	5	17
Fluoroquinolones	24	0	19
Carbapenems	26	0	17
Penicillin+βlac	16		27
Monobactam	18	0	25
Folate inhibitor	11		31

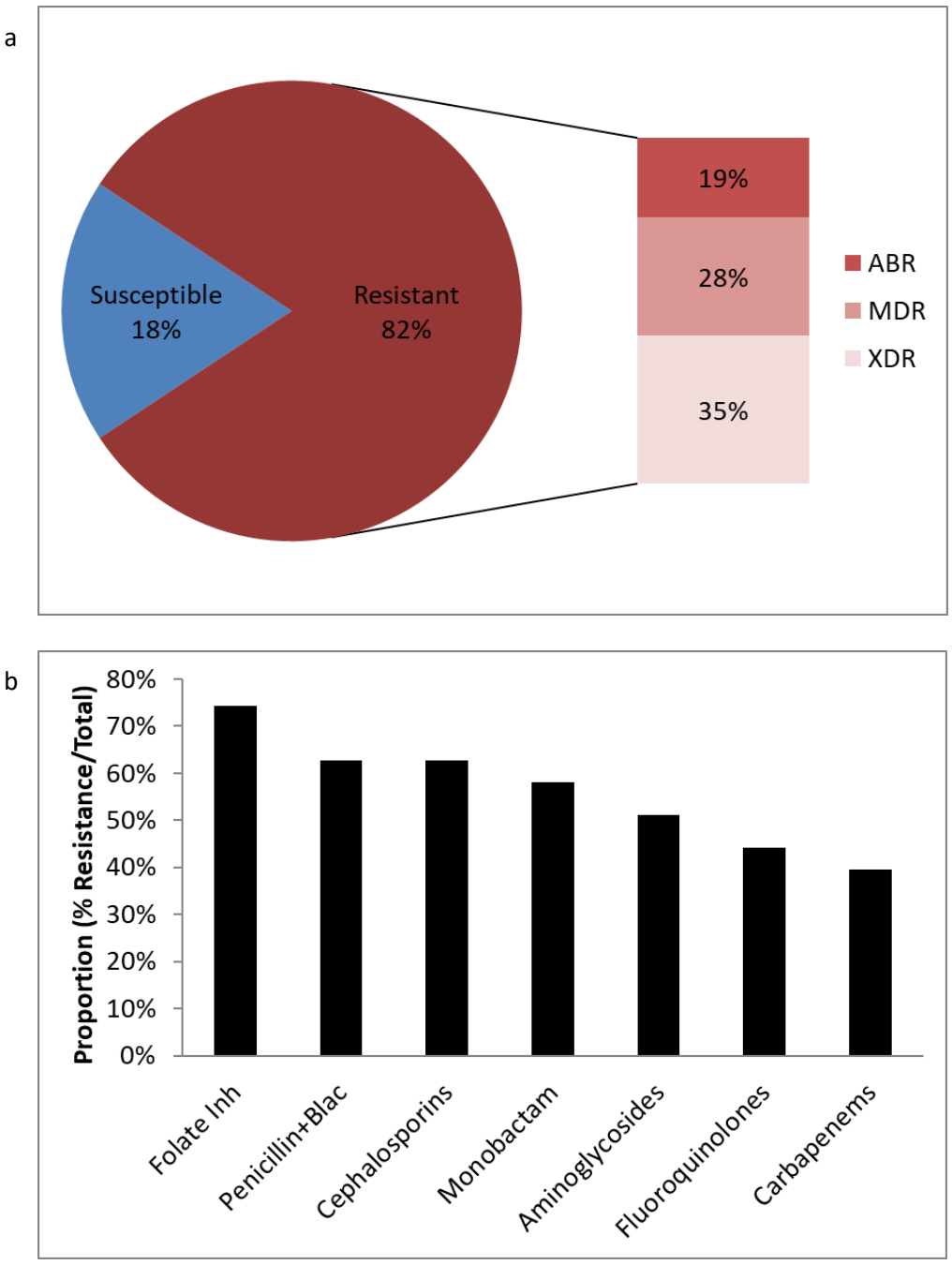


Figure 17 – Summary of antibiotic resistance phenotypes of *K. pneumoniae* isolates. a) Percent of strains that are antibiotic susceptible, ABR (dark red), MDR (pink) or XDR (light pink); b) Percent of strains with resistance to at least one therapeutic agent in indicated classes. (n = 43)

Over 70% of the strains used in this analysis were resistant to folate inhibitor (Figure 17b). In examining prevalence of resistance to antimicrobial classes among MDR strains, we again observed certain combinations of antimicrobial classes were more common (Figure 18a-d). Notably, two-thirds of the *K. pneumoniae* strains were resistant to the combination of cephalosporins + monobactam + folate inhibitor (n = 8/12, Figure 18a – d). Similarly, two-thirds of strains were resistant to the combination of cephalosporins + monobactam + penicillin/β-lactamase combination drugs (n = 8/12, Figure 18a – d). In this way, ABR, MDR and XDR phenotypes were prevalent in the *K. pneumoniae* population used for this analysis.

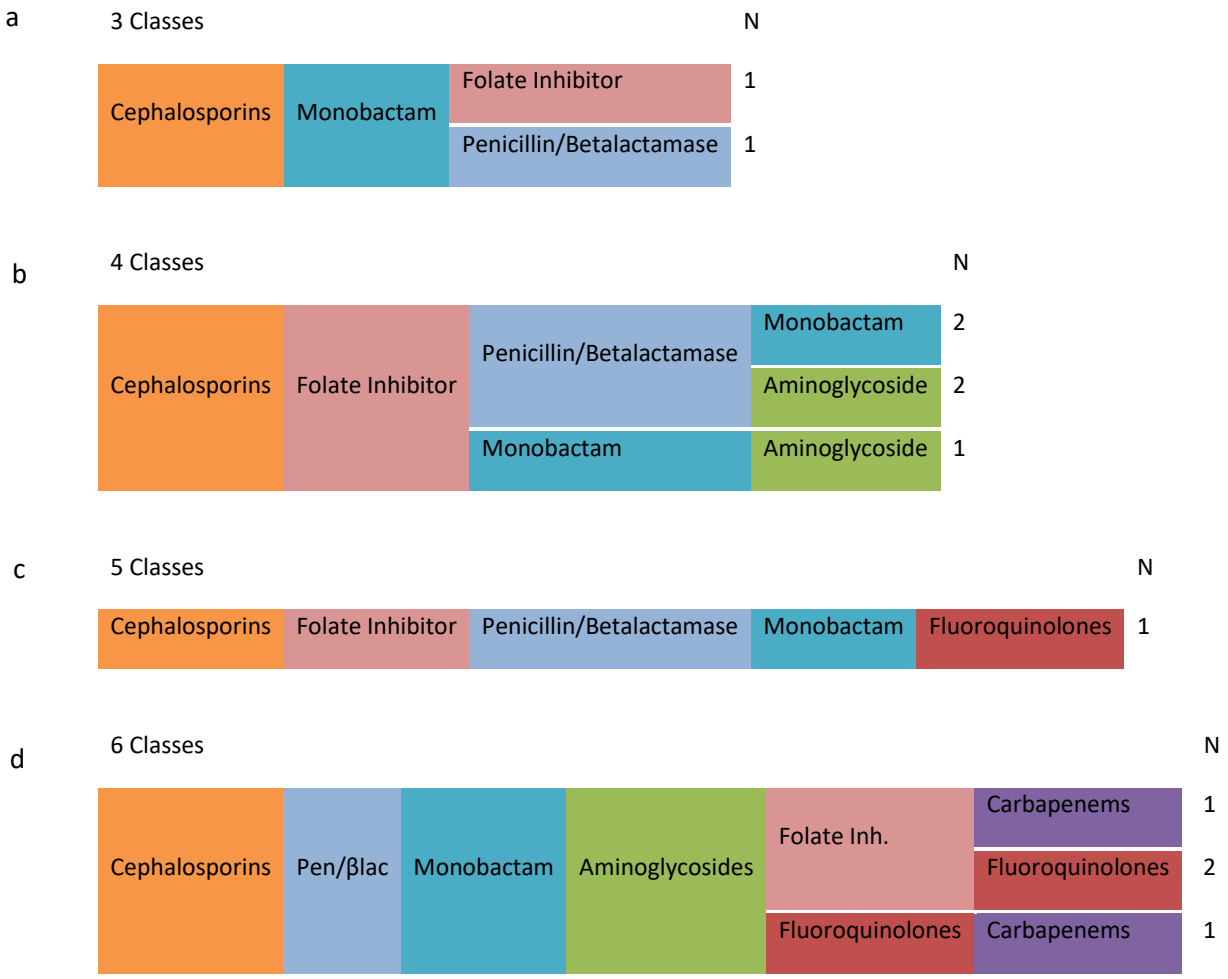


Figure 18 – Combinations of resistance to antimicrobial classes in MDR *K. pneumoniae*. a) Strains resistant to 3 antimicrobial classes, b) 4 antimicrobial classes, c) 5 antimicrobial classes, or d) 6 antimicrobial classes. Rows of each table represent indicated number of strains with the same pattern of antimicrobial resistance. Columns indicate antimicrobial class. Depth of blocks in each column represent antimicrobial classes shared between multiple rows.

Relative virulence of K. pneumoniae

The *K. pneumoniae* strains used in this analysis had been challenged in a mouse model of pneumonia as part of a separate study (unpublished data). We collected data from that study to create relative virulence scores for each strain. Specifically, strains were scored based on the bacterial dose that was required for greater than 50% but less than 100% pre-lethal illness in the mouse model of disease. Virulence scores ranged between 1 and 3, with 1 being the lowest virulent strains (requiring the highest bacterial dose to induce disease) and 3 being the most virulent strains (requiring the lowest bacterial dose to cause severe illness). Figure 19a depicts the distribution of strains across virulence score groups. Overall, the majority of strains received a low virulence score of 1 (n = 26, Figure 19a). It must be noted that these strains were not selected from serial infections like the *P. aeruginosa* studies analyzed above. These strains were selected as representative isolates of antimicrobial resistance phenotypes (carbapenem resistant strains, strains producing extended-spectrum β-lactamases [ESBLs], etc) without regard or pre-conceived notions of their virulence.

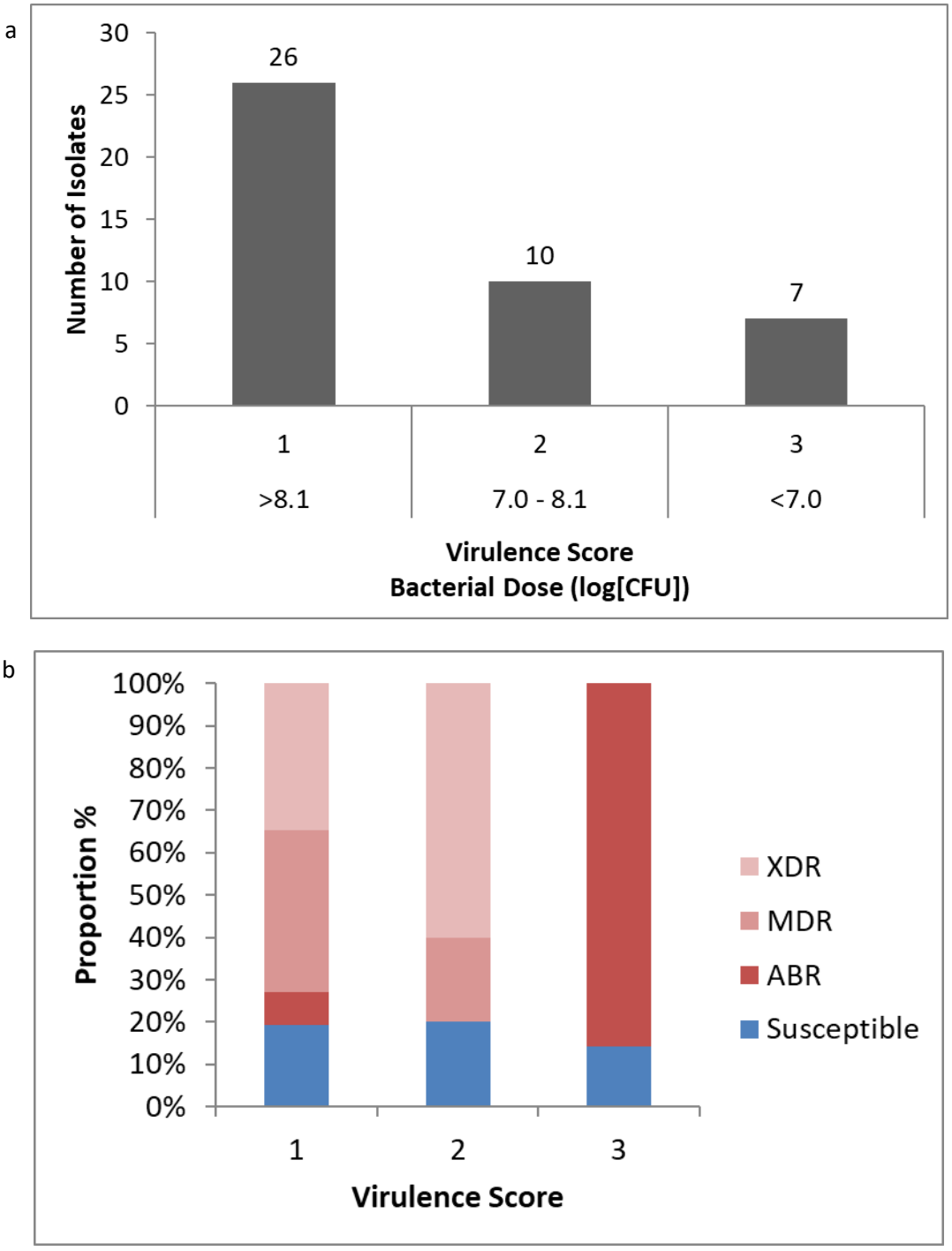


Figure 19 – Distribution of *K. pneumoniae* antimicrobial resistance phenotypes by strain virulence (n = 43). a) Histogram of strains by virulence score where 1 represents the least virulent group and 3 represents the most virulent group. b) Proportion of antibiotic susceptible (blue), ABR (dark red), MDR (pink) and XDR (light pink) strains stratified by virulence score.

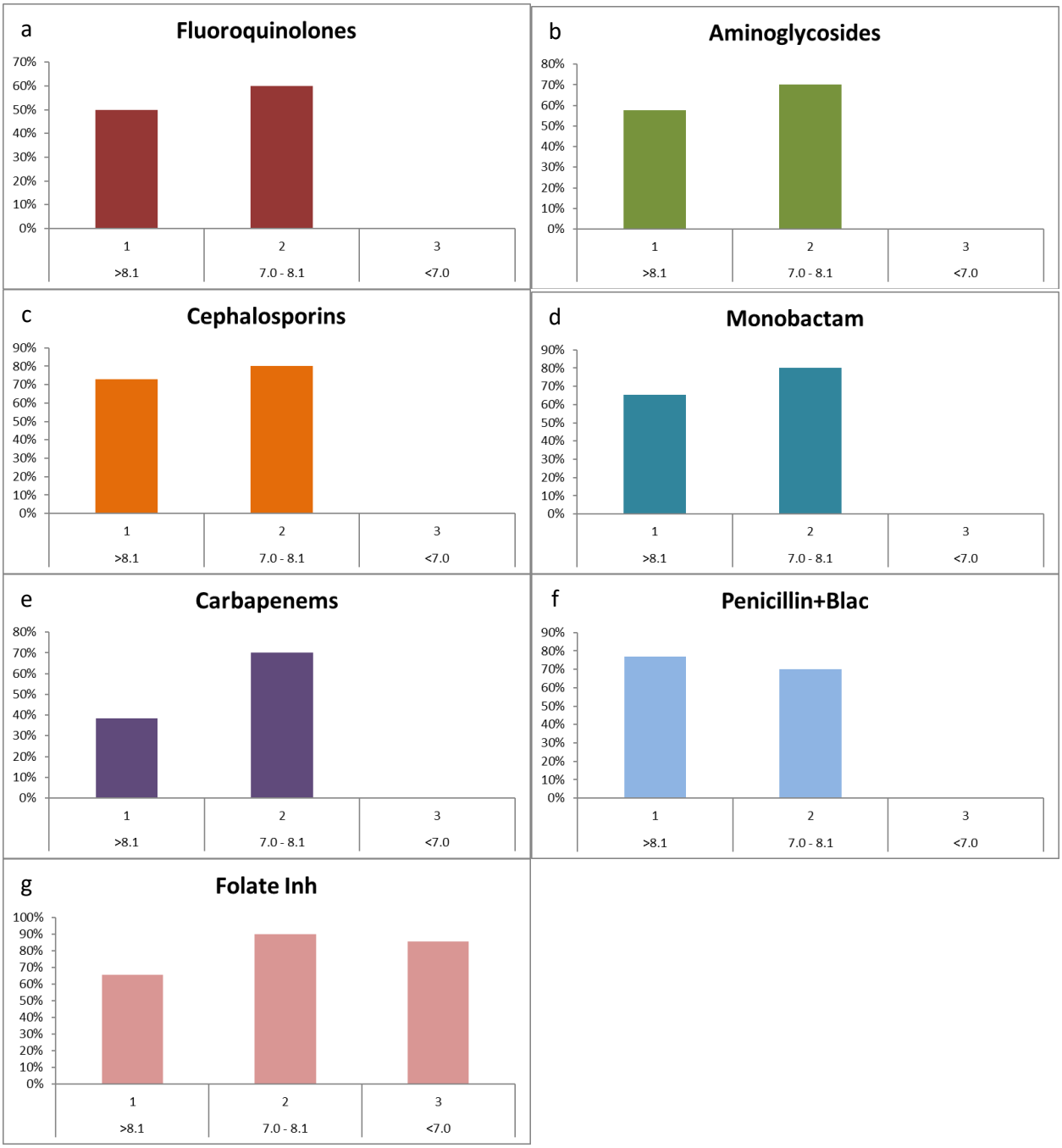


Figure 20 – Percent of *K. pneumoniae* strains resistant to at least on therapeutic agent in indicated antimicrobial class stratified by virulence rank. a) Fluoroquinolone resistance stratified by virulence rank. b) Aminoglycoside resistance stratified by virulence rank. c) Cephalosporin resistance stratified by virulence rank. d) Monobactam resistance stratified by virulence rank. e) Carbapenem resistance stratified by antibiotic resistance. f) Penicillin/tazobactam resistance stratified by virulence rank. g) Folate inhibitor resistance stratified by virulence rank.

Antibiotic resistance is inversely correlated with K. pneumoniae virulence

To assess the relationship between antimicrobial resistance and virulence of *K. pneumoniae*, we stratified strains by their resistance phenotype and virulence score. Antimicrobial susceptible strains were generally equally distributed among all virulence groups (Figure 19b). None of the most virulent strains exhibited MDR or XDR phenotypes (Figure 19b). We again used logistic regression modeling to calculate the odds ratio of a strain exhibiting a resistance phenotype based on increasing virulence score (Table 5, Figure 21). The logistic regression model including the MDR/XDR phenotypes had an odds ratio of 0.279 (0.11-0.73 95% CI, Table 5, Figure 21b). This indicates that increased virulence is associated with a decreased odds of a strain having an MDR or XDR phenotype. Although the logistic regression model of the XDR phenotype also had an odds ratio less than 1, this model was not statistically significant (Table 5, Figure 21c).

The above analysis relied on inclusive categorical grouping by antibiotic resistance phenotype (eg. ABR, MDR/XDR or XDR). We repeated the analysis by evaluating the number of antibiotic classes that each strain was resistant to versus its virulence score. These data are represented in Figure 21d where increasing weight is given to points that represent a greater number of strains. All of our most virulent *K. pneumoniae* strains were antimicrobial susceptible or resistant to only one antimicrobial class. Conversely, a number of strains receiving the lowest virulence score of 1 were resistant to 4 or more antimicrobial classes (Figure 21d). This trend was statistically significant by linear regression (β coefficient = -1.25, $p = 0.031$, Figure 21d). This suggests that there is a reduction of 1.25 in virulence score for each additional antibiotic class that a strain is resistant to. Taken together, our statistical models demonstrate that there is an inverse relationship between bacterial virulence and antibiotic

resistance of *K. pneumoniae* such that strains with increasing virulence have lower odds of exhibiting antimicrobial resistance than strains of lower virulence.

Table 5 – Logistic regression models of association between *K. pneumoniae* antibiotic resistance and virulence score. *Indicates statistically significant models

	Univariate	
	OR	95% CI
ABR	1.137	0.4-3.24
MDR/XDR	0.279	0.11-0.73*
XDR	0.634	0.26-1.56

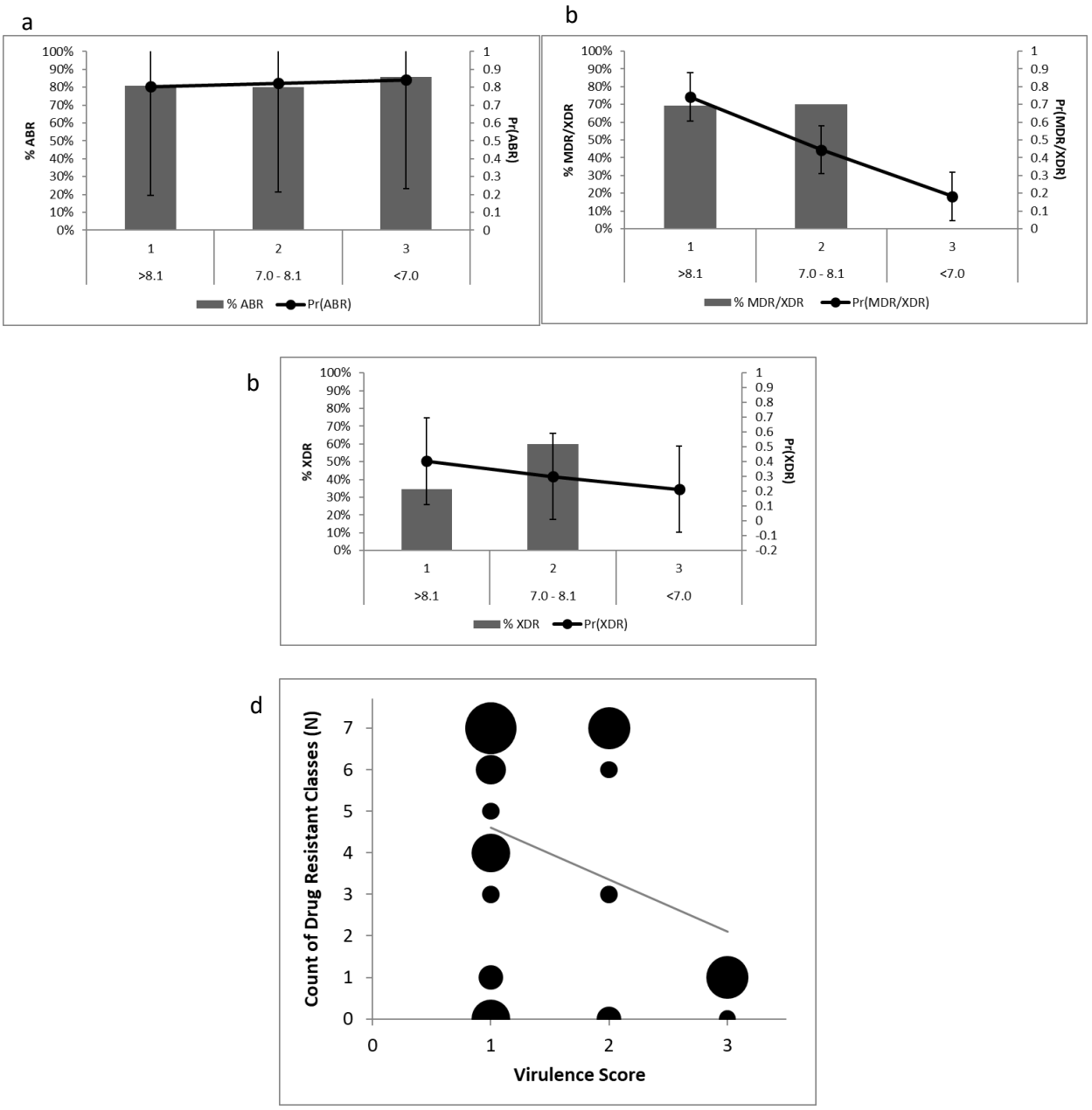


Figure 21 – Regression models of *K. pneumoniae* antibiotic resistance and virulence score. Percent of strains that are resistant to a) at least one antibiotic (ABR) in each virulence group, b) at least 3 classes of antibiotics (MDR/XDR, inclusive) in each virulence group, c) at least one antibiotic in all antimicrobial classes tested (XDR) in each virulence group. Bars represent percent of strains resistant to the specified minimum number of drug classes (left axis). Circles represent the calculated probability of antibiotic resistance from regression models by virulence score (right axis). d) Number of drug classes a strain is resistant to versus virulence score where each circle represents a strain and the area of each circle is weighted for the number of strains it represents. Line represents the linear regression model fit to the data represented.

DISCUSSION

In the current chapter, we analyzed the relationship between antimicrobial resistance and bacterial virulence of *K. pneumoniae* using statistical modeling. We found a significant inverse association between antibiotic resistance phenotypes and bacterial virulence. *K. pneumoniae* isolates of increased virulence had lower odds of exhibiting an MDR phenotype. Similarly, we analyzed the association between increasing number of antimicrobial classes to which a strain was resistant and bacterial virulence. Again, we found a statistically significant inverse correlation between increasing antimicrobial resistance and bacterial virulence. Importantly, these results are consistent with the published literature [118] and with our prior analysis of *P. aeruginosa* antimicrobial resistance and virulence. Collectively, our models suggest that the virulence cost of antimicrobial resistance may be more broadly applicable to other Gram-negative bacteria.

To our knowledge, this is the first study examining large populations of *P. aeruginosa* and *K. pneumoniae* clinical isolates and comprehensively challenging each strain in mouse models of disease. Previous studies screening a large number of antibiotic resistant strains used *C. elegans, Dictysotelium* or *Galleria* infection models [107, 112, 118]. When mouse models of infection were utilized, they were limited in scope and number to assessment of targeted antibiotic resistant mutants [100, 106]. In the current study, we utilized virulence data from previously conducted mouse models of infection to assign a virulence score. Mouse models of infection are arguably more informative as representations of clinical illness because the animals also have an intact immune response (which is lacking in lower models of infection) that the bacteria must overcome to cause illness. Each strain was challenged in mice at a minimum of 2 bacterial doses. In this way, we were able to robustly identify the bacterial dose required to induce severe illness for each strain.

The cephalosporin cefazolin was excluded from analysis in designating ABR, MDR and XDR phenotypes to the *K. pneumoniae* strains. All of the *K. pneumoniae* strains tested were resistant to cefazolin even though some strains were susceptible to all other antimicrobial classes tested. Cefazolin is listed as a clinically relevant antimicrobial agent for treatment of *K. pneumoniae* infections in the citation referenced here [91]. Notably, cefazolin has two breakpoints for resistance in urine and blood. We utilized the breakpoint for blood, which is lower than the breakpoint for urine, because it is more relevant to the current study [119]. Many of the isolates would be susceptible to cefazolin in the urine. Using the blood breakpoint, all of our *K. pneumoniae* isolates were non-susceptible to cefazolin. To our knowledge, this is the first report of intrinsic resistance to cefazolin for *K. pneumoniae*. The resistance of our strains to this agent may be a phenomenon specific to the facility where isolates were collected. Alternatively, this may illustrate emerging resistance patterns among *K. pneumoniae* such that cefazolin may no longer be clinically relevant for treatment of *K. pneumoniae* infections with the exception of urinary tract infections.

As noted above, the *K. pneumoniae* strains used in this study were skewed towards increased prevalence of antimicrobial resistance (82%) and a lower bacterial virulence score. This study was a post-hoc analysis of antimicrobial resistance and bacterial virulence using a pre-existing population of *K. pneumoniae* clinical isolates. These strains were selected for the purposes of another study without consideration or foreknowledge of their relative virulence. The skewness of the population may have limited our statistical power in finding statistical significance in the ABR and XDR logistic regression models. It is encouraging that we were able to find a statistically significant correlation between increasing antimicrobial resistance and lower bacterial virulence by linear regression despite the skewness of the population.

CHAPTER 5: **Conclusions and Future Directions**

The ability for a pathogen to cause disease is a balance of bacterial virulence in causing illness, the host immune defenses in combating infection and efficacy of antimicrobial agents in treating infection. *P. aeruginosa* is a nosocomial pathogen of concern due to its propensity for causing severe illness and due to emergence of antimicrobial resistance [2, 93, 109, 110]. In the present work, we evaluated factors contributing to *P. aeruginosa* pathogenesis including the relationship between the T3SS and activation of the inflammasome-mediated immune response in addition to the relationship between antimicrobial resistance and bacterial virulence.

We uncovered a novel role for the inflammasome adaptor protein ASC in promoting severe infections *in vivo.* Using a mouse model of acute pneumonia, we showed that mice lacking ASC had improved survival and that 80% of these mice had reduced numbers of bacteria in their lungs. These results demonstrate that ASC is detrimental to infection outcomes because bacterial clearance and host survival are improved in the absence of ASC. Importantly, these results did not correspond to those of mice lacking caspase-1/11, indicating that these inflammasome components impact the host to *P. aeruginosa* in different ways. To explore how ASC was impacting infection *in vivo*, we examined multiple aspects of inflammasome activation *in vitro,* including caspase-1-mediated cell death, cytokine release and the contributions of the non-canonical caspase-11 inflammasome pathway. We found that *P. aeruginosa* infection of BMDMs resulted in caspase-1-dependent cell death and release of IL-18 and IL-1β indicative of inflammasome activation. While cell death was partially reduced in the absence of ASC, cytokine release was nearly abrogated in the absence of ASC. These results are consistent with reports that *P. aeruginosa* activates the NLRC4 inflammasome, which requires ASC for caspase-1-

dependent proteolytic processing of IL-18 and IL-1β but which is capable of initiating pyroptotic cell death in the absence of ASC [8, 15, 53]. These findings, however, did not explain our *in vivo* results. This led us to the conclusion that *P. aeruginosa* engages an alternate ASC pathway that is detrimental to infection outcomes and is independent of the caspase-1 inflammasome.

In evaluating activity of other caspases, we found caspase-3/7 was activated during PSE9 infection in the caspase-1/11$^{-/-}$ BMDMs as well as in the context of caspase-1/11 in B6 BMDMs. However, caspase-3/7 activity was not detected in the absence of ASC during PSE9 infection of BMDMs. Notably, the caspase-3 inhibitors, antibodies and assays used in this study and others have cross reactivity with caspase-7 so we cannot exclude a contribution of caspas-7 to these results. We focus on the role of caspase-3 in *P. aeruginosa* infection based on the body of evidence in the published literature. Others have reported that ASC-dependent caspase-3 activity is also dependent on the adaptor protein NLRP3 upon stimulation with Shiga toxin [88]. NLRP3 is a receptor protein upstream of ASC in the inflammasome complex. If NLRP3 were required for ASC-dependent caspase-3 reported in the current study, we would predict that NLRP3$^{-/-}$ BMDMs would phenocopy ASC$^{-/-}$ BMDMs. While we observed decreased cell death in the absence of ASC, we did not observe a role for NLRP3 in cell death upon infection with PSE9 as BMDMs lacking NLRP3 continued to exhibit high levels of cell death. Our results further the understanding of the cross-talk between cell death pathways during *P. aeruginosa* infection and add to the expanding body of literature indicating that inflammasome responses extend beyond the simple canonical caspase-1 inflammasome to include multiple receptors [24-26], non-canonical caspase-11 [63, 116], and caspase-1 independent IL-1β release [47, 113].

Our results expand on recent findings that ASC is capable of interacting with caspases other than caspase-1. Having identified a role for caspase-3 in cell death during *P. aeruginosa* infection, we expected that an initiator caspase would also play a role caspase-3-dependent apoptosis to facilitate cleavage of caspase-3 to the active form. ASC has been implicated in the function of caspase-8, caspase-12 [87, 88]. We did not observe a role for either caspase-8 or caspase-12 activation during PSE9 infection of BMDMs *in vitro.* This was surprising as caspase-8 can function upstream of caspase-3 in cell death pathways [120]. We did not find evidence of casase-9 activation during PSE9 infection *in vitro* which also acts upstream of caspase-3 [52, 120]. It is possible that caspase-9 could function in the absence of cleavage similar to pro-caspase-1-dependent cell death [52, 53]. Further experiments are required to determine if unprocessed caspase-9 is functioning as the initiator caspase downstream of ASC and upstream of caspase-3. Thus, we have identified ASC-dependent activation of caspase-3 during *P. aeruginosa* infection but the initiator caspase remains unknown.

We reported 3 mechanisms by which *P. aeruginosa* may cause death of macrophages – 1) secretion of ExoS that leads to cell rounding [6, 10], 2) caspase-1-dependent pyroptosis and 3) caspase-3-dependent apoptosis. We found that activation of both caspase-1 and caspase-3 were dependent on ASC, although caspase-1-dependent cell death was independent of ASC. One may assume that bacterial activation of programmed cell death in macrophages would promote infection by attenuating propagation of the host immune response. Yet, cell death may play a protective role in immune defenses by restricting intracellular pathogens or phagocytosed bacteria as well as by releasing antimicrobial peptides and inflammatory cytokines into the extracellular milieu [16, 26, 30]. Indeed, the inflammasome may be required for immune clearance of *P. aeruginosa* in certain contexts [7, 8, 14, 15, 26, 43] despite an association with pyroptosis. Our findings, however, do not implicate caspase-1 in promoting immune

clearance of PSE9 or in exacerbating infection outcomes. Mice lacking casapase-1 were equally susceptible to infection as B6 mice *in vivo* despite a reduction in cell death *in vitro*. Although cell death by pyroptosis is one of the outcomes of inflammasome activation, our findings suggest that infection outcomes *in vivo* are not related to caspase-1-dependent pyroptosis of macrophages. Conversely, mice lacking ASC exhibited improved survival relative to caspase-$1^{-/-}$ mice and B6 mice *in vivo* despite an intermediate cell death phenotype *in vitro.* Activation of an ASC-dependent caspase-3 pathway may provide an explanation for the partial loss of cell death in $ASC^{-/-}$ BMDMs and may represent an alternate pathway by which ASC may contribute to severe infection outcomes *in vivo*.

Several questions remain as to how ASC-dependent caspase-3 activation is contributing to infection outcomes *in vivo*. Others have shown that ExoS activates caspase-3 and results in cell death by apoptosis [51, 114]. Our results demonstrated that the T3SS, rather than the effector protein ExoS, was sufficient to cause ASC-dependent activation of caspase-3. ASC assembles with the inflammasome complex upon recognition of a DAMP by a NAIP and an NLR in the cytosol of mammalian cells. PscF and PscI proteins of the T3SS apparatus are ligands for the inflammasome activation cascade. It is unknown if these proteins are also ligands for activation of alternate ASC pathways or if other bacterial stimuli signal activation of the ASC-dependent caspase-3 pathway. Additionally, it is unknown which eukaryotic receptors may be acting upstream of ASC that lead to the caspase-3 activation pathway in parallel to caspase-1 activation. While others have shown NLRP3 interacts with ASC to activate caspase-3, we did not find a role for NLRP3 in the context of PSE9 pneumonia [88]. Thus, although we have identified a novel role for ASC in activating caspase-3 during PSE9 infection, significant work remains to understand how this pathway is activated, how the signal is transduced and what implications the role of ASC-

mediated caspase-3 activation may have for assembly or function of the caspase-1 inflammasome complex.

Another area for future exploration is how ASC-dependent mechanisms of programmed cell death may be involved in phagocytosis and bacterial clearance. We observed reduced bacterial burden in the lungs of $ASC^{-/-}$ mice by 30 hpi. This suggests that an ASC-dependent pathway may interfere with the cellular processes of phagocytosis and bacterial clearance. Our results indicate ASC is required for caspase-3 activity and programmed cell death by apoptosis. Cell rounding and formation of membrane blebs are morphologic changes that the cell undergoes during apoptosis [52]. By initiating a process of cell rounding, caspase-3 may inhibit phagocytic uptake of bacteria during infection. ExoS is known to inhibit phagocytosis and cause cell rounding [114]. However, ExoS is not required for the interaction between ASC and caspase-3 during PSE9 infection. Thus, activation of caspase-3 through ASC may represent an alternate strategy for the bacteria to promote severe infection by initiating a mechanism of programmed cell death that causes cell rounding and reduces phagocytosis to promote bacterial persistence. Further studies are required in this area.

Upon infection with *P. aeruginosa*, BMDMs activate parallel cell death pathways through caspase-1 and caspase-3. We were able to demonstrate that both pathways are activated upon PSE9 infection using DEVD to inhibit caspase-3 and YVAD to inhibit caspase-1. Thus, both caspase-3 and caspase-1 are sufficient to cause cell death. However, only an ASC-dependent pathway is necessary to promote severe infection outcomes *in vivo.* The hypothesis above proposes that caspase-3 cell death has specific implications on the outcome of infection independent of caspase-1 by inhibiting bacterial clearance by phagocytosis. Although we know that both pathways are active by the evidence presented here, we

don't know if both pathways are active within a single cell. It is equally possible that while one cell activates caspase-1 pyroptosis, the neighboring cell activates caspase-3 apoptosis. The net effect would be consistent with conclusions drawn in the current study. Single cell microscopy would be required to determine if caspase-1 and caspase-3 are activated within a single cell or in neighboring cells of a population of BMDMs. Indeed, if caspase-3 is active in a neighboring cell, it may also dampen the immune response promoted by inflammasome-dependent cytokine release if caspase-1 is not also active in the same cell. That outcome would be masked in the current experiments as all of the experiments that eliminated ASC-dependent caspase-3 activation also eliminated caspase-1 dependent cytokines. But, the possibility that directing ASC towards a caspase-3 pathway may dampen caspase-1 cytokines cannot be excluded. Others reported a similar effect of caspase-3 where caspase-1-dependent IL-1β was reduced when caspase-3 was active [51]. However, the authors of that study reported caspase-3 activity was dependent on ExoS. We did not find a role for ExoS in caspase-3 in the current experiments. Thus, more work is required to elucidate the implications of caspase-3 and caspase-1-dependent pathways and how activation of each influences or alters infection outcomes *in vivo*.

Recent findings on the role of gasdermin D in pyroptosis also provide an intriguing avenue for future studies. Caspase-1 cleaves gasdermin D in the eukaryotic cell membrane, resulting in gasdermin D pore formation and cleavage of membrane lipids [55]. It has also been proposed that caspase-3 may activate gasdermin D during late stages of apoptosis if apoptotic bodies are not taken up by neighboring cells [55]. This would suggest temporal regulation of gasdermin D during pyroptosis (rapid activation) and apoptosis (late-stage activation). Our findings also demonstrate that caspase-3-dependent cell death occurs later in the absence of caspase-1 *in vitro* during PSE9 infection of caspase-1/11$^{-/-}$ BMDMs. One

may hypothesize that ASC-dependent activation of caspase-3 could delay activation of gasdermin D thereby reducing the number of membrane pores, limiting cytokine release and affecting cell death. All of these outcomes are consistent with our combined *in vivo* and *in vitro* results. If this hypothesis proves true, it may provide an explanation for how activation of parallel cell death pathways alters infection outcomes *in vivo* by modulating the role of gasdermin D in the inflammasome-mediated immune response.

In the current study, we used a highly virulent clinical isolate, PSE9, to evaluate the relationship between *P. aeruginosa* and the host inflammasome response during acute pneumonia. In this regard, PSE9 is a relevant strain, as it was cultured from a patient with ventilator-associated pneumonia. In addition, PSE9 was noted to be the most virulent of 35 ventilator-associated pneumonia isolates tested in a mouse model [115], which suggests that it has multiple mechanisms to enhance disease progression. Although clinical isolates of *P. aeruginosa* have been reported to activate the inflammasome and induce IL-1β [121], most prior studies of inflammasome activation used lab-adapted strains of *P. aeruginosa* [8, 20, 24-26, 53]. Therefore, further studies are necessary to determine whether our findings apply to lab-adapted or less virulent strains of *P. aeruginosa*. In this regard, it is reassuring that PSE9 behaved similarly in *in vitro* cell death and cytokine assays to lab-adapted strains reported in the literature.

In summary, *P. aeruginosa* T3SS affects disease pathogenesis in multiple ways: 1) by direct toxicity and manipulation of cellular components by the effector proteins ExoU, ExoS, ExoT and ExoY [6], 2) by enhancing the host immune response through the caspase-1-dependent inflammasome [7, 17-20], and 3) by inducing caspase-3-dependent cell death (Figure ##). The last form of cell death is activated either by ExoS [51, 114] or a T3SS-dependent but ExoS-independent process. Understanding these interactions

may have clinical relevance. Indeed, a recent report demonstrated that treatment of *P. aeruginosa* infection with macrolides ameliorated infection by reducing the inflammasome response [21]. Additional efforts have been directed at developing inhibitors of the *P. aeruginosa* T3SS [122-125]. Further understanding of the pathways activated by *P. aeruginosa* T3SS may aid the development and optimal use of these therapies.

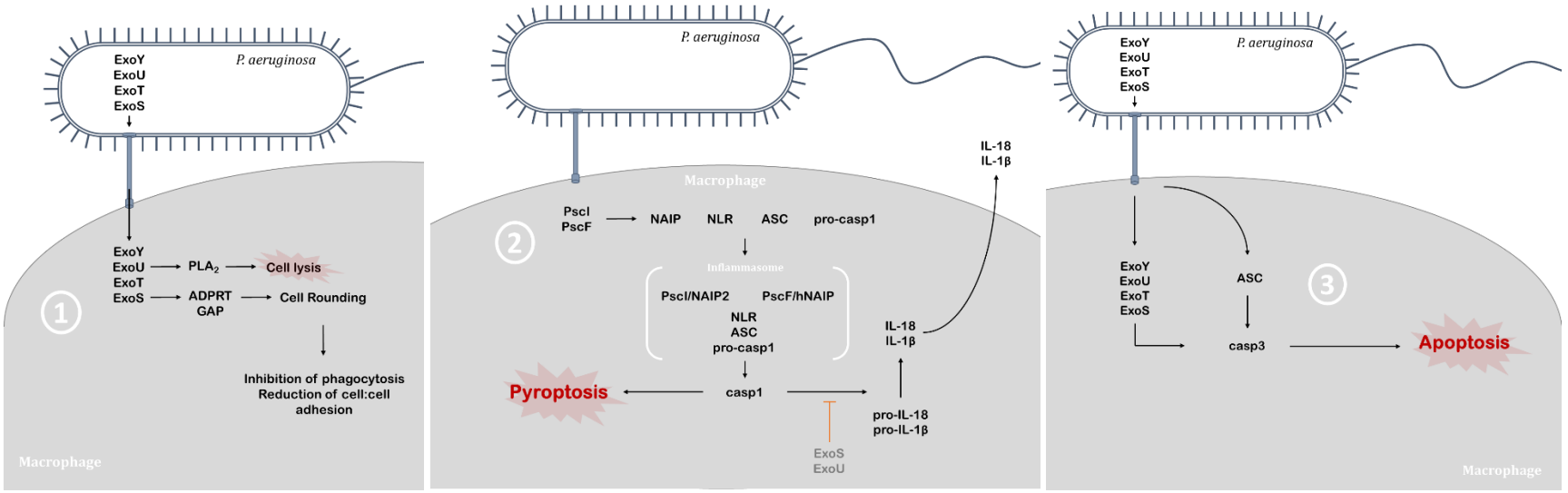


Figure 22 – Model of T3SS roles in virulence and cell death. 1) T3SS injects virulence factors directly into the target cell. 2) Needle apparatus activates the host immune response and cell death by caspase-1-dependent pyroptosis. 3) Capase-3-dependent apoptosis is activated by ExoS or through the T3SS via ASC.

We next examined the relationship between antimicrobial resistance and bacterial virulence of *P. aeruginosa*. Prevalence of *P. aeruginosa* antimicrobial resistance has been increasing rapidly in recent years [2] and infections due to MDR-*P. aeruginosa* strains are reported to have more severe infection outcomes [2, 93, 94]. There have been limited studies examining differences in bacterial fitness and bacterial virulence related of antibiotic resistant *Pseudomonas*. Nearly all of the studies that have been conducted utilize lab-adapted strains of *P. aeruginosa* that have been genetically manipulated by allelic exchange, transposon insertion or spontaneous mutation under antibiotic stress to compare antibiotic resistant strains to antibiotic susceptible parental strains [98, 100, 101, 106, 107, 111, 112]. Differences between antimicrobial mechanisms examined, strains utilized and experimental methods may have contributed to confounding and divergent conclusions of these studies, particularly where MexAB-OprM [107, 112] and *oprD* mutations are concerned [100, 106]. In the current study, we utilized a library of clinical isolates that acquired or developed antimicrobial resistance without designed genetic alterations. The benefit of this approach allowed us to examine overall bacterial virulence in the context of diverse antimicrobial resistance pathways. In this way, we were able to describe the relationship between antimicrobial resistance and bacterial virulence. Future studies are required to identify specific target mechanisms that may explain the associations we describe herein.

Another aspect of previous studies examining relationships between *P. aeruginosa* virulence and antimicrobial resistance is the use of lower infection models in *C. elegans* or *G. mellonella.* These studies infected multiple strains of bacteria into one host and evaluated relative numbers of bacteria after infection to describe persistence defects of antibiotic resistant strains. The authors utilized the hosts as *in vivo* competition assays. It must be noted that our general conclusions regarding the relationship between bacterial virulence and antimicrobial resistance are consistent with the findings of

preliminary studies in *G. mellonella* [118]. This suggests that lower infection models may be options for inexpensive preliminary analysis or screening of bacterial strains in relation to other strains by coinfection. However, using a mouse model of virulence allowed us to examine bacterial virulence in the context of a clinically relevant infection model and the host immune response. Thus, the collective studies examined bacterial virulence using different measures of stress during infection (bacterial competition versus overcoming the host immune response). The progression from simpler infection models such as *C. elegans* and *G. mellonella* to complex infection models such as mouse models of infection is a necessary step towards drawing conclusions and hypotheses for impacting human illness.

We utilized a pre-existing library of 89 *P. aeruginosa* clinical isolates to evaluate the relationship between antimicrobial resistance and bacterial virulence. These strains were collected and evaluated in a mouse model of disease for other studies. Using logistic regression, we identified an inverse association between antimicrobial resistance and increasing bacterial virulence. *P. aeruginosa* strains with higher virulence scores had lower odds of ABR and MDR phenotypes than strains receiving lower virulence scores. Furthermore, linear regression analysis of the number of antimicrobial classes to which a strain was resistant versus the bacterial virulence score revealed an inverse correlation between the variables. The statistical models predicted a virulence cost of 0.71 virulence points for each additional antimicrobial class a strain becomes resistant to. This relationship was highly statistically significant.

The inverse relationship between antimicrobial resistance and bacterial virulence suggests that strains of increasing antimicrobial resistance are less capable of establishing severe illness. It is noteworthy that all of the strains used in this analysis caused illness in humans [109]. But, in the absence of

antimicrobial stress in a mouse model of disease, antimicrobial resistant strains required larger bacterial doses to establish illness than antimicrobial susceptible strains (data not shown). In retrospect, this correlation was surprising as antimicrobial resistant strains were found to have an increased odds of secreting ExoU compared to antimicrobial susceptible strains. ExoU is a robust virulence factor that leads to rapid lysis of target cells and is a marker for highly virulent strains of *P. aeruginosa* [6, 12, 115]. Thus, how can it be reconciled that ExoU is associated with virulence and with antimicrobial resistance but antimicrobial resistance is inversely correlated with virulence? In fact, ExoU was not associated with virulence in the current study, although functional T3SS was associated with virulence (data not shown). This was surprising because others have reported ExoU is associated with worse infection outcomes [110, 115]. One possible explanation for this relationship could be if the magnitude of ExoU secretion is altered by acquisition or evolution of antimicrobial resistance mechanisms. In this way, antimicrobial resistant strains may continue to secrete ExoU but to a lesser extent than strains with less extensive antimicrobial resistance phenotypes. Alternatively, ExoU may be genetically linked to a specific antimicrobial resistance pathway, thus establishing an inherent association with antibiotic resistance phenotypes. It is clear that additional work is required in this area to clarify how virulence is modulated in the context of antimicrobial resistance.

In addition to *P. aeruginosa*, we utilized a pre-existing library of *K. pneumoniae* to evaluate antimicrobial resistance and bacterial virulence. This allowed us to determine if our conclusions regarding the virulence cost of antimicrobial resistance in *P. aeruginosa* were generally applicable to other Gram-negative bacteria. Unlike the *P. aeruginosa* isolates, which had a normal distribution of virulence scores and a higher proportion of antimicrobial susceptible strains, the *K. pneumoniae* population was skewed towards lower virulence scores and had a higher proportion of XDR strains. The limited number of

strains in the *K. pneumoniae* population and the skewness of the virulence and resistance phenotypes created limitations to the statistically significant relationships that we could find. However, we were able to identify an inverse correlation between the number of antimicrobial classes to which a strain is resistant and increased bacterial virulence. In this way, the linear regression model of *K. pneumoniae* predicted a virulence cost of increasing antimicrobial resistance similar to our conclusions from the *P. aeruginosa* analyses. Thus, the virulence cost of antimicrobial resistance is not a phenomenon relevant only to *P. aeruginosa* but may be more broadly applicable to other Gram-negative bacterial species.

We began this work by placing bacterial infections in the context of 3 general factors: bacterial virulence, the host immune response and therapeutic antimicrobial agents. The balance of these factors determines infection outcomes that promote disease or resolution of infection. In the current work, we have provided evidence that *P. aeruginosa* expresses the T3SS to establish severe infection in a mouse model of pneumonia. The host inflammasome-mediated immune response is activated in response to infection. Rather than promoting bacterial clearance and host survival, the inflammasome adaptor protein contributes to severe infection outcomes in mice. We also provide evidence that ASC is required for a caspase-3-dependent mechanism of programmed cell death. With regards to therapeutic antimicrobial agents, *P. aeruginosa* utilizes multiple mechanisms to reduce efficacy of antibiotics. Yet, increasing antimicrobial resistance was correlated with a virulence cost. Collectively, we have described how the *P. aeruginosa* T3SS enhances bacterial virulence, how expression of the T3SS exacerbates the host immune defense and how the bacterial virulence is reduced in favor of antimicrobial resistance in clinical isolates. The diversity of strategies that *P. aeruginosa* employs to promote illness illustrates how this bacterium has become a successful nosocomial pathogen. Future studies of these processes will contribute to development and efficacious use of novel therapeutic strategies.

CHAPTER 6: **Materials and Methods**

Mice

Mice for these experiments included 6 to 10-week-old C57BL/6NJ (Jackson Laboratories, Bar Harbor, ME), Casp1/11$^{-/-}$ (Jackson Laboratories), IL-18$^{-/-}$ (Jackson Laboratories), NLRP3$^{-/-}$ (Jackson Laboratories) and Caspase11$^{-/-}$ (Jackson Laboratories) and ASC$^{-/-}$ (gift from Genentech, South San Francisco). Control mice were age and gender matched. All experiments were conducted under supervision and with approval of the Northwestern University Animal Care and Use Committee.

Bacterial Strains

Summary of strains used in this work may be found in Table 3. *P. aeruginosa* strains were plated on Vogel-Bonner medium (VBM) agar and incubated at 37°C overnight before inoculation into Luria-Bertani (LB) liquid media or MINS media. The gene encoding *pscF* was deleted from strain PSE9 by allelic exchange according to published methods [126]. Briefly, an in-frame deletion was created by cloning 100 base pairs of the 5' and 3' gene regions with overlapping complementary regions using PCRSoeing (refer to Table 4 for primer sequences). After isolating the cloned gene fragment by gel electrophoresis, the fragment was digested with restriction enzymes HindIII (New England Biolabs, Ipswich, MA) and EcorV (New England Biolabs) and ligated into a similarly digested pEX18Gm vector containing a gentamicin resistance gene and the *sacB* gene with T4 DNA ligase (New England Biolabs). The resultant pEX18:Δ*pscF* plasmid was transformed into competent S17.1+ *E. coli* and mated for approximately 12 hours with PSE9 on LB agar plates. LB agar plates containing 50 µg/mL gentamicin were used to select PSE9 strains that had integrated the deletion vector. The vector backbone was resolved by selection on LB containing 6% sucrose. PSE9 strains that retained the Δ*pscF* gene truncation were verified by sequencing the gene region and by whole-genome sequencing.

Mouse infections

Bacterial cultures were grown at 37°C with shaking overnight in MINS media. A sample of 350 µL was then subcultured into fresh 5 mL medium and allowed to grow for an additional 2-4 hours until appropriate bacterial density was reached during exponential growth phase. Bacteria were re-suspended in sterile PBS to determine bacterial numbers by optical density, aiming for approximately 1.2×10^7 CFU per mL. Prior to infection, mice were anesthetized by i.p. injection of 100 mg/mL ketamine and 20 mg/mL xylazine cocktail. Approximately 3.5×10^5 (or 5.5 logs) CFU in 30 µL volume were administered to the nares of anesthetized mice. Bacterial numbers were verified by counting serial dilutions of the inoculum on LB plates. For survival experiments during acute pneumonia, mice were monitored for illness over a period of 96 hours. Mice that demonstrated severe illness in accordance with approved animal protocols were humanely euthanized and scored as mortalities. For bacterial persistence assays, mice were humanely euthanized at the indicated times and lungs were removed and homogenized in 5 mL sterile PBS. Serial dilutions of lung homogenates were plated on LB agar plates. CFU were counted to quantify bacteria present in the organs.

Cell Culture

Macrophages were differentiated from bone marrow as previously described [8]. Briefly, bone marrow was isolated from the femur of mature mice (>6 weeks old) and cultured in petri dishes with RPMI medium supplemented with 30% L cell supernatants and 20% fetal bovine serum (FBS). Cells were incubated at 37°C and 5% CO_2 with humidity. Fresh growth media was added after 3 days. After 7 days of growth, non-adherent cells were washed away with spent medium. Adherent cells were scraped from the culture dish and re-suspended in fresh RMPI medium supplemented with 10% L cell

supernatants and 10% FBS. Cells were counted and seeded at a density of 1 x 10^6 cells for 100 mm plates, 5 x 10^5 cells per well for 6-well plates or 2.5 x 10^5 cells per well for 12-well plates. Plates were incubated for an additional 72 hours until BMDMs were adherent to the bottom of the wells. Cell differentiation was verified by visual inspection of cell morphology under an upright microscope.

Cell culture infections

Bacterial cultures for cell infections were prepared with the same method described for animal experiments. Within 30 minutes to 1 hour prior to infection, cell medium was removed and replaced with serum-free RPMI medium. Cells were infected with appropriate volumes to result in MOIs of 25 and mixed thoroughly by gentle pipetting. Bacterial numbers were verified by counting serial dilutions of the inoculation volume. Samples of infected culture supernatants were collected at the indicated times for cytotoxicity assays or cytokine quantitation. Cytotoxicity assays were conducted in 12-well plates and quantified by measuring LDH release using Cytotox-96 kit (Promega, Fitchburg, WI) according to the manufacturer's instructions. Cytotoxicity was normalized to 100% cell lysis by treatment with 10% Triton-X100. Cytokine release assays were conducted in 96-well plates and quantified by IL-18 ELISA (MBL International, Woburn, MA) or IL-1β ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Where indicated, cells were pre-incubated for 1 hour prior to bacterial inoculation with YVAD (Cayman Chem, Ann Arbor, MI), DEVD (Cayman Chem), IETD (R&D Systems) or ATAD (R&D Systems) in DMSO at a final concentration of 100 μM in serum-free RPMI medium. Bioluminescent reporter assays were conducted in 12-well plates using Caspase-Glo 1 and Caspase-Glo 3 Inflammasome Assay kits (Promega) according to the manufacturer's instructions.

Immunoblot

Active and inactive caspases in BMDMs were detected by immunoblot. Briefly, cells were plated in full-sized plates (for caspase-1) or 6-well plates (all other immunoblots) and infected for the indicated time at an MOI of 25. The full volume of culture supernatant was collected and proteins were precipitated by 10% trichloroacetic acid (TCA) at 4°C overnight with mixing. Precipitated proteins were re-suspended in 1.5 mL 10 mM NaCl. Cells were rinsed with cold PBS and re-suspended in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.2% Triton X100, 0.3% NP40, 25 mM NaF, 0.1Mm Na orthovanadate, 0.25% Na deoxycholate) with a dissolved cOmplete protease inhibitor tablet (Sigma-Aldrich, St. Louis, MO). Cells were then lysed by passage through a needle and lysate was placed on ice. Cell debris was pelleted. Forty milliliters of either cell lysate or culture supernatant was mixed with 6x protein loading dye, boiled for 2 min and loaded onto a 15% SDS polyacrylamide gel. After electrophoresis, samples were transferred to a nitrocellulose membrane for detection of protein bands. Blots were blocked with either 10% milk buffer or bovine serum albumin (BSA), according to the antibody supplier recommendation. Primary antibodies used included: Rabbit anti-caspase-1 (5 µg, EMD Millipore, Darmstadt, Germany), rabbit anti-βactin (1:1,000 dilution, Abcam, Cambridge, MA), rabbit anti-caspase-3 (1:500 dilution, Cell Signaling Technologies, Danvers, MA), rabbit anti-caspase-8 (1:500 dilution, Cell Signaling), rabbit anti-caspase-9 (1:500 dilution, Cell Signaling) and rabbit anti-caspase-12 (1:500 dilution, Cell Signaling). Goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (1:5,000 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) was used for secondary detection followed by incubation with Super Signal West Dura (Thermo/Fisher, Waltham, MA) for development.

P. aeruginosa Type III *Secretion Status*

Secretion was observed by immunoblot according to previously described methods [115]. Briefly, bacterial cultures were grown in MINS medium that is known to induce T3SS *in vitro*. Proteins secreted into the culture supernatants were concentrated by ammonium sulfate, separated by denaturing gel electrophoresis and transferred to nitrocellulose membranes. Proteins were detected by immunoblot using antisera with secondary detection with goat anti-rabbit IgG conjugated to HRP as described above. The proteins of the T3S apparatus, PopB and PopD, as well as the effectors ExoU or ExoS were detected with antisera mixture. Strains were called secretion positive (T3SS+) if the PopB/D proteins were detected in addition to one or more of the toxin proteins.

Antibiotic Resistance Study Population

A retrospective cohort study was conducted to evaluate relationships between bacterial virulence and antibiotic resistance. Archived specimens and clinical data from previous studies were used [109]. Strains were originally studied in mouse models of disease to identify novel genetic elements that contribute to virulence (unpublished data). These specimens came from adult patients at Northwestern Memorial Hospital. This study was conducted with oversight and approval from the Institutional Review Board at Northwestern University.

P. aeruginosa – A total of 108 non-clonal, serial isolates from patients diagnosed *P. aeruginosa* bacteremia were collected between 1999 and 2003. Nineteen isolates were excluded because the patients were under 16 years of age (5), previously enrolled in the study (3), or the medical chart was unavailable for review (1). Eight more patients were excluded because their infecting strains were identified as bacteria other than *P. aeruginosa* by whole genome sequencing or due to other

contamination. Additionally, 2 cystic fibrosis patients were excluded because *P. aeruginosa* chronically infects these patients and the strains are phenotypically different from acutely infecting strains [72]. The final study population included 89 *P. aeruginosa* strains.

K. pneumoniae – A total of 37 non-clonal, non-sequential isolates from patients infected or colonized with *K. pneumoniae* were collected from Northwestern Medicine. An additional 6 strains collected from the following locations were included in this study as well as the original study: NTUH-K2044 from Taipei, Taiwan, hvKP1-3 from Buffalo, NY, kvKP4 from Minneapolis, MN, KPPR1 (ATCC 43816). The final study population included 43 *K. pneumoniae* strains.

Antibiotic Susceptibility Data

Minimum inhibitory concentration (MICs) for relevant antibiotics were determined at the time of collection by the Clinical Microbiology laboratory at NMH. The vast majority of MICs were determined by the Vitek2 with the exception of *P. aeruginosa* MICs for aztreonam and *K. pneumoniae* MICs for ertapenem which were determined by Etest according to the manufacturers specifications (BioMerieux). Additionally, certain *K. pneumoniae* strains were tested for resistance to piperacillin/tazobactam by the Kirby Bauer method, as was standard practice at the time of collection. At least one antibiotic in each relevant drug class was tested for every strain. Where information was missing or incomplete, samples were retested by the appropriate method.

Antibiotic Resistance Definitions

Antibiotic resistance was coded as susceptible or non-susceptible (intermediate and resistant) according to 2014 CLSI breakpoints [119]. *K. pneumoniae* resistance to cefazolin has to two breakpoints for urine

and blood. In this case, the breakpoint for blood was utilized because it is more relevant to the infection models used in the current study. For discussion purposes, the term "resistant" is used to mean non-susceptibility to an antibiotic. Strains were characterized as antibiotic resistant (ABR) if they were non-susceptible to at least 1 antibiotic typically used to treat infections caused by the species. Definitions for multi-drug resistant (MDR) and extensively-drug resistant (XDR) were previously described by Magiorakos, *et al*. [91]. Briefly, strains were characterized as MDR if they were non-susceptible to at least 1 antibiotic in 3 different drug classes. Strains were characterized as XDR if they were non-susceptible to at least 1 antibiotic in every drug class tested for each organism. Pan-drug resistance could not be assessed because an insufficient number of drug classes were tested for each organism according to the cited definitions [91]. Furthermore, cefazolin and ampicillin were excluded from the analysis of *K. pneumoniae* because 100% of the strains were resistant to these drugs, suggesting that these drugs are not clinically relevant for this organism.

Virulence Ranking

Bacterial virulence was characterized for other studies by Egon Ozer, Abbey Goodyear, Jon Allen and Andrew Morris of the Hauser Lab. Briefly, *P. aeruginosa* virulence was assessed in a mouse model of bacteremia by tail vein injection. *K. pneumoniae* virulence was assessed in a mouse model of pneumonia by intranasal delivery. The virulence rank of a particular isolate was defined as the dose of bacteria required to cause >50% but less than 100% pre-lethal illness. Pre-lethal illness is severe disease that is indicative of imminent death. Mice that exhibited signs of severe illness according to approved protocols were humanely euthanized and scored as mortalities. Strains were then grouped by virulence according to biologically relevant cutoffs for each organism and the number of strains in the populations. *P. aeruginosa* scoress were assigned based on the following bacterial doses (ranges are

noted as the log CFU of bacterial burden): (most virulent) Rank 5 = less than 6.5, Rank 4 = 6.5 – 6.9, Rank 3 = 7.0 – 7.4, Rank 2 = 7.5 – 7.9, Rank 1 = 8.0 and greater (least virulent). *K. pneumonia* scores were assigned based on the following bacterial doses: (most virulent) Rank 3 = less than 7.0, Rank 2 = 7.0-8.1, Rank 1 = greater than 8.1 (least virulent).

Statistical Analysis

Statistical significance of differences in mouse survival were determined by Kaplan-Meier log-rank test for equality. Bacterial burdens in the lungs were evaluated with the Kruskal-Wallis equality of populations rank test; pairwise comparisons between groups were evaluated by Fisher's exact test of the medians. Figures represent combined data from at least 2 independent experiments. Cell death and cytokine release *in vitro* were analyzed by ANOVA with Bonferroni adjustment of pairwise comparisons. In all cases, tests with a p value < 0.05 were deemed statistically significant results. For data figures, bars represent mean of conditions in triplicate and error bars are standard deviation.

Statistical tests for antibiotic resistance studies were conducted with STATA v12 analysis software. Because there is intrinsic ordering in the bacterial virulence ranking system, virulence rank was treated as a continuous variable. Categorical variables (ABR, MDR, XDR and secretion status) were evaluated by univariate and multiple logistic regression. Linear regression was used to evaluate the count of drug-resistant classes. Results from logistic regression models are reported as Odds Ratio (OR) with 95% confidence interval (CI) while β coefficient and standard error (SE) are reported for linear regression models.

TABLES

Table 6 – Bacterial strains used in this study

Species	Strain Name	Reference
P. aeruginosa	PSE9	Schulert, Kung
P. aeruginosa	PSE9ΔpscF	This study
P. aeruginosa	PA99S	Shaver
P. aeruginosa	PA99null	Shaver
P. aeruginosa	PA99Δ*pscJ*	Shaver
E. coli	S17.1	
P. aeruginosa	89 Bloodstream Isolates (see Table 8)	This study, unpublished data
K. pneumoniae	43 Clinical Isolates (see Table 9)	This study, unpublished data

Table 7 – Primers used in this study

Primer Name	Sequence
PSE9delpscF Forward 1	aaaaaagcttcagcgctacctcgatggcag
PSE9delpscF Reverse 1	aaaaaagcttcagcgctacctcgatggcag
PSE9delpscF Forward 2	aaaaaagcttcagcgctacctcgatggcag
PSE9delpscF Reverse 2	aaaaaagcttcagcgctacctcgatggcag

Table 8 – Summary of strains in the *P. aeruginosa* antibiotic resistance population. MIC interpretation scored as susceptible (0) versus non-susceptible (1). ND = no data. Res_sum = sum of antimicrobial classes to which each strain is resistant. Secretion phenotype denoted as ExoU (U), ExoS (S), ExoT (T) or combinations of effectors. Exclusions denoted as no = 0, yes = 1.

	MICs											MIC Interpretation							Resistance Phenotype					
BL	Pip/Tazo	Levofloxacin	Ciprofloxacin	Ceftazidime	Cefepime	Amikacin	Tobramycin	Gentamicin	Aztreonam	Imipenem	Meropenem	Fluoroquinolones	Aminoglycosides	Carbapenems	Monobactam	Cephalosporin	Pip/Tazo	Res_Sum	ABR	MDR	XDR	Secretion Phenotype	Virulence Score	Exclude
1	8	1	0.25		2	2	1	2	4		0.5	0	0	0	0	0	0	0	0	0	0	ST	4	0
2	8	1	0.25		1	2	1	1	4		0.5	0	0	0	0	0	0	0	0	0	0	UT	4	0
3	8	1	0.25		2	8	1	2	4		0.25	0	0	0	0	0	0	0	0	0	0	ST	4	0
4	64	16	4		64	8	1	4	64		4	1	0	1	1	1	1	5	1	1	0	ST	2	0
6	64	16	4	4	16	8	1	8	6		4	1	1	1	0	1	1	5	1	1	0	UT	4	0
7	8	1	0.25		2	8	1	2	4		0.25	0	0	0	0	0	0	0	0	0	0	ST	4	0
9	8	1	0.25		2	8	1	2	4		0.25	0	0	0	0	0	0	0	0	0	0		3	0
10	128	8	4		8	16	4	16	48		4	1	1	1	1	0	1	5	1	1	0	ST	2	0
11	8	2	0.5	4	2	8	1	2	4		0.25	0	0	0	0	0	0	0	0	0	0		2	0
12	8	1	0.25		2	2	1	2	3		0.5	0	0	0	0	0	0	0	0	0	0	ST	5	0
13	8	1	0.25		1	8	1	2	3		0.25	0	0	0	0	0	0	0	0	0	0	UT	4	0
14	8	8	2		2	8	1	4	6		0.5	1	0	0	0	0	0	1	1	0	0	UT	3	0
15	8	16	4	4	8	16	4	8	6		1	1	1	0	0	0	0	2	1	0	0	ST	3	0
16	8	0.5	0.25		2	8	1	4	4		0.25	0	0	0	0	0	0	0	0	0	0	UT	5	0
17	8	1	0.25		4	8	1	4	3		1	0	0	0	0	0	0	0	0	0	0	ST	4	0
18	128	1	0.25		64	8	1	4	24		0.5	0	0	0	1	1	1	3	1	1	0	ST	3	0
20	64	16	4		8	16	4	16	16		16	1	1	1	1	0	1	5	1	1	0	UT	2	0
21	64	16	4	4	64	32	8	16			16	1	1	1	ND	1	1	5	1	1	0	UT	2	0
22	8	1	0.25		2	8	1	4	1.5		4	0	0	1	0	0	0	1	1	0	0	UT	4	0
23	8	2	0.5		4	8	1	4	8		1	0	0	0	0	0	0	0	0	0	0	ST	3	0
24	8	0.5	0.25		2	2	1	2	1		0.5	0	0	0	0	0	0	0	0	0	0	ST	4	0
26	16	1	0.25		1	8	1	2	3		0.25	0	0	0	0	0	0	0	0	0	0	ST	4	0
27	16	0.5	0.25		1	2	1	2	3		1	0	0	0	0	0	0	0	0	0	0		1	0

28	8	0.5	0.25		4	8	1	8	6		0.5	0	1	0	0	0	0	1	1	0	0	ST	2	0
29	128	16	4		8	8	8	16	8		4	1	1	1	0	0	1	4	1	1	0	ST	4	0
31	8	0.5	0.25		1	2	1	2	4		0.25	0	0	0	0	0	0	0	0	0	0	UST	3	0
32	8	1	0.5	1		8	1	4	8	1		0	0	0	0	0	0	0	0	0	0	ST	3	0
33	8	0.5	0.5	4	16	2	4	2	64		1	0	0	0	1	1	0	2	1	0	0		3	0
34	8	0.5	0.5	1		2	1	2	2	1		0	0	0	0	0	0	0	0	0	0	ST	4	0
36	64	8	4	4		2	8	8	32	1		1	1	0	1	0	1	4	1	1	0	UT	3	0
37	128	8	4	64		16	16	16	32	16		1	1	1	1	1	1	6	1	1	1	ST	3	0
38	8	4	1	1		2	1	2	16	1		1	0	0	1	0	0	2	1	0	0	ST	2	0
41	8		0.25		1	2	1	1	3		0.5	0	0	0	0	0	0	0	0	0	0	UT	4	0
42	8	0.5	0.5	4		2	1	1	8	1		0	0	0	0	0	0	0	0	0	0		2	0
43	128		4		32	4	1	4	4		0.25	1	0	0	0	1	1	3	1	1	0		1	0
44	128		4		8	2	8	8	16		0.25	1	1	0	1	0	1	4	1	1	0	ST	3	0
46	64	8	4	32		16	1	16	32	4		1	1	1	1	1	1	6	1	1	1	UT	3	0
47	8	8	4	1		2	1	2	8	1		1	0	0	0	0	0	1	1	0	0	ST	3	0
48	64	8	4	4		16	16	16	32	4		1	1	1	1	0	1	5	1	1	0	UT	3	0
49	8	2	0.5	4		2	1	1	32	2		0	0	0	1	0	0	1	1	0	0	ST	5	0
51	8	0.5	0.5	4		2	1	2	8	1		0	0	0	0	0	0	0	0	0	0	ST	2	0
52	8	8	2	1		2	1	1	2	1		1	0	0	0	0	0	1	1	0	0		1	0
53	128	8	4	16		16	2	8	32	4		1	1	1	1	1	1	6	1	1	1	UT	3	0
55	8	0.5	0.5	1		2	1	1	8	1		0	0	0	0	0	0	0	0	0	0	ST	3	0
56	64	8	4	4		2	16	16	32	4		1	1	1	1	0	1	5	1	1	0	UT	2	0
57	8	0.5	0.5	1		4	1	4	16	1		0	0	0	1	0	0	1	1	0	0	ST	3	0
58	128	8	4	128		2	1	16	32	16		1	1	1	1	1	1	6	1	1	1		2	0
59	8	1	0.5	1		2	1	2	8	1		0	0	0	0	0	0	0	0	0	0	ST	2	0
60	8	0.5	0.5	1		2	1	1	8	1		0	0	0	0	0	0	0	0	0	0	ST	3	0
62	8	0.5	0.5	1	1	2	1	2	8		0.25	0	0	0	0	0	0	0	0	0	0	ST	2	0
63	16	8	4	4		4	1	4	32		1	1	0	0	1	0	0	2	1	0	0	UT	3	0
64	8	8	4	1		4	16	16	16		2	1	1	0	1	0	0	3	1	1	0	ST	2	0
66	8	0.5	0.5	1		2	1	1	8		1	0	0	0	0	0	0	0	0	0	0		2	0
67	64	4	4	4		4	1	2	32		4	1	0	1	1	0	1	4	1	1	0	UT	2	0

68	8	0.5	0.5	1		2	1	2	16		1	0	0	0	1	0	0	1	1	0	0	UT	3	0
69	8	2	0.5	1		2	1	1	16		2	0	0	0	1	0	0	1	1	0	0	ST	3	0
70	8	0.5	0.5		2	2	1	2	3		0.25	0	0	0	0	0	0	0	0	0	0	ST	4	0
71	8	2	4	4		16	2	16	16		1	1	1	0	1	0	0	3	1	1	0	UT	2	0
72	64	8	4	4	8	4	1	2	32		1	1	0	0	1	0	1	3	1	1	0	UT	3	0
73	8	1	0.5	1		4	1	2	8		1	0	0	0	0	0	0	0	0	0	0	ST	3	0
74	8	1	0.5	1		2	1	2	16		1	0	0	0	1	0	0	1	1	0	0	ST	2	0
75	8	0.5	0.5	1		2	1	1	8		1	0	0	0	0	0	0	0	0	0	0	ST	3	0
76	8	0.5	0.5	1		2	1	2	8		1	0	0	0	0	0	0	0	0	0	0	ST	3	0
77	8	0.5	0.5	1	2	2	1	2	8		0.25	0	0	0	0	0	0	0	0	0	0	ST	3	0
78	8	1	0.5	4		2	1	2	8		1	0	0	0	0	0	0	0	0	0	0	ST	4	0
79	8	1	0.5	1		2	1	2	8		1	0	0	0	0	0	0	0	0	0	0	UT	3	0
80	8	0.5	0.5	1	2	2	1	2	8		0.5	0	0	0	0	0	0	0	0	0	0	ST	3	0
81	64	4	1	8	16	2	1	2	48		1	1	0	0	1	1	1	4	1	1	0		2	0
82	8	1	0.5	1		4	1	4	8		1	0	0	0	0	0	0	0	0	0	0		1	0
83	8	0.5	0.5	4		4	1	4	8		1	0	0	0	0	0	0	0	0	0	0	UT	4	0
85	8	0.5	0.5	1		2	1	1	8		1	0	0	0	0	0	0	0	0	0	0	UT	4	0
86	128	8	4	4	16	16	16	16	32		1	1	1	0	1	1	1	5	1	1	0	ST	3	0
88	64	8	2	4		4	16	16	32		8	1	1	1	1	0	1	5	1	1	0	UT	2	0
89	8	0.5	0.5	2		2	1	2	8		1	0	0	0	0	0	0	0	0	0	0	ST	2	0
90	128	8	4	64	64	64	16	16	4		16	1	1	1	0	1	1	5	1	1	0	ST	4	0
91	128	8	4	128		32	1	8	32		16	1	1	1	1	1	1	6	1	1	1	ST	2	0
92	64	8	4	8	88	16	1	4	16		8	1	0	1	1	1	1	5	1	1	0	ST	1	0
93	16	8	4	4		4	1	4	32		2	1	0	0	1	0	0	2	1	0	0	UT	2	0
94	8	8	4	2		16	1	8	16		1	1	1	0	1	0	0	3	1	1	0	ST	3	0
95	8	8	4	4		2	1	2	16		4	1	0	1	1	0	0	3	1	1	0	ST	4	0
96	8	0.5	0.5	2		2	1	2	8		1	0	0	0	0	0	0	0	0	0	0	ST	4	0
97	64	8	4	2		16	1	4	8		1	1	0	0	0	0	1	2	1	0	0	UT	3	0
98	16	2	0.5	4		2	1	2	32		1	0	0	0	1	0	0	1	1	0	0	ST	3	0
101	8	0.5	0.5	2		2	1	2	8		1	0	0	0	0	0	0	0	0	0	0		3	0
102	8	1	0.5	2		4	1	4	8		1	0	0	0	0	0	0	0	0	0	0	ST	4	0

104	128	8	4	32		16	2	16	16		16	1	1	1	1	1	1	6	1	1	1	ST	1	0
105	16	0.5	0.5	2		16	1	8	16		1	0	1	0	1	0	0	2	1	0	0		2	0
107	8	0.5	0.5	2		4	1	4	8		1	0	0	0	0	0	0	0	0	0	0	UT	4	0
108	8	8	4	2		16	2	16	128		1	1	1	0	1	0	0	3	1	1	0	UT	4	0
5												ND	ND	ND	ND	ND	ND						0	1
8	8	0.5				8	1	4				0	0	ND	ND	ND	0	0	0	0	0		0	1
19	128	2				32	4	16				0	1	ND	ND	ND	1	2	1	0	0		0	1
25	8	0.5		4		2	1	2				0	0	ND	ND	0	0	0	0	0	0		0	1
30												ND	ND	ND	ND	ND	ND					ST	0	1
35												ND	ND	ND	ND	ND	ND					ST	0	1
39												ND	ND	ND	ND	ND	ND						0	1
40												ND	ND	ND	ND	ND	ND					UT	0	1
45	16	2	4	64		64	16	16	32	1		1	1	0	1	1	0	4	1	1	0		0	1
50												ND	ND	ND	ND	ND	ND						0	1
54												ND	ND	ND	ND	ND	ND					ST	0	1
61	8	0.5	0.5	1		2	1	2	8			0	0	ND	0	0	0	0	0	0	0		0	1
65												ND	ND	ND	ND	ND	ND						0	1
84												ND	ND	ND	ND	ND	ND					UT	0	1
87	128	8	4	4		16	16	16	32		4	1	1	1	1	0	1	5	1	1	0		0	1
99												ND	ND	ND	ND	ND	ND						0	1
100												ND	ND	ND	ND	ND	ND					ST	0	1
103												ND	ND	ND	ND	ND	ND						0	1
106												ND	ND	ND	ND	ND	ND					UT	0	1

Table 9 – Summary of strains in the *K. pneumoniae* antibiotic resistance population. MIC interpretation scored as susceptible (0) versus non-susceptible (1). Ceph_no_cefazolin = resistance to cephalosporins excluding cefazolin. Res_sum = sum of antimicrobial classes to which each strain is resistant.

	MIC Interpretation																MIC Interpretation										Resistance Phenotype				
Strain	Ampicillin/Sulbactam	Aztreonam	Cefazolin	Cefepime	Ceftriaxone	Ciprofloxacin	Gentamicin	Amikacin	Tobramycin	Imipenem	Meropenem	Ertapenem	Nitrofurantoin	Trimethoprim/Sulfamethoxazole	Tigecycline	Piperacillin/Tazobactam	Fluoroquinolones	Aminoglycosides	Carbapenems	Cephalosporins	Monobactam	Penicillin+Blac	Ceph_no_cefazolin	Folate Inhibitor	ABR_sum	ABR	MDR	XDR	Virulence Score
hvKP1	8	1	4	1	1	0.25	1	2	1		0.25	0.5	32	20	0.5	4	0	0	0	1	0	0	0	1	1	1	0	0	3
hvKP2	4	1	4	1	1	0.25	1	2	1		0.25	0.5	32	20	0.5	4	0	0	0	1	0	0	0	1	1	1	0	0	3
hvKP3	8	1	4	1	1	0.25	1	2	1		0.25	0.5	32	20	0.5	4	0	0	0	1	0	0	0	1	1	1	0	0	3
hvKP4	4	1	4	1	1	1	1	2	1		0.25	0.5	128	20	4	8	0	0	0	1	0	0	0	1	1	1	0	0	3
KPPR1	4	1	4	1	1	0.25	1	2	1		0.25	0.5	64	20	0.5	4	0	0	0	1	0	0	0	1	1	1	0	0	3
K2044	8	1	4	1	1	0.25	1	2	1		0.25	0.5	64	20	0.5	4	0	0	0	1	0	0	0	1	1	1	0	0	3
OC1035	32	64	64	8	64	4	1	16	16	8	16		512	>=16/304		R	1	1	1	1	1	1	1	1	7	1	1	1	1
OC1052	32	64	64	64	64	4	1	16	8	16	16			>=16/304		R	1	1	1	1	1	1	1	1	7	1	1	1	1
OC1092	32	64	64	4	16	4	16	16	16	16	16			>=16/304	4	R	1	1	1	1	1	1	1	1	7	1	1	1	1
OC1116	32	64	64	2	16	1	1	16	8	4	16		64	>=16/304		R	0	1	1	1	1	1	1	1	6	1	1	0	2
OC1136	32	64	64	64	64	4	1	16	16	16	16	32		>=16/304	6	R	1	1	1	1	1	1	1	1	7	1	1	1	1
OC1163	32	64	64	8	64	4	1	64	16	4	16			<=1/19	4	R	1	1	1	1	1	1	1	0	6	1	1	0	1
OC1186	32	64	64	2	16	4	2	64	16	2	16		256	>=16/304		R	1	1	1	1	1	1	1	1	7	1	1	1	1
OC1191	32	64	64	8	16	4	8	2	16	4		16	256	>=16/304		R	1	1	1	1	1	1	1	1	7	1	1	1	2
OC356	32	64	64	2	16	4	1	16	8	4	2	8		>=16/304	1	R	1	1	1	1	1	1	1	1	7	1	1	1	1
OC740	32	64	64	8	64	4	16	16	16	4		32		>=16/304		R	1	1	1	1	1	1	1	1	7	1	1	1	2
OC743	32	64	64	32	32	4	1	64	16	16		32	64	>=16/304		R	1	1	1	1	1	1	1	1	7	1	1	1	2
OC810	32	64	64	8	64	4	1	16	16	8	16	32	512	>=16/304		R	1	1	1	1	1	1	1	1	7	1	1	1	1

OC835	32	64	64	8	32	4	1	16	8	8	16		256	>=16/304		R	1	1	1	1	1	1	1	1	7	1	1	1	2
OC951	32	64	64	2	16	4	1	4	8	16	16	32		<=1/19	1	R	1	1	1	1	1	1	1	1	7	1	1	1	1
OC968	32	64	64	8	16	4	1	16	16	8	16	32	256	>=16/304		R	1	1	1	1	1	1	1	1	7	1	1	1	1
OC970	32	64	64	8	32	4	1	16	16	4	16			>=16/304	1.5	R	1	1	1	1	1	1	1	1	7	1	1	1	2
OC981	32	64	64	8	64	4	8	2	16	32	16	32	256	>=16/304		R	1	1	1	1	1	1	1	1	7	1	1	1	2
S001	8	1	4	1	1	0.25	1	2	1		0.25			<=1/19		S	0	0	0	1	0	0	0	0	0	0	0	0	1
S002	4	1	4	1	1	0.25	1	2	1		0.25			<=1/19		S	0	0	0	1	0	0	0	0	0	0	0	0	1
S003	4	1	4	1	1	0.25	1	2	1		0.25			<=1/19		S	0	0	0	1	0	0	0	0	0	0	0	0	2
S004	4	1	4	1	1	0.25	1	2	1		0.25			<=1/19		S	0	0	0	1	0	0	0	0	0	0	0	0	3
S005	2	1	4	1	1	0.25	1	2	1		0.25			<=1/19		S	0	0	0	1	0	0	0	0	0	0	0	0	1
S006	4	1	4	1	1	0.25	1	2	1		0.25			<=1/19		S	0	0	0	1	0	0	0	0	0	0	0	0	1
S007	32	1	4	1	1	0.25	1	2	1		0.25			<=1/19		S	0	0	0	1	0	1	0	0	1	1	0	0	1
S008	4	1	4	1	1	0.25	1	2	1		0.25			<=1/19		S	0	0	0	1	0	0	0	0	0	0	0	0	2
S009	32	1	4	1	1	0.25	1	2	1		0.25			<=1/19		S	0	0	0	1	0	1	0	0	1	1	0	0	1
S010	2	1	4	1	1	0.25	1	2	1		0.25						0	0	0	1	0	0	0		0	0	0	0	1
Z4146	16	64	64	1	8	0.5	1	2	1		0.25	0.5	64	320	0.5	4	0	0	0	1	1	1	1	1	4	1	1	0	1
Z4147	32	64	64	1	8	1	1	2	1	1	0.25			<=1/19		S	0	0	0	1	1	1	1	0	3	1	0	0	1
Z4149	32	64	64	32	64	4	1	8	16		0.25	0.5	64	320	0.5	32	1	1	0	1	1	1	1	1	6	1	1	0	1
Z4155	32	16	64	2	64	4	1	2	1		0.25	0.5	512	320	8	32	1	0	0	1	1	1	1	1	5	1	1	0	1
Z4157	32	4	64	2	32	1	16	2	4		0.25	0.5	64	320	1	32	0	1	0	1	0	1	1	1	4	1	1	0	1
Z4158	32	2	64	2	16	1	16	2	8		0.25	0.5	64	20	2	4	0	1	0	1	0	1	1	1	4	1	1	0	1
Z4160	32	16	64	2	64	1	1	2		1	0.25			>=16/304		S	0	0	0	1	1	1	1	1	4	1	1	0	1
Z4174	8	64	64	1	8	0.5	1	2	1		0.25		64	>=16/304			0	0	0	1	1	0	1	1	3	1	0	0	2
Z4175	8	64	64	1	8	0.5	16	2	8		0.25	0.5	64	320	1	4	0	1	0	1	1	0	1	1	4	1	1	0	1
Z4179	32	64	64	64	64	4	8	64	16		0.25		256	>=16/304			1	1	0	1	1	1	1	1	6	1	1	0	1

REFERENCES

1. Pier, G.B. and R. Ramphal, *Pseudomonas aeruginosa*, in *Mandell, Douglas, and Bennett's Principles and Practices of Infectious Disease*, G.L. Mandell, J.E. Bennett, and R. Dolin, Editors. 2010, Elsevier/Churchill Livingstone. p. 2587-2615.
2. 2013, Antibiotic Resistance Threats in the United States, 2013.
3. Klockgether, J., N. Cramer, L. Wiehlmann, C.F. Davenportand B. Tummler, 2011, *Pseudomonas aeruginosa* Genomic Structure and Diversity. *Front Microbiol*. 2: p. 150.
4. Kung, V.L., E.A. Ozerand A.R. Hauser, 2010, The accessory genome of *Pseudomonas aeruginosa. Microbiol Mol Biol Rev*. 74(4): p. 621-41.
5. Filloux, A., 2011, Protein Secretion Systems in *Pseudomonas aeruginosa*: An Essay on Diversity, Evolution, and Function. *Front Microbiol*. 2: p. 155.
6. Hauser, A.R., 2009, The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nat Rev Microbiol*. 7(9): p. 654-65.
7. Wangdi, T., L.A. Mijaresand B.I. Kazmierczak, 2010, In vivo discrimination of type 3 secretion system-positive and -negative *Pseudomonas aeruginosa* via a caspase-1-dependent pathway. *Infect Immun*. 78(11): p. 4744-53.
8. Franchi, L., J. Stoolman, T.D. Kanneganti, A. Verma, R. Ramphaland G. Nunez, 2007, Critical role for Ipaf in *Pseudomonas aeruginosa*-induced caspase-1 activation. *Eur J Immunol*. 37(11): p. 3030-9.
9. Miao, E.A., D.P. Mao, N. Yudkovsky, R. Bonneau, C.G. Lorang, S.E. Warren, I.A. Leafand A. Aderem, 2010, Innate immune detection of the type III secretion apparatus through the NLRC4 inflammasome. *Proc Natl Acad Sci U S A*. 107(7): p. 3076-80.
10. Shaver, C.M. and A.R. Hauser, 2004, Relative contributions of *Pseudomonas aeruginosa* ExoU, ExoS, and ExoT to virulence in the lung. *Infect Immun*. 72(12): p. 6969-77.
11. Feltman, H., G. Schulert, S. Khan, M. Jain, L. Petersonand A.R. Hauser, 2001, Prevalence of type III secretion genes in clinical and environmental isolates of *Pseudomonas aeruginosa. Microbiology*. 147(Pt 10): p. 2659-69.
12. Tyson, G.H. and A.R. Hauser, 2013, Phosphatidylinositol 4,5-bisphosphate is a novel coactivator of the *Pseudomonas aeruginosa* cytotoxin ExoU. *Infect Immun*. 81(8): p. 2873-81.
13. Davis, B.K., H. Wenand J.P. Ting, 2011, The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu Rev Immunol*. 29: p. 707-35.
14. Strowig, T., J. Henao-Mejia, E. Elinavand R. Flavell, 2012, Inflammasomes in health and disease. *Nature*. 481(7381): p. 278-86.
15. Sutterwala, F.S., L.A. Mijares, L. Li, Y. Ogura, B.I. Kazmierczakand R.A. Flavell, 2007, Immune recognition of *Pseudomonas aeruginosa* mediated by the IPAF/NLRC4 inflammasome. *J Exp Med*. 204(13): p. 3235-45.
16. Descamps, D., M. Le Gars, V. Balloy, D. Barbier, S. Maschalidi, M. Tohme, M. Chignard, R. Ramphal, B. Manouryand J.M. Sallenave, 2012, Toll-like receptor 5 (TLR5), IL-1beta secretion, and asparagine endopeptidase are critical factors for alveolar macrophage phagocytosis and bacterial killing. *Proc Natl Acad Sci U S A*. 109(5): p. 1619-24.
17. Schultz, M.J., A.W. Rijneveld, S. Florquin, C.K. Edwards, C.A. Dinarelloand T. van der Poll, 2002, Role of interleukin-1 in the pulmonary immune response during *Pseudomonas aeruginosa* pneumonia. *Am J Physiol Lung Cell Mol Physiol*. 282(2): p. L285-90.

18. Schultz, M.J., S. Knapp, S. Florquin, J. Pater, K. Takeda, S. Akiraand T. van der Poll, 2003, Interleukin-18 impairs the pulmonary host response to *Pseudomonas aeruginosa. Infect Immun*. 71(4): p. 1630-4.
19. Thakur, A., R.P. Barrett, J.A. Hobdenand L.D. Hazlett, 2004, Caspase-1 inhibitor reduces severity of *Pseudomonas aeruginosa* keratitis in mice*. Invest Ophthalmol Vis Sci*. 45(9): p. 3177-84.
20. Cohen, T.S. and A.S. Prince, 2013, Activation of inflammasome signaling mediates pathology of acute *P. aeruginosa* pneumonia*. J Clin Invest*. 123(4): p. 1630-7.
21. Fan, L.C., J.L. Lin, J.W. Yang, B. Mao, H.W. Lu, B.X. Ge, A.M.K. Choiand J.F. Xu, 2017, Macrolides protect against *Pseudomonas aeruginosa* infection via inhibition of inflammasomes*. Am J Physiol Lung Cell Mol Physiol*. 313(4): p. L677-L686.
22. Aloush, V., S. Navon-Venezia, Y. Seigman-Igra, S. Cabiliand Y. Carmeli, 2006, Multidrug-resistant *Pseudomonas aeruginosa*: risk factors and clinical impact*. Antimicrob Agents Chemother*. 50(1): p. 43-8.
23. Gales, A.C., R.N. Jones, J. Turnidge, R. Rennieand R. Ramphal, 2001, Characterization of *Pseudomonas aeruginosa* isolates: occurrence rates, antimicrobial susceptibility patterns, and molecular typing in the global SENTRY Antimicrobial Surveillance Program, 1997-1999*. Clin Infect Dis*. 32 Suppl 2: p. S146-55.
24. Kofoed, E.M. and R.E. Vance, 2011, Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity*. Nature*. 477(7366): p. 592-5.
25. Zhao, Y., J. Yang, J. Shi, Y.N. Gong, Q. Lu, H. Xu, L. Liuand F. Shao, 2011, The NLRC4 inflammasome receptors for bacterial flagellin and type III secretion apparatus*. Nature*. 477(7366): p. 596-600.
26. Miao, E.A., R.K. Ernst, M. Dors, D.P. Maoand A. Aderem, 2008, *Pseudomonas aeruginosa* activates caspase 1 through Ipaf*. Proc Natl Acad Sci U S A*. 105(7): p. 2562-7.
27. Arlehamn, C.S., V. Petrilli, O. Gross, J. Tschoppand T.J. Evans, 2010, The role of potassium in inflammasome activation by bacteria*. J Biol Chem*. 285(14): p. 10508-18.
28. Sahoo, M., I. Ceballos-Olvera, L. del Barrioand F. Re, 2011, Role of the inflammasome, IL-1beta, and IL-18 in bacterial infections*. ScientificWorldJournal*. 11: p. 2037-50.
29. Miao, E.A., I.A. Leaf, P.M. Treuting, D.P. Mao, M. Dors, A. Sarkar, S.E. Warren, M.D. Wewersand A. Aderem, 2010, Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria*. Nat Immunol*. 11(12): p. 1136-42.
30. Bergsbaken, T., S.L. Fink, A.B. den Hartigh, W.P. Loomisand B.T. Cookson, 2011, Coordinated host responses during pyroptosis: caspase-1-dependent lysosome exocytosis and inflammatory cytokine maturation*. J Immunol*. 187(5): p. 2748-54.
31. Hughes, A.J. and A.R. Hauser, *Pseudomonas* Activation of the Inflammasome, in *Pseudomonas*, J. Ramos, J.B. Goldberg, and A. Filloux, Editors. 2015, Springer. p. 51-74.
32. Puren, A.J., G. Fantuzziand C.A. Dinarello, 1999, Gene expression, synthesis, and secretion of interleukin 18 and interleukin 1beta are differentially regulated in human blood mononuclear cells and mouse spleen cells*. Proc Natl Acad Sci U S A*. 96(5): p. 2256-61.
33. Bauernfeind, F.G., G. Horvath, A. Stutz, E.S. Alnemri, K. MacDonald, D. Speert, T. Fernandes-Alnemri, J. Wu, B.G. Monks, K.A. Fitzgerald, V. Hornungand E. Latz, 2009, Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression*. J Immunol*. 183(2): p. 787-91.
34. Franchi, L., R. Munoz-Planilloand G. Nunez, 2012, Sensing and reacting to microbes through the inflammasomes*. Nat Immunol*. 13(4): p. 325-32.

35. Arlehamn, C.S. and T.J. Evans, 2011, *Pseudomonas aeruginosa* pilin activates the inflammasome. *Cell Microbiol*. 13(3): p. 388-401.
36. Mariathasan, S., K. Newton, D.M. Monack, D. Vucic, D.M. French, W.P. Lee, M. Roose-Girma, S. Ericksonand V.M. Dixit, 2004, Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature*. 430(6996): p. 213-8.
37. Miao, E.A., C.M. Alpuche-Aranda, M. Dors, A.E. Clark, M.W. Bader, S.I. Millerand A. Aderem, 2006, Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf. *Nat Immunol*. 7(6): p. 569-75.
38. Sun, Y.H., H.G. Rolanand R.M. Tsolis, 2007, Injection of flagellin into the host cell cytosol by *Salmonella enterica* serotype Typhimurium. *J Biol Chem*. 282(47): p. 33897-901.
39. Lightfield, K.L., J. Persson, S.W. Brubaker, C.E. Witte, J. von Moltke, E.A. Dunipace, T. Henry, Y.H. Sun, D. Cado, W.F. Dietrich, D.M. Monack, R.M. Tsolisand R.E. Vance, 2008, Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin. *Nat Immunol*. 9(10): p. 1171-8.
40. Yang, J., Y. Zhao, J. Shiand F. Shao, 2013, Human NAIP and mouse NAIP1 recognize bacterial type III secretion needle protein for inflammasome activation. *Proc Natl Acad Sci U S A*. 110(35): p. 14408-13.
41. Amiel, E., R.R. Lovewell, G.A. O'Toole, D.A. Hoganand B. Berwin, 2010, *Pseudomonas aeruginosa* evasion of phagocytosis is mediated by loss of swimming motility and is independent of flagellum expression. *Infect Immun*. 78(7): p. 2937-45.
42. Lovewell, R.R., R.M. Collins, J.L. Acker, G.A. O'Toole, M.J. Wargoand B. Berwin, 2011, Step-wise loss of bacterial flagellar torsion confers progressive phagocytic evasion. *PLoS Pathog*. 7(9): p. e1002253.
43. Patankar, Y.R., R.R. Lovewell, M.E. Poynter, J. Jyot, B.I. Kazmierczakand B. Berwin, 2013, Flagellar motility is a key determinant of the magnitude of the inflammasome response to *Pseudomonas aeruginosa. Infect Immun*. 81(6): p. 2043-52.
44. Grandjean, T., A. Boucher, M. Thepaut, L. Monlezun, B. Guery, E. Faudry, E. Kipnisand R. Dessein, 2017, The human NAIP-NLRC4-inflammasome senses the *Pseudomonas aeruginosa* T3SS inner-rod protein. *Int Immunol*. 29(8): p. 377-384.
45. Burrows, L.L., 2012, *Pseudomonas aeruginosa* twitching motility: type IV pili in action. *Annu Rev Microbiol*. 66: p. 493-520.
46. Lee, J.K., S.H. Kim, E.C. Lewis, T. Azam, L.L. Reznikovand C.A. Dinarello, 2004, Differences in signaling pathways by IL-1beta and IL-18. *Proc Natl Acad Sci U S A*. 101(23): p. 8815-20.
47. Karmakar, M., Y. Sun, A.G. Hise, A. Rietschand E. Pearlman, 2012, Cutting edge: IL-1beta processing during *Pseudomonas aeruginosa* infection is mediated by neutrophil serine proteases and is independent of NLRC4 and caspase-1. *J Immunol*. 189(9): p. 4231-5.
48. Hirata, T. and S. Narumiya, 2012, Prostanoids as regulators of innate and adaptive immunity. *Adv Immunol*. 116: p. 143-74.
49. Funk, C.D., 2001, Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science*. 294(5548): p. 1871-5.
50. von Moltke, J., N.J. Trinidad, M. Moayeri, A.F. Kintzer, S.B. Wang, N. van Rooijen, C.R. Brown, B.A. Krantz, S.H. Leppla, K. Gronertand R.E. Vance, 2012, Rapid induction of inflammatory lipid mediators by the inflammasome in vivo. *Nature*. 490(7418): p. 107-11.

51. Galle, M., P. Schotte, M. Haegman, A. Wullaert, H.J. Yang, S. Jinand R. Beyaert, 2008, The *Pseudomonas aeruginosa* Type III secretion system plays a dual role in the regulation of caspase-1 mediated IL-1beta maturation. *J Cell Mol Med*. 12(5A): p. 1767-76.
52. Fink SL, C.B., 2005, Apoptosis, pyroptosis, necrosis: Mechanistic description of dead and dying eukaryotic cells. *Infect Immun*. 73(4): p. 1907-1916.
53. Broz, P., J. von Moltke, J.W. Jones, R.E. Vanceand D.M. Monack, 2010, Differential requirement for Caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing. *Cell Host Microbe*. 8(6): p. 471-83.
54. Motani, K., H. Kushiyama, R. Imamura, T. Kinoshita, T. Nishiuchiand T. Suda, 2011, Caspase-1 protein induces apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC)-mediated necrosis independently of its catalytic activity. *J Biol Chem*. 286(39): p. 33963-72.
55. Kovacs, S.B. and E.A. Miao, 2017, Gasdermins: Effectors of Pyroptosis. *Trends Cell Biol*. 27(9): p. 673-684.
56. Huang, X., L.D. Hazlett, W. Duand R.P. Barrett, 2006, SIGIRR promotes resistance against *Pseudomonas aeruginosa* keratitis by down-regulating type-1 immunity and IL-1R1 and TLR4 signaling. *J Immunol*. 177(1): p. 548-56.
57. Veliz Rodriguez, T., F. Moalli, N. Polentarutti, M. Paroni, E. Bonavita, A. Anselmo, M. Nebuloni, S. Mantero, S. Jaillon, A. Bragonzi, A. Mantovani, F. Rivaand C. Garlanda, 2012, Role of Toll interleukin-1 receptor (IL-1R) 8, a negative regulator of IL-1R/Toll-like receptor signaling, in resistance to acute *Pseudomonas aeruginosa* lung infection. *Infect Immun*. 80(1): p. 100-9.
58. Qu, Y., S. Misaghi, A. Izrael-Tomasevic, K. Newton, L.L. Gilmour, M. Lamkanfi, S. Louie, N. Kayagaki, J. Liu, L. Komuves, J.E. Cupp, D. Arnott, D. Monackand V.M. Dixit, 2012, Phosphorylation of NLRC4 is critical for inflammasome activation. *Nature*. 490(7421): p. 539-42.
59. Stehlik, C. and A. Dorfleutner, 2007, COPs and POPs: modulators of inflammasome activity. *J Immunol*. 179(12): p. 7993-8.
60. Stehlik, C., M. Krajewska, K. Welsh, S. Krajewski, A. Godzikand J.C. Reed, 2003, The PAAD/PYRIN-only protein POP1/ASC2 is a modulator of ASC-mediated nuclear-factor-kappa B and pro-caspase-1 regulation. *Biochem J*. 373(Pt 1): p. 101-13.
61. Bedoya, F., L.L. Sandlerand J.A. Harton, 2007, Pyrin-only protein 2 modulates NF-kappaB and disrupts ASC:CLR interactions. *J Immunol*. 178(6): p. 3837-45.
62. Dorfleutner, A., N.B. Bryan, S.J. Talbott, K.N. Funya, S.L. Rellick, J.C. Reed, X. Shi, Y. Rojanasakul, D.C. Flynnand C. Stehlik, 2007, Cellular pyrin domain-only protein 2 is a candidate regulator of inflammasome activation. *Infect Immun*. 75(3): p. 1484-92.
63. Kayagaki, N., S. Warming, M. Lamkanfi, L. Vande Walle, S. Louie, J. Dong, K. Newton, Y. Qu, J. Liu, S. Heldens, J. Zhang, W.P. Lee, M. Roose-Girmaand V.M. Dixit, 2011, Non-canonical inflammasome activation targets caspase-11. *Nature*. 479(7371): p. 117-21.
64. Blander, J.M., 2014, A long-awaited merger of the pathways mediating host defence and programmed cell death. *Nat Rev Immunol*. 14(9): p. 601-18.
65. Anderson, D.M., K.M. Schmalzer, H. Sato, M. Casey, S.S. Terhune, A.L. Haas, J.B. Feixand D.W. Frank, 2011, Ubiquitin and ubiquitin-modified proteins activate the *Pseudomonas aeruginosa* T3SS cytotoxin, ExoU. *Mol Microbiol*. 82(6): p. 1454-67.
66. Shafikhani, S.H., C. Moralesand J. Engel, 2008, The *Pseudomonas aeruginosa* type III secreted toxin ExoT is necessary and sufficient to induce apoptosis in epithelial cells. *Cell Microbiol*. 10(4): p. 994-1007.

67. Brodsky, I.E., N.W. Palm, S. Sadanand, M.B. Ryndak, F.S. Sutterwala, R.A. Flavell, J.B. Bliskaand R. Medzhitov, 2010, A Yersinia effector protein promotes virulence by preventing inflammasome recognition of the type III secretion system. *Cell Host Microbe*. 7(5): p. 376-87.
68. Schotte, P., G. Denecker, A. Van Den Broeke, P. Vandenabeele, G.R. Cornelisand R. Beyaert, 2004, Targeting Rac1 by the Yersinia effector protein YopE inhibits caspase-1-mediated maturation and release of interleukin-1beta. *J Biol Chem*. 279(24): p. 25134-42.
69. Evdokimov, A.G., J.E. Tropea, K.M. Routzahnand D.S. Waugh, 2002, Crystal structure of the *Yersinia pestis* GTPase activator YopE. *Protein Sci*. 11(2): p. 401-8.
70. Frithz-Lindsten, E., Y. Du, R. Rosqvistand A. Forsberg, 1997, Intracellular targeting of exoenzyme S of *Pseudomonas aeruginosa* via type III-dependent translocation induces phagocytosis resistance, cytotoxicity and disruption of actin microfilaments. *Mol Microbiol*. 25(6): p. 1125-39.
71. Stotland, P.K., D. Radziochand M.M. Stevenson, 2000, Mouse models of chronic lung infection with *Pseudomonas aeruginosa*: models for the study of cystic fibrosis. *Pediatr Pulmonol*. 30(5): p. 413-24.
72. Jain, M., D. Ramirez, R. Seshadri, J.F. Cullina, C.A. Powers, G.S. Schulert, M. Bar-Meir, C.L. Sullivan, S.A. McColleyand A.R. Hauser, 2004, Type III secretion phenotypes of *Pseudomonas aeruginosa* strains change during infection of individuals with cystic fibrosis. *J Clin Microbiol*. 42(11): p. 5229-37.
73. Mahenthiralingam, E., M.E. Campbelland D.P. Speert, 1994, Nonmotility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. *Infect Immun*. 62(2): p. 596-605.
74. Luzar, M.A., M.J. Thomassenand T.C. Montie, 1985, Flagella and motility alterations in *Pseudomonas aeruginosa* strains from patients with cystic fibrosis: relationship to patient clinical condition. *Infect Immun*. 50(2): p. 577-82.
75. Bucior, I., J.F. Pielageand J.N. Engel, 2012, *Pseudomonas aeruginosa* pili and flagella mediate distinct binding and signaling events at the apical and basolateral surface of airway epithelium. *PLoS Pathog*. 8(4): p. e1002616.
76. Parker, D. and A. Prince, 2013, Epithelial uptake of flagella initiates proinflammatory signaling. *PLoS One*. 8(3): p. e59932.
77. Williams, B.J., J. Dehnbosteland T.S. Blackwell, 2010, *Pseudomonas aeruginosa*: host defence in lung diseases. *Respirology*. 15(7): p. 1037-56.
78. Chadha, V., F.S. Schaeferand B.A. Warady, 2010, Dialysis-associated peritonitis in children. *Pediatr Nephrol*. 25(3): p. 425-40.
79. Szeto, C.C., K.M. Chow, C.B. Leung, T.Y. Wong, A.K. Wu, A.Y. Wang, S.F. Luiand P.K. Li, 2001, Clinical course of peritonitis due to *Pseudomonas* species complicating peritoneal dialysis: a review of 104 cases. *Kidney Int*. 59(6): p. 2309-15.
80. Siva, B., C.M. Hawley, S.P. McDonald, F.G. Brown, J.B. Rosman, K.J. Wiggins, K.M. Bannisterand D.W. Johnson, 2009, *Pseudomonas* peritonitis in Australia: predictors, treatment, and outcomes in 191 cases. *Clin J Am Soc Nephrol*. 4(5): p. 957-64.
81. Stapleton, F. and N. Carnt, 2012, Contact lens-related microbial keratitis: how have epidemiology and genetics helped us with pathogenesis and prophylaxis. *Eye (Lond)*. 26(2): p. 185-93.
82. Rudner, X.L., K.A. Kernacki, R.P. Barrettand L.D. Hazlett, 2000, Prolonged elevation of IL-1 in *Pseudomonas aeruginosa* ocular infection regulates macrophage-inflammatory protein-2

production, polymorphonuclear neutrophil persistence, and corneal perforation. *J Immunol*. 164(12): p. 6576-82.

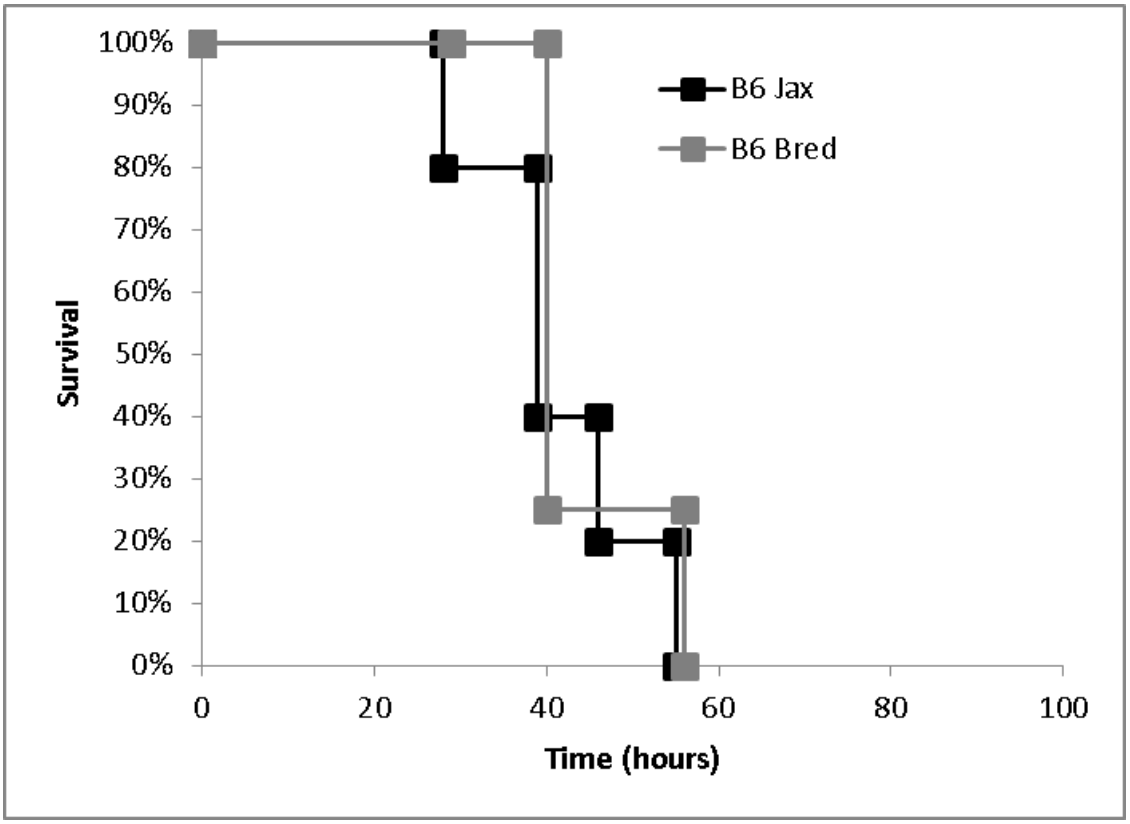
83. Sun, Y., M. Karmakar, P.R. Taylor, A. Rietschand E. Pearlman, 2012, ExoS and ExoT ADP ribosyltransferase activities mediate *Pseudomonas aeruginosa* keratitis by promoting neutrophil apoptosis and bacterial survival. *J Immunol*. 188(4): p. 1884-95.
84. Sun, Y., M. Karmakar, S. Roy, R.T. Ramadan, S.R. Williams, S. Howell, C.L. Shive, Y. Han, C.M. Stopford, A. Rietschand E. Pearlman, 2010, TLR4 and TLR5 on corneal macrophages regulate *Pseudomonas aeruginosa* keratitis by signaling through MyD88-dependent and -independent pathways. *J Immunol*. 185(7): p. 4272-83.
85. Sievert, D.M., P. Ricks, J.R. Edwards, A. Schneider, J. Patel, A. Srinivasan, A. Kallen, B. Limbagoand S. Fridkin, 2013, Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010. *Infect Control Hosp Epidemiol*. 34(1): p. 1-14.
86. Tait, S.W. and D.R. Green, 2013, Mitochondrial regulation of cell death. *Cold Spring Harb Perspect Biol*. 5(9).
87. Chung, H., A. Vilaysane, A. Lau, M. Stahl, V. Morampudi, A. Bondzi-Simpson, J.M. Platnich, N.A. Bracey, M.C. French, P.L. Beck, J. Chun, B.A. Vallanceand D.A. Muruve, 2016, NLRP3 regulates a non-canonical platform for caspase-8 activation during epithelial cell apoptosis. *Cell Death Differ*. 23(8): p. 1331-46.
88. Lee, M.S., H. Kwon, E.Y. Lee, D.J. Kim, J.H. Park, V.L. Tesh, T.K. Ohand M.H. Kim, 2015, Shiga Toxins Activate the NLRP3 Inflammasome Pathway To Promote Both Production of the Proinflammatory Cytokine Interleukin-1beta and Apoptotic Cell Death. *Infect Immun*. 84(1): p. 172-86.
89. Lage, S.L., C.L. Buzzo, E.P. Amaral, K.C. Matteucci, L.M. Massis, M.Y. Icimoto, A.K. Carmona, M.R. D'Imperio Lima, M.M. Rodrigues, L.C. Ferreira, G.P. Amarante-Mendesand K.R. Bortoluci, 2013, Cytosolic flagellin-induced lysosomal pathway regulates inflammasome-dependent and -independent macrophage responses. *Proc Natl Acad Sci U S A*. 110(35): p. E3321-30.
90. Fleiszig, S.M., T.S. Zaidiand G.B. Pier, 1995, *Pseudomonas aeruginosa* invasion of and multiplication within corneal epithelial cells in vitro. *Infect Immun*. 63(10): p. 4072-7.
91. Magiorakos, A.P., A. Srinivasan, R.B. Carey, Y. Carmeli, M.E. Falagas, C.G. Giske, S. Harbarth, J.F. Hindler, G. Kahlmeter, B. Olsson-Liljequist, D.L. Paterson, L.B. Rice, J. Stelling, M.J. Struelens, A. Vatopoulos, et al., 2012, Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect*. 18(3): p. 268-81.
92. 2015, Antimicrobial resistance surveillance in Europe: Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2014.
93. Appaneal, H.J., A.R. Caffrey, L. Jiang, D. Dosa, L.A. Mermeland K.L. LaPlante, 2017, Antibiotic resistance rates for *Pseudomonas aeruginosa* clinical respiratory and bloodstream isolates among the Veterans Affairs Healthcare System from 2009 to 2013. *Diagn Microbiol Infect Dis*.
94. Nathwani, D., G. Raman, K. Sulham, M. Gavaghanand V. Menon, 2014, Clinical and economic consequences of hospital-acquired resistant and multidrug-resistant *Pseudomonas aeruginosa* infections: a systematic review and meta-analysis. *Antimicrob Resist Infect Control*. 3(1): p. 32.

95. Chatterjee, M., C.P. Anju, L. Biswas, V. Anil Kumar, C. Gopi Mohanand R. Biswas, 2016, Antibiotic resistance in *Pseudomonas aeruginosa* and alternative therapeutic options. *Int J Med Microbiol*. 306(1): p. 48-58.
96. Lister, P.D., D.J. Wolterand N.D. Hanson, 2009, Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin Microbiol Rev*. 22(4): p. 582-610.
97. Poole, K., 2005, Aminoglycoside resistance in *Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy*. 49(2): p. 479-487.
98. Kohler, T., C. van Delden, L.K. Curty, M.M. Hamzehpourand J.C. Pechere, 2001, Overexpression of the MexEF-OprN multidrug efflux system affects cell-to-cell signaling in *Pseudomonas aeruginosa. J Bacteriol*. 183(18): p. 5213-22.
99. Kos, V.N., R.E. McLaughlinand H.A. Gardner, 2016, Identification of unique in-frame deletions in OprD among clinical isolates of *Pseudomonas aeruginosa. Pathog Dis*. 74(4): p. ftw031.
100. Skurnik, D., D. Roux, V. Cattoir, O. Danilchanka, X. Lu, D.R. Yoder-Himes, K. Han, T. Guillard, D. Jiang, C. Gaultier, F. Guerin, H. Aschard, R. Leclercq, J.J. Mekalanos, S. Lory, et al., 2013, Enhanced in vivo fitness of carbapenem-resistant oprD mutants of *Pseudomonas aeruginosa* revealed through high-throughput sequencing. *Proc Natl Acad Sci U S A*. 110(51): p. 20747-52.
101. Sun, Q., Z. Ba, G. Wu, W. Wang, S. Linand H. Yang, 2016, Insertion sequence ISRP10 inactivation of the oprD gene in imipenem-resistant *Pseudomonas aeruginosa* clinical isolates. *Int J Antimicrob Agents*. 47(5): p. 375-9.
102. Cabot, G., L. Zamorano, B. Moya, C. Juan, A. Navas, J. Blazquezand A. Oliver, 2016, Evolution of *Pseudomonas aeruginosa* Antimicrobial Resistance and Fitness under Low and High Mutation Rates. *Antimicrob Agents Chemother*. 60(3): p. 1767-78.
103. Kallel, H., F. Mahjoubi, H. Dammak, M. Bahloul, C.B. Hamida, H. Chelly, N. Rekik, A. Hammamiand M. Bouaziz, 2008, Correlation between antibiotic use and changes in susceptibility patterns of *Pseudomonas aeruginosa* in a medical-surgical intensive care unit. *Indian J Crit Care Med*. 12(1): p. 18-23.
104. Carmeli, Y., N. Troillet, G.M. Eliopoulosand M.H. Samore, 1999, Emergence of antibiotic-resistant *Pseudomonas aeruginosa*: comparison of risks associated with different antipseudomonal agents. *Antimicrob Agents Chemother*. 43(6): p. 1379-82.
105. Melnyk, A.H., A. Wongand R. Kassen, 2015, The fitness costs of antibiotic resistance mutations. *Evol Appl*. 8(3): p. 273-83.
106. Abdelraouf, K., S. Kabbara, K.R. Ledesma, K. Pooleand V.H. Tam, 2011, Effect of multidrug resistance-conferring mutations on the fitness and virulence of *Pseudomonas aeruginosa. J Antimicrob Chemother*. 66(6): p. 1311-7.
107. Sanchez, P., J. Linares, B. Ruiz-Diez, E. Campanario, A. Navas, F. Baqueroand J. Martinez, 2002, Fitness of in vitro selected *Pseudomonas aeruginosa* nalB and nfxB multidrug resistant mutants. *Journal of Antimicrobial Chemotherapy*. 50: p. 657-664.
108. Rossi Goncalves, I., R.C.C. Dantas, M.L. Ferreira, D. Batistao, P.P. Gontijo-Filhoand R.M. Ribas, 2017, Carbapenem-resistant *Pseudomonas aeruginosa*: association with virulence genes and biofilm formation. *Braz J Microbiol*. 48(2): p. 211-217.
109. Scheetz, M.H., M. Hoffman, M.K. Bolon, G. Schulert, W. Estrellado, I.G. Baraboutis, P. Sriram, M. Dinh, L.K. Owensand A.R. Hauser, 2009, Morbidity associated with *Pseudomonas aeruginosa* bloodstream infections. *Diagn Microbiol Infect Dis*. 64(3): p. 311-9.

110. Pena, C., G. Cabot, S. Gomez-Zorrilla, L. Zamorano, A. Ocampo-Sosa, J. Murillas, B. Almirante, V. Pomar, M. Aguilar, A. Granados, E. Calbo, J. Rodriguez-Bano, F. Rodriguez-Lopez, F. Tubau, L. Martinez-Martinez, et al., 2015, Influence of virulence genotype and resistance profile in the mortality of *Pseudomonas aeruginosa* bloodstream infections*. Clin Infect Dis*. 60(4): p. 539-48.
111. Linares, J., J. Lopez, E. Camafeita, J. Albar, F. Rojoand J. Martinez, 2005, Overexpression of the Multidrug Efflux Pumps MexCD-OprJ and MexEF-OprN Is Associated with a Reduction of Type III Secretion in *Pseudomonas aeruginosa. Journal of Bacteriology*. 187(4): p. 1384-1391.
112. Cosson, P., L. Zulianello, O. Join-Lambert, F. Faurisson, L. Gebbie, M. Benghezal, C. Van Delden, L.K. Curtyand T. Kohler, 2002, *Pseudomonas aeruginosa* virulence analyzed in a Dictyostelium discoideum host system*. J Bacteriol*. 184(11): p. 3027-33.
113. Patankar, Y.R., R. Mabaeraand B. Berwin, 2015, Differential ASC requirements reveal a key role for neutrophils and a noncanonical IL-1beta response to *Pseudomonas aeruginosa. Am J Physiol Lung Cell Mol Physiol*. 309(8): p. L902-13.
114. Kaufman, M.R., J. Jia, L. Zeng, U. Ha, M. Chowand S. Jin, 2000, *Pseudomonas aeruginosa* mediated apoptosis requires the ADP-ribosylating activity of exoS*. Microbiology*. 146 (Pt 10): p. 2531-41.
115. Schulert, G.S., H. Feltman, S.D. Rabin, C.G. Martin, S.E. Battle, J. Relloand A.R. Hauser, 2003, Secretion of the toxin ExoU is a marker for highly virulent *Pseudomonas aeruginosa* isolates obtained from patients with hospital-acquired pneumonia*. J Infect Dis*. 188(11): p. 1695-706.
116. Rathinam, V.A., S.K. Vanaja, L. Waggoner, A. Sokolovska, C. Becker, L.M. Stuart, J.M. Leongand K.A. Fitzgerald, 2012, TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria*. Cell*. 150(3): p. 606-19.
117. Bogiel, T., A. Deptula, J. Kwiecinska-Pirog, M. Prazynska, A. Mikuckaand E. Gospodarek-Komkowska, 2017, The Prevalence of Exoenzyme S Gene in Multidrug-Sensitive and Multidrug-Resistant *Pseudomonas aeruginosa* Clinical Strains*. Pol J Microbiol*. 66(4): p. 427-431.
118. McLaughlin, M.M., M.R. Advincula, M. Malczynski, G. Barajas, C. Qiand M.H. Scheetz, 2014, Quantifying the clinical virulence of *Klebsiella pneumoniae* producing carbapenemase Klebsiella pneumoniae with a *Galleria mellonella* model and a pilot study to translate to patient outcomes*. BMC Infect Dis*. 14: p. 31.
119. CLSI, 2014, MIC Interperative Standards for *Pseudomonas aeruginosa* and *Acinetobacter spp.*
120. Zamaraev, A.V., G.S. Kopeina, B. Zhivotovskyand I.N. Lavrik, 2015, Cell death controlling complexes and their potential therapeutic role*. Cell Mol Life Sci*. 72(3): p. 505-517.
121. Anantharajah, A., J.M. Buyck, E. Faure, Y. Glupczynski, H. Rodriguez-Villalobos, D. De Vos, J.P. Pirnay, F. Bilocq, B. Guery, P.M. Tulkens, M.P. Mingeot-Leclercqand F. Van Bambeke, 2015, Correlation between cytotoxicity induced by *Pseudomonas aeruginosa* clinical isolates from acute infections and IL-1beta secretion in a model of human THP-1 monocytes*. Pathog Dis*. 73(7).
122. Bowlin, N.O., J.D. Williams, C.A. Knoten, M.C. Torhan, T.F. Tashjian, B. Li, D. Aiello, J. Mecsas, A.R. Hauser, N.P. Peet, T.L. Bowlinand D.T. Moir, 2014, Mutations in the *Pseudomonas aeruginosa* needle protein gene pscF confer resistance to phenoxyacetamide inhibitors of the type III secretion system*. Antimicrob Agents Chemother*. 58(4): p. 2211-20.
123. Anantharajah, A., E. Faure, J.M. Buyck, C. Sundin, T. Lindmark, J. Mecsas, T.L. Yahr, P.M. Tulkens, M.P. Mingeot-Leclercq, B. Gueryand F. Van Bambeke, 2016, Inhibition of the Injectisome and Flagellar Type III Secretion Systems by INP1855 Impairs *Pseudomonas aeruginosa* Pathogenicity and Inflammasome Activation*. J Infect Dis*. 214(7): p. 1105-16.

124. Wang, C., X. Liu, J. Wang, J. Zhou, Z. Cuiand L.H. Zhang, 2016, Design and characterization of a polyamine derivative inhibiting the expression of type III secretion system in *Pseudomonas aeruginosa. Sci Rep*. 6: p. 30949.
125. Thanabalasuriar, A., B.G. Surewaard, M.E. Willson, A.S. Neupane, C.K. Stover, P. Warrener, G. Wilson, A.E. Keller, B.R. Sellman, A. DiGiandomenicoand P. Kubes, 2017, Bispecific antibody targets multiple *Pseudomonas aeruginosa* evasion mechanisms in the lung vasculature*. J Clin Invest*. 127(6): p. 2249-2261.
126. Hoang, T.T., R.R. Karkhoff-Schweizer, A.J. Kutchmaand H.P. Schweizer, 1998, A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants*. Gene*. 212(1): p. 77-86.

APPENDIX



Appendix Figure 1 – Comparison of survival between B6 mice bred in house vs mice purchased from Jackson Laboratories. (n=5 mice per group)