Understanding Inflammatory Mechanisms during Interactions between *Pseudomonas aeruginosa* and Host Cells in the Context of Cystic Fibrosis

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ABSTRACT

Cystic fibrosis (CF) is one of the most common genetic diseases in Europe and North America. Chronic bacterial infections with *Pseudomonas aeruginosa* (*P. aeruginosa*) are common among CF patients and are associated with increased disease progression among patients. While inflammation is considered to be a key driver of lung function decline, the precise mechanisms at play have remained unclear. The objective of this thesis was to evaluate the role of inflammatory signalling components that result in host cell death during respiratory infections observed in CF. First, I investigated the differences in inflammatory mechanisms and cytokine expression induced by *P. aeruginosa* isolated from early versus chronic infections in CF. I found that early respiratory isolates of *P. aeruginosa* from CF patients induced inflammasome signalling, cell death, and IL-1β expression by THP-1 macrophages, yet little expression of other proinflammatory cytokines. However, *P. aeruginosa* isolates from chronic infections induced relatively less THP-1 macrophage inflammasome signalling, cell death, and IL-1β expression but greater production of other cytokines. Using laboratory reference strains and various mutants of *P. aeruginosa*, I validated how due to their inability to induce early and extensive host cell death, isolates from chronic infections are able to induce sustained levels of proinflammatory cytokines, which may contribute to the pathogenesis observed in CF. I then investigated one specific virulence factor identified among clinical *P. aeruginosa* isolates, the effector protein ExoU. ExoU is known to induce rapid host cell death and has previously been described to be an inhibitor of caspase-1, limiting IL-1β secretion in immune cells. Using relevant laboratory reference strains, I have shown that ExoU is able to induce IL-1β expression at lower multiplicities of infection or at earlier time points than described in previous reports when infecting THP-1 macrophages and NuLi-1 bronchial epithelial cells. Through immunoblotting and the use of relevant inhibitors, it was found

that this observed difference could be partially dependent on the activation of various caspases, including ones that induced canonical and non-canonical inflammasome activation. Overall, this described work adds to our understanding of respiratory infections observed among CF patients and could shed light on possible therapeutic options to reduce disease progression.

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PREFACE

The following sections of this thesis have been previously published in *Cell Death and Disease* in March 2021 (*1*) with some modifications having been completed thereafter: Section 3.0 Materials and Methods, Tables S1 to S4, Section 4.1 of Results, Figures 6 to 28 (as well as respective captions), and Section 5.1 of Discussion. Specific contributions from co-authors and collaborators are outlined in Table 8.

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LIST OF ABBREVIATIONS

All abbreviations are defined at first mention in the text. For quick reference, an alphabetical list of high yield abbreviations is presented here:

ASC	Apoptosis-associated speck-like protein containing CARD
ASL	Air surface liquid
Bax	Bcl-2-associated X protein
BALF	Bronchoalveolar lavage fluid
BMDM	Bone marrow-derived macrophage
BSA	Bovine serum albumin
CARD	Caspase activating and recruitment domain
CF	Cystic fibrosis
CFU	Colony forming units
Cl^-	Chloride
DAMPs	Damage associated molecular patterns
DD	Death Domain
ER	Endoplasmic reticulum
FADD	Fas-associated death domain protein
FasL	Fas ligand
FBS	Fetal bovine serum
GSDMD	Gasdermin - D
$HCO3^-$	Bicarbonate
hTERT	Human telomerase reverse transcriptase
HRP	Horse radish peroxidase
IAPs	Inhibitor of apoptosis proteins
IL-(1...)	Interleukin
IL-1R	IL-1 receptor
IKK	Inhibitor of Nuclear Factor Kappa-B Kinase
K+	Potassium ion
LB	Lysogeny broth
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LUBAC	Llinear ubiquination chain assembly complex
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage-colony stimulating factor
MK-2	MAPK activated protein kinase 2
MKK	MAP kinase kinase
MLKL	Mixed lineage kinase domain-like
MOI	Multiplicity of infection
Na^+	Sodium ion
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
NIK	NF-κB-inducing kinase
NLR	NOD-like receptors

NLRC4	NOD, LRR family CARD domain-containing protein 4
NLRP3	NOD, LRR family pyrin domain-Containing protein 3
NOD	Nucleotide-binding oligomerization domain family
OD_{600}	Optical density at 600 nm
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PCD	Programmed cell death
PCR	Polymerase chain reaction
PI	Propidium iodide
PMA	Phorbol 12-myristate 13-acetate
PRRs	Pattern recognition receptors
PVDF	Polyvinylidene fluoride
PYD	Pyrin domain
qPCR	Quantitative polymerase chain reaction
R8	RPMI 1640 with 8% fetal bovine serum
RIPK1	Receptor-interacting protein kinase I
RPM	Revolutions per minute
ROS	Reactive oxygen species
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SOD1	Cu^{2+}, Zn^{2+}-superoxide dismutase
T3SS	Type III secretion system
TBST	Tris buffered saline with 0.1% Tween-20
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TRADD	TNF-receptor associated death domain protein
WT	Wild-type

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1.0 INTRODUCTION

1.1 Cystic fibrosis

1.1.1 Overview

Cystic fibrosis (CF) is a common autosomal recessive genetic disease, especially among North American and European populations (*2*). Recent epidemiological reports found that the incidence of CF in Canada was 1 in every 3,600 live births, with a total of over 4,300 Canadians currently living with CF (*3*). CF is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, which is located on human chromosome 7 (*4*, *5*). CFTR itself is a chloride (Cl^-) and bicarbonate ($HCO3^-$) channel, which plays an important role in modulating electrolyte balance and fluid homeostasis (*6*). Of the nearly 2,000 CFTR mutations previously reported, by far the most common is the ΔF508 nutation, which is found in approximately 70-90% of CF patients (*3*, *7*, *8*). These mutations are categorized in different classes which, in brief, can be summarized as: no CFTR transcription, no CFTR trafficking, impaired CFTR gating, decreased CFTR conductance, less CFTR protein, and less CFTR stability (*9*). The ΔF508 mutation results in impaired processing and trafficking of CFTR to the cell surface (*9*, *10*). As CFTR is expressed in a range of cell types throughout the human body, particularly epithelial cells, its impairment could give rise to various complications among patients (*6*, *11*). As a multisystem disease, it is common for CF patients to experience lung disease and chronic infections, pancreatic insufficiency, nutrient malabsorption, and infertility (*6*, *11*). Given the complexity of the disease, life expectancy for those with CF has historically been 20-30 years, although that has increased to as high as 50 years due to advancements in healthcare and treatment options (*12*, *13*). Regardless, the morbidity and mortality due to CF is often attributed to progressive pulmonary disease caused by respiratory pathophysiology (*6*, *14*, *15*). Specifically, loss of CFTR function results in altered

airway surface liquid (ASL) composition and impaired secretion clearance by the airways, resulting in patients entering a cycle of chronic infection and further respiratory decline (*6*).

1.1.2 Respiratory system composition and CFTR

Epithelial cells cover bodily surfaces, and they are defined as being polarized, meaning that there is an apical side of the cell that is exposed to an external environment, while the basolateral membrane is in contact with the interstitium (*16*). The basolateral membrane allows for the passage of nutrients, oxygen, and waste products through the cell. In contrast, the apical membrane provides a physical and chemical barrier that, especially in the respiratory system, serves to prevent invasive pathogens or toxins from invading the body (*16*).

Ion transport between epithelial cells is a frequent occurrence for homeostasis, and there is an interplay between different ion gradients. Cl^- transcellular secretion, which is mainly controlled by the ion channel CFTR, across the apical membrane modulates salt secretion (*16, 17*). Cl^- movement in turn creates an electrochemical gradient to drive sodium ion (Na^+) movement across the epithelium (*16, 17*). Elevated salt concentration on the apical surface drives water secretion via osmosis (*18, 19*). In addition to modulating Cl^- transport, CFTR is involved in $HCO3^-$ transport, which also impacts fluid secretion, albeit to a lesser extent than Cl^- (*4, 20, 21*). CFTR itself is a transmembrane glycoprotein and as a member of the ATP-binding cassette gene family, its function is regulated by ATP hydrolysis and phosphorylation (*4, 20, 21*).

The respiratory system can be divided into two sections: the conducting portion and the gas-exchange portion. The conducting portion consists of structures allowing inhaled air to pass through and be warmed prior to gas exchange (*22*). The anatomical structures which comprise the conducting portion include the nasal cavity, trachea, bronchi, and bronchioles (*22*). This portion is characterized by ciliated, pseudostratified columnar epithelial cells as well as mucus-secreting

goblet cells (*22*, *23*). These cells contribute to the mucociliary escalator, where the mucus secreted by goblet cells is able to trap pathogens, while ciliated cells are able to beat the trapped pathogens up along the respiratory tract away from the vulnerable lungs (*22*, *24*). The gas-exchange portion consists of incredibly thin, squamous epithelium, called pneumocytes (*22*). Type I pneumocytes allow for gas exchange to occur, while type II pneumocytes secrete surfactant, a substance which decreases surface tension to prevent alveolar collapse (*22*). In this portion of the respiratory system, oxygen is supplied to the blood to be delivered to organs throughout the rest of the body, and accumulated carbon dioxide is expelled (*22*).

1.1.3 CFTR dysfunction and increased susceptibility to chronic respiratory infections in CF

There are many prevailing theories proposing why CFTR dysfunction causes complications in the respiratory system. Indeed, signs of permanent lung architecture can be observed among CF patients as young as 5 years old (*2*, *25*). Often these signs can be appreciated through computed tomography imaging even prior to the occurrence of acute and chronic infections among patients (*25*, *26*). Therefore, it is suggested that independently of invasive pathogens, structural and physiological changes occur among CF patients, ultimately making them more susceptible to infections.

The increased susceptibility to respiratory infections reported among CF patients can be due to a number of factors, and one of the most well-established contributors is disruption in function of the mucociliary escalator in the respiratory tract (*6*, *27*). In healthy patients, the mucociliary escalator consists of ciliated epithelium that serves to discourage the colonization of pathogens in the upper respiratory tract (*22*). CFTR dysfunction causes changes in this physiology due to impaired secretion of chloride and bicarbonate ions into the airway lumen (*6*, *28*, *29*). This "dehydration hypothesis" proposes that without functioning CFTR, sodium hyperreabsorption

occurs, causing an increased uptake of water along with it as well as dehydrating the ASL and mucus (*30*). Dehydrated ASL impairs the mucociliary escalator by making it more challenging to move the mucus and entrapped pathogens up the respiratory tract, and this impaired clearance increases patients' susceptibility to respiratory infections. This pathophysiology is believed to drive the occurrence of "mucus plugs" reported in CF, which is a hallmark sign of the disease (*31*). General dehydration due to CFTR dysfunction also impacts the lower airways, where normally, pneumocytes must remove water from their surfaces to facilitate gas exchange (*32*). A study that performed *in situ* perfusion of lungs in wild-type (WT) mice as well as experiments using primary human type II pneumocytes demonstrated that the inhibition of Cl^- transport reduced this fluid clearance (*33*).

An alternate theory describes how reduced bicarbonate secretion into the airway lumen results in more acidic ASL, altering both mucus composition and mucociliary escalator function. A more acidic baseline ASL has been well-established in various CF *in vitro* cultures and models in comparison to WT controls (*29*, *34*, *35*). In addition, acidic ASL impairs cationic antimicrobial peptides, which aids in bacterial clearance and prevents their propagation (*36*). Acidic ASL also alters mucus viscosity (*36*).

Other than being an ion transporter, CFTR is known to be a transporter of glutathione, which is an antioxidant that plays a role in the immune response in the respiratory system (*37*). Reactive oxygen species (ROS) are molecules that can be produced either endogenously from the oxidative-burst response of phagocytes in response to insults or can come from exogenous sources, such as environmental toxins (*38*). Endogenously produced ROS can aid in the immune response during acute infections, but persistent production can further increase inflammation and cause tissue damage (*38*). Consequently, antioxidants produced by epithelial cells, such as glutathione,

are key to regulate these processes. Interestingly, CFTR dysfunction has been shown to reduce levels of glutathione in the ASL, and this subsequent abnormal antioxidant composition may result in the exacerbation of inflammatory processes in the lung (*39*).

CF patients are susceptible to a wide range of polymicrobial infections, including those caused by bacteria, viruses, and fungi. Bacteria most often isolated from CF patients include: *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus aureus* (*S. aureus*), *Haemophilus influenzae* (*H. influenzae*), and *Burkholderia* species (*40*). *P aeruginosa*, a species of Gram-negative bacilli ubiquitously found in the environment, are certainly among one of the more insidious organisms to cause complications among patients, especially during chronic infections, which is associated with worse patient morbidity (*6*, *14*, *15*). Further details about *P. aeruginosa* infections in CF will be discussed in **Section 1.4**. However, prior to reviewing the implications of chronic respiratory *P. aeruginosa* infections and their consequences, it is first necessary to review innate immunity, relevant signalling cascades, and how these defense mechanisms may be dysregulated in CF.

1.2 Innate immunity

1.2.1 Overview

Given the prevalence of acute and chronic respiratory infections in CF, it stands to reason that immunity, and likely its impairment, are important in the study of CF disease progression. Upon first encountering foreign pathogens in the lung environment, the physical barriers provided by the epithelial cells and resident leukocytes are significant defense mechanisms to protect the host. The innate immune system is one of the earliest responders to pathogens (*6*, *30*, *41*, *42*). It consists of many cells, such as macrophages and neutrophils, which recognize pathogens through pattern recognition receptors (PRR) (*43*). Examples of PRRs include the Toll-like receptors

(TLRs), complement receptors, Fc receptors, nucleotide-binding oligomerization domain family (NOD)-like receptors, and many more (*44*). TLRs are able to recognize a number of microbial components, such as TLR4 recognizing liposaccharide (LPS) (*45*) and TLR5 recognizing flagellin (*46*). TLRs are expressed on airway epithelial cells, including tracheal, bronchial, and alveolar epithelium, with many antibacterial TLRs being expressed on the apical layer of the airway lumen (*43*). Airway epithelium appear to express mostly TLR2 on the surface, with TLR3, TLR4, and TLR5 being expressed at low levels on the cell membrane or present intracellularly (*43*). Macrophages tend to express numerous TLRs including TLR3, TLR4, TLR5, TLR7, TLR8, and TLR9 (*47–49*).

Although not the focus of this thesis, it would be remiss to discuss CF and the innate immune system without briefly touching on the pathological contributions of neutrophils in the CF lung. Neutrophils are known to be recruited in large numbers in response to respiratory infections. Indeed, CF children with ongoing respiratory infections have been found to have elevated neutrophil counts (*50*, *51*). Despite this exaggerated immune response, this recruitment of neutrophils is puzzlingly unable to adequately control bacterial infections in CF, suggesting aberrations in their function due to CFTR impairment (*51*, *52*). Consequently, one of the often cited contributors to CF lung pathology is neutrophil extracellular trap (NET) formations, the process of which is termed NETosis (*51*, *52*). In this process, neutrophils release their cellular contents that contain antimicrobial granular proteins in response to infections, creating a highly inflammatory extracellular milieu that is associated with worsening lung pathology in CF (*51*, *52*). In addition to neutrophils, epithelial cells and macrophages in the lung environment also contribute to CF immunity, which will be discussed below.

1.2.2 Role of lung epithelium and alveolar macrophages in CF immunity

Epithelial cells, as previously discussed, are one of the first host cells which would encounter invading pathogens. Upon pathogen recognition, inflammatory cascades to recruit immune cells, such as neutrophils, into the airway lumen occur (*53*, *54*). In addition to the aforementioned TLR expression among epithelial cells, these cells are also known to express Nod-like receptors (NLR), such as NLRP3 and NLRC4 (*53*, *54*). Although these will be discussed in greater detail, in brief, these receptors can recognize sterile stress stimuli as well as components of *P. aeruginosa* to initiate inflammatory cascades. Aside from these sensors of pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), the role that epithelial cells play in immunity tend to be in relation to the physical and chemical approaches previously mentioned when discussing CFTR physiology and dysfunction.

Alveolar macrophages are one of the identified macrophage populations found in the lung, and they are an important component of the innate immune system in the lung environment. Although they are generally considered to have suppressive functions so as to not inflict excessive inflammation in the delicate lung environment, as with other macrophages, they are still able to phagocytize bacteria and apoptotic cells as well as initiate signalling cascades to facilitate neutrophil migration and activation of the adaptive immune system (*55*, *56*). It should also be noted that a second population, termed interstitial macrophages, is known to reside in the lung environment. While these are not as well-characterized as alveolar macrophages, it is possible they contribute to the secretion of IL-10 (*57*). Finally, in addition to macrophage populations that reside in the lung, circulatory monocytes are also known to be recruited to the lung space upon the presence of an infection due to the release of proinflammatory cytokines (*57*).

As previously mentioned, macrophages express a wide range of TLRs and NLRs in order to respond to invasive pathogens as well as sterile stressors (*58–60*). If and how CFTR impairment in CF impacts innate immunity and macrophage function as well as whether CFTR dysfunction creates a hyperinflammatory environment are still being investigated. Various studies have validated the expression of CFTR and its function among immune cells for both human (*61*) and murine macrophages (*62*, *63*). As well, a distinct CF phenotype can be replicated by inhibiting CFTR function in WT macrophages (*64*, *65*). Whether CFTR dysfunction impacts macrophage polarization in the lung environment as well as the precise mechanisms behind any observed changes are not as well established. Indeed, it is likely that given how dynamic the macrophage polarization process is and how dependent it is on a patient's overall health and disease status, truly understanding how macrophage polarization is impacted in CF patients and the implications for disease processes are challenging to study (*57*). Typically, macrophages can adopt the proinflammatory M1 or the M2 phenotype, which is associated with tissue repair (*57*). Standard protocols developed for *in vitro* experiments will induce M1 polarization by exposing M_0 macrophages to interferon gamma (IFN-γ) and LPS, while M2 polarization is induced by IL-4 or IL-13 (*66*). Although it is suspected that the proinflammatory CF lung environment would favour the M1 phenotype (*57*, *67*), $CFTR^{-/-}$ murine models demonstrate enhanced levels of both M1 and M2 populations in response to LPS treatment (*68*). Human studies have demonstrated contrary findings as to which macrophage population dominates the respiratory tract (*69–71*). Dysregulated M2 polarization, but not M1 polarization, upon using peripheral blood monocytes from CF patients has also been observed (*72*).

Previous reports have suggested that CFTR dysfunction is able to impair immune cells, like macrophages, by encouraging a hyperinflammatory state independent of infection. Various *in*

vitro and *ex vivo* studies of sterile CF cell cultures or tissues as well as animal models demonstrate elevated proinflammatory markers compared to non-CF specimens (*73–75*). Bronchoalveolar lavage fluid (BALF) from CF patients, even from those who were negative for common pathogens, was found to contain higher concentrations of proinflammatory cytokines (e.g., IL-6, IL-8, TNF) compared to BALF collected from healthy controls (*6, 76–80*). Immune cells with impaired CFTR function have also been found to overproduce ROS. This increase in ROS and oxidative stress may contribute to subsequent tissue damage that is seen in CF (*39, 81, 82*). If a hyperinflammatory environment is indeed maintained due to CFTR dysfunction, this may further predispose patients to bacterial infections due to the recruitment of inflammatory cells, such as neutrophils, which can release proteases that contribute to lung pathology (*6, 83, 84*). Serine proteases released by neutrophils can degrade antimicrobial peptides and components of the complement system, further impairing the immune system (*6, 83*, *84*).

Upon the establishment of respiratory infections, CF patients also face challenges when mounting an appropriate immune response and demonstrate dysregulation of these processes. CF human studies have shown that CFTR impairment impacts bacterial clearance early in life (*61–65, 85*). CF children have also been found to have a higher number of alveolar macrophages and higher levels of the chemoattractant chemokine MCP-1 in their BALF compared to non-CF controls (*86, 87*). Macrophages from CF patients have been found to be hyperresponsive when exposed to LPS. Namely, there seems to be elevated activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) pathways (*65, 85*) and secretion of proinflammatory cytokines (*65*). The dysregulation of proinflammatory cytokine secretion is also believed to be dependent on the extent of CFTR dysfunction, as macrophages isolated from CF carriers (*85*) demonstrated a more moderate phenotype. It has been suggested

that CFTR impairment causes defective phagolysosome acidification due to dysregulated Cl^- concentrations which impair bacteria killing activities (*62, 88, 89*), although some have argued that CFTR channel activity has no impact on this process (*30, 89*).

It has also been demonstrated that macrophages from CF patients have dysregulated TLR4 trafficking, causing enhanced TLR4 signalling and proinflammatory cytokine secretion compared to WT cells (*90*). Macrophages are known to be scavengers of cellular debris, which include apoptotic neutrophils, through a process called efferocytosis (*30, 91, 92*). This interplay between these two innate immune cells is especially important in CF, as efferocytosis regulates the activation of proinflammatory cascades, such as secondary necrosis (*30, 91, 93*). Studies have described efferocytosis impairment in the CF lung environment due to elastase accumulation (*30, 91, 93*).

1.2.3 Role of cytokines

Cytokines are proteins secreted by cells that induce various signalling cascades that impact any number of cellular functions, such as immune cell activation, recruitment, and cell death. In CF, cytokines are released in response to engagement of pathogen-associated molecular patterns (PAMPs) and play a vital role in regulating innate immunity (*94–96*). Tumor necrosis factor (TNF) and the IL-1 family (i.e., IL-1α and IL-1β) are classic examples of proinflammatory cytokines that are released by macrophages (*97*). Given how TNF and IL-1β have been detected in the serum and BALF of CF patients (*98, 99*), this suggests that alveolar macrophages continuously produce cytokines. Admittedly, the contribution of TNF, which is often viewed as an "early release" cytokine, to CF lung pathology and patient morbidity has not been consistently established (*100, 101*). However, IL-1β levels have been correlated with the presence of chronic respiratory infections with *P. aeruginosa* (*100, 102–104*), where the resolution of infection has been shown

to correlate with a decrease in measured IL-1β levels (*105*). Taken together, this suggests that IL-1β secretion is tied with *P. aeruginosa* respiratory infections.

A subsection of cytokines, called chemokines, are released by epithelial cells and macrophages in the lung environment to recruit and activate leukocytes, making them areas of interest for CF research given the hyperinflammatory disease state (*106–108*). One such chemokine is IL-8 (CXCL8), which acts on the CXCR1 and CXCR2 surface receptors to initiate neutrophil recruitment and induce degranulation and superoxide production (*109*). As with TNF and IL-1β, IL-8 has been reported to be detected at elevated levels in the BALF and sputum of CF patients (*94, 102, 110, 111*). Additional studies demonstrated that CXCR1 is involved in neutrophil antibacterial activities. However, in the CF lung environment, CXCR1 is proteolytically cleaved, resulting in its impairment (*112*). IL-8 has also been found to correlate with *P. aeruginosa* colonization and lung function (*113–115*).

1.3 Immune recognition and associated pathways

Initiation of immune cascades typically begin with the recognition of a diverse range of DAMPs or PAMPs. DAMPs, such as adenosine triphosphate (ATP) or the cytokine IL-1α, are endogenous molecules that are released upon necrotic cell death, resulting in "sterile inflammation" (*103*). PAMPs are components of infiltrating pathogens which are recognized by PRRs, including nucleic acids, flagellin, and LPS (*103*).

1.3.1 NF-κB pathway

NF-κB is a transcription factor that, upon activation, leads to the expression of numerous proinflammatory and anti-inflammatory mediators, including cytokines. There are two NF-κB signalling pathways: the canonical (also known as classical) pathway and the non-canonical (also known as alternative) pathway (*116, 117*). The canonical pathway is rapidly activated by

inflammatory cytokines, PAMPs, and DAMPs (*116*, *117*). The non-canonical pathway is slower, as it depends on the synthesis of NF-κB-inducing kinase (NIK) (*118*), and it is activated by a subset of cytokines from the TNF family, such as receptor activator of NF-κB ligand (RANKL) and CD40 ligand (CD40L) (*119–121*). The canonical pathway mainly targets p50–p65 dimers, while the non-canonical pathway results in activation of p52–RELB dimers (*116*, *119*, *120*). The IKK subunits involved in both pathways also differ in that the IKKα–IKKβ heterodimers are involved in the canonical pathway, but the non-canonical pathway uses IKKα–IKKα homodimers (*116*, *119*, *120*). As a general overview (**Fig. 1**), NF-κB activation begins with a PRR or cytokine receptor recognizing their respective ligand. TNF receptor-1 (TNFR-1) and TNF as well as TLR4 and LPS are common examples (*122*). While there can be variation in the exact cascade depending on the stimulus, ultimately the IKK complex is phosphorylated, which then phosphorylates IκB, an inhibitory protein of NF-κB (*122*). Phosphorylation-driven ubiquitination of IκB results in the proteasomal degradation of IκB, allowing for the release of the NF-κB dimers, which can then be translocated to the nucleus and initiate the transcription of gene targets (*122*). Typical gene targets for canonical NF-κB signalling include those which encode proinflammatory cytokines (e.g., TNF, IL-1 family, and IL-6); chemokines like IL-8; and inhibitors of NF-κB signalling as part of a self-regulatory mechanism (i.e., IκB) (*116*, *123*, *124*). It should also be emphasized that while NF-κB signalling is implicated in the production of cytokines and chemokines, these same cytokines are also known to act as mediators of NF-κB activation, such as the aforementioned TNF (*117*, *125–127*). Therefore components of the NF-κB signalling participate in either positive or negative feedback loops.

Non-canonical activation of NF-κB also begins with the engagement of a ligand with its respective receptor, such as CD40L with CD40R (*119*, *128*, *129*). Upon non-canonical activation,

NIK is activated, resulting in the phosphorylation of p100. This event leads to the activation of IKKα, which leads to p100 ubiquitination (*119, 128, 129*). Finally, degradation of p100 into p52 occurs and allows the p52–RELB dimer to translocate to the nucleus for gene transcription (*129*).

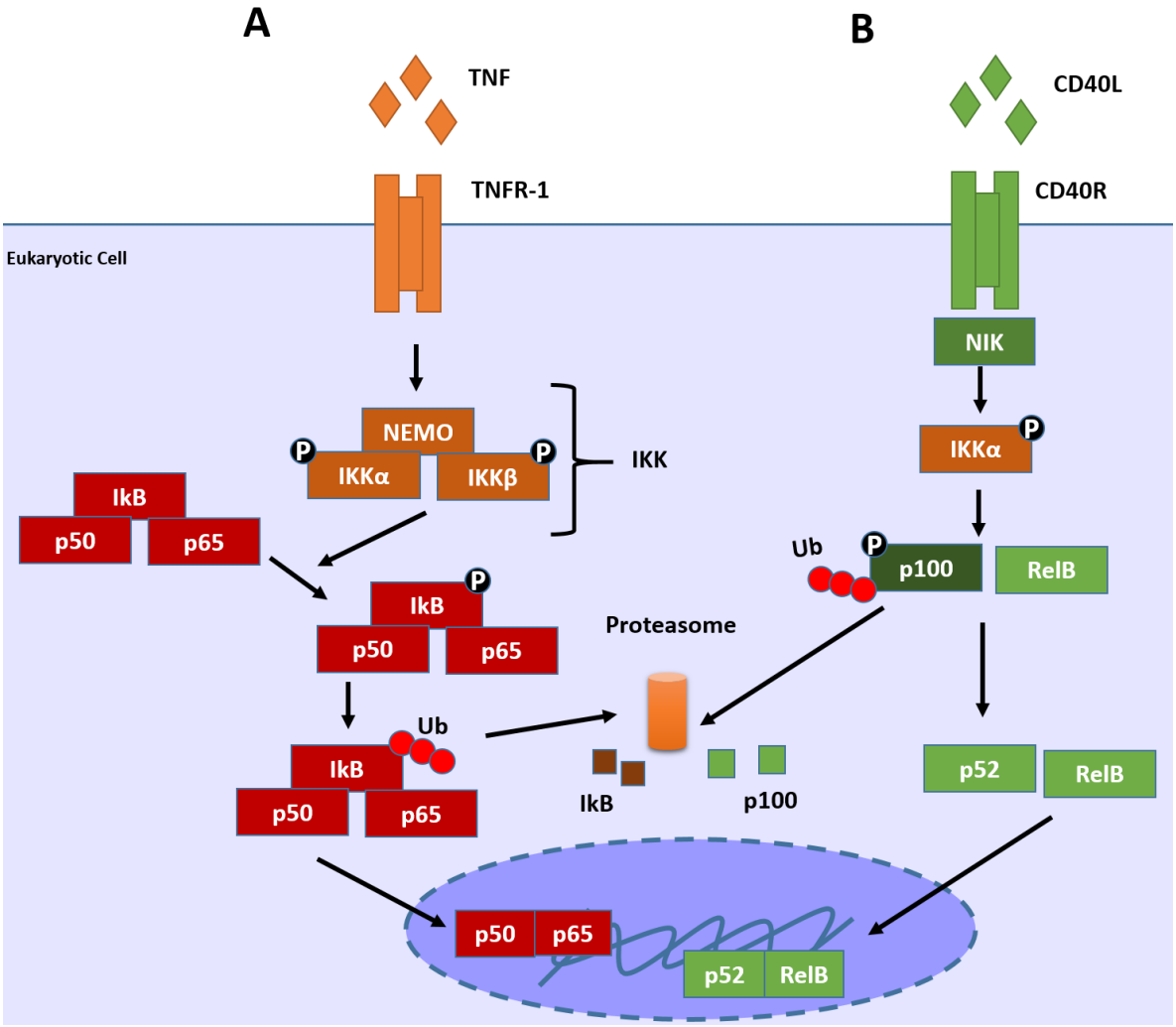


Fig. 1. The canonical and non-canonical NF-κB pathways. (**A**) The canonical pathway can be triggered by a number of different stimuli (shown is TNFR-1 recognizing TNF). The recruitment of various proteins then ensues, which induces the phosphorylation and activation of IKKβ. IKKβ mediates phosphorylation of IkB, which is an inhibitor of NF-κB. This phosphorylation of IkB leads to polyubiquitination and subsequent degradation of IkB. p50 and p65 are then free to translocate to the nucleus and begin transcription of gene targets. (**B**) The non-canonical pathway can be triggered by stimuli such as CD40L activating CD40R, resulting in the activation of NIK and phosphorylation of IKKα. p100 is then phosphorylated and partially degraded, thereby allowing the subsequent p52 and RelB complex to translocate to the nucleus to transcribe gene targets. These pathways have been previously described (*119*, *128*, *129*).

1.3.2 MAPK pathway

The MAPK pathway plays a significant role in cell proliferation, differentiation, and apoptosis (*130, 131*). Each MAPK pathway consists of at least three members: MAPK kinase kinase (MAP3K), MAPK kinase (MAPKK), MAPK, and MAPK-activated protein kinases (MAPKAPK) (*132*, *133*). There are three main MAPK cascades commonly under study: ERK1/2, JNK/stress-activated protein kinase, and p38 MAPK (with p38α, p38β, p38γ, and p38δ isoforms) (*130*, *133*).

Investigating the involvement of the p38 MAPK pathway is of importance in the following thesis work, and thus will be described in greater detail. p38α (p38) was the first isoform identified, and it has been extensively studied (*134–137*). Interestingly, much like with the NF-κB pathway, p38 activation can occur in response to extracellular stimuli, including inflammatory cytokines (*136*, *138, 139*), and result in the activation of transcription of various inflammatory cytokines, such as TNF, IL-1β, and IL-6 (*140*). As with other MAP kinases, p38 kinases are activated by dual kinases called MAP kinase kinases (MKKs), of which MKK3 and MKK6 act on p38. Following activation, one known substrate of p38 is MAP kinase-activated protein kinase 2 (MAPKAPK2 or MK2) (*137*, *138*). MK2 acts on various substrates, including phosphorylating tristetraprolin (TTP) in order to increase, among others, TNF and IL-1β mRNA stability (*141–146*).

1.3.3 Programmed cell death

Cell death is a process often used by multicellular organisms for the purposes of homeostasis or for cellular defense against insults. However, there is a range of cell death mechanisms that can be induced in cells, often depending on the type of stimulant that a given cell is exposed to. Traditional thinking had these mechanisms divided into programmed cell death (e.g., apoptosis and autophagy) and necrosis (i.e., inflammatory cell death caused by external trauma)

due to differences in cell morphology while undergoing these processes. Namely, apoptosis is characterized by cell shrinkage, extensive plasma membrane blebbing, condensation of chromatin, and nuclear fragmentation (*147*, *148*). Necrosis is characterized by the induction of rapid cellular swelling, plasma membrane dissolution, and release of intracellular contents to induce inflammation in the neighbouring extracellular environment (*147*). However, we have now begun to appreciate a greater variety of programmed cell death mechanisms that share some features with apoptosis but result in necrotic cell death. As some mechanisms of cell death can lead to the amplification of inflammatory responses during infections, some of the relevant mechanisms of cell death that will be discussed for the purposes of this thesis include apoptosis, necroptosis, and pyroptosis.

Apoptosis can be initiated via one of 2 distinct pathways: the intrinsic and extrinsic pathways (**Fig. 2 A, B**). As their names imply, each pathway responds to different types of stimuli. The intrinsic pathway is activated by intracellular stimuli, while the extrinsic pathway is activated upon engagement of death receptors (*147*). One example of the extrinsic pathway is the Fas cell-surface death receptor, which is activated following engagement with the Fas ligand (FasL) (*147*). It has been reported that Fas is expressed on various cell types found in the respiratory tract, including but not limited to bronchial epithelial cells, neutrophils, and alveolar macrophages (*147*). Similarly, epithelial cells, neutrophils, and monocytes express FasL (*147*, *149*). Another well-studied pathway of extrinsic apoptosis follows engagement of TNFR-1 by the cytokine TNF, which leads to the activation of Fas-associated protein with death domain (FADD) and subsequent recruitment of pro-caspase-8 (*126*, *147*). Downstream executioner caspases are then activated, resulting in apoptotic cell death (*126*, *147*). Alternatively, upon activation of TNFR-1, the fate of the cell can depend on posttranslational modifications of receptor-interacting protein kinase I

(RIPK1), which is associated with the TNFR-1 complex (*147*). If TNFR1-associated death domain (TRADD) is recruited, RIPK1 is ubinquinated by the inhibitor of apoptosis proteins (IAPs) and linear ubiquination chain assembly complex (LUBAC) (*147*). This promotes cell survival by activating IKK-dependent NF-κB activation (*147*). However, inhibition of IAPs by other mechanisms results in deubiquitination of RIPK1 by cylindromatosis (CYLD), allowing RIPK1 to form a complex with FADD and caspase-8, leading to the processing and activation of caspase-8 and cell death by apoptosis (*147*).

Intrinsic apoptosis is regulated by the B-cell lymphoma 2 (Bcl-2) protein family (*147*). Within this group of proteins, one subgroup has a single Bcl-2 homology 3 domain (BH3-only proteins) and promotes apoptosis (*126*, *147*). The other protein subsets have more than one BH domains, one of which also promotes apoptosis, while the other inhibits apoptosis (*126*, *147*). The pro-apoptotic Bcl-1 proteins induce permeabilization of the outer mitochondrial membrane, which leads to an efflux of cytochrome c and causes the adaptor Apaf-1 to bind to caspase-9 (*126*, *147*). Subsequently, the apoptosome complex is formed, activating initiator caspases (i.e., caspase-2, -8, -9, and -10) and other effector caspases, including caspase-3, caspase-6, and caspase-7 (*147*). Caspase-3 and caspase-7 promotes the typical morphological changes seen in apoptosis, including DNA fragmentation (*147*). Caspase-6 initiates processes that disintegrate cytoskeletal structures (*147*). The mitochondrial pathway entails Bcl family members binding to the mitochondrial membrane, including pro-apoptotic Bcl-2-associated X protein (Bax) and anti-apoptotic Bcl-2 (*147*).

Apoptosis is usually silent in the sense that it does not impact inflammation significantly, and it has been previously reported to be an anti-inflammatory mechanism of programmed cell death (*147*, *150*). Immediately after apoptosis, surrounding macrophages in the tissue rapidly

engulf the apoptotic blebs, which eliminates any possibility that intracellular contents are released (*147*, *150*). In situations where efferocytosis does not occur, secondary necrosis can ensue, contributing to the initiation of inflammation cascades (*150–152*).

In contrast to apoptosis, pyroptosis and necroptosis have been revealed to result in cell rupture and have consequently been deemed as inflammatory mechanisms of cell death (*147*). Necroptosis shares some of the triggers as apoptosis in that both can begin with the ligation of death receptors such as TNFR-1 (**Fig. 2 C**) (*147*). As well, it is understood that necroptosis proceeds when IAPs and caspase-8 are inhibited, which dissociates RIPK1 from the TNFR-1 complex and facilitates its interaction with RIPK3. Consequently, m-lineage kinase domain-like pseudokinase (MLKL) is phosphorylated, leading to the formation of the necrosome complex (*147*, *153*). Phosphorylation of MLKL leads to its oligomerization, which promotes its translocation to the cell membrane (*147*, *153*). Pore formation and cellular swelling result, causing the release of intracellular contents such as DAMPs to further promote inflammation (*147*, *153*). In addition, RIPK3 is able to activate IL-1β and IL-18 through its interaction with caspase-8 (*147*, *154*).

For pyroptosis, DAMPs or PAMPs activate NOD-like receptors, resulting in the oligomerization of a multiprotein complex called the inflammasome, which consists of the NLR, an adaptor molecule called apoptosis-associated speck-like protein (ASC), and caspase-1 (**Fig. 3**) (*147*, *155*). NOD-like receptors have been categorized based on the N-terminal domain, which can include a caspase recruitment domain (CARD) or pyrin domain (PYD), and the receptor nomenclature reflects this (*147*, *155*). For *P. aeruginosa* infections, NLRC4 (i.e., CARD domain-containing) and NLRP3 (i.e., PYD domain-containing) are of importance for phagocytes, with NLRP3 being the NOD-like receptor that has been most well characterized in studies of pyroptosis

(*156–158*). After formation of the inflammasome, procaspase-1 is recruited and cleaved (*147, 155*). Activated caspase-1 cleaves the N terminus of gasdermin D (GSDMD), which generates pores in the host plasma membrane (*147, 155*). Active caspase-1 is also able to cleave pro-IL-1β and pro-IL-18, allowing for the mature forms of these cytokines to be secreted by the dying cell (*147, 155*). Non-canonical paths of pyroptosis can also occur in that for human cells, caspase-4 or -5 (i.e., homolog for caspase-11 in murine cells) can be activated by intracellular LPS without the involvement of caspase-1, ultimately leading to the cleavage of GSDMD (*159*). Caspase-4/5 and capase-8 has also been implicated in promoting NLRP3 expression and activation, respectively, resulting in pro-IL-1β and GSDMD cleavage during bacterial infections (*160, 161*).

1.3.4 Mechanisms of cell death induced by P. aeruginosa

During *P. aeruginosa* respiratory infections, evidence points to the induction of a diversity of different cell death mechanisms, including apoptosis and pyroptosis, some of which are summarized in **Table 1**. Namely, *P. aeruginosa* possesses the ability to induce apoptosis in a multitude of ways, such as through the quorum-sensing molecule 3-oxo-C12-HSL activating TNFR-1 for phagocytes as well as disrupting mitochondrial structure to induce ROS generation in epithelial cells (*155, 162*). Various secreted proteins, such as protease and pyocyanin, have also been implicated in inducing apoptosis among mammalian cells (*163–165*). In addition, *P. aeruginosa* has been found to increase expression of Fas/FasL in the extrinsic apoptosis pathway for epithelial cells, likely predisposing cells to engage subsequent signalling mechanisms (*155*).

As previously mentioned, *P. aeruginosa* is known to activate the assembly of the NLRC4 inflammasome in macrophages via the type III secretion system (T3SS), a needle-like apparatus that secretes exotoxins and which will be described in greater detail below. Likewise, the NLRC4 inflammasome is activated by the presence of *P. aeruginosa* flagellin in a manner that is dependent

on K^+ efflux in both macrophages and neutrophils (*30*, *155*, *166*). *P. aeruginosa* flagellin is also known to stimulate NF-κB activity via activation of TLR5 as well as stimulate caspase-1 activity (*30*, *155*). The various effector proteins of the T3SS have been reported to induce different mechanisms of cell death. The T3SS effector protein ExoT and ExoS have also been shown to induce intrinsic apoptosis through their enzymatic activity, which may be related to their ability to disrupt the host cytoskeleton (*167–170*). ExoU-induced cell death, which has been described as necrotic, is also known to occur due to its phospholipase A2 activity that causes host plasma membrane disruption (*171–173*). The NLRP3 inflammasome has been known to be activated by pore-forming bacterial components and subsequent mitochondrial dysfunction (*174*, *175*), which could feasibly implicate the *P. aeruginosa* T3SS and cytotoxin ExoU as triggers of this mechanism. This may be further corroborated by data demonstrating that mitochondrial Ca^{2+}-dependent NLRP3 activation exacerbates the inflammatory response during *P. aeruginosa* infections upon using CF models (*176*). Finally, although *P. aeruginosa* has been described to induce necroptosis through the use of *in vivo* models and sublethal doses (*177*), this particular cell death mechanism has not been implicated in *in vivo* models of pneumonia or in CF pathogenesis in the literature to date.

P. aeruginosa is known to induce other mechanisms of programmed cell death that are not within the scope of this doctoral work. As previously described, *P. aeruginosa* can stimulate the initiation of NETosis, which results in the production of NETs and subsequent host cell death (*155*). *P. aeruginosa* can also induce autophagy upon infecting alveolar macrophages and epithelial cells, which may be aid the bacterium in evading eradication through downregulating phagocytosis and intracellular killing (*155*, *178*, *179*). Anoikis apoptosis and ferroptosis are two

additional mechanisms of programmed cell death that *P. aeruginosa* have been found to induce upon infecting epithelial cells (*155*).

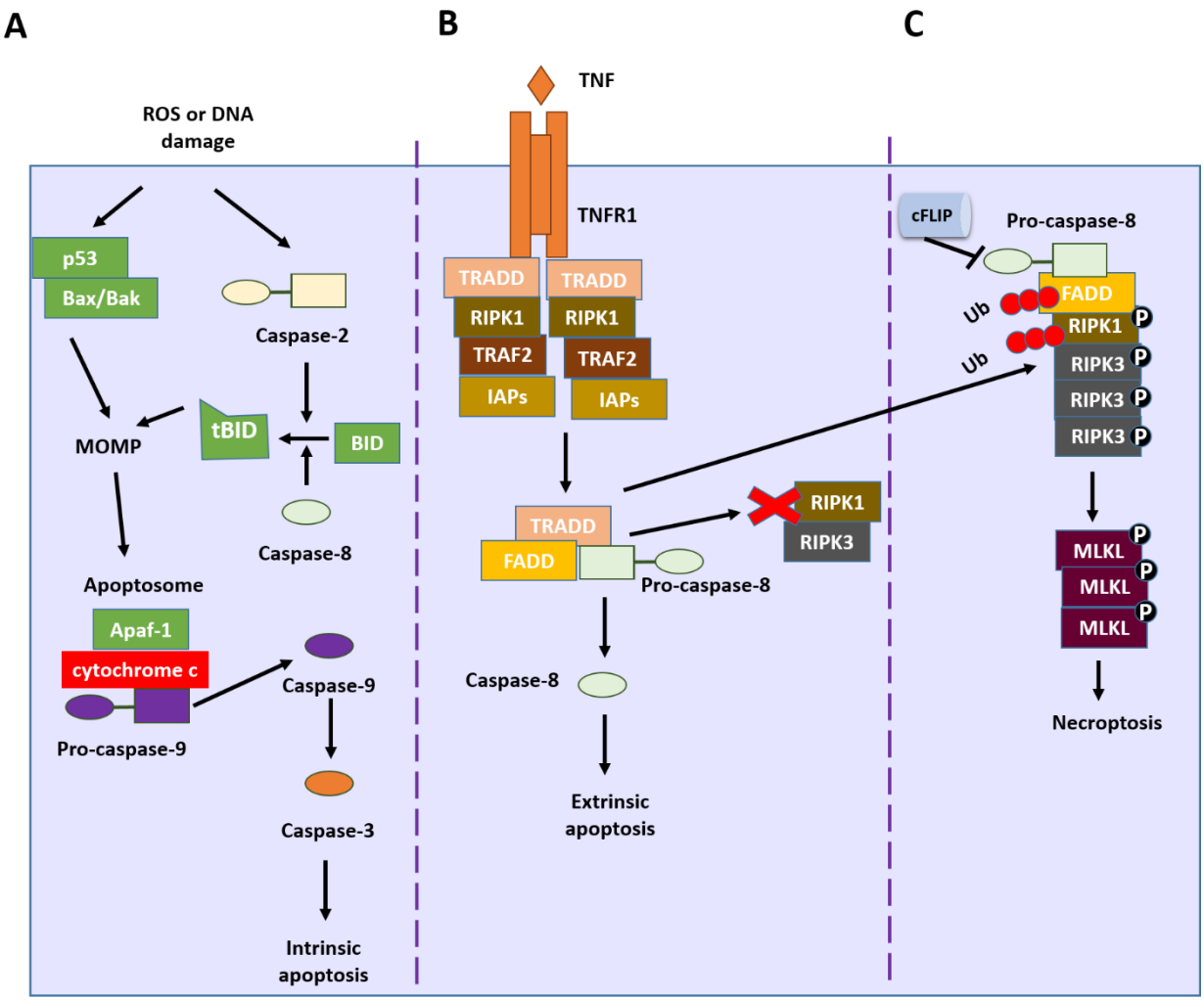

A
B
C
ROS or DNA damage
p53
Bax/Bak
Caspase-2
MOMP
tBID
BID
Caspase-8
Apoptosome
Apaf-1
cytochrome c
Pro-caspase-9
Caspase-9
Caspase-3
Intrinsic apoptosis
TNF
TNFR1
TRADD
RIPK1
TRAF2
IAPs
TRADD
FADD
Pro-caspase-8
RIPK1
RIPK3
Caspase-8
Extrinsic apoptosis
cFLIP
Pro-caspase-8
Ub
FADD
RIPK1
RIPK3
MLKL
Necroptosis

Fig. 2. Apoptosis and necroptosis pathways. (**A**) Intrinsic apoptosis is initiated upon the activation of p53 and pro-apoptotic Bcl-2 proteins (e.g., Bax and Bak). This leads to mitochondrial outer membrane permeabilization (MOMP), release of cytochrome c, and activation of the apoptosome. Caspase-9 is then cleaved, and it activates other effector caspases such as caspase-3. Alternatively, caspase-2 or caspase-8 can be activated and cause Bid to induce MOMP. (**B**) Extrinsic apoptosis involves the formation of a complex with death-domain-containing proteins, which vary based on the stimuli, and activation of caspase-8. Using the example of activated TNFR-1, TRADD, RIPK1, TRAFs and IAPs are recruited. Subsequent removal of polyubiquitin on RIPK1 dissociates this complex, allowing TRADD, procaspase-8, and FADD to form a complex. This results in caspase-8 activation, which also activates effector caspases and inactivates RIPK1 and RIPK3, leading to apoptosis. (**C**) In certain conditions when caspase activity is inhibited, RIPK1 and RIPK3 are stabilized and form complexes called necrosomes. The recruitment of the MLKL complex leads to pore formation and necroptotic cell death. These pathways have been previously described (*128, 129, 180*).

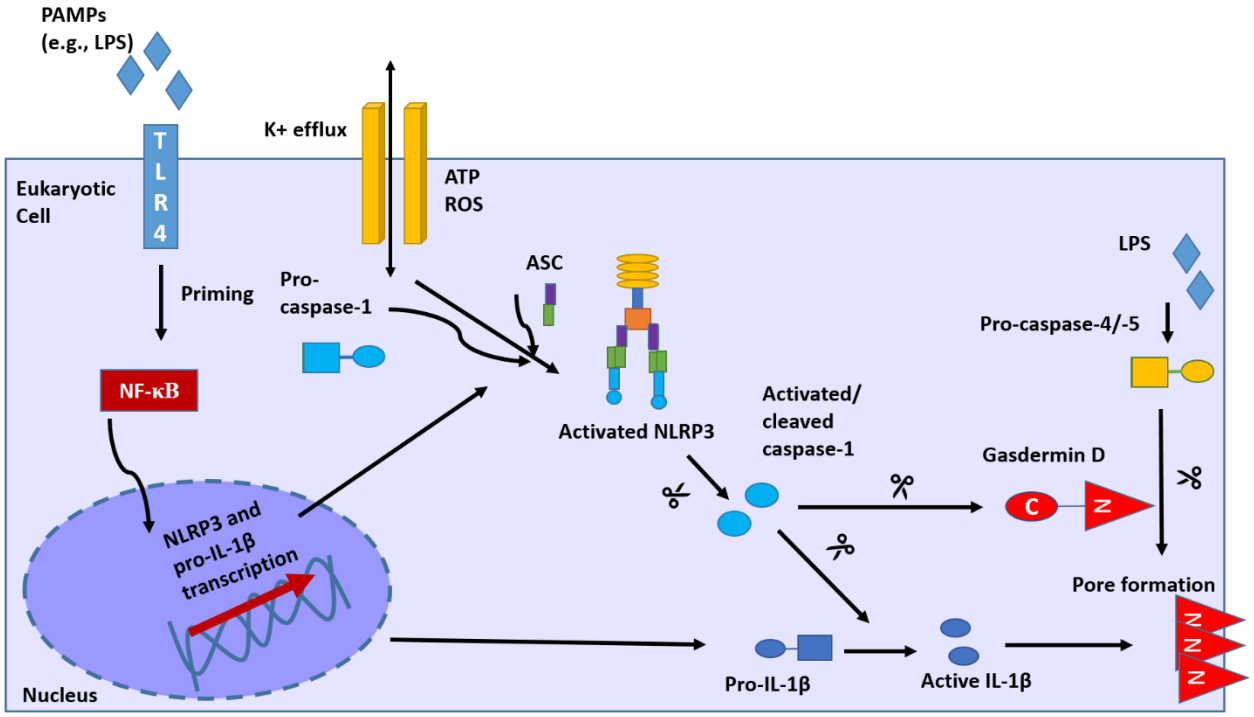


Fig. 3. Mechanisms for canonical and non-canonical inflammasome activation. Shown is one of the predominantly studied inflammasomes NLRP3. Canonical NLRP3 inflammasome activation requires activation of a TLR, which then induces activation of NF-κB and subsequent expression of pro-IL-1β and NLRP3. A variety of stimuli can activate NLRP3, including potassium (K+) efflux, and the activation of NLRP3 then cleaves caspase-1. Cleaved caspase-1 is able to cleave pro-IL-1β to its mature form, as well as cleave GSDMD. The N-terminal domains of GSDMD oligomerize to form pores in the plasma membrane, allowing the release of cellular contents, including mature IL-1β. In the non-canonical inflammasome pathway, caspase-4/5 is activated by cytosolic LPS. Active caspase-4/5 is then able to cleave GSDMD, allowing for IL-1β secretion and inducing pyroptotic cell death. These pathways have been previously described (*181–183*).

Table 1. *P. aeruginosa* factors known to induce programmed cell death

***P. aeruginosa* factor**	**Cell Death**	**Cell Type**	**Reference**
T3SS injector/needle complex	Apoptosis (caspase-3)	Macrophages and neutrophils	(*164*, *184–186*)
	Pyroptosis (NLRC4 inflammasome)	Macrophages	(*187*, *188*)
T3SS effector proteins	Apoptosis (caspase-3, mitochondrial pathway)	Macrophages and epithelial cells	(*164*, *187*, *188*)
Pyocyanin	Apoptosis (reactive oxygen intermediate, cAMP)	Neutrophils	(*163*, *164*)
Protease	Apoptosis (caspase-3)	Macrophages	(*164*, *165*)
N-3-(oxododecanoyl)-l-homoserine lactone	Apoptosis (caspase-3/-8, mitochondrial pathway, cytochrome c)	Macrophages, neutrophils, T cells, and airway epithelial cells	(*164*, *190–192*)
Flagellin	Pyroptosis (NLRC4 inflammasome)	Macrophages and neutrophils	(*30*, *155*, *166*, *186*)

1.4 *P. aeruginosa* in CF respiratory infections

1.4.1 Overview

As previously mentioned, CF patients are frequently vulnerable to *P. aeruginosa* infections. Indeed, anywhere from 10-50% of patients can be expected to be colonized with *P. aeruginosa* at a given time (*193–195*). *P. aeruginosa* is a Gram-negative bacterium ubiquitously found in the environment (*196*). It is characterized as an opportunistic pathogen that has been implicated in acute and chronic infections in patients that are hospitalized or otherwise immunocompromised (*197, 198*). *P. aeruginosa* has therefore been isolated in a variety of human infections, including those in the skin or soft tissue, the respiratory system, and the urinary tract (*197, 198*). This bacterium is also a causative agent of ventilator-associated pneumonia, where it is the one pathogen that is associated with the highest mortality rate (*199*).

P. aeruginosa respiratory infections in CF, especially chronic infections, are correlated with poorer clinical outcomes, such as respiratory function decline, which is often measured by a reduction in the forced expiratory volume (FEV) during the first second (FEV_1) (*156, 200*). It is therefore recommended that the sputum of CF patients above the age of 6 years old should be regularly monitored for bacteria colonization in the respiratory tract every 3 months (*201*). Given the regular recommended surveillance, the Leeds criteria was developed in order to categorize patients based on their *P. aeruginosa* status and provide a definition for the term "chronic *P. aeruginosa* infection" (*202, 203*). In brief, there are 4 groups which are defined based on obtained cultures within the previous 12 months (*202, 203*). The first group is "never infected", which is self-explanatory (*202, 203*). The second group is "free of *P. aeruginosa*", which includes patients who are currently *P. aeruginosa*-negative in their cultures but who have been colonized by *P. aeruginosa* in the past (*202, 203*). "Intermittent infection" is assigned to patients who have fewer

than 50% of their cultures within the past year test positive for *P. aeruginosa* (*202*, *203*). Finally, the "chronic infection" group refers to patients who have had more than 50% of their cultures grow *P. aeruginosa* in the last year (*202*, *203*).

If a CF patient is found to be colonized with *P. aeruginosa* or is acutely ill, early eradication is typically attempted in an effort to prevent the establishment of chronic infections. First-line treatment is typically inhaled tobramycin for 28 days, although there are other nebulized antibiotic options (*202*). If a patient is diagnosed with a chronic infection, inhaled tobramycin with aztreonam is prescribed for a 1-month on and 1-month off regimen until eradication is achieved (*202*, *204*).

1.4.2 Type III secretion system

As with other bacteria, *P. aeruginosa* is associated with a number of acute virulence factors that are segregated into pathogenicity islands (*184*, *205*). The T3SS is considered one of the dominant virulence apparatuses that is found in bacteria such as *Salmonella*, *Yersinia* and *Pseudomonas* (*184*, *205*). First described in *P. aeruginosa* in 1996 (*206*) and visualized in 2005 (*207*), the T3SS is a macromolecular complex often described as "needle-like", allowing it to inject effector proteins from the bacterial cytosol into host cell cytosol (*184*, *205*, *208*). The T3SS consists of structural proteins, regulatory proteins, chaperone proteins, and exotoxins (*184*, *205*). As a result, the apparatus consists of several proteins that are able to span the inner bacterial membrane, periplasmic space, peptidoglycan layer, outer bacterial membrane, extracellular space, and host cell membrane (*184*, *205*). *P. aeruginosa* T3SS transcription is regulated by the activator ExsA, and it is inhibited by ExsC and ExsD during non-secretory conditions (*184*, *205*, *209–211*). Under secretory conditions, ExsA is freed from the inhibitor ExsD, which results in transcription of T3SS genes, ultimately leading to the secretion of T3SS cytotoxins (*184*, *205*, *212*, *213*). The

translocator proteins PopB and PopD are also important components of the T3SS apparatus (*184*, *205*). In brief, they can oligomerize to form a complex that recognizes lipid rafts on the plasma membrane of host cells, allowing for effector protein secretion through the formation of a translocon channel (*184*, *205*, *214*, *215*). This process may also directly cause host cell death independent of effector protein activity (*184*, *205*, *214*, *215*).

Rapid death of host cells is mediated mainly by the toxins secreted through the T3SS of bacteria, including *P. aeruginosa* (**Fig. 4**). Identified effector proteins include: ExoU, ExoS, ExoT, and ExoY (*184*, *205*). The toxins ExoS and ExoT resemble one another and have dual functions as GTPase activating proteins (GAP) and adenosine diphosphate ribosyltransferases (ADPRT) (*184*, *205*). GAP activity is associated with the disruption of the host cytoskeleton, possibly impacting phagocytosis (*184*, *205*). ADPRT activity may also disrupt the host cytoskeleton but plays an additional role in the inhibition of DNA synthesis and endocytosis (*184*, *205*, *216*, *217*). The toxin ExoY is an adenylate cyclase which can impact gene expression through increasing intracellular cyclic adenosine monophosphate (cAMP) concentration in host cells (*184*, *205*, *218*). Much like ExoS and ExoT, it is speculated that this can result in disruption of the host cell cytoskeleton and phagocytosis (*184*, *205*).

Finally, the effector protein which has been studied for its potency and clinical impact is ExoU, a phospholipase A2 (PLA2) with activity against phospholipids, lysophospholipids, and neutral lipids. PLA2 activity operates by hydrolyzing the ester bond of the acyl group at the *sn*-2 position of phospholipids, allowing for the release of free fatty acids and lysophopspholipids (*171*, *172*, *184*, *205*). ExoU is characterized as a highly potent cytotoxin which can induce rapid necrosis-like cell death within a couple hours of infecting host cells (*171*, *172*, *184*, *205*). *In vivo*, this activity may possibly contribute to promoting bacterial dissemination (*172*, *184*, *205*). The

cytoxicity of ExoU is of importance given how it is prevalent in approximately 20% of clinical *P. aeruginosa* (*219*) isolates and was found in as many as 30-60% in studies of CF patients (*220, 221*). While ExoU is of clinical relevance, it is also of interest for inflammasome studies because ExoU has been reported to inhibit caspase-1 and subsequent IL-1β and IL-18 production, possibly influencing the early inflammatory response from host cells (*158*). As well, ExoU may be able to increase the levels of arachidonic acid in host cells, allowing for the production of proinflammatory eicosanoids (*171*, *205*, *222*). ExoU, as with other *P. aeruginosa* T3SS effector proteins, may also impact lung compliance and promote bacterial and cytokine dissemination *in vivo* due to their ability to disrupt cytoskeletal structures of cells and epithelial integrity (*205*). Therefore, there are simultaneous immune-suppressive and inflammatory properties associated with this effector protein.

For these effector proteins to exert targeted activity towards host cells alone, all effector proteins must interact with host cell cofactors which further modify their activity, many of which have been identified with the exception of those associated with ExoY (*184*, *205*). For ExoU, eukaryotic Cu^{2+}, Zn^{2+}-superoxide dismutase (SOD1) has been identified as either a direct or indirect cofactor, although the precise mechanism is unknown (*184*, *205*). ExoS, ExoT, and ExoU are also associated with chaperone proteins which may aid in the translocation of proteins through the T3SS apparatus, optimizing secretion. Specifically, SpcS for ExoS and ExoT as well as SpcU for ExoU have been identified as chaperone proteins (*184*, *205*, *223*).

Interestingly, it has been observed that chronic infections in CF may be dominated by *P. aeruginosa* strains that do not express T3SS (*224*). Therefore, *P. aeruginosa* T3SS can play critical roles in exacerbating inflammatory conditions during acute infections (*189, 225*). However, with time, there is likely an evolutionary advantage for *P. aeruginosa* to downregulate T3SS proteins

(*205*, *224*). The prevailing accepted paradigm is that downregulating these acute virulence factors permits the bacteria to chronically persist in the CF lung environment without activating an overt inflammatory host response (*184*, *205*, *225*).

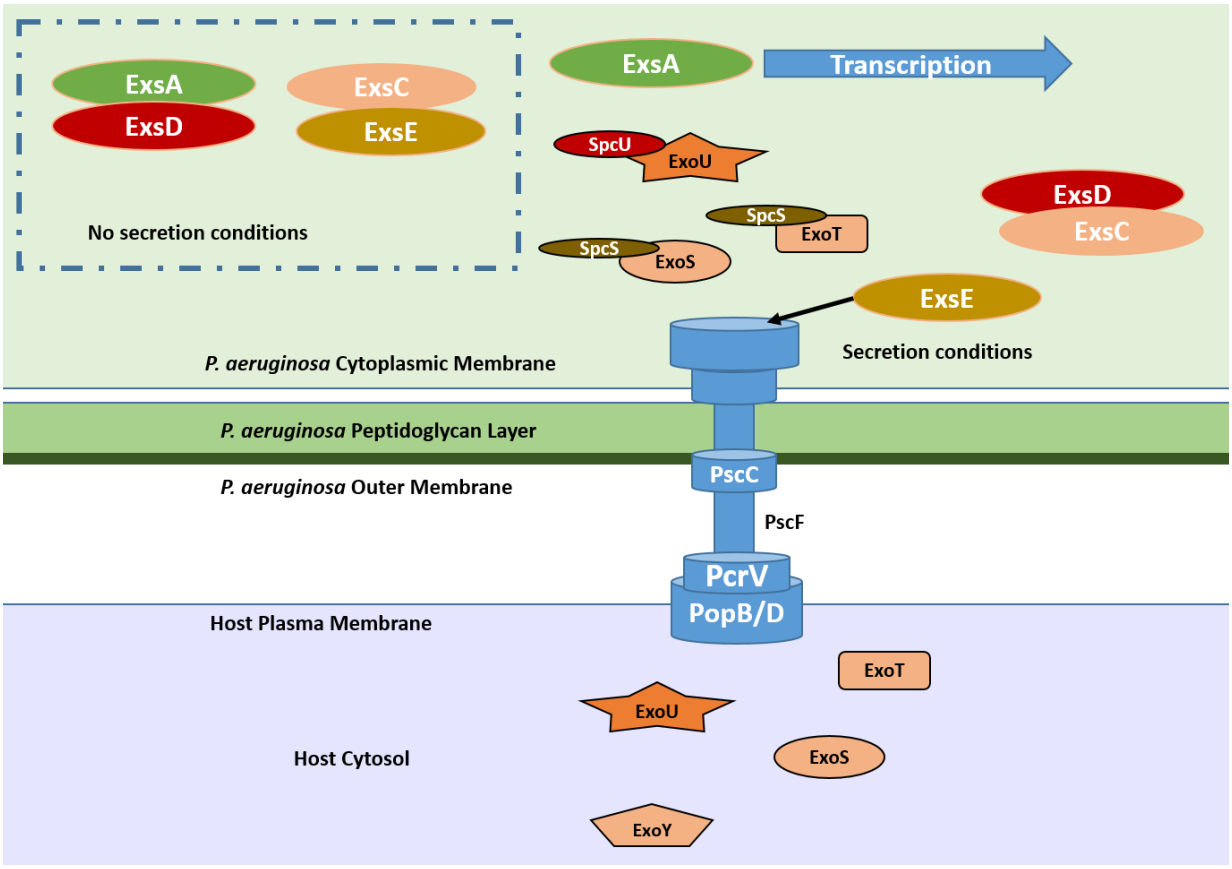


Fig. 4. *P. aeruginosa* type III secretion system. Under typical nonsecretory conditions, ExsA transcription is inhibited by the binding of ExsD. ExsC, which is able to bind to negatively regulate ExsD, is also bound to ExsE during these conditions. When secretion of T3SS proteins is promoted, ExsE is exported from the bacterium. Reduced levels of ExsE frees ExsC, allowing it to bind to ExsD. This frees ExsA to promote transcription of T3SS genes. Chaperone proteins are able to bring their respective effector proteins to the transmembrane T3SS apparatus for secretion into host cells. This description of T3SS regulation has been previously described (*205*).

1.4.3 P. aeruginosa *adaptations during chronic CF respiratory infections*

For *P. aeruginosa* to persist in the CF lung and evade the host immune system during chronic infections, there is a survival benefit for the pathogen to undergo a number of adaptations. The nature of these adaptations during chronic infections may occur at the genetic or phenotypic level (*224*). Genetic adaptations may occur intrinsically among *P. aeruginosa* strains that are more prone to mutations or due to the CF lung containing inflammatory oxidative stresses (*226*, *227*). Commonly identified mutations among *P. aeruginosa* isolated from CF patients include, but are not limited to: acute virulence factors (e.g., T3SS), immunogenicity factors (e.g., LPS), motility via flagellum, and antibiotic resistance (*224*, *228*). Most of these recognized mutations for virulence factors result in loss of function, possibly to promote immune evasion, and suggesting the role in selective pressure driving *P. aeruginosa* to undergo these adaptations (*224*, *229*, *230*). In contrast, *P. aeruginosa* components that are upregulated during chronic infection states tend to include the exopolysaccharides Pel and Psl (*224*, *231*). Psl and Pel are polysaccharides produced by *P. aeruginosa* to facilitate self- or surface-adherence (*232*),

A commonly described adaptation during chronic infections in general, but especially CF, is the formation of biofilms. As an alternative to growing as free-living bacteria (i.e., planktonically), *P. aeruginosa* can grow in a multicellular community, called biofilms, which are formed when bacteria adhere to surfaces and become embedded in an extracellular matrix (*233*–*235*). Studies of *in vivo* host response to *P. aeruginosa* biofilms are, unfortunately, limited due to poor available models that accurately recapitulate human infection (*224*). Findings from *in vitro* work, however, suggest that the expression of flagellin and T3SS is downregulated or physically unavailable for host detection when embedded in the extracellular matrix during biofilm growth (*224*, *236*, *237*).

The RetS/GacS sensor pathway has been implicated in modulating the switch in state from planktonic to biofilm living in acute and chronic infections, respectively (**Fig. 5**). The RetS and GacS signalling cascades have opposing functions in regulating the expression of proteins related to motility and T3SS versus Pel and Psl (*231*). Evidence of the RetS/GacS sensor pathway involvement in CF is also apparent via identified genetic mutations from *P. aeruginosa* isolates. Mutations compromising RetS function have been shown to promote the more avirulent phenotype, while mutations causing GacS dysfunction have been found to cause enhanced T3SS activity (*238*, *239*).

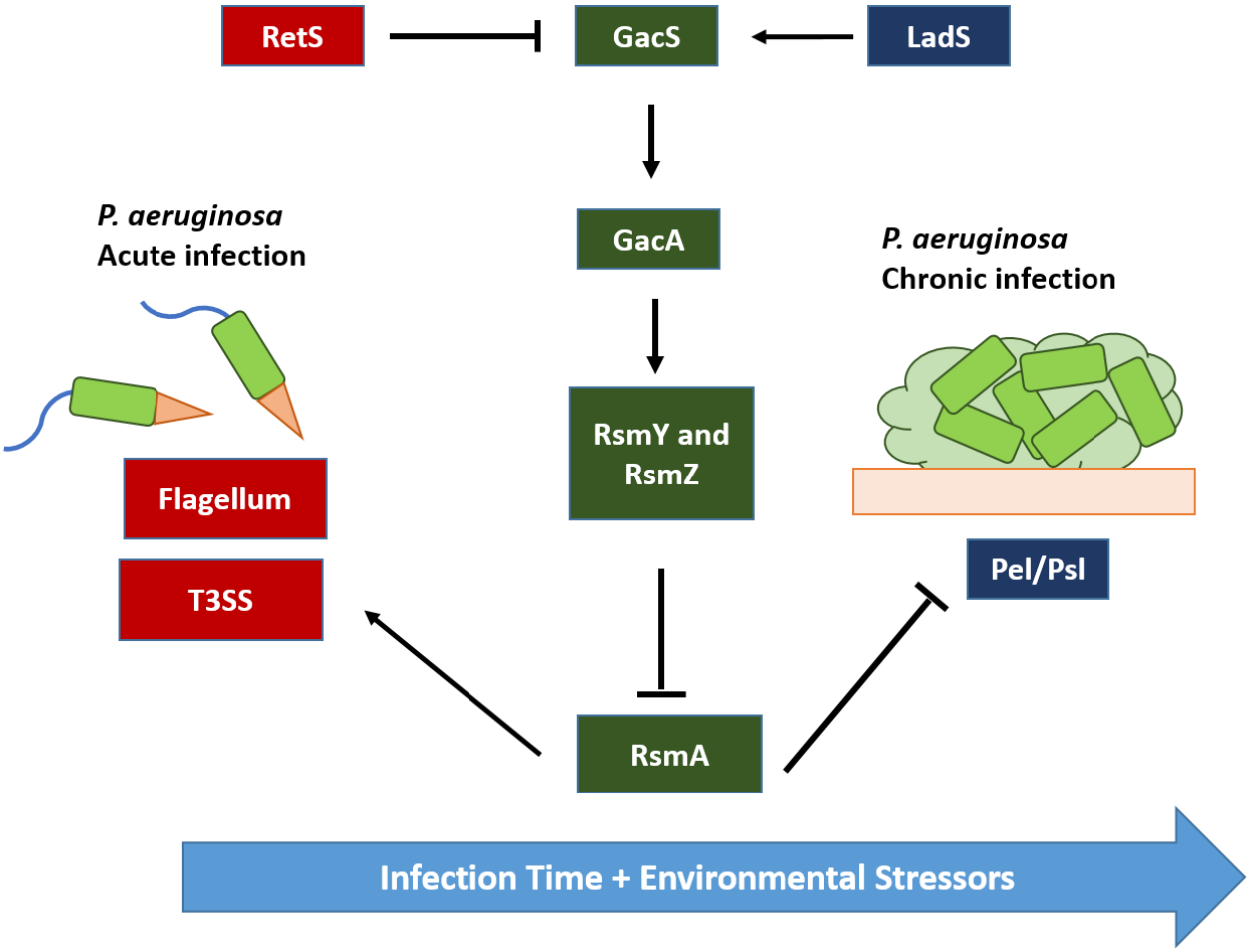


Fig. 5. Regulatory mechanisms implicated for acute infection versus chronic infection states of *P. aeruginosa*. RsmA is a regulatory protein that is able to bind to the promoter regions of various genes and is able to enhance the expression of acute virulence factors as well as repress the expression of genes associated with biofilm formation (e.g., Psl). LadS and GacS activity results in the phosphorylation of GacA, which then triggers the production of the small RNAs RsmZ and RsmY to inhibit RsmA. The lack of free RsmA allows for the expression of genes associated with chronic infections. RetS drives the expression of acute virulence factors by forming a complex with GacS to inhibit RsmY and RsmZ production. This paradigm has been previously described (*231*).

2.0 RATIONALE, HYPOTHESIS, AND AIMS

2.1 Rationale

The CF lung is frequently colonized with pathogenic bacteria due to aberrations in the host immune system and respiratory system architecture (*240*). Early in life, the CF lung is colonized with bacteria such as *S. aureus* and *H. influenza* (*241*). *P. aeruginosa*, however, is known to be a dominant causative agent in chronic infections (*241*). There are a wide range of sources which can drive this change in the lung microbiome, including the age of patients and antibiotic treatment used in response to acute infections (*242–245*). Indeed, early infections themselves may be a contributor, as they are associated with increased production of inflammatory markers, loss of diversity in the lung microbiome, lasting damage to the architecture of the respiratory system, and a decline in lung function (*246–250*). Thus, as the CF lung microbiome becomes less diverse with time, chronic infections dominated by *P. aeruginosa* eventually become common among CF patients (*251*). These chronic infections are considered to drive further decline in pulmonary function, including causing more frequent episodes of pulmonary exacerbations and mortality with 90% of CF patient deaths attributed to respiratory complications (*14*, *15*, *252*, *253*). Therefore, *P. aeruginosa* infections in CF are a significant concern that requires further understanding in the underlying pathogenesis in order to advance the development of available therapies.

P. aeruginosa from chronic respiratory infections among CF patients suggest that the pathogens undergo adaptations with time (*254*). Traditionally, the bacteria have been thought to downregulate acute virulence factors in order to exploit a vulnerable host and promote their persistence in the CF lung (*224*). Indeed, many of the known downregulated bacterial products are also cytotoxic, as determined in *in vitro* experiments, and are recognizable targets for the immune system (*224*, *228*, *255*, *256*). However, the very nature of chronic infections causing further decline

in lung function and worsening disease pathogenesis appears to be paradoxical (*14*, *15*). As well, chronic infections with *P. aeruginosa* are associated with sustained inflammation, where increased levels of inflammatory mediators have been reported in CF patients' sputum and BALF (*252*, *257–259*). These inflammatory markers have also been shown to decline after antibiotic treatment, suggesting a strong correlation with inflammation and the presence of *P. aeruginosa* in the lungs (*252*, *257–259*). Therefore, I aimed to elucidate if and how *P. aeruginosa* of supposedly lower virulence could stimulate inflammatory cascades that could possibly be correlated with the respiratory system decline seen in clinical observations.

Related to this inquiry, ExoU is a potent cytotoxin that induces rapid, necrosis-like host cell death. It has, however, also been reported to possess immunosuppressive qualities in that it inhibits caspase-1 and IL-1β production. Given how ExoU is seen among clinical *P. aeruginosa* isolates (*219–221*), expanding our understanding of what mechanisms this cytotoxin induces is worthwhile to comprehend its role in respiratory infections.

2.2 Hypothesis

I hypothesize that despite *P. aeruginosa* strains downregulating virulence factors or otherwise characterized as evading the immune system, these strains are still able to engage inflammatory mechanisms upon infecting host cells.

2.3 Aims

In **Section 4.1** of this thesis, I investigated the relationship between pathogen-induced host cell death and cytokine production during infections with *P. aeruginosa* isolated from early and chronic CF respiratory infections. I found that *P. aeruginosa* from chronic infections induce poor host cell death yet are able to stimulate potent secretion of proinflammatory cytokines. This work proposes a mechanism for how chronic respiratory infections contribute to increased disease

progression in CF given the ability for these pathogens to contribute to a proinflammatory milieu in the lung environment. Observations from **Section 4.1** drove further investigations which are described in **Section 4.2**, where I examined the novel role of ExoU in inducing IL-1β production in THP-1 macrophages and NuLi-1 bronchial epithelial cells. ExoU has been previously characterized as an inhibitor of caspase-1 and IL-1β production, although the mechanism of this inhibition has not been further elucidated (*158*). However, I found that under certain experimental conditions, ExoU-expressing *P. aeruginosa* is able to stimulate signalling cascades relevant to IL-1β secretion. Therefore, both sections shed light on how *P. aeruginosa* strains that are thought to evade the host immune system are still able to activate inflammatory mechanisms that may be detrimental to the host.

3.0 MATERIALS AND METHODS

3.1 Ethics

Clinical bacterial isolates were provided by the Cystic Fibrosis Isolate Core, which is a repository approved by the Seattle Children's Hospital Institutional Review Board to provide de-identified clinical isolates to researchers. The use of these isolates for *in vitro* experiments was approved by the Ethics Board of the University of Ottawa.

3.2 Cell culture

THP-1 monocytes (ATCC® TIB-202™) were grown in RPMI 1640 medium (Thermo Fosher, 31800089) supplemented with 8% fetal bovine serum (FBS) (R8) and 50 µg/mL of gentamicin. To detect NF-κB activation upon exposure to heat-killed bacteria, the reporter cell line THP1-Lucia™ NF-κB (InvivoGen, thpl-nfkb) was used. THP1-defCASP1 (InvivoGen, thp-dcasp1), a cell line that is 7-fold deficient in caspase-1, was used to determine caspase-1 involvement during infections. Both cell lines were grown according to recommended conditions. In brief, the cells were cultured in R8 that was further supplemented with 25 mM HEPES, 100 µg/mL of Normocin™ (InvivoGen), and 50 µg/mL gentamicin. On alternating passages, 100 µg/mL of Zeocin™ (InvivoGen) was added to the culture medium to maintain selective pressure. To differentiate the THP-1 cells into a macrophage phenotype (i.e., THP-1-derived macrophages or THP-1 macrophages), 50 ng/mL of phorbol 12-myristate 13-acetate (PMA) was added to the culture medium, and the cells were incubated at 37°C with 5% CO_2 for 72 h. Afterwards, the cells were washed with phosphate buffered saline (PBS), seeded in a 96- or 24-well plate, and maintained in R8 without antibiotics and PMA for 24 hours.

To generate primary human macrophages, peripheral blood mononuclear cells (PBMCs) were isolated from the blood of a healthy donor using SepMate™ tubes (STEMCELL

Technologies, 85450) and density gradient medium, Lymphoprep™ (STEMCELL Technologies, 07801) according to manufacturer's instructions. After washing the PBMCs twice with PBS, 10^7 cells were added to a polystyrene petri dish (100mm x 15mm) coated with 100 µL of 1µg/mL recombinant human macrophage-colony stimulating factor (M-CSF) (R&D Systems, 216-MC). RPMI 1640 medium supplemented with 10% FBS was added up to 10mL per dish. Cells were incubated at 37°C in a 5% CO_2 incubator for 6 days to allow macrophage differentiation to occur. The entire cell culture was harvested on day 6 and seeded in a 96-well plate at $5x10^4$ cells per well.

NuLi-1 (ATCC® CRL-4011™) and CuFi-1 (ATCC® CRL-4013) are human bronchial epithelial (HBE) human telomerase reverse transcriptase (hTERT)-immortalized cell lines which have been previously described and validated (*260*). Both were grown in Bronchial Epithelial Cell Growth Medium (BEGM™) (Lonza, CC-3171) supplemented with SingleQuots Supplements and Growth Factors™ (Lonza, CC-4175) and 50 µg/mL of gentamicin. Flasks and culture plates were coated in 50 µg/mL of collagen Type 4 (Sigma, C7521) prior to use.

All cells were maintained in flasks and were grown at 37°C with 5% CO_2.

3.3 Bacterial sample collection and selection

Clinical isolates of *P. aeruginosa* were collected retrospectively after the isolation and identification of *P. aeruginosa* from routine CF patient respiratory cultures performed in the clinical microbiology laboratories at Seattle Children's Hospital and the University of Washington Medical Center. The clinical standard for sputum collection is approximately every 3 months for CF patients with the option of an oropharyngeal throat swab for patients who cannot produce sputum. More frequent cultures are collected if patients are acutely ill.

Both bacterial isolates and the associated data were coded and de-identified by the CF Isolate Core prior to transfer. *P. aeruginosa* isolates from early (n=15) and chronic (n=10)

infections from CF patients were obtained from the Seattle CF Isolate Core (**Table 2**). Isolates from early infections were identified as the first positive *P. aeruginosa* culture for patients seen at Seattle Children's, whereas isolates from chronic infections were identified in patients whose sputum cultures were positive for *P. aeruginosa* for most samples for at least 4 years. A second longitudinal, clonally-related cohort of isolates was also obtained from the Seattle CF Isolate Core in which phylogenic relationships were established using previous pulsed-field gel electrophoresis data from the PASA collection of *P. aeruginosa* isolates (*261*). For this group, early (n=15) and chronic (n=16) isolates were collected at least 1.5 years apart from 7 patients (**Table 3**).

Environmental and acute, non-CF infection *P. aeruginosa* isolates were previously acquired from Dr. G. Perron (Department of Biology, University of Ottawa) and have been used by our lab (*262*). Randomly selected isolates were used to validate the relationship between induced host cell death and cytokine production, and the isolates as well as their origins are listed (**Table 4**).

WT PA14, PAO1, and *Staphylococcus aureus* subsp. *aureus* Rosenbach (ATCC® 6538™) (*S. aureus* 6538) were used as reference strains. Both PA14 and PAO1 are commonly used reference strains for pathogenesis studies, and PA14 was originally isolated from a burn patient (*263*) while PAO1 was isolated from a wound (*264*). In addition, transposon mutants of PA14 (i.e., PA14 *popB*::tn, *popD*::tn, *exoU*::tn, *exoT*::tn, *exoY*::tn, *fliC*::tn, and *exsA*::tn) were used for *in vitro* infections and were acquired from the PA14 Transposon Insertion Mutant Library (*265*). Transposon mutants were grown in LB supplemented with 20 μg/ml of gentamicin to maintain insertion.

For work investigating pathways induced by ExoU, ExoU-expressing PAO1 as well as relevant controls (i.e., WT PAO1 and PAO1+pUCP19 plasmid) were kindly provided by Drs.

Gregory Priebe and Gerald Pier from Boston Children's Hospital and Brigham and Women's Hospital. In their previously described work (*266*), PAO1 was transformed with the plasmid pUCP19exoUspcU, which contained the *exoU* gene and the gene of its associated chaperone protein *spcU*. Transformed strains were grown in LB supplemented with 400 μg/ml of carbenicillin to maintain selective pressure.

PAO1 transposon mutants for ExoS, ExoT, and ExoY as well as WT PAO1 were also acquired from the *Pseudomonas aeruginosa* PAO1 Transposon Mutant Library (*267*). Specific strain names are indicated in the relevant figure.

Table 2. *P. aeruginosa* isolates from CF patients' with early and chronic infections.

Early Isolates	Chronic Isolates
AMT0474-1	AMT0028-84
AMT0481-5	AMT0030-74
AMT0522-5	AMT0108-26
AMT0527-4	AMT0118-46
AMT0529-5	AMT0130-24
AMT0530-4	AMT0209-82
AMT0536-2	AMT0302-77
AMT0538-2	AMT0321-29
AMT0540-1	AMT0322-66
AMT0544-1	AMT0494-9
AMT0545-1	
AMT0547-1	
AMT0548-1	
AMT0549-1	
AMT0550-1	

Early isolates were defined as those having been acquired from CF patients' first positive sputum culture. Chronic isolates were defined as those having been acquired from CF patients with a 4-year history of positive sputum cultures.

Table 3. Clonally-related *P. aeruginosa* isolates from CF patients.

Early Isolates	Chronic Isolates
AMT0085-2 AMT0085-3	AMT0085-22 AMT0085-23 AMT0085-24
AMT0086-2 AMT0086-3	AMT0086-24
AMT0102-2	AMT0102-38 AMT0102-39 AMT0102-40
AMT0114-2	AMT0114-47
AMT0116-2 AMT0116-3	AMT0116-27 AMT0116-29 AMT0116-31
AMT0159-1 AMT0159-2 AMT0159-3	AMT0159-25 AMT0159-26 AMT0159-27
AMT0166-1 AMT0166-2 AMT0166-3 AMT0166-4	AMT0166-23 AMT0166-24

Early and chronic isolates were acquired longitudinally from 7 CF patients and were collected at least 1.5 years apart. Isolates were confirmed to be clonally-related using pulsed-field gel electrophoresis.

Table 4. **Isolates of *P. aeruginosa* from the environment and from clinical, non-CF infections.**

Isolate ID	Type of Isolate	Source	Location of Isolation
PR230	Environment	River Water	Woluwe, Belgium
PR231	Environment	River Water	Woluwe, Belgium
PR232	Environment	River Water	Woluwe, Belgium
PR233	Environment	River Water	Woluwe, Belgium
PR234	Environment	River Water	Woluwe, Belgium
PR235	Environment	River Water	Woluwe, Belgium
PR236	Environment	River Water	Woluwe, Belgium
PR237	Environment	River Water	Woluwe, Belgium
PR2	Clinical	Wound	Paris, France
PR3	Clinical	Wound	Paris, France
PR5	Clinical	Wound	Paris, France
PR7	Clinical	Wound	Paris, France
PR9	Clinical	Leg ulcer	Paris, France
PR80	Clinical	Urine	London, UK
PR331	Clinical	Blood	Lwiro, Congo

The type of isolate, source, and location of isolation are presented.

3.4 Bacteria growth conditions and infection of cells

All acquired bacterial isolates were streaked on LB agar and grown at 37ºC overnight. For each isolate, a single colony was selected to grow in overnight culture in LB. The liquid cultures were then diluted 1/10 to 1/25 and grown to the mid-log phase as determined by regular monitoring of the optical density measured at a wavelength of 600 nm (OD_{600}) using an Ultrospec 1100 Pro spectrophotometer (i.e., final OD_{600} ~ 0.5 for *P. aeruginosa* and ~ 0.6 for *S. aureus*). Glycerol was added to a final concentration of 20%, and the stocks were stored in -80ºC.

Prior to *in vitro* infections, bacterial stocks were streaked on LB agar, grown at 37ºC overnight, and a single colony was used to grow an overnight culture in LB. On the day of the experiment, the overnight culture was diluted 1/10 to 1/25 and grown to the mid-log phase at 37ºC. The liquid bacterial cultures were then pelleted, resuspended in R8, serially diluted, and the macrophages or bronchial epithelial cells were infected with the desired multiplicity of infection (MOI) of bacteria. When exposing cells to heat-killed bacteria, the liquid bacterial culture was boiled at 95ºC for 10 min prior to the preparation of serial dilutions. After centrifugation of cell culture with the bacteria at 1,174 x *g* for 7 min, the infections were terminated at 3 h postinfection for THP-1 cells and 6 h postinfection for bronchial epithelial cells, unless otherwise indicated in the figure legends. For time course experiments, concurrent infections were also terminated at 15-30 min or 1-h intervals for up to 3 h or 6 h, as indicated in figure legends. MOIs was confirmed by plating the greatest prepared dilution of *P. aeruginosa* on LB agar and counting the CFUs after incubation at 37°C for 24–48 h. For infections with heat-killed bacteria, the boiled culture was plated on LB agar to confirm absence of viable bacteria after the plates were incubated at 37ºC for 48 h.

3.5 Cell viability and cytokine assays

THP-1 macrophages and HBE cells were seeded in a 96-well plate at 100,000 cells/well or 30,000 cells/well, respectively. Cell viability was evaluated by measuring the lysosomal uptake of neutral red dye postinfection, as has been previously described (*262*, *268*). In brief, commercially available 0.33% neutral red solution in PBS (N2889, Sigma) was further diluted 1:20 in R8 with gentamicin. The diluted neutral red was added to the cell culture postinfection, and the plate was returned to the incubator for up to 15-20 min with periodic monitoring for dye uptake. After sufficient neutral red uptake by cell cultures was visually confirmed, the cells were washed once in PBS, and then solubilized using a solution of 1:1 deionized water:ethanol with 1% acetic acid. The absorbance of the solubilized dye was measured at 570 nm using a FilterMax F5 plate reader (Molecular Devices).

Hoechst and propidium iodide (PI) staining and fluorescent microscopic imaging were also conducted to evaluate induced host cell death. THP-1 macrophages and NuLi-1 cells were seeded in a 24-well plate at 500,000 cells/well or 150,000 cells/well, respectively. 2 h postinfection, the cells were washed with PBS, and staining was initiated. The staining solution consisted of 1:7000 dilution of Hoechst 33342 (ThermoFisher, H3570) and 1:10 dilution of PI (BD Pharmingen, 550825) in phenol red-free RPMI 1640 medium (Gibco, 11-835-030). After the staining solution was added, the plate was covered in foil and incubated at 37ºC for 25 minute. Afterwards, the cells were washed twice with PBS, and 1% paraformaldehyde was added to each well to fix the cells. Imaging using a ZOE Fluorescent Cell Imager was completed immediately, with at least 3 images captured per well. Representative images are included in this thesis.

Postinfection, supernatants were collected and frozen at -80ºC for storage. Cytokines were measured using human IL-1β (R&D Systems: DY201 or BD Biosciences: 557953), TNF (R&D

Systems: DY210 or BD Biosciences: 555212), IL-8 (R&D Systems: DY208), IL-6 (R&D: DY206) and IL-10 (BD Biosciences: 555157) ELISA kits according to the manufacturers' instructions. The final optical density was measured at 450 nm using a FilterMax F5 plate reader (Molecular Devices). All cell viability and cytokine assay experiments were performed in triplicates.

3.6 Antibodies and immunoblotting

Cell lysates were obtained using 1% sodium dodecyl sulfate (SDS) lysis buffer containing 1% β-mercaptoethanol (2-ME) at indicated time intervals for up to 3 h or 6 h for THP-1 macrophages or NuLi-1 bronchial epithelial cells, respectively. Lysis buffer containing 50 µM dithiothreitol (DTT) and 1% SDS was also used for caspase immunoblotting for samples infected with lower MOIs (*269*). For measuring protein in supernatants, concentrated lysis buffer was added to collected supernatant to final concentrations previously mentioned. Cell lysates and supernatant with lysis buffer were then boiled for 5 min, stored at -20ºC and subjected to western blot analysis using a standard protocol. 8-15% polyacrylamide gel (SDS-PAGE) were poured and set with stacking gels. 15-25µL of sample per well were loaded, and the gel was run at 135 V for up to 1.5 h. The proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane via wet transfer using 20% methanol transfer buffer. Membranes were blocked in 5% bovine albumin serum (BSA) in Tris buffered saline (TBS, 0.5M Tris 1.5M sodium chloride pH 7.6) with 0.1% Tween-20 (TBST) for 1 h at room temperature while on a rocker. Membranes were then left in 4ºC overnight on a rocker in diluted primary antibody. Following overnight incubation, washes were conducted with TBST, and the membranes were allowed to incubate in secondary antibody diluted in the same blocking buffer for 40 min to 1 h at room temperature while on a rocker. Finally, washes with the same buffer were conducted, and the blots were developed with an enhanced chemiluminescence substrate (Thermo Scientific), including Pierce™ ECL Western

Blotting Substrate (32109), SuperSignal™ West Pico PLUS Chemiluminescent Substrate (34579), or SuperSignal™ West Femto Maximum Sensitivity Substrate (34094). Bands were identified by exposure of the membrane to X-ray film (Kodak, CareStream Health), and any densitometry performed was completed with Image-J 1.48 software (Maryland, USA).

Primary and secondary antibodies used for immunoblotting can be found in **Table 5**. All primary antibodies were diluted 1:1000 in 5% BSA in TBST and 0.02% sodium azide, while secondary antibodies were diluted to 1:5000 in 5% BSA in TBST.

Table 5. Primary and secondary antibodies used for immunoblot analysis.

Antibody	Source	Catalog #	Dilution	Buffer	Expected Band Size (kDa)
Rabbit anti-phospho-NF-κB p65 (Ser536) (93H1)	Cell Signaling	#3033	1:1000	5% BSA	65
Rabbit anti-NF-κB p65 (D14E12)	Cell Signaling	#8242	1:1000	5% BSA	65
Rabbit anti-phospho-MAPKAPK-2 (Thr334) (27B7)	Cell Signaling	#3007	1:1000	5% BSA	49
Rabbit anti-MAPKAPK-2	Cell Signaling	#3042	1:1000	5% BSA	47, 49
Rabbit anti-phospho-p38 MAPK (Thr180/Tyr182) (D3F9)	Cell Signaling	#9211	1:1000	5% BSA	43
Rabbit anti-p38 MAPK	Cell Signaling	#9212	1:1000	5% BSA	43
Rabbit anti-cleaved caspase-1(Asp297) (D57A2)	Cell Signaling	#4199	1:1000	5% BSA	20, 22
Rabbit anti-caspase 1 (D7F10)	Cell Signaling	#3866	1:1000	5% BSA	48, 20
Rabbit anti-caspase-4	Cell Signaling	#4450	1:1000	5% BSA	45, 20
Rabbit anti-caspase-5 (D3G4W)	Cell Signaling	#46680	1:1000	5% BSA	50, 44, 35, 20
Mouse anti-caspase-8 (8CSP03)	Santa Cruz Biotechnology	sc-56070	1:1000	5% BSA	55, 18, 10
Rabbit anti-Gasdermin D (E9S1X)	Cell Signaling	#39754	1:1000	5% BSA	53, 30
Mouse anti-β-actin (8H10D10)	Cell Signaling	#3700	1:1000	5% BSA	45
Anti-rabbit IgG, HRP-linked	Cell Signaling	#7074	1:5000	5% BSA	
Anti-mouse IgG, HRP-linked	Cell Signaling	#7076	1:5000	5% BSA	

3.7 NF-κB detection with reporter cell line

In vitro treatments with heat-killed *P. aeruginosa* were conducted with the THP1-Lucia™ NF-κB macrophages seeded in 96-well plates, as previously described for the THP-1 cells, and performed in triplicates. 3 h postinfection, 20 µL of the supernatant from each well was transferred to a white opaque plate (ThermoFisher Scientific, 236105). In each well, 50 µL of QUANTI-Luc™ (InvivoGen, rep-qlc1) was added, and luminescence was immediately measured using a FilterMax F5 plate reader (Molecular Devices).

3.8 RNA extraction and quantitative reverse transcription PCR (RT-qPCR) analysis

THP-1 macrophages were infected with select *P. aeruginosa* isolates from CF patients for 1 h. RNA extractions were conducted using an RNeasy Mini Kit (Qiagen, 74004) as per manufacturer's instructions, and eluted RNA was stored at -80°C prior to use. cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, 1708890) according to manufacturer's instructions. Quantitative real-time PCR (qPCR) reactions were conducted in triplicates with a total volume of 20 µl per reaction using: 2 µl cDNA, 10 µl SYBR green PCR master mix (Bio-Rad), and 100 pmol of forward and reverse primers for each gene of interest (**Table 6**). The following thermal cycler conditions were used: 10 min at 95°C followed by 35 cycles of 15 s at 95°C, 30 s at 55-58°C, and 30 s at 72°C. mRNA levels of target genes were normalized to human β-actin for each sample, and the fold-change in mRNA postinfection was compared with the uninfected control using the $2^{-\Delta\Delta}CT$ method.

Table 6. Primers for cytokine and reference gene targets.

Gene Target	Primers
Human IL-1β	Forward: 5'-CCACAGACCTTCCAGGAGAATG-3' Reverse: 5'-GTGCAGTTCAGTGATCGTACAGG-3'
Human TNF	Forward: 5'-CTCTTCTGCCTGCTGCACTTTG-3' Reverse: 5'-ATGGGCTACAGGCTTGTCACTC-3'
Human IL-8	Forward: 5'-GAGAGTGATTGAGAGTGGACCAC-3' Reverse: 5'-CACAACCCTCTGCACCCAGTTT-3'
Human IL-10	Forward: 5'-TCTCCGAGATGCCTTCAGCAGA-3' Reverse: 5'-TCAGACAAGGCTTGGCAACCCA-3'
Human β-actin	Forward: 5'-GACTTCGAGCAAGAGATGGC-3' Reverse: 5'-CACAGGACTCCATGCCCAG-3'

3.9 Inhibitors

A comprehensive list of inhibitors, their function, and final concentrations used can be found in **Table 7**. All inhibitors were dissolved in DMSO. MCC950 and ralimetinib were both added to the cell culture at the start of *in vitro* infections for the 3 h or 6 h duration of the experiment. For treatments with GSK872, Z-IETD-FMK, Ac-YVAD-cmk, and emricasan, THP-1 macrophages and NuLi-1 were pre-treated for 2 h prior to the start of the infection as well as treated over the course of infections. $CFTR_{inh}$-172 was used at a concentration of 10 μM as previously reported (*270–272*) for overnight treatment prior to *in vitro* infections as well as during the 3 h infection for THP-1 cells and 6 h infections for NuLi-1 cells. A DMSO vehicle control was used for all experiments at a 1:1000 dilution in R8 or BEGM.

3.10 Statistical analyses

Each experiment was conducted at least twice with experimental triplicates for cell viability assays and ELISAs. The data shown throughout this thesis are derived from representative experiments. GraphPad Prism 8 (GraphPad Software, CA, USA) was used for statistical analyses. Means were compared using either unpaired Student's t-test or Mann–Whitney U test for comparing two groups or one-way ANOVA (with post-hoc Dunnett's multiple comparison tests) for comparing multiple groups. Simple linear regressions were conducted to determine correlations between percent cell viability and the production of cytokines. Densitometric analyses were completed using with Image-J 1.48 software (Maryland, USA), where the signal of phosphorylated or cleaved proteins were compared with the signal of the respective total protein. The level of significance was set at $P < 0.05$. The following P value codes were used for all figures: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

Table 7. Inhibitors used in experiments.

Inhibitor	Source and Catalog #	Final Concentration Used (μM)	Notes
MCC950	Calbiochem (256373-96-3)	10	NLRP3 inhibitor
Ralimetinib (LY2228820)	Selleckchem (S1494)	0.1	p38 MAPK inhibitor
GSK872 (GSK2399872A)	Glixx Laboratories (GLXC-03990)	5	RIP3 kinase inhibitor
Z-IETD-fmk	Apexbio (B3232)	10	Caspase-8 inhibitor
Ac-YVAD-cmk	InvivoGen (inh-yvad)	10	Caspase-1 inhibitor
Emricasan	SelleckChem (IDN-6556)	10	Pan-caspase inhibitor
$CFTR_{inh}$-172	Sigma (C2992)	10	CFTR inhibitor

4.0 RESULTS

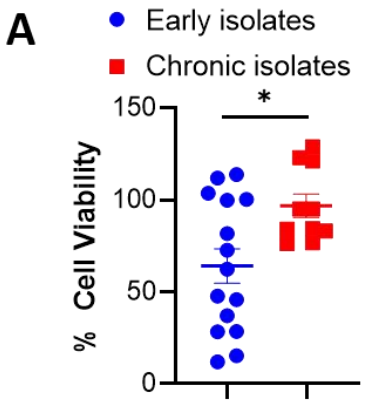
4.1 Differences in inflammasome activation and cytokine expression for *P. aeruginosa* isolated from early versus chronic CF respiratory infections

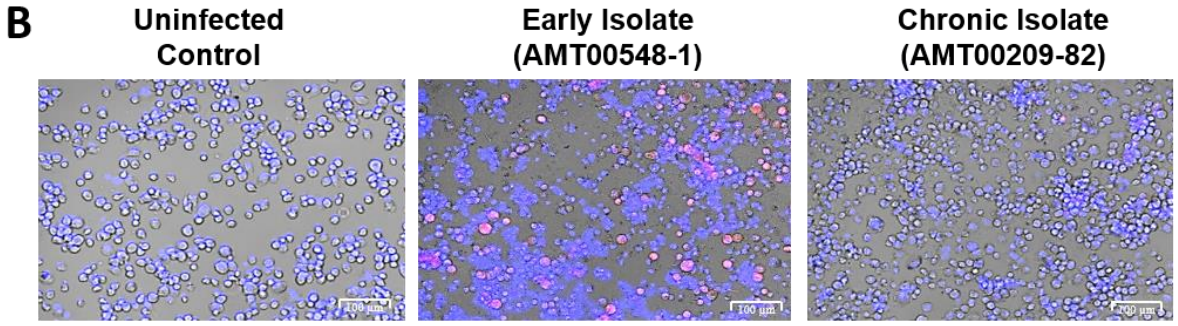
4.1.1 P. aeruginosa *isolates from chronically infected CF patients induce relatively little macrophage and bronchial epithelial cell death but higher cytokine expression compared with early isolates*

P. aeruginosa isolates from patients with CF who either had their first positive sputum culture for *P. aeruginosa* (i.e., early isolates) (n=15 patients), or at least a 4-year history of positive sputum cultures (i.e., chronic isolates) (n=10 patients) were obtained (**Table 2**). As a primary *in vitro* infection model, I used the THP-1 monocyte cell line and differentiated the cells with PMA into a macrophage phenotype to simultaneously test all bacterial samples obtained from CF patients. Infections of THP-1-derived macrophages (i.e., THP-1 macrophages) *in vitro* with early or chronic isolates of *P. aeruginosa* elicited significant differences in host cell death and cytokine expression. Namely, isolates from chronic respiratory infections induced less cell death and lower IL-1β expression than did early isolates (**Fig. 6 A-C**). In contrast, the production of other inflammatory cytokines (i.e., TNF, IL-6 and IL-8) was increased upon infection by chronic infection isolates compared with early isolates (**Fig. 6 C**). It was also of interest to observe the expression of anti-inflammatory cytokines in response to *P. aeruginosa* isolated from different stages of respiratory infections. Expression of the anti-inflammatory cytokine IL-10 was also increased during infections with chronic isolates (**Fig. 6 C**). To observe whether this trend could be seen in primary cells, primary human macrophages from the PBMCs of healthy volunteers were generated and infected with PA14 or select clinical isolates, 2 each from the early and chronic groups. PA14 is a commonly used laboratory reference strain for bacteriology and pathogenesis

studies, which were originally isolated from a burn patient (*263*). I found that at 3 h postinfection, PA14 and early clinical isolates induced greater levels of cell death and IL-1β expression, but poor expression of TNF, IL-8, and IL-6 (**Fig. 7**).

Given the range of host cell types found in the lung environment, I also used an available bronchial epithelial cell line NuLi-1 for this work. NuLi-1 cells are an immortalized cell line derived from a donor who was homozygous for WT CFTR (*273*). After infecting NuLi-1 cells for 6 h at an MOI of 100 to induce adequate levels of host cell death, it was found that early isolates also induced more cell death than infections with chronic isolates (**Fig. 8 A**). As with infection with THP-1 macrophages, NuLi-1 cells infected with chronic *P. aeruginosa* isolates induced less IL-1β production but more IL-6 production (**Fig. 8 B, C**). No statistically significant difference was seen for IL-8 production, and TNF as well as IL-10 production were undetectable (**Fig. D-F**). Therefore, given how the NuLi-1 bronchial epithelial cells failed to express appreciable levels of inflammatory cytokines, THP-1 macrophages were predominantly used for further study in comparing inflammatory mechanisms induced by isolates from early versus chronic infections.


A
Early isolates
Chronic isolates
*
% Cell Viability
150
100
50
0


B
Uninfected Control
Early Isolate (AMT00548-1)
Chronic Isolate (AMT00209-82)

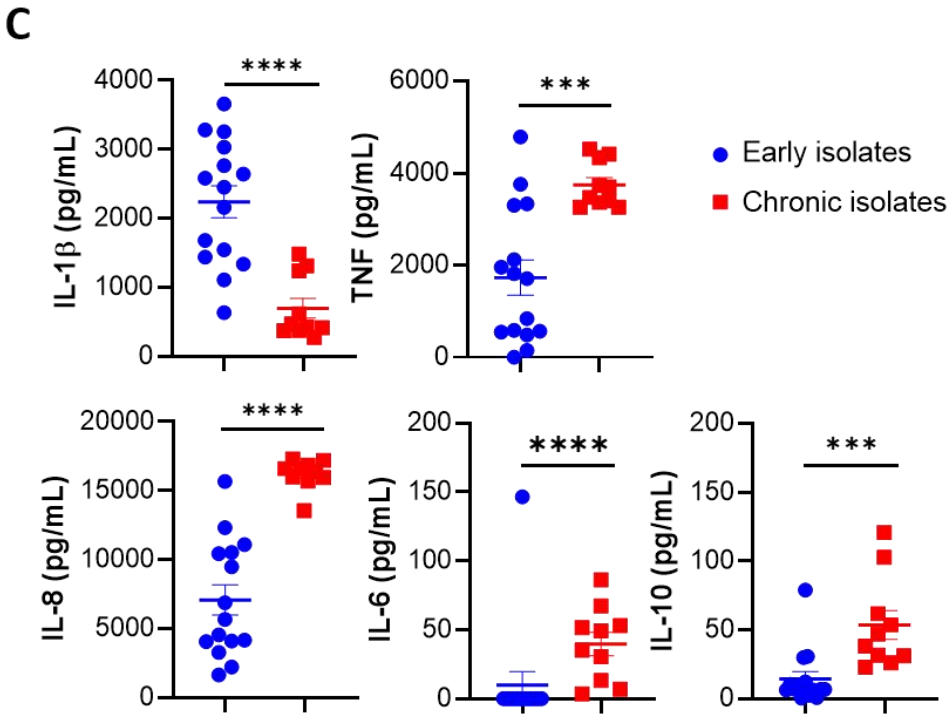

C
IL-1β (pg/mL)
4000
3000
2000
1000
0
****
TNF (pg/mL)
6000
4000
2000
0
***
Early isolates
Chronic isolates
IL-8 (pg/mL)
20000
15000
10000
5000
0
****
IL-6 (pg/mL)
200
150
100
50
0
****
IL-10 (pg/mL)
200
150
100
50
0
***

Fig. 6. *P. aeruginosa* isolates from chronic CF infections induce less THP-1 macrophage cell death but increased expression of TNF, IL-8, and IL-6 compared with early isolates. Early isolates were defined as isolates from the first positive *P. aeruginosa*-positive sputum culture, while chronic isolates were from patients who had 4 years of positive sputum cultures. THP-1 macrophages were seeded in fresh R8 at 100,000 cells/well in a 96-well plate and infected with P. aeruginosa isolates from CF patients with early or chronic respiratory infections at 1 MOI and in experimental triplicates. (**A, C**) At 3 h postinfection, cell viability was evaluated by neutral red assay, and cytokine production was measured in the supernatants by ELISA. Mean ± SEM are shown. Student's t-tests were used to compare the means for cell viability, TNF, and IL-8 while Mann–Whitney U tests were used to compare the mean ranks for IL-1β, IL-6, and IL-10. THP-1 macrophages were seeded at 500,000 cells per well and infected with P. aeruginosa (early or chronic isolate) at 10 MOI. (**B**) At 2 h postinfection, cells were stained with Hoechst/PI to distinguish live (blue) from dead (pink) cells. Data shown are representative of 3 independent experiments. (**P* < 0.05, ****P* < 0.001, *****P* < 0.0001).

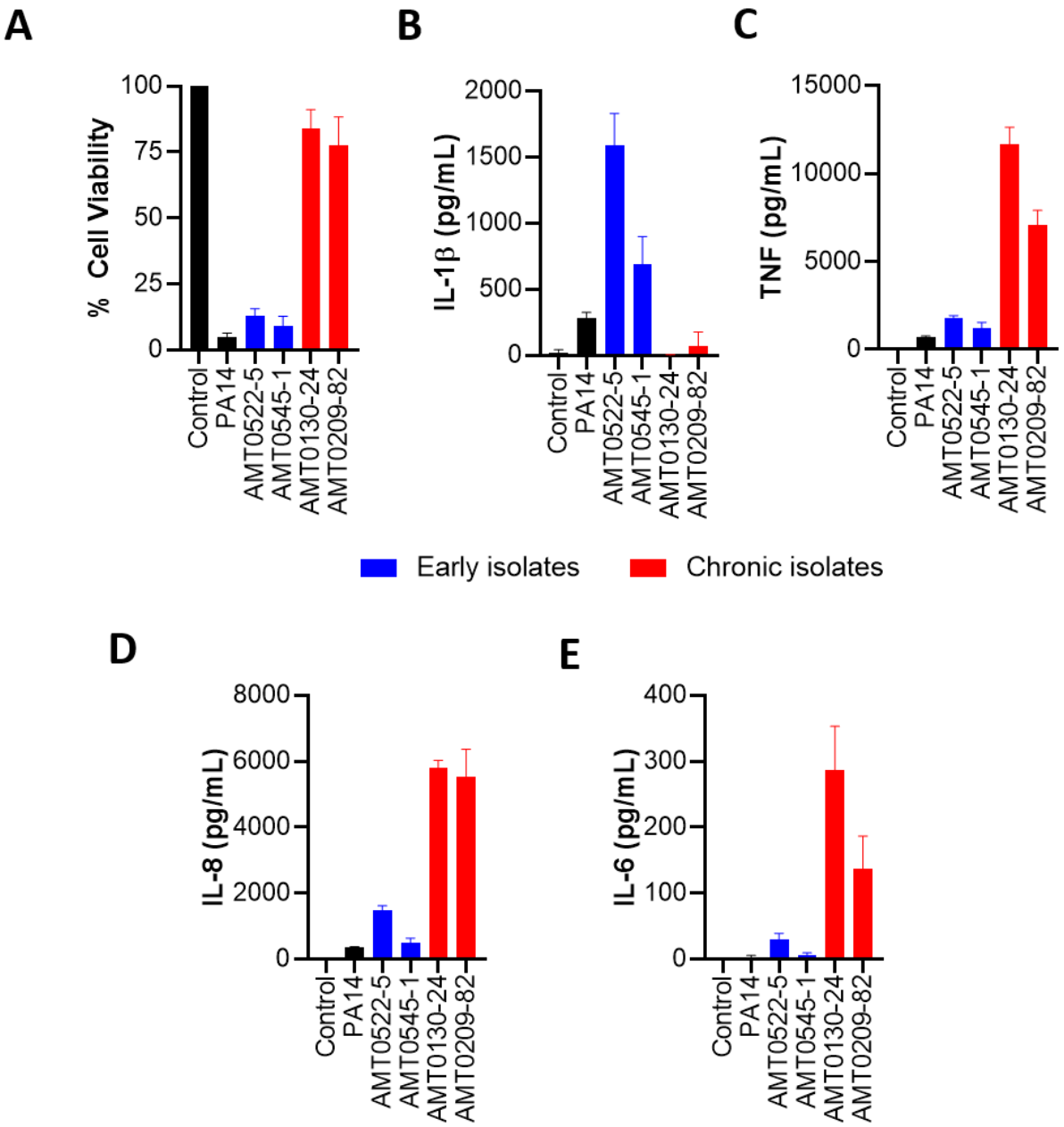


Fig. 7. *P. aeruginosa* isolates from chronic CF infections induce less primary macrophage cell death but increased expression of TNF, IL-8, and IL-6 compared with early isolates. Primary human macrophages were seeded at 50,000 cells/well and infected with the indicated early and chronic isolates of *P. aeruginosa* or PA14 (10 MOI). (**A**) Cell viability was measured via neutral red assay and (**B**-**E**) cytokine production was measured via ELISA at 3 h postinfection. Mean ± SD of experimental triplicates are shown from a representative experiment.

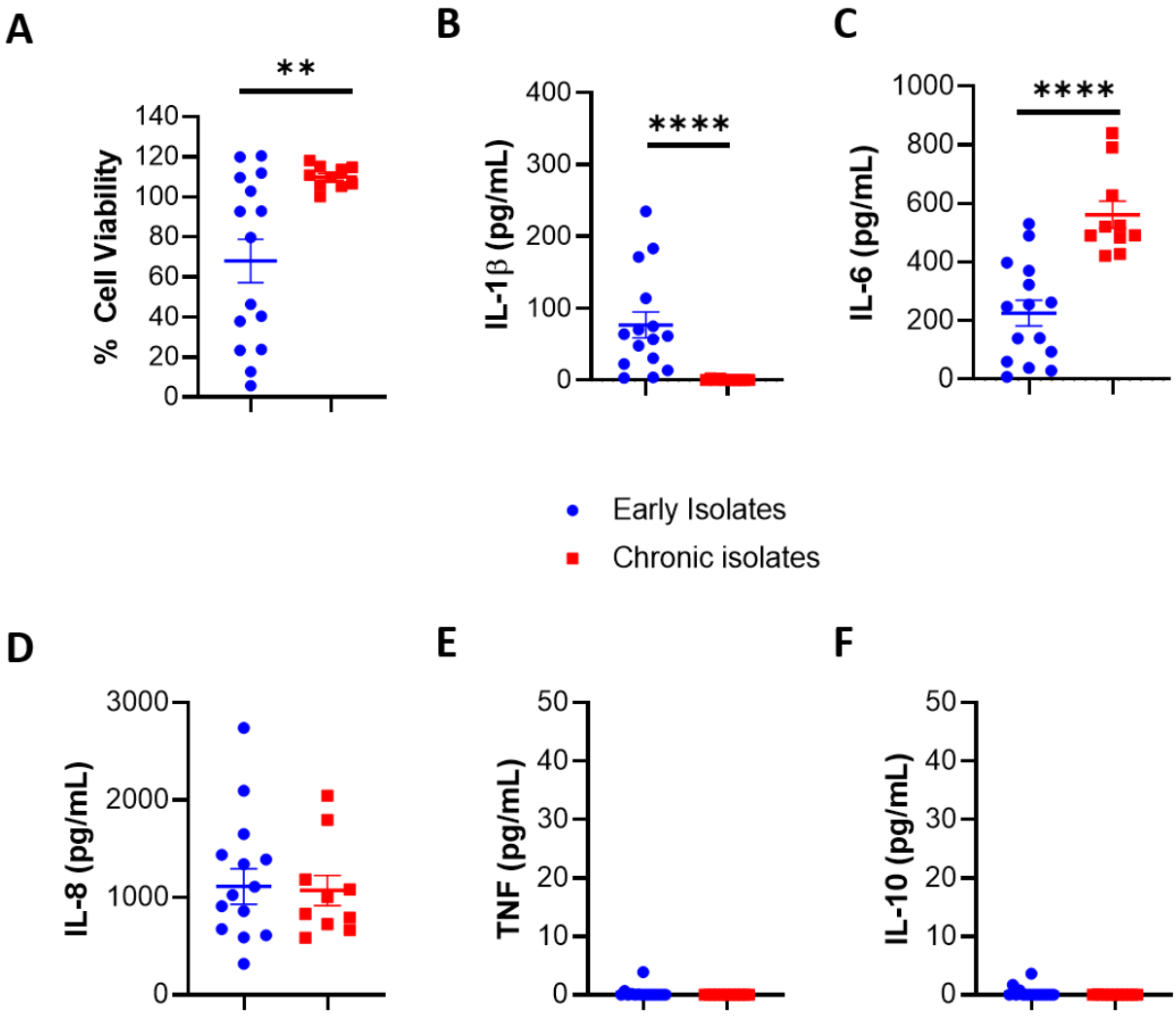


Fig. 8. *P. aeruginosa* isolates from chronic CF infections induce less NuLi-1 cell death and IL-1β secretion but increased expression of IL-6 compared with early isolates. NuLi-1 bronchial epithelial cells were seeded at 30,000 cells/well in a collagen-coated 96-well plate and infected with the same cohort of early and chronic *P. aeruginosa* isolates at 100 MOI and in experimental triplicates. (**A**) Cell viability via neutral red assay and (**B-F**) cytokine production in supernatants via ELISA were measured 6 h postinfection. Mean ± SEM are shown. Mann–Whitney U tests were used to compare the mean ranks for IL-6 while Student's t-tests were used to compare the means for cell viability and IL-1β. Data shown are representative of 3 independent experiments. (**$P < 0.01$, ****$P < 0.0001$).

While it could be appreciated that *P. aeruginosa* from early infections could induce relatively higher levels of cell death, I sought to determine how quickly this host cell death could occur. Conducting a time course experiment revealed that some isolates from early infections induced cell death within 1 h postinfection at an MOI of 10 (**Fig. 9 A)**. IL-1β production was generally correlated with level of cell death (**Fig. 9 B**). Once again, among this group of isolates, those that induced relatively high levels of THP-1 macrophage death also demonstrated limited expression of TNF and IL-8 compared with isolates that did not induce as high a magnitude of cell death (**Fig. 9 C, D**).

To determine if indeed host cell death could be a main contributor to the observed differences in cytokine production, THP-1 macrophages were also exposed to heat-killed bacteria. This adequately controlled for cell death, as I observed that any differences in cell death and cytokine production were reduced (**Fig. 10**) with the exception of TNF expression (**Fig. 10 C**). This suggests the possibility that there are heat-resistant bacterial factors which could induce TNF expression during *P. aeruginosa* infections, where isolates from chronic respiratory infections were still able to induce higher TNF levels. As well, it was necessary to determine if *P. aeruginosa* isolates from chronic infections induced greater transcription of cytokines or if it was that isolates from early infections interfered with cytokine processing and secretion through inducing rapid host cell death. Using randomly selected clinical isolates to infect THP-1 macrophages (5 from early infections and 5 from chronic infections), I measured mRNA levels of IL-1β, TNF, IL-8, and IL-10 through qRT-PCR analysis. It was found that at 1 h postinfection, there were no differences between the levels of mRNA induced during infections using early or chronic isolates (**Fig. 11 A-D**), suggesting that any differences in the protein levels of the measured cytokines in the supernatant of infected cells may be due to interruptions in cytokine processing.

I also used a collection of *P. aeruginosa* isolates from a second cohort of CF patients, where the isolates were obtained from 7 patients longitudinally (i.e., at different time intervals) to see if the same trends could be observed using isolates collected from a single group of patients over time (**Table 3**). In identifying these isolates, the "chronic" isolates was collected at least 1.5 years after the "early" isolates and were confirmed to be clonally-related. After infecting THP-1 macrophages with these isolates, I observed that the isolates reflecting chronic infection stages induced less cell death and IL-1β but increased expression of TNF and IL-8 (**Fig. 12**). Therefore, consistency in cell death and cytokine production trends between these two cohorts of *P. aeruginosa* from CF patients was seen. The observed magnitude in differences between the two groups was smaller than observed for the first cohort of isolates from CF patients. This may have been due to the fact that less time may have passed for this second cohort than the first, where it was at least 1.5 years as opposed to at least 4 years, allowing fewer *P. aeruginosa* adaptations to evade inducing host cell death to take place. As clearer differences were seen upon using the first cohort of *P. aeruginosa* isolates, most of the following work continued to use this group or the randomly selected subset of this group.

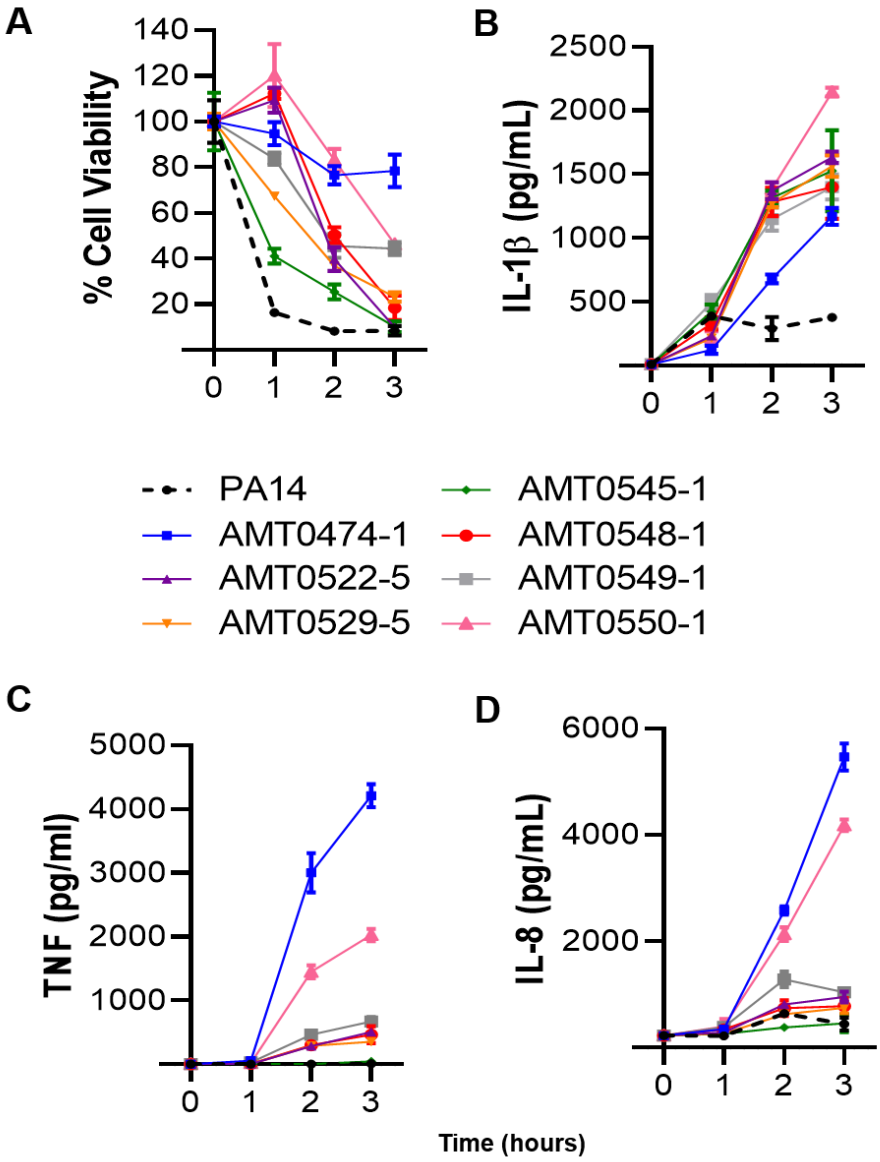


Fig. 9. Early CF infection *P. aeruginosa* isolates induce death as early as 1 h postinfection. THP-1 macrophages were seeded at 100,000 cells/well in a 96-well plate and infected with the indicated early infection isolates of *P. aeruginosa* at an MOI of 10. (**A**) At the time intervals shown, cell viability was evaluated by neutral red assay. (**B-D**) Expression of cytokines was measured in supernatants at various time intervals by ELISA. Mean ± SD of experimental triplicates are shown. Data shown are representative of 3 independent experiments.

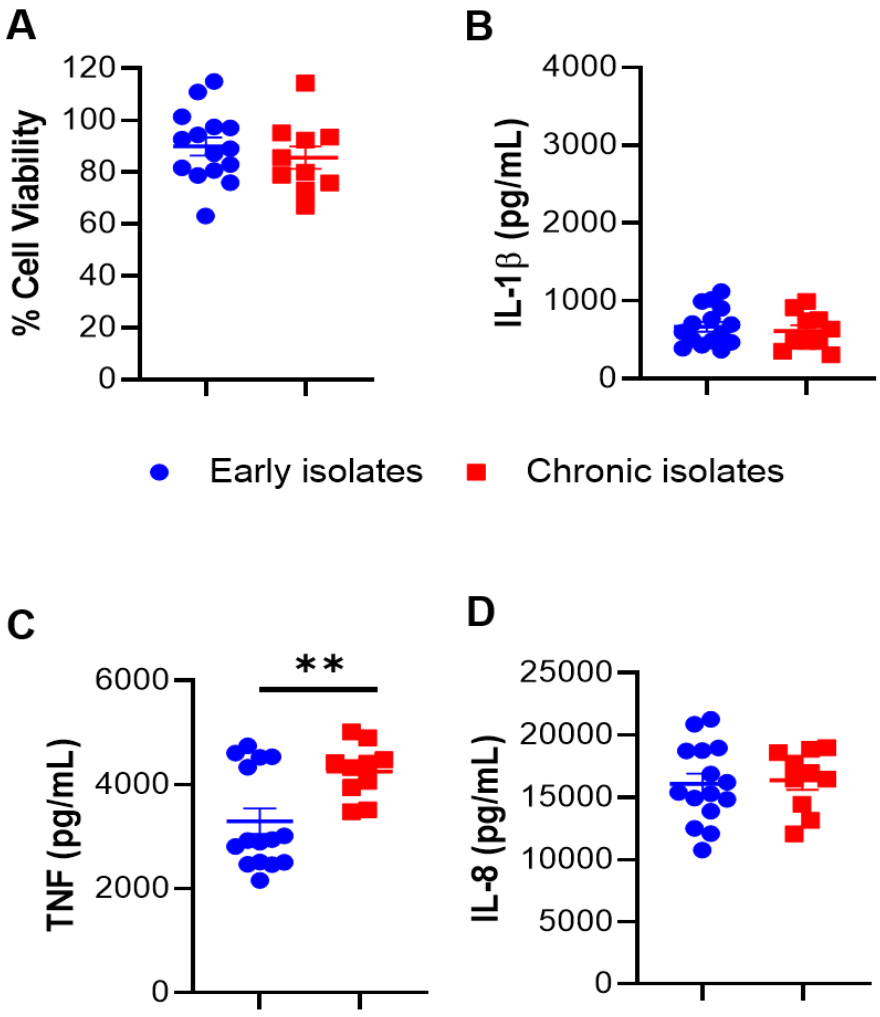


Fig. 10. The inverse correlation of cell death versus expression of inflammatory cytokines was abrogated with heat-killed bacteria. Clinical *P. aeruginosa* isolates described in Methods and Fig. 1 were heat-killed and incubated with THP-1 macrophages seeded at 100,000 cells/well in a 96-well plate (1 MOI). (**A**) Cell viability was measured via neutral red assay and (**B-D**) cytokine production was measured via ELISA at 3 h postinfection. Mean ± SEM are shown, and Student's t-tests were used to compare the means. Data shown are representative of 3 independent experiments. (**$P < 0.01$).

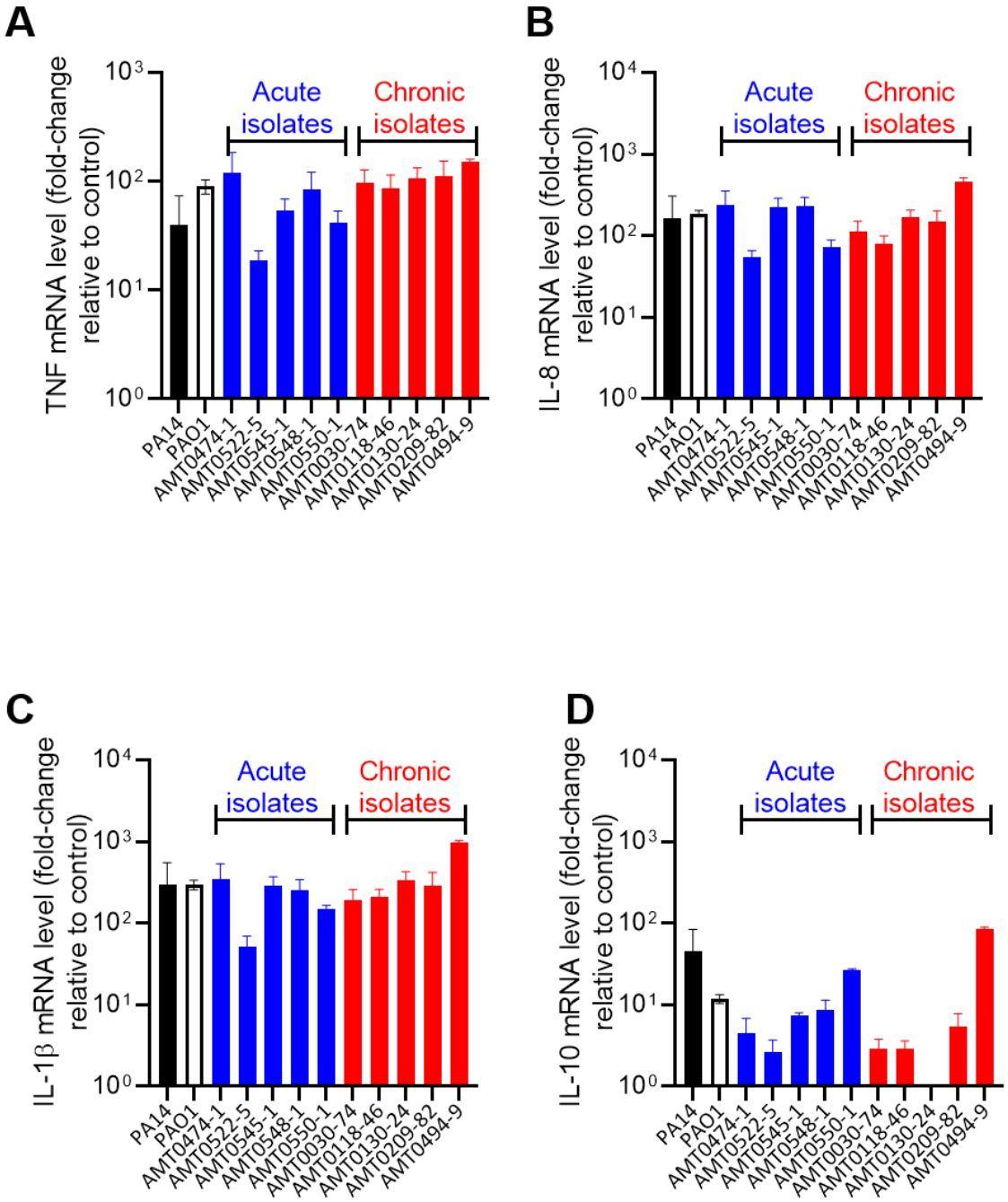

A
TNF mRNA level (fold-change relative to control)
Acute isolates
Chronic isolates
PA14
PAO1
AMT0474-1
AMT0522-5
AMT0545-1
AMT0548-1
AMT0550-1
AMT0030-74
AMT0118-46
AMT0130-24
AMT0209-82
AMT0494-9
B
IL-8 mRNA level (fold-change relative to control)
C
IL-1β mRNA level (fold-change relative to control)
D
IL-10 mRNA level (fold-change relative to control)

Fig. 11. Chronic *P. aeruginosa* isolates do not induce different levels of cytokine mRNA expression compared with early isolates. THP-1 macrophages were seeded at 500,000 cells/well in a 24-well plate. At 1 h postinfection (1 MOI) with the indicated acute and chronic isolates of *P. aeruginosa* from CF patients. RNA was extracted and cDNA was synthesized. (**A-D**) qPCR was conducted on samples targeting genes for various cytokines, mRNA levels were normalized to β-actin, and fold-change expression was compared with the uninfected control. Mean ± SD of experimental triplicates from a representative experiment are shown.

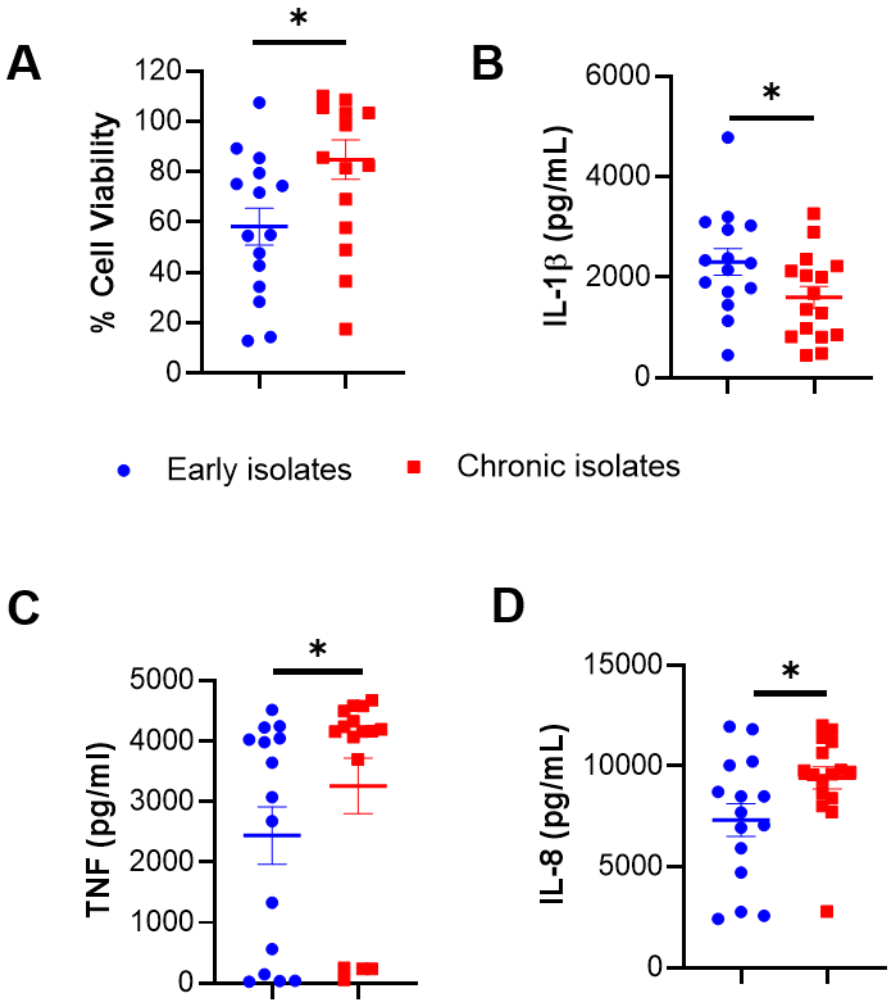


Fig. 12. *P. aeruginosa* isolates from chronic CF infections that were longitudinally acquired induce less THP-1 macrophage death but increased expression of TNF, IL-8, and IL-6 compared with early isolates. THP-1 macrophages were seeded at 100,000 cells/well in a 96-well plate and infected with early and chronic longitudinal isolates of *P. aeruginosa* from CF patients collected at least 1.5 years apart. Infections were performed in experimental triplicates. At 3 h postinfection with live bacteria (1 MOI), (**A**) cell viability and (**B-D**) cytokines were measured via ELISA. Student's t-tests were used to compare the means for cell viability and IL-1β, while Mann–Whitney U tests were used to compare TNF and IL-8. Mean ± SEM are shown. Data shown are representative of 3 independent experiments. (*$P < 0.05$).

4.1.2 Chronic P. aeruginosa *isolates from CF patients poorly activate inflammasome signalling*

Previous work from our lab demonstrated that *P. aeruginosa* isolated from CF patients induces both inflammasome-dependent and -independent cell death in murine macrophage (*262*). Therefore, I evaluated the involvement of caspase-1 activation in cell death and IL-1β production in human THP-1 macrophages infected with early and chronic CF infection isolates of *P. aeruginosa*. Caspase-1 and caspase-5 were potently activated by the two laboratory reference strains PA14 and PAO1 and the select early CF respiratory infection isolates of *P. aeruginosa* (**Fig. 13 A**). In contrast, there was poorer activation of caspase-1, caspase-5 and caspase-8 in macrophages infected by the chronic *P. aeruginosa* isolates compared with early isolates (**Fig. 13 A, B**). Caspase-8 plays a role in driving inflammasome-dependent and inflammasome-independent cell death, suggesting that poor activation of caspase-8 by chronic isolates may impact cell death of infected cells through multiple mechanisms. Given the prominent role of caspase-1 in inflammasome signalling in general and the differences observed in the activation of caspase-1 with early and chronic isolates, I used a THP-1 cell line that is 7-fold deficient in caspase-1 (THP1-defCASP1). To validate the knockdown of active caspase-1 in this cell line, I conducted an immunoblot using the reference strains PA14 and PAO1. Activation of caspase-1 was detectable in the cell culture of WT THP-1 cells but not in THP-1-defCASP1 cells following infection with PA14 and PAO1 (**Fig. 14 A**). Using the clinical CF isolates, the early *P. aeruginosa* isolates induced more cell death of both WT THP-1 and THP1-defCASP1 cells in comparison to chronic isolates. Considering how this induced cell death by early isolates was similar between the WT THP-1 macrophages and THP1-defCASP1 macrophages, the observed cell death appeared to be primarily mediated by a non-caspase-1 mechanism regardless of whether the cells were infected with early or chronic isolates (**Fig. 14 B**). The activation of inflammatory caspases that were of

interest for this work, particularly caspase-1 during canonical inflammasome activation, results in the maturation of IL-1β through cleavage of pro-IL-1β. THP-1-defCASP1 cells demonstrated significant reductions in IL-1β expression in comparison to WT THP-1 cells upon infection with either early or chronic infection isolates (**Fig. 14 B**). Therefore, while caspase-1 likely does not play a major role in cell death during infection with clinical *P. aeruginosa* isolates from CF patients, canonical inflammasome activation appears to play a role in IL-1β maturation and secretion. To further validate these findings using the reference strains, I observed that reducing the availability of active caspase-1 reduced the observed cell death of THP-1 cells at lower MOIs and more appreciably for the PAO1 strain (**Fig. 14 C, D**). In contrast to cell death, caspase-1 played a significant role in IL-1β secretion during *P. aeruginosa* infection for both PA14 and PAO1 (**Fig. 14 C, D**).

Experimental procedures described here involving THP-1 cells involved examining inflammatory mechanisms that occured for up to 3 h postinfection, as this is often a sufficient amount of time to observe appreciable levels of cell death by more virulent strains of *P. aeruginosa*. However, I sought to see if and how cell death trends and cytokine expression could change if the amount of time for the *in vitro* infections was extended past 3 h, especially given the length of time these infections likely happen in more clinical settings. To do so, I used the same randomly selected 10 *P. aeruginosa* isolates from early and chronic infections (5 in each group) to see how host cell death and cytokine production could change with increased infection time to 6 h. Interestingly, increasing the length of infection from 3 h to 6 h resulted in increased cell death for infections with some of the early isolates (3 out of 5) but not for infections with chronic isolates (4 out of 5) (**Fig. 15 A**). With the increased infection time, IL-1β and TNF levels did not consistently increase at 6 h postinfection compared with 3 h, whereas IL-6 and IL-10 production

did increase (**Fig. 15 B-F**). This suggests IL-1β and TNF are produced fairly early in response to *P. aeruginosa* infection and that expression is not continuously sustained as the infection is prolonged. Trends for IL-8 were more subtle upon studying this particular subset of isolates (**Fig. 15 D**). Otherwise, it was noted that trends between early and chronic infection isolates in cell death and cytokine expression that were previously observed remained relatively stable even with an increased infection time among this subset of isolates (**Fig. 15 A-F**).

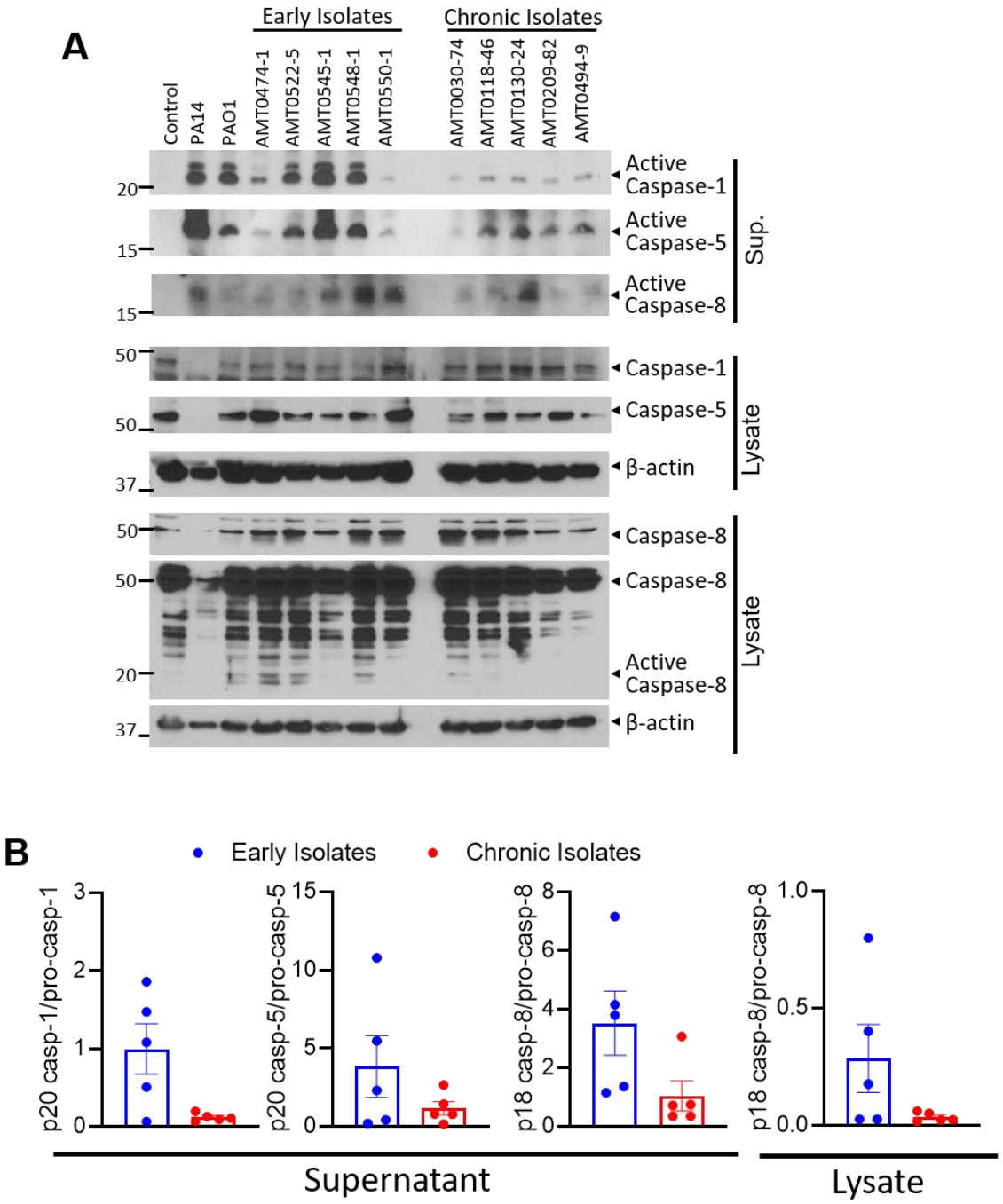

A
Early Isolates
Chronic Isolates
Control
PA14
PAO1
AMT0474-1
AMT0522-5
AMT0545-1
AMT0548-1
AMT0550-1
AMT0030-74
AMT0118-46
AMT0130-24
AMT0209-82
AMT0494-9
20
15
15
50
50
37
50
50
20
37
Active Caspase-1
Active Caspase-5
Active Caspase-8
Sup.
Caspase-1
Caspase-5
β-actin
Lysate
Caspase-8
Caspase-8
Active Caspase-8
β-actin
Lysate
B
Early Isolates
Chronic Isolates
p20 casp-1/pro-casp-1
p20 casp-5/pro-casp-5
p18 casp-8/pro-casp-8
p18 casp-8/pro-casp-8
Supernatant
Lysate

Fig. 13. *P. aeruginosa* infections from early CF infections potently induce activation of caspase-1, -5, and -8. (**A**) THP-1 macrophages were seeded at 500,000 cells/well in a 24-well plate. At 3 h postinfection (1 MOI) with the indicated acute and chronic isolates of *P. aeruginosa* from CF patients, western blotting was performed on cell supernatants or extracts using specific antibodies shown in the figure. (**B**) Densitometric analysis of western blots shown in panel A were completed, and mean ± SEM are shown. Immunoblot shown is a representative figure from 3 independent experiments.

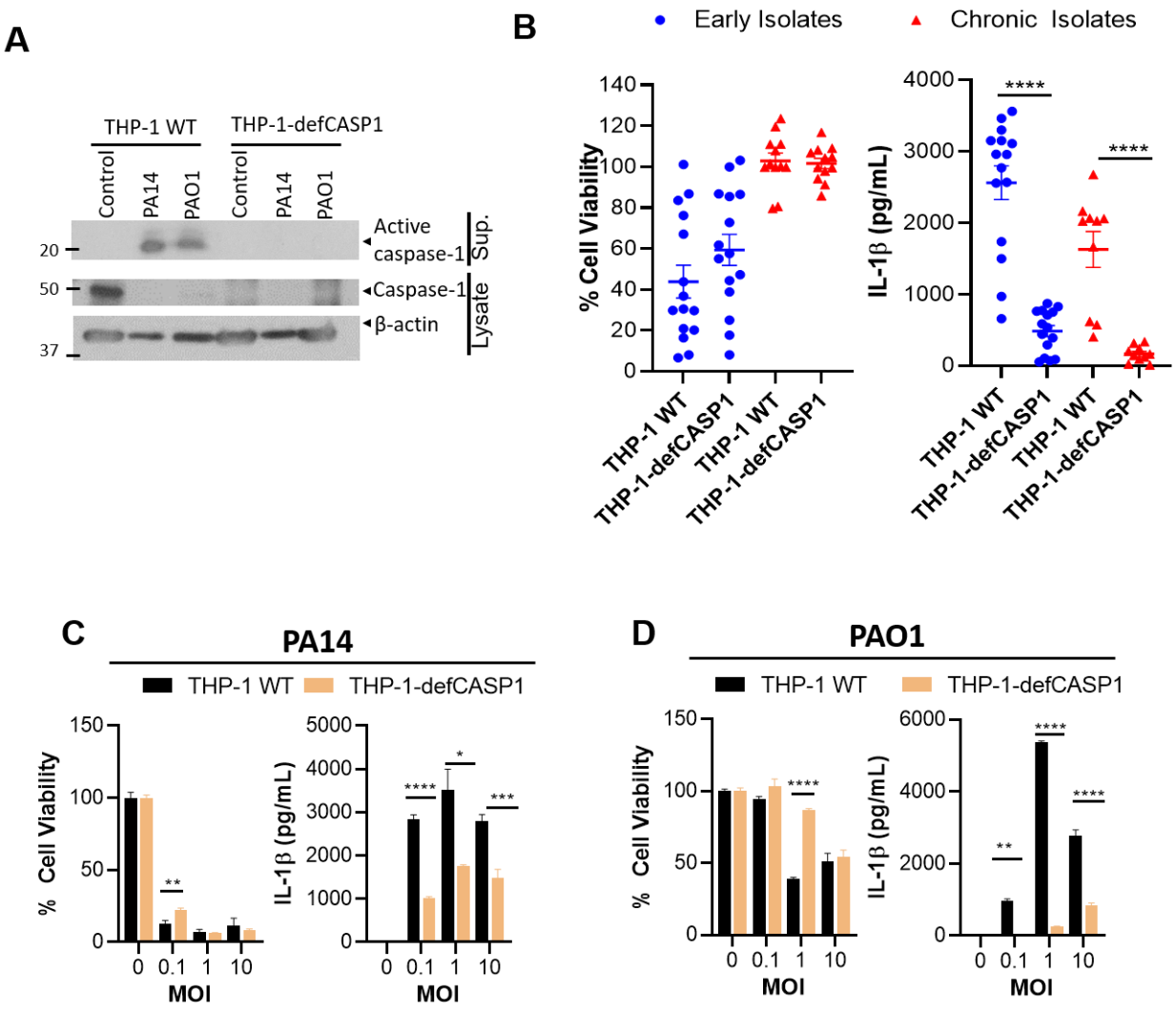

A
THP-1 WT
THP-1-defCASP1
Control
PA14
PAO1
Control
PA14
PAO1
20
50
37
Active caspase-1
Caspase-1
β-actin
Sup.
Lysate
B
Early Isolates
Chronic Isolates
% Cell Viability
IL-1β (pg/mL)
****
****
THP-1 WT
THP-1-defCASP1
THP-1 WT
THP-1-defCASP1
C
PA14
THP-1 WT
THP-1-defCASP1
% Cell Viability
IL-1β (pg/mL)
MOI
MOI
D
PAO1
THP-1 WT
THP-1-defCASP1
% Cell Viability
IL-1β (pg/mL)
MOI
MOI

Fig. 14. Cell death induced by *P. aeruginosa,* but not IL-1β expression, is independent of caspase-1. (**A**) THP-1 WT and THP1-defCASP1 were seeded at 500,000 cells/well in a 24-well plate and infected with PA14 or PAO1 (1 MOI). Immunoblotting was performed on supernatants or cell extracts at 3 h postinfection. (**B**) THP-1 WT and THP1-defCASP1 were seeded at 100,000 cells/well in a 96-well plate and infected with early and chronic *P. aeruginosa* CF isolates (1 MOI) in experimental triplicates. Cell viability was evaluated at 3 h postinfection by neutral red assay, and IL-1β production was measured in supernatants by ELISA. Mean ± SEM are shown. Mann–Whitney U tests were conducted. THP-1 WT and THP-1-defCASP1 macrophages were seeded at 100,000 cells/well and infected with (**C**) PA14 or (**D**) PAO1 (1 MOI), and cell viability and cytokine production were measured as mentioned above. Mean ± SD of experimental triplicates are shown, and mean values were compared by using Student's t-tests. Data shown are representative from 3 independent experiments. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

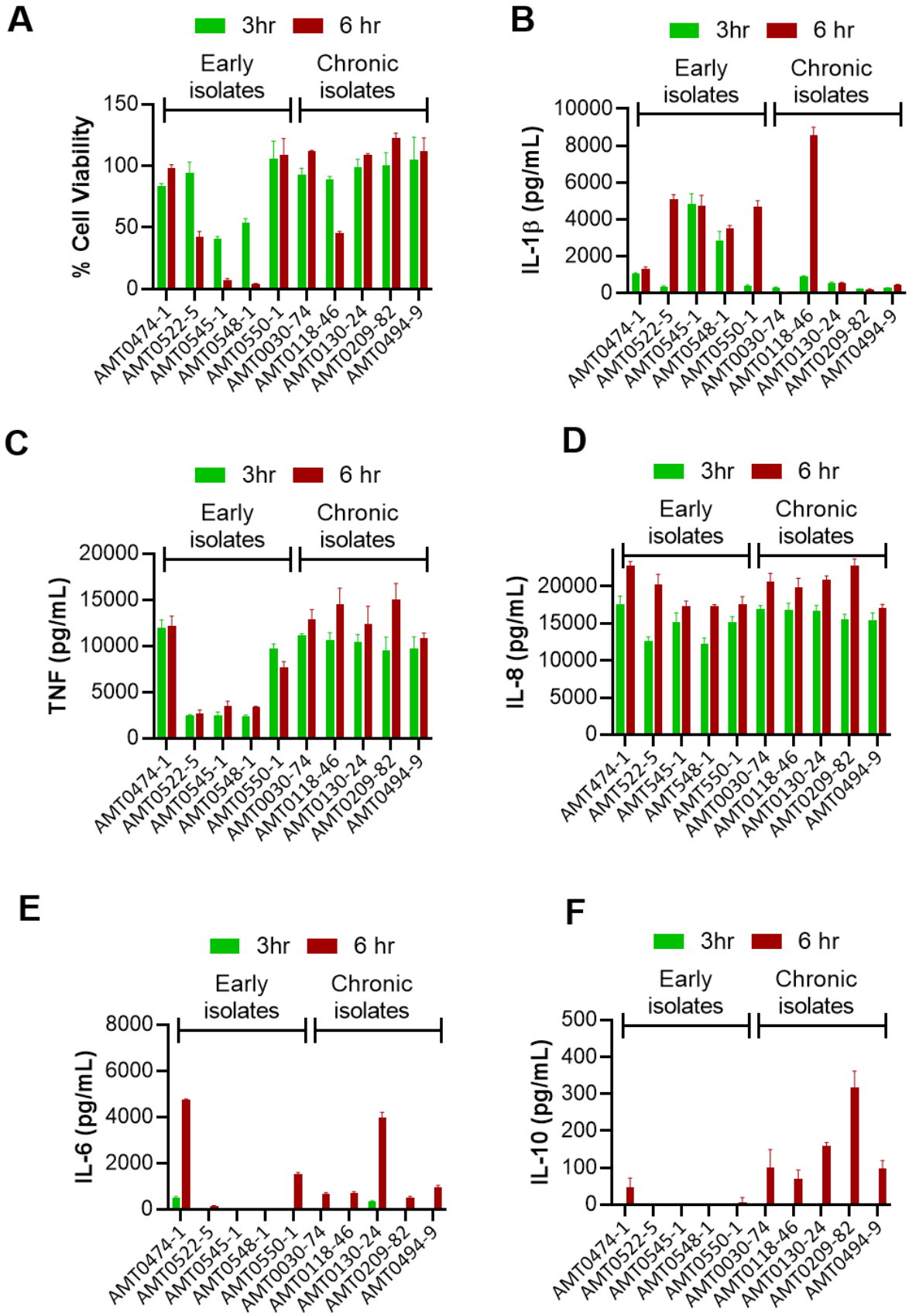

A
3hr
6 hr
Early isolates
Chronic isolates
% Cell Viability
150
100
50
0
AMT0474-1
AMT0522-5
AMT0545-1
AMT0548-1
AMT0550-1
AMT0030-74
AMT0118-46
AMT0130-24
AMT0209-82
AMT0494-9
B
3hr
6 hr
Early isolates
Chronic isolates
IL-1β (pg/mL)
10000
8000
6000
4000
2000
0
C
3hr
6 hr
Early isolates
Chronic isolates
TNF (pg/mL)
20000
15000
10000
5000
0
D
3hr
6 hr
Early isolates
Chronic isolates
IL-8 (pg/mL)
20000
15000
10000
5000
0
E
3hr
6 hr
Early isolates
Chronic isolates
IL-6 (pg/mL)
8000
6000
4000
2000
0
F
3hr
6 hr
Early isolates
Chronic isolates
IL-10 (pg/mL)
500
400
300
200
100
0

Fig. 15. An inverse relationship between cell death and cytokine expression is observed with increased time of infection. THP-1 macrophages were seeded at 100,000 cells/well in a 96-well plate. *In vitro* infections with indicated clinical *P. aeruginosa* isolates were conducted for 3 and 6 h. (**A**) Cell viability was assessed via neutral red assay and (**B-F**) cytokine levels were measured in the supernatant using ELISAs. Mean ± SD of experimental triplicates from a representative experiment are shown.

4.1.3 The NLRP3 inflammasome plays a role in inducing IL-1β secretion in response to P. aeruginosa *isolates*

Previous reports suggest that while *P. aeruginosa* typically activates the NLRC4 inflammasome, the NLRP3 inflammasome may play a role in exacerbating the host response (*274*). To look into this further, I used the NLRP3 inhibitor MCC950 to evaluate the role of NLRP3 in the induction of cell death and IL-1β secretion by THP-1 cells. MCC950 is described to inhibit both the canonical and non-canonical NLRP3 inflammasome activation by specifically preventing oligomerization of the ASC scaffold protein (*275*). I found that the inhibition of NLRP3 reduced the induction of both cell death (4 out of 5 isolates) and IL-1β expression (5 out of 5 isolates) by early isolates (**Fig. 16 A, B)**. These results indicate that NLRP3 promotes both cell death and IL-1β production during macrophage infections by clinical *P. aeruginosa* isolates. To assess the role of necroptosis and apoptosis during *P. aeruginosa* infections, I pre-treated THP-1 macrophages with GSK872 (an inhibitor of RIPK3) or Z-IETD-FMK (a caspase-8 inhibitor) for 2 hours prior to infection. These treatments were also continued during infections with select clinical isolates from early infections. We found that while neither inhibitor impacted cell death, inhibition of caspase-8 reduced IL-1β expression while GSK872 reduced TNF expression for 3 isolates (**Fig. 16 C-E**). This further adds evidence that THP-1 macrophages can engage multiple mechanisms to induce IL-1β expression during *P. aeruginosa* infections.

Similar trends were observed when using laboratory reference strains PA14 and PAO1. Inhibition of NLRP3 reduced the level of induced cell death more consistently for PAO1 than for PA14 (**Fig. 17 A, D)**. We also found that the secretion of IL-1β (**Fig. 17 B, E**), but not TNF (**Fig. 17 C, F**), was consistently reduced by cells treated with MCC950. Furthermore, inhibition of caspase-8 did not impact cell death or TNF expression induced by either reference strain but did

reduce IL-1β expression (**Fig. 17 G-I**). GSK872 also did not impact cell death and appeared to impact TNF and IL-1β expression for infections with PA14, perhaps suggesting that there are indeed different inflammatory mechanisms induced by PA14 compared with PAO1, given previously described differences between the strains (*238*) (**Fig. 17 G-I**).

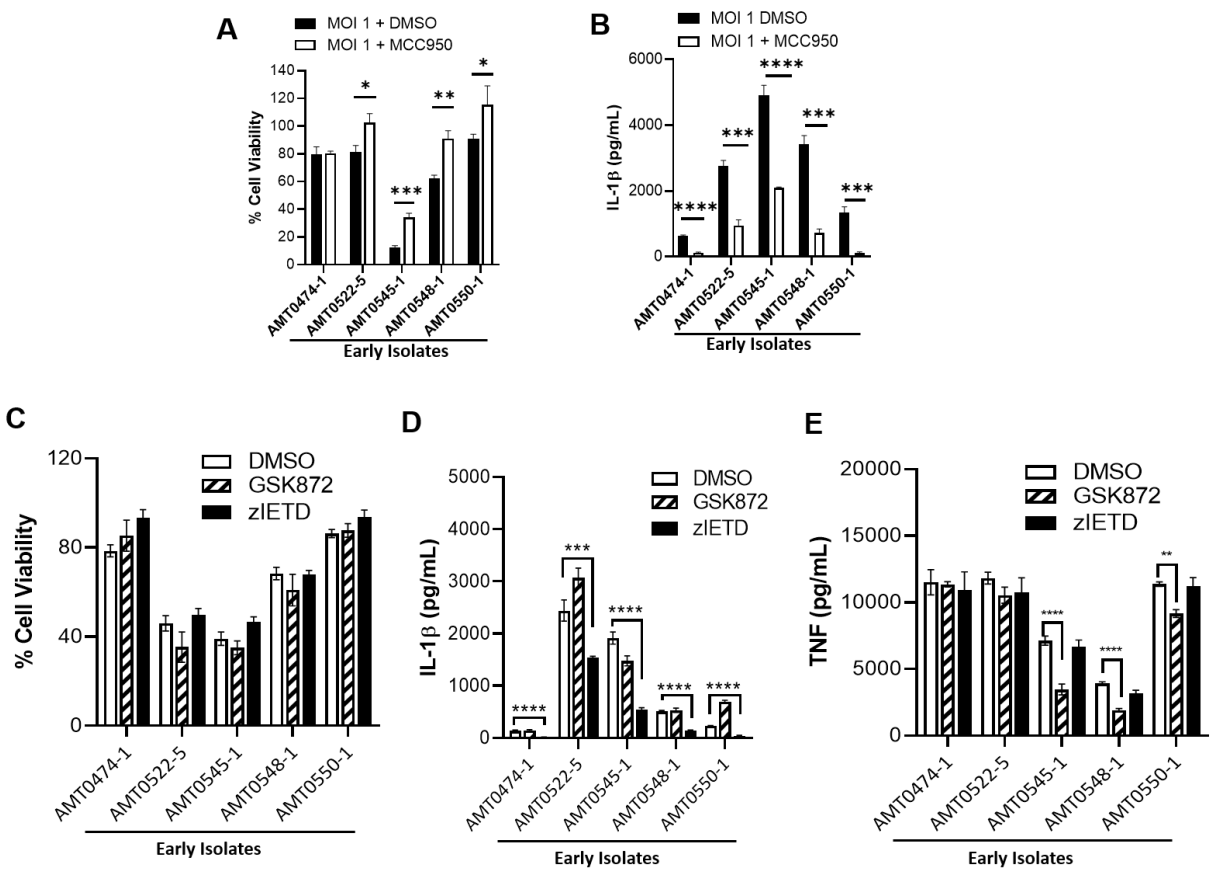

A
MOI 1 + DMSO
MOI 1 + MCC950
% Cell Viability
AMT0474-1
AMT0522-5
AMT0545-1
AMT0548-1
AMT0550-1
Early Isolates
B
MOI 1 DMSO
MOI 1 + MCC950
IL-1β (pg/mL)
C
DMSO
GSK872
zIETD
% Cell Viability
D
E
TNF (pg/mL)

Fig. 16. Early CF infection *P. aeruginosa* isolates are partially dependent on NLRP3 to induce cell death and IL-1β production. THP-1 macrophages were seeded at 100,000 cell/well in a 96-well plate. THP-1 macrophages were treated with a DMSO vehicle control or 10 μM of MCC950 while infected with early *P. aeruginosa* isolates (1 MOI) from CF patients to inhibit NLRP3 activation. At 3 h postinfection, (**A**) cell viability was evaluated by neutral red assay and (**B**) IL-1β production was measured in cell supernatants by ELISA. THP-1 macrophages were seeded at 100,000 cell/well in a 96-well plate. THP-1 macrophages were pre-treated with 5 μM GSK872 (RIPK3 inhibitor), 10 μM Z-IETD-FMK (caspase-8 inhibitor) or a DMSO control prior to infection. Respective treatments were continued during THP-1 macrophage infections with indicated early *P. aeruginosa* isolates (1 MOI). At 3 h postinfection, (**C**) cell viability was evaluated by neutral red assay and (**D**) IL-1β and (**E**) TNF production were measured in cell supernatants by ELISA. Mean ± SD of experimental triplicates from one experiment are shown. (**A, B**) Student's t-tests and (**D, E**) one-way ANOVAs followed by Dunnett's multiple comparisons tests were conducted. (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001).

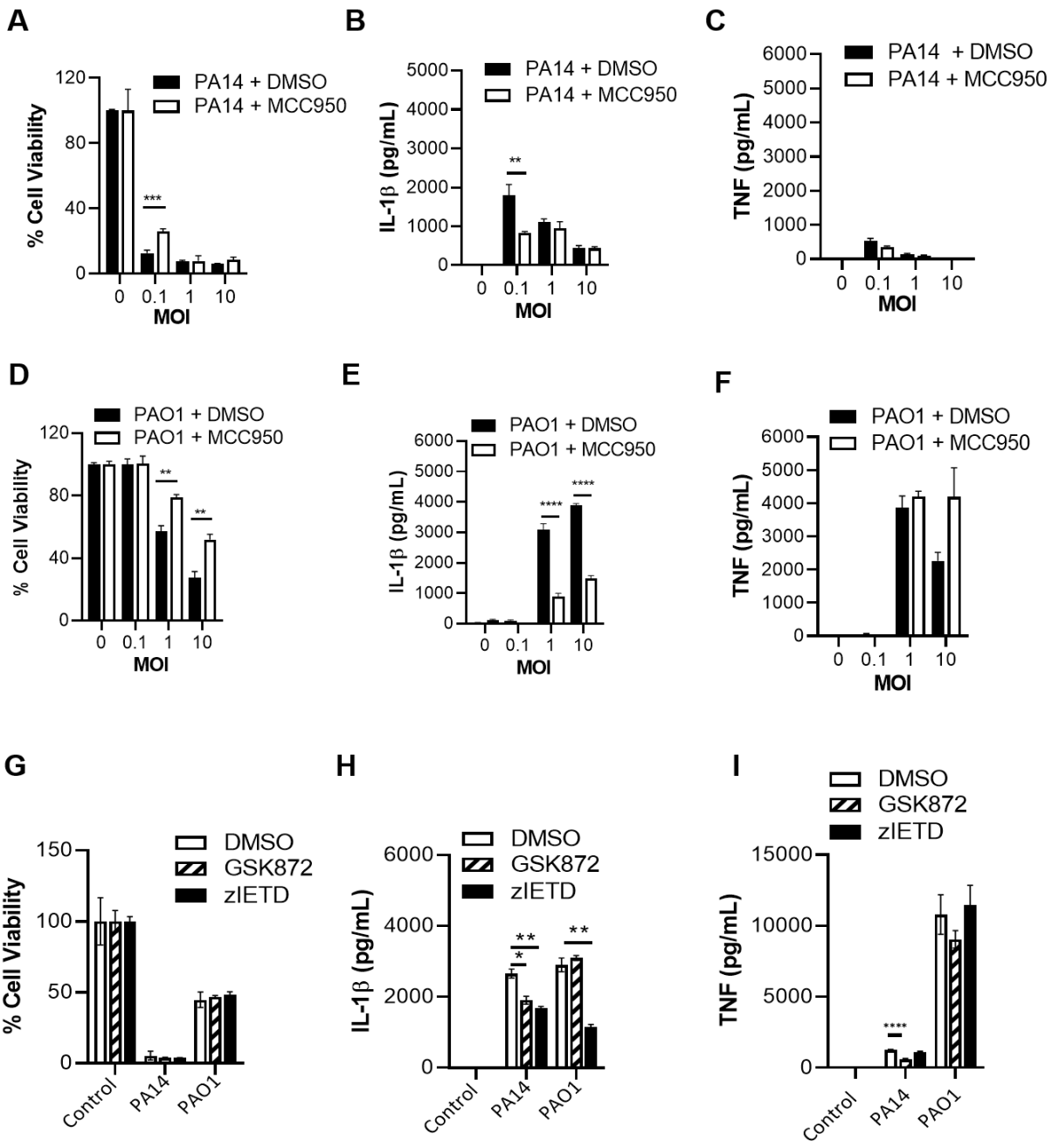

A
% Cell Viability
PA14 + DMSO
PA14 + MCC950
MOI
B
IL-1β (pg/mL)
C
TNF (pg/mL)
D
PAO1 + DMSO
PAO1 + MCC950
E
F
G
DMSO
GSK872
zIETD
Control
PA14
PAO1
H
I

Fig. 17. NLRP3 and RIP3 promote IL-1β/cell death and TNF respectively during infection with reference strains of *P. aeruginosa*. THP-1 macrophages were seeded at 100,000 cells/well in a 96-well plate. *In vitro* infections with indicated PA14 and PAO1 reference strains of *P. aeruginosa* were then conducted with the addition of either 10 μM MCC950 (NLRP3 inhibitor), 5 μM GSK872 (RIPK3 inhibitor), 10 μM Z-IETD-FMK (caspase-8 inhibitor), or a DMSO control. THP-1 macrophages were pre-treated with GSK872, Z-IETD-FMK, or a DMSO control prior to infection. Respective treatments were continued during THP-1 macrophage infections with indicated *P. aeruginosa* strains (MOI 1 unless otherwise indicated). At 3 h postinfection, (**A, D, G**) cell viability was evaluated by neutral red assay and (**B, E, H**) IL-1β and (**C, F, I**) TNF production were measured in cell supernatants by ELISA. (**A, B, D, E**) Student's t-tests (**H, I**) and one-way ANOVAs followed by Dunnett's multiple comparisons tests (**D, E**) were conducted. (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001).

4.1.4 Chronic isolates of P. aeruginosa *strongly activate NF-κB and MAPK than early isolates*

As differences in TNF, IL-6, and IL-8 expression were observed between the early and chronic respiratory isolates, signalling pathways that are known to induce expression of these cytokines were explored. Immunoblotting of THP-1 macrophage extracts at 1 h postinfection revealed an enhanced activation of the NF-κB and p38 MAPK pathways by the chronic isolates of *P. aeruginosa* (**Fig. 18**). Activation of the p38 MAPK by chronic isolates of *P. aeruginosa* was further validated by increased activation of MK2, the downstream target of p38 MAPK (**Fig. 18 A, E**). Using a commercially available THP-1 NF-κB reporter cell line, I observed that the heat-killed chronic isolates stimulated more NF-κB activation than early isolates (**Fig. 18 C**). This difference further suggests that chronic isolates of *P. aeruginosa* from CF patients may differ from early isolates in the expression of heat-stable PAMPs that activate the NF-κB pathway in host cells.

Using ralimetinib, a selective inhibitor of p38α and p38β MAPKs (*276*), I also observed that there was no impact on THP-1 macrophage death and IL-1β secretion (**Fig. 19 A, B**), but the expression of TNF and IL-8 was decreased upon inhibiting p38 MAPK signalling (**Fig. 19 C, D**). These same trends were observed when exposing THP-1 macrophages to ralimetinib during infections with the laboratory reference strain PAO1 (**Fig. 20**).

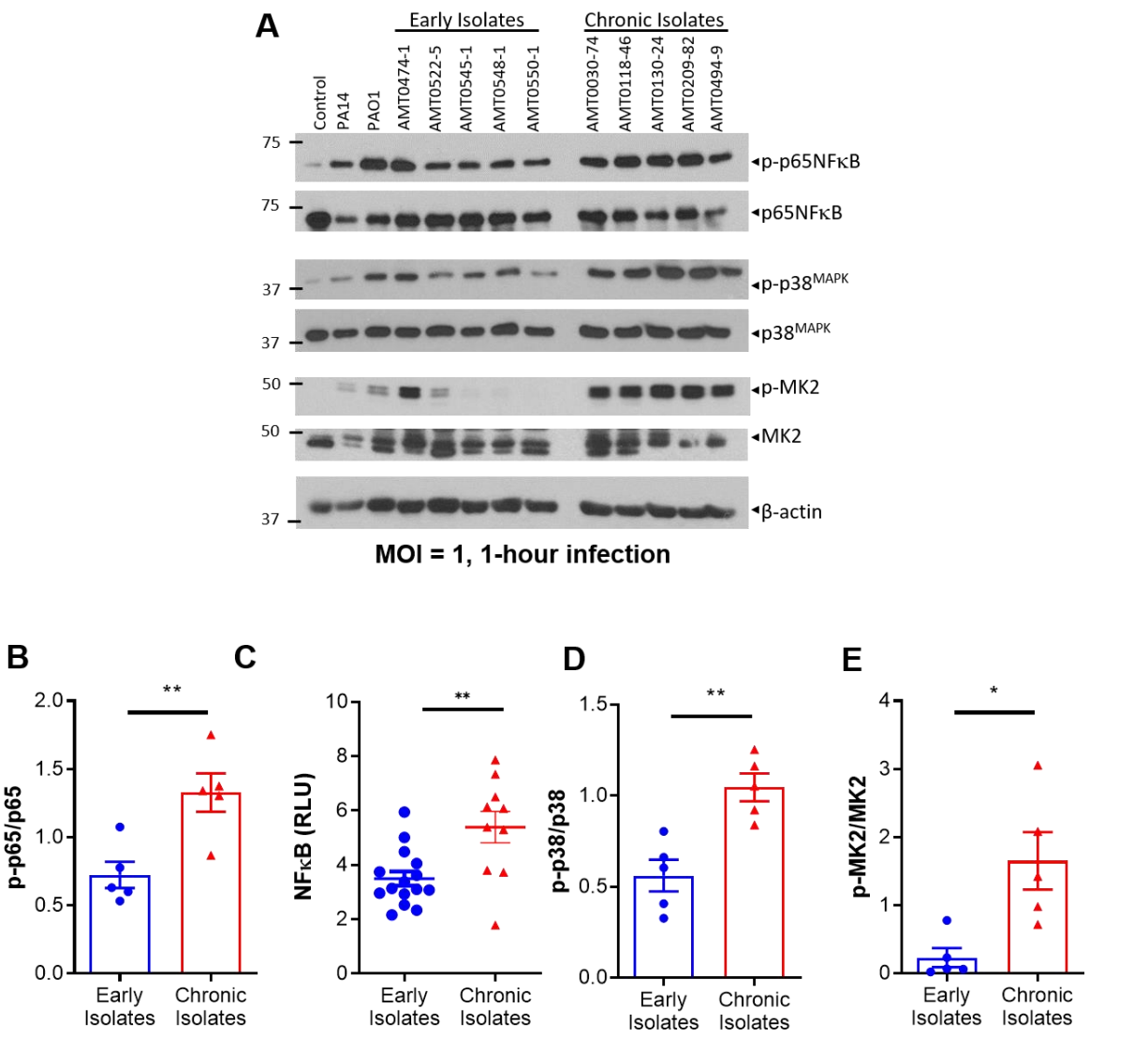
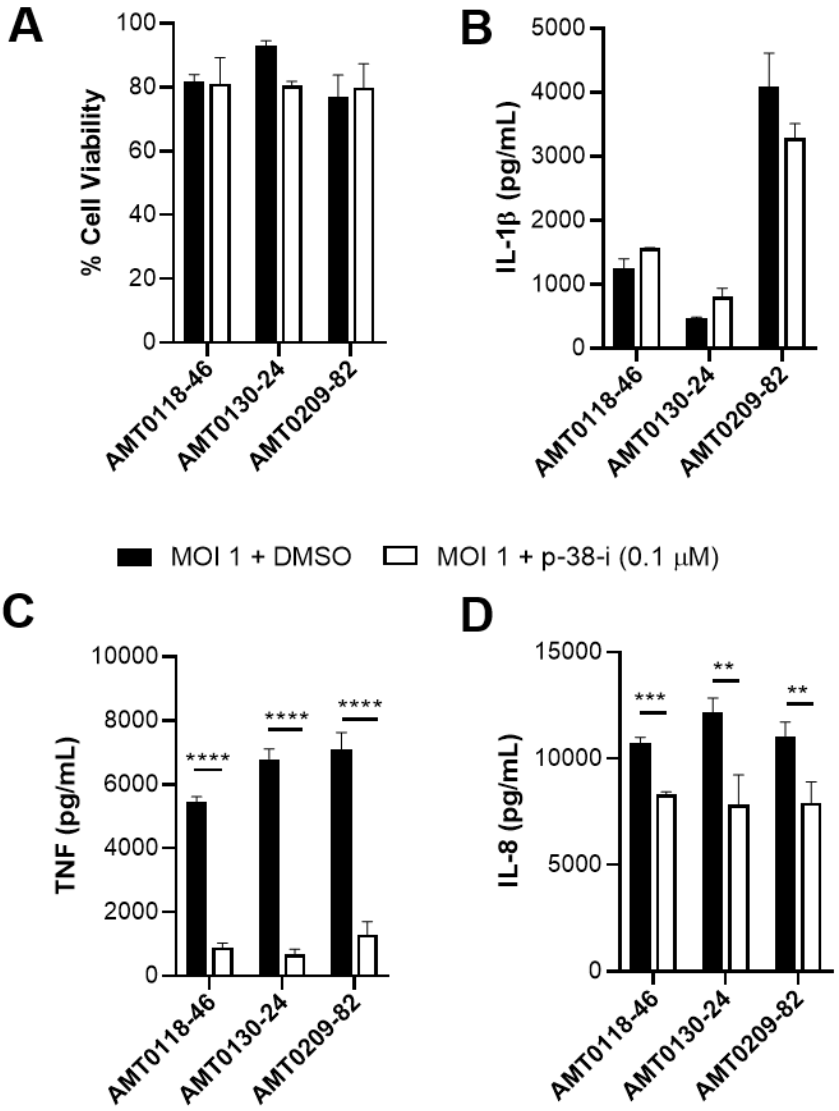


Fig. 18. Chronic CF infection *P. aeruginosa* isolates induce increased activation of NF-κB and MAPK more than the early infection isolates. THP-1 macrophages were seeded at 500,000 cells/well in a 24-well plate. (**A**) Immunoblotting was conducted on lysates collected at 1 h postinfection with the indicated CF *P. aeruginosa* isolates (1 MOI). (**B, D, E**) Densitometric analysis of western blots shown in panel A was performed. Mean ± SD are shown. THP-1 Lucia™ NF-κB cells were differentiated, seeded at 100,000 cells/well in a 96-well plate, and incubated with 1 MOI of heat-killed early or chronic CF *P. aeruginosa* isolates in experimental triplicates. (**C**) At 3 h, supernatant luciferase activity (relative light units, RLUs) was measured to assess NF-κB activation. Mean ± SEM from a representative of 3 independent experiments are shown. Mean values were compared by Student's t-tests. (*$P < 0.05$, **$P < 0.01$).



Chronic Isolates

Fig. 19. p38 MAPK plays a role in TNF and IL-8 expression during infections with *P. aeruginosa* isolates from chronic CF infections. THP-1 macrophages were seeded at 100,000 cells/well in a 96-well plate. Ralimetinib, a p38 MAPK inhibitor, or a DMSO control was added during infections with select chronic *P. aeruginosa* isolates from CF patients to a final concentration of 0.1 μM. (**A**) Cell viability via neutral red assay and (**B-D**) cytokine production via ELISA were then measured 3 h postinfection. Mean ± SD of experimental triplicates from a representative experiment are shown. Mean values were compared by Student's t-tests. (***P* < 0.01, ****P* < 0.001, *****P* < 0.0001).

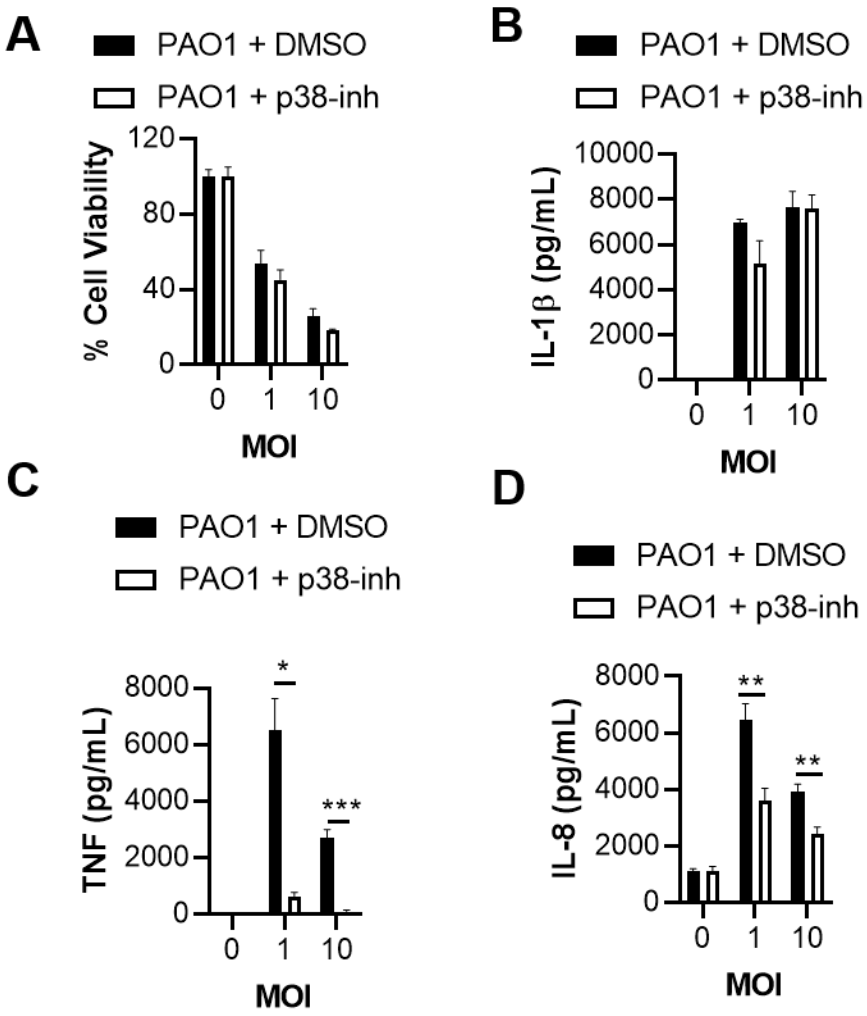


Fig. 20. Inhibition of p38 MAPK significantly reduces TNF and IL-8 expression during infections with PAO1. THP-1 macrophages were seeded at 100,000 cells/well in a 96-well plate. *In vitro* infections with PAO1 were then conducted at MOI 1 and 10 with the addition of a DMSO control or 0.1 µM ralimetinib, a specific p38 MAPK inhibitor. At 3 h postinfection, (**A**) cell viability was evaluated via neutral red assay and (**B-D**) cytokine expression was measured via ELISA. Mean ± SD of experimental triplicates from a representative of 3 independent experiments are shown. (*$P < 0.05$, **$P < 0.01$).

4.1.5 Inverse correlation between cell death and expression of inflammatory cytokine by environmental and non-CF isolates of P. aeruginosa

Given how the relationship between cell death and cytokine expression was consistently observed using isolates from CF respiratory infections, I sought to determine how THP-1 macrophages would respond to infection by *P. aeruginosa* from the environment (i.e., river water) and non-CF clinical infections (e.g., wounds, urinary tract infections) (**Table 4**). I observed that for both groups, isolates that induced greater cell death failed to elicit enhanced expression of TNF and IL-8 (**Fig. 21**). Therefore, the inverse relationship between the magnitude of host cell death and select proinflammatory cytokine expression applies generally to *P. aeruginosa* when infecting macrophages and not just clinical isolates from CF patients.

4.1.6 Mutants of P. aeruginosa *provide mechanistic insights into the inverse relationship between cytokine expression and macrophage cell death*

To provide more insight into which virulence factors could be at play to drive these differences in induced cell death and cytokine expression, I infected macrophages with *P. aeruginosa* reference strains and mutants of varying virulence in the PA14 background. PA14 is a highly virulent strain of *P. aeruginosa*, while PAO1 is moderately virulent (*277*). Some genetic differences between PA14 and PAO1 are predicted to impact mechanisms of host cell death, including the presence in PA14 but not PAO1 of the *exoU* gene (*277*), which has been previously reported to induce caspase-1-independent cell death, and the deletion of the *ladS* gene, resulting in enhanced T3SS activity (*238*). Therefore, when comparing the two strains, host cell death induced by PAO1 is likely to be predominantly inflammasome-dependent. As expected, PA14 induced potent cell death and poor expression of inflammatory cytokines in contrast to PAO1, which induced reduced cell death but enhanced expression of inflammatory cytokines when

infecting primary human macrophages (**Fig. 22 A**) and THP-1 macrophages (**Fig. 22 B**). THP-1 macrophage death when infected with PA14 occurred relatively early, nearing 90% cell death after 1 h when using an MOI of 10, with only limited TNF and IL-8 expression (**Fig. 22 C**). In contrast, cell death induction by PAO1 was both delayed and reduced compared with PA14, and the expression of TNF and IL-8 was greater (**Fig. 22 C**). Similar results were also observed with the NuLi-1 bronchial epithelial cells infected with either PA14 or PAO1 (**Fig. 23**).

The T3SS is a key virulence needle-like apparatus of *P. aeruginosa* that injects effector proteins and toxins into the host cell cytoplasm and is often a significant inducer of host cell death (*278*). We therefore used the PA14 Transposon Mutation Library to evaluate the impact of various T3SS genes on cell death versus cytokine production. Infection with the flagellar mutant PA14 *fliC*::tn induced similar levels of THP-1 macrophage death as WT PA14 (**Fig. 24 A**). PA14 T3SS mutants *popB*::tn and *popD*::tn (translocator proteins of T3SS) and *exsA*::tn (a T3SS transcriptional regulator) induced minimal cell death (**Fig. 24 A**). Expression of IL-1β was found to positively correlate with cell death at a lower MOI of 0.1, which may be because effector proteins of T3SS have been described to limit IL-1β production (**Fig. 24 A**). Both WT PA14 and PA14 *fliC*::tn potently induced cell death yet poor expression of TNF and IL-8, consistent with the inverse patterns of early cell death and cytokine production (**Fig. 24 A**). WT PA14 and PA14 *fliC*::tn in comparison to other transposon mutants also demonstrated extensive THP-1 cell death within 1 h of infection, which resulted in compromised production of TNF and IL-8 by both strains (**Fig. 24 B**).

Much like what was explored for the clinical CF isolates, I wanted to observe if these trends were consistent upon extending the infection time. Ultimately, cell death and cytokine expression trends observed among the PA14 transposon mutants and WT PA14 were consistent upon

extending the course of *in vitro* infections from 3 h to 6 h at an MOI of 1 (**Fig. 25**). Namely, among infections with WT PA14 or the transposon mutants, extremes of either extensive THP-1 macrophage death or minimal death were observed. This resulted in very little changes in cell death occurring upon extending the infection time from 3 to 6 h (**Fig. 25 A**). Once again, IL-1β and TNF was observed to not change much with the extended period of time, suggesting that they are secreted fairly early over the course of infections and are not continuously secreted (**Fig. 25, B, C**). IL-8, IL-6, and IL-10 production was found to increase over time, especially for transposon mutants that did not induce as much host cell death (**Fig. 25, D, E, F**). As well, the inverse relationship between cell death and the expression of some proinflammatory cytokines was still observed at 6 h postinfection.

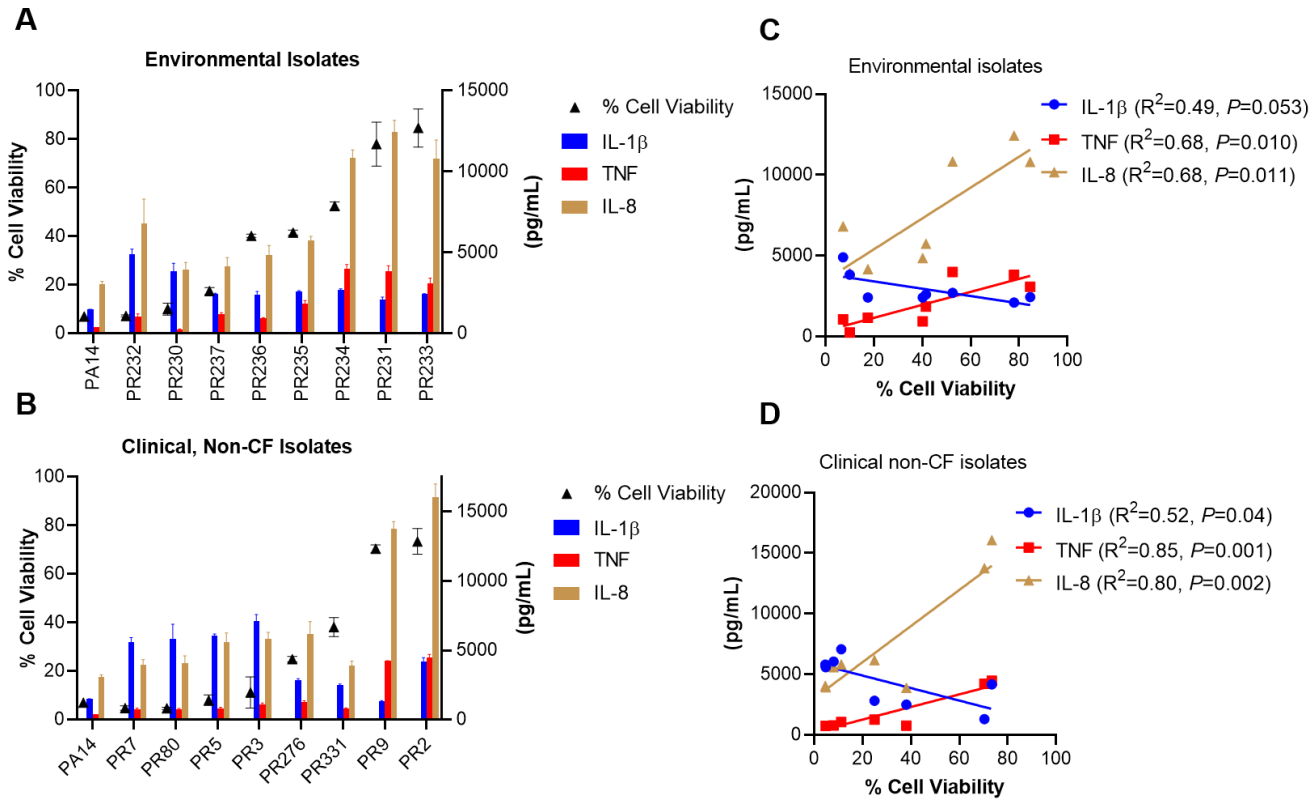
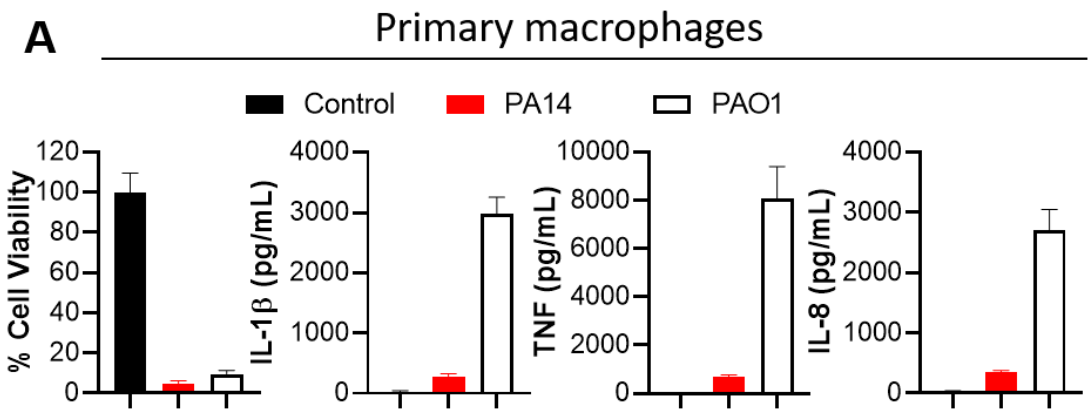
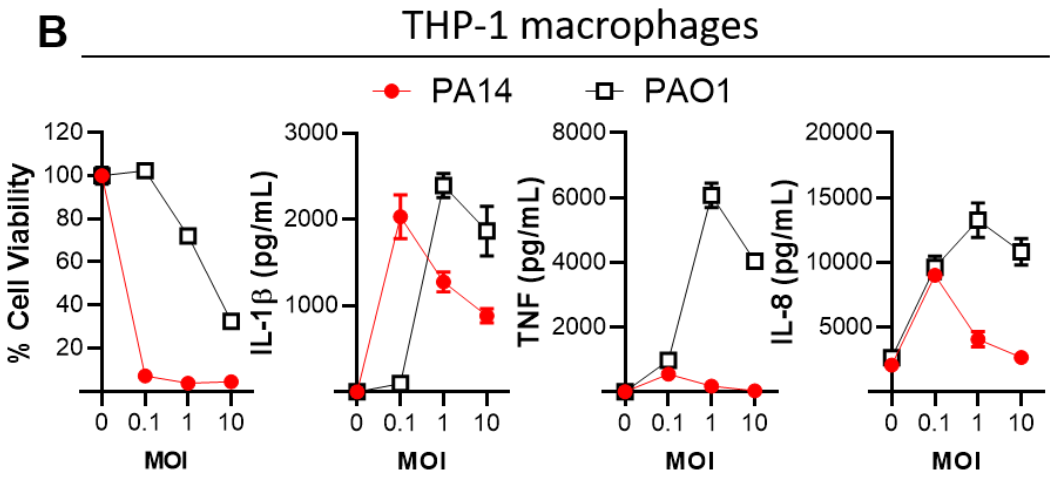


Fig. 21. Environmental and clinical, non-CF *P. aeruginosa* isolates also generally exhibit an inverse relationship between induction of cell death and TNF and IL-8 expression. THP-1 macrophages were seeded at 100,000 cells/well in a 96-well plate. Environmental *P. aeruginosa* isolates from (**A**) river water and (**B**) clinical *P. aeruginosa* isolates from non-CF patients (i.e. blood, wound, and urine infections) were used to infect THP-1 macrophages at an MOI of 1. Cell viability was evaluated by neutral red assay and IL-1β, TNF, and IL-8 production were measured by ELISA at 3 h postinfection (**A, B**). Simple linear regression analyses were conducted to determine the relationship between cell viability and cytokine expression for infections with (**C**) environmental and (**D**) non-CF isolates. Mean ± SD of experimental triplicates from a representative experiment are shown.


A
Primary macrophages
Control
PA14
PAO1
% Cell Viability
IL-1β (pg/mL)
TNF (pg/mL)
IL-8 (pg/mL)


B
THP-1 macrophages
PA14
PAO1
% Cell Viability
IL-1β (pg/mL)
TNF (pg/mL)
IL-8 (pg/mL)
MOI

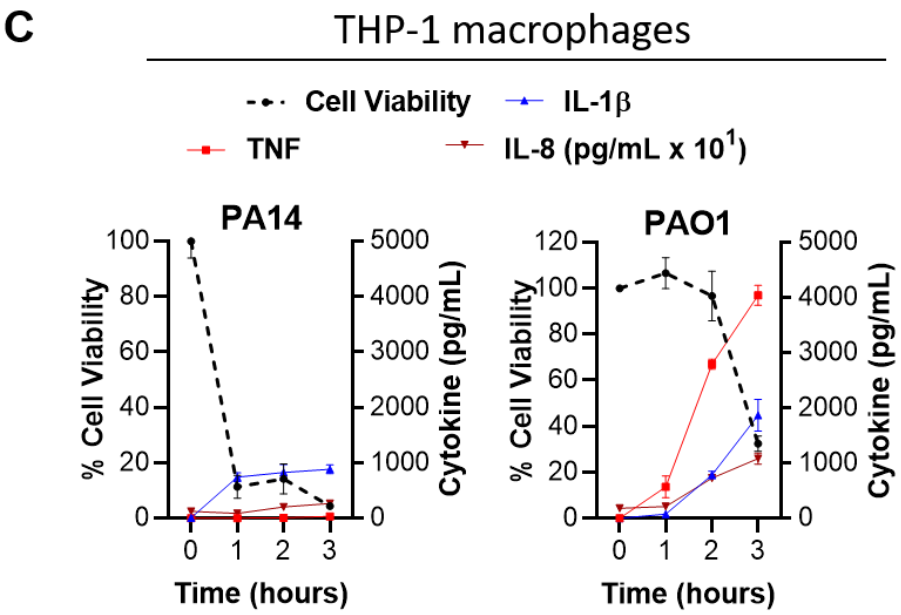

C
THP-1 macrophages
Cell Viability
IL-1β
TNF
IL-8 (pg/mL x 10^1)
PA14
PAO1
% Cell Viability
Cytokine (pg/mL)
Time (hours)

Fig. 22. PAO1 induces less THP-1 macrophage death but higher expression of TNF and IL-8 compared with WT PA14. Primary human macrophages were seeded at 50,000 cells/well in a 96-well plate and infected with PA14 or PAO1 at an MOI of 10. (**A**) Cell viability via neutral red assay and cytokine production via ELISA were measured 3 h postinfection. THP-1 macrophages were seeded at 100,000 cells/well in a 96-well plate. *In vitro* infections were conducted with PA14 or PAO1. Cell viability was measured by neutral red assay and cytokine production was evaluated by ELISA at (**B**) 3 h or at (**C**) 1-h intervals. Mean ± SD of experimental triplicates are shown. Data shown are a representative from 3 independent experiments.

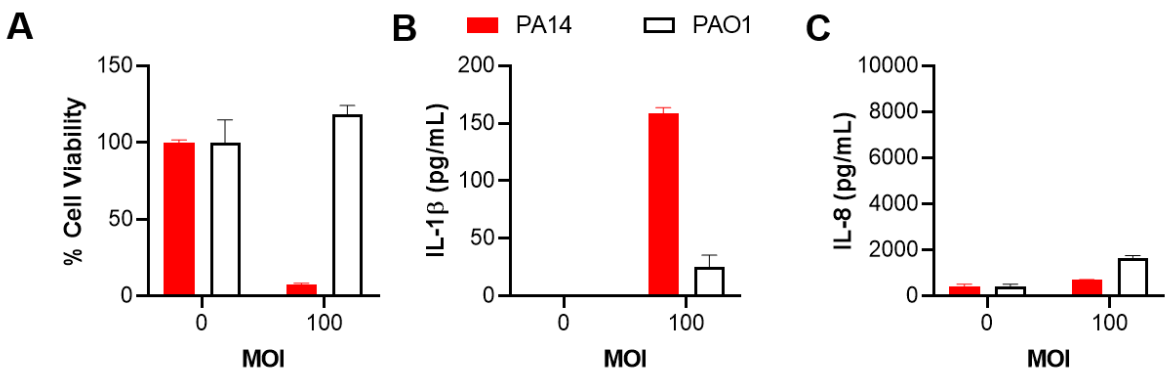
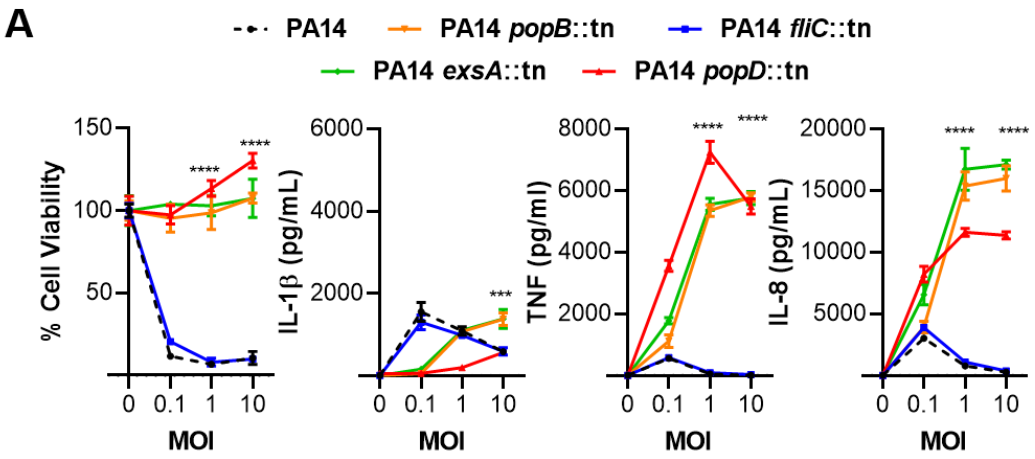


Fig. 23. PAO1 induces less NuLi-1 cell death and IL-1β expression but higher expression of IL-8 compared with WT PA14. Bronchial epithelial NuLi-1 cells were seeded at 30,000 cells/well in a 96-well plate and infected with either PA14 or PAO1 at 100 MOI. (**A**) Cell viability via neutral red assay and (**B, C**) cytokine production via ELISA were measured 6 h postinfection. Mean ± SD of experimental triplicates are shown. Data shown are a representative from 3 independent experiments.


A
PA14
PA14 popB::tn
PA14 fliC::tn
PA14 exsA::tn
PA14 popD::tn
% Cell Viability
IL-1β (pg/mL)
TNF (pg/ml)
IL-8 (pg/mL)
MOI
****
***

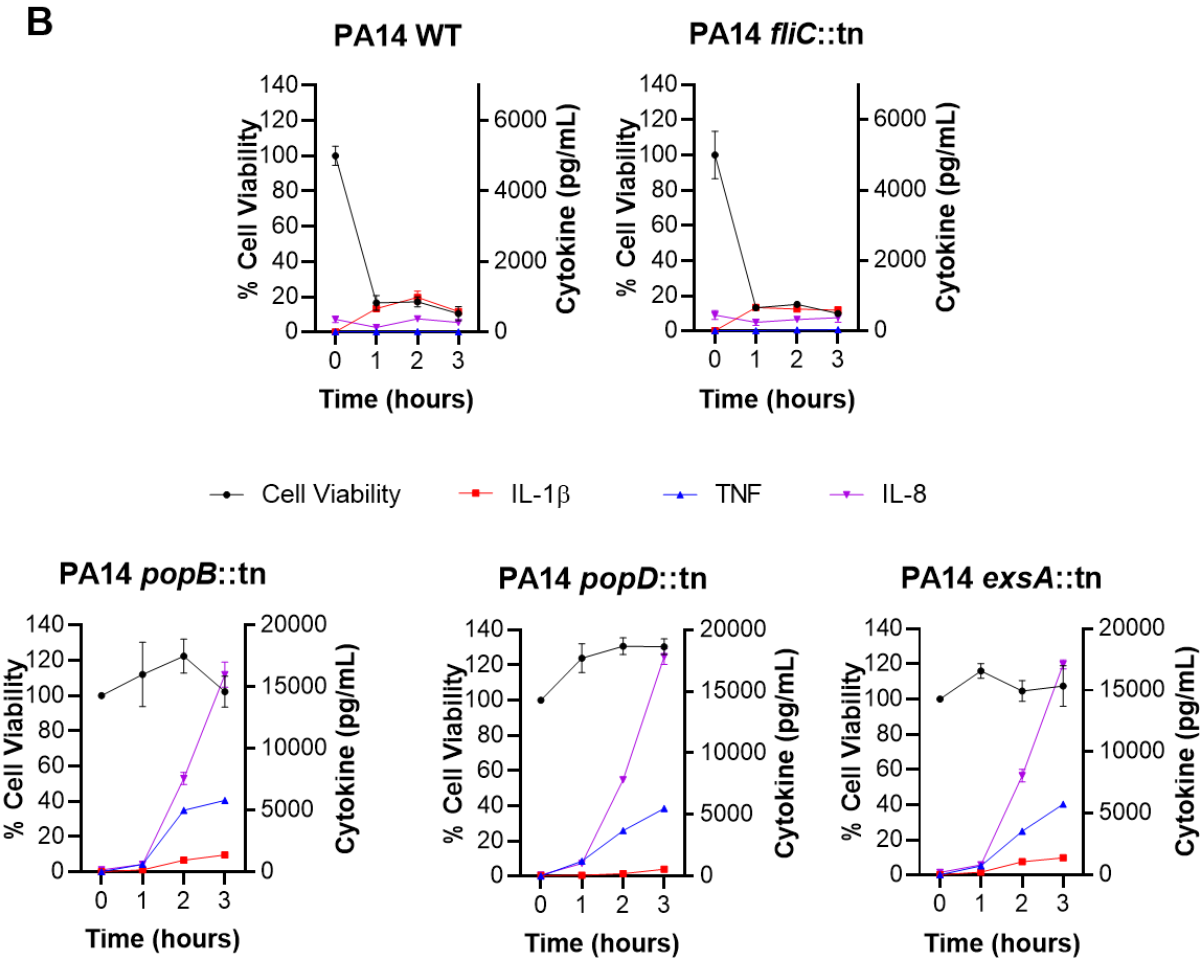

B
PA14 WT
PA14 fliC::tn
PA14 popB::tn
PA14 popD::tn
PA14 exsA::tn
Cell Viability
IL-1β
TNF
IL-8
% Cell Viability
Cytokine (pg/mL)
Time (hours)

Fig. 24. PA14 mutants with impaired T3SS induce less cell death but higher expression of TNF and IL-8 compared with wild-type PA14. THP-1 macrophages were seeded at 100,000 cells/well in a 96-well plate. *In vitro* infections were conducted with WT PA14 and the various transposon mutants (e.g., PA14 *fliC*::tn, PA14 *popB*::tn, PA14 *popD*::tn, and PA14 *exsA*::tn) of PA14. Cell viability was measured by neutral red assay and cytokine production was evaluated by ELISA at (**A**) 3 h or (**B**) 1-h intervals. Mean ± SD of experimental triplicates are shown. One-way ANOVAs were conducted to compare mean values at MOIs of 1 and 10. Data shown are from a representative of three experiments. (****$P < 0.0001$).

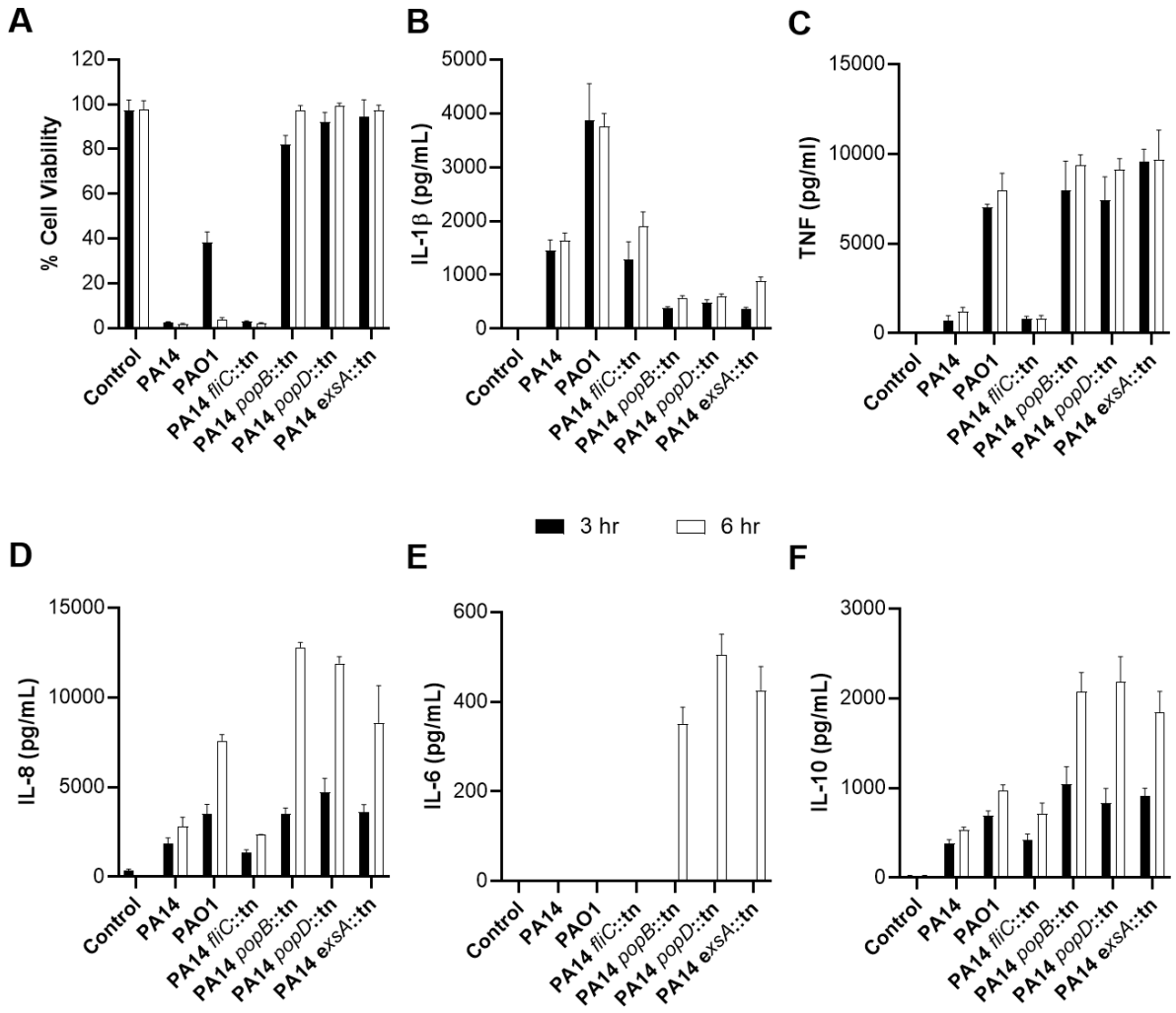


Fig. 25. An inverse relationship between cell death and cytokine expression among WT PA14 and PA14 transposon mutants is observed with increased time of infection. THP-1 macrophages were seeded at 100,000 cells/well in a 96-well plate. *In vitro* infections were conducted with WT PA14, PAO1, or indicated transposon mutants of PA14. (**A**) At 3 h and 6 h postinfection, cell viability was evaluated by neutral red assay. (**B-F**) Cytokine production was measured in cell supernatants by ELISA. Mean ± SD of experimental triplicates from a representative experiment are shown.

4.1.7 Inhibition of CFTR does not impact the inverse relationship between cell death and cytokine expression during P. aeruginosa *infections*

It is still unclear how CFTR dysfunction, as seen in CF, may impact immune cell function (*42*, *279*, *280*). Therefore, it was important to see if trends in cell death and cytokine production could still be observed if CFTR function was inhibited in both THP-1 macrophages and NuLi-1 bronchial epithelial cells. To do so, I treated THP-1 macrophages overnight with the CFTR-selective inhibitor ($CFTR_{inh}$-172) using a previously reported concentration of 10 μM (*270*, *272*, *281*) and infected the THP-1 cells with PA14 or PAO1. No clear impact of the CFTR inhibitor was observed in cell death or cytokine expression during PA14 (**Fig. 26 A**) and PAO1 (**Fig. 26 B**) infections. Furthermore, greater expression of IL-1β, TNF, and IL-8 was still observed during infection with PAO1 than PA14 with or without the presence of $CFTR_{inh}$-172 at 1 MOI. The same treatments and conditions were applied to the bronchial epithelial cells but with a lengthier infection time of 6 h. As with THP-1 macrophages, there were no significant differences observed with NuLi-1 cells between treatment groups during infections with PA14 (**Fig. 27 A**) or PAO1 (**Fig. 27 B**) and previously observed trends were still appreciated even after $CFTR_{inh}$-172 treatment.

4.1.8 Inverse correlation between host cell death and cytokine expression is observed between P. aeruginosa *and* S. aureus

Staphylococcus aureus (*S. aureus*) is another pathogen that often colonizes the lungs of CF patients, particularly in young CF patients (*282*). Therefore, I wanted to explore whether the inverse relationship between host cell death and proinflammatory cytokine expression could be appreciated across different pathogens known to cause respiratory infections. Using laboratory reference strains *S. aureus* 6538 and PA14 to infect THP-1 macrophages, I found that *S. aureus*

6538 did not induce cell death as rapidly as PA14, which allowed for higher levels of TNF and IL-1β expression at specific MOIs and over time (**Fig. 28**). However, when the *S. aureus* MOI was increased to 10 MOI, cell death was increased and there was a reduction of TNF production (**Fig. 28 A**). Therefore, it may be that the trends presented here may be applicable across different species of bacteria, including those that are frequently seen in CF.

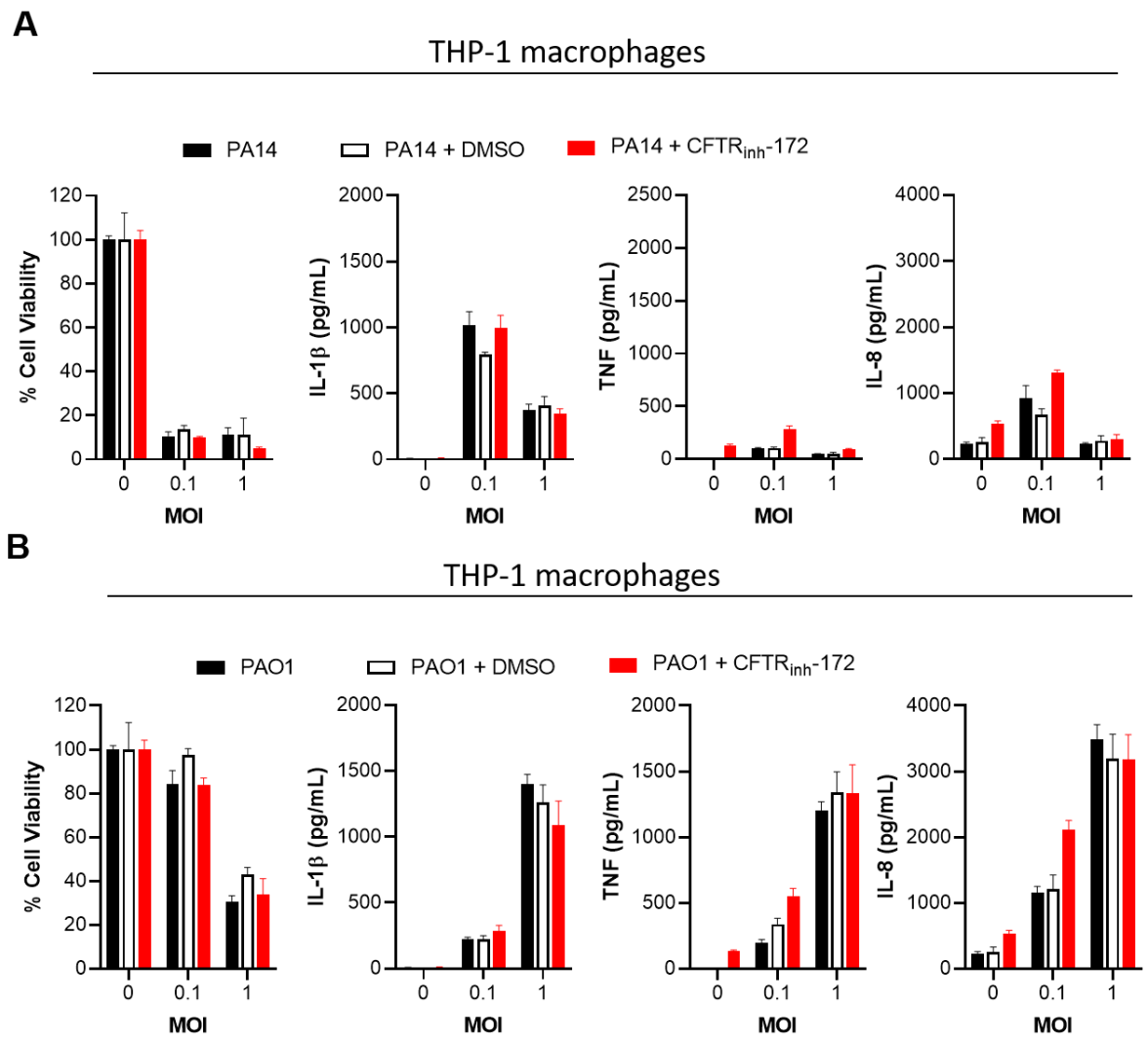


Fig. 26. CFTR inhibition in THP-1 macrophages does not impact cell death or cytokine production. THP-1 macrophages were seeded at 100,000 cells/well in a 96-well plate with the addition of either a DMSO vehicle control or 10 µM $CFTR_{inh}$-172 overnight. *In vitro* infections were conducted with (**A**) PA14 or (**B**) PAO1 with the addition of either treatment. At 3 h postinfection, cell viability was evaluated by neutral red assay and cytokine production was measured by ELISA. Mean ± SD of experimental triplicates from a representative of 3 experiments are shown.

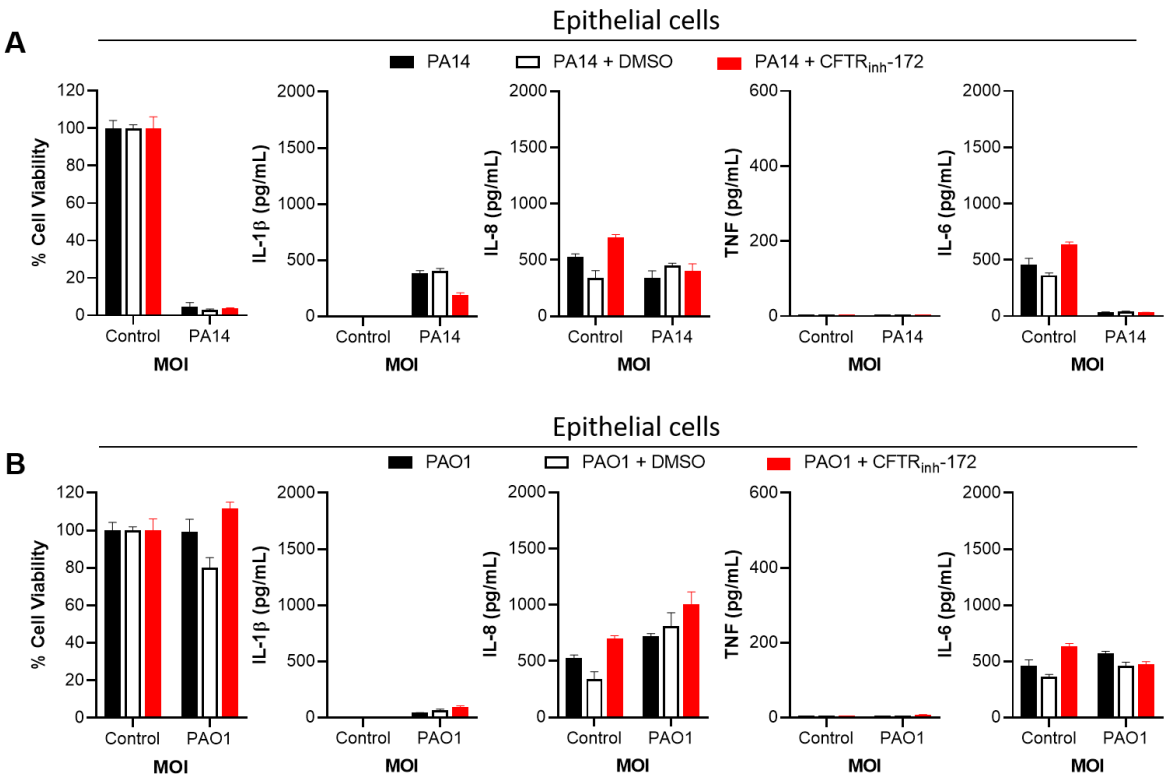


Fig. 27. Inhibition of CFTR in NuLi-1 cells does not impact cell death or cytokine production. NuLi-1 cells were seeded at 30,000 cells/well in a 96-well plate with the addition of either a DMSO vehicle control or 10 µM $CFTR_{inh}$-172 overnight. *In vitro* infections were conducted with (**A**) PA14 or (**B**) PAO1 with the addition of either treatment. At 6 h postinfection, cell viability was evaluated by neutral red assay and cytokine production was measured by ELISA. Mean ± SD of experimental triplicates from a representative of 3 experiments are shown.

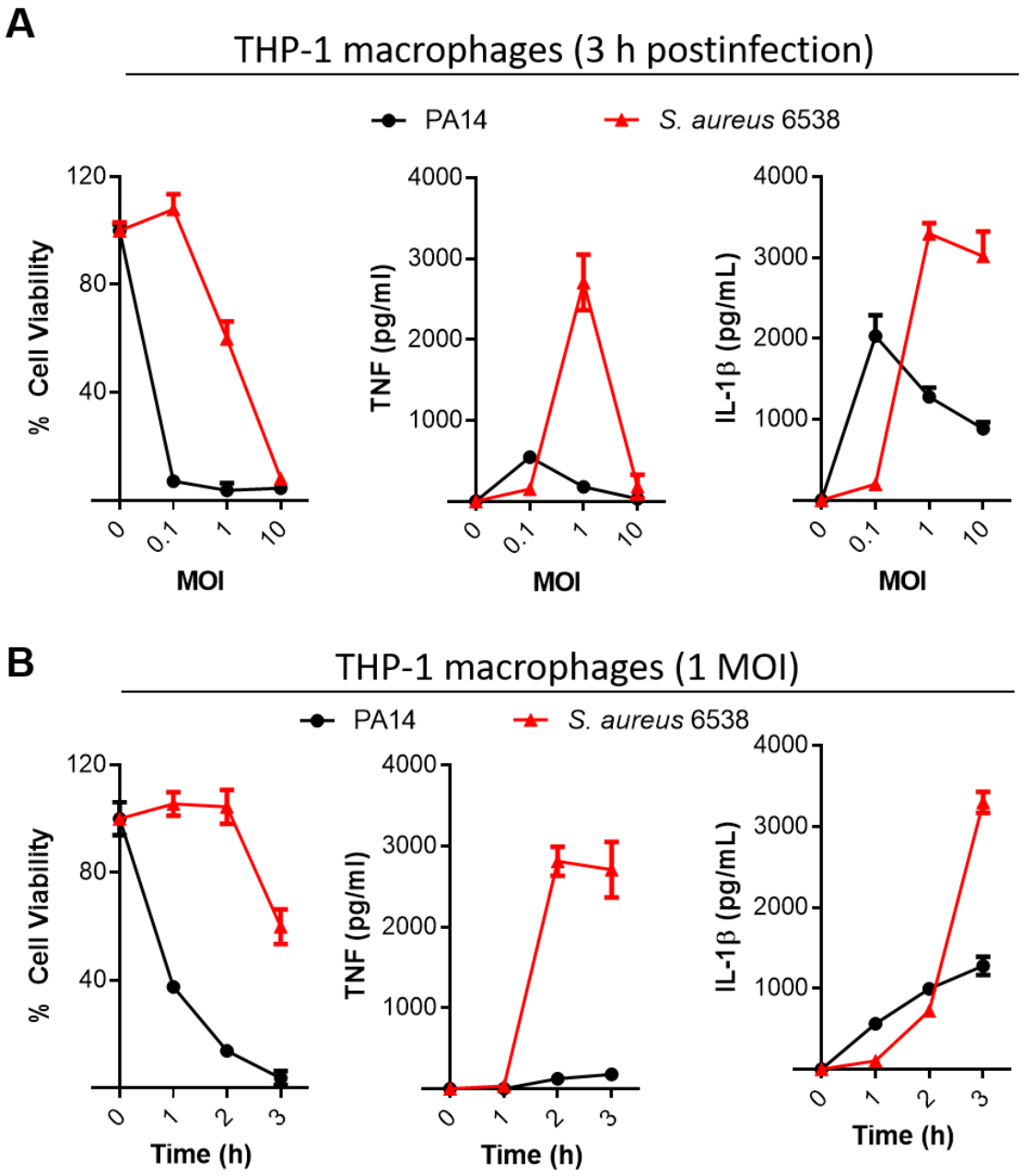


Fig. 28. PA14 and *S. aureus* demonstrate an inverse relationship between cell death and cytokine expression. THP-1 macrophages were seeded at 100,000 cells/well in a 96-well plate and infected with either PA14 or *S. aureus* 6538 at various MOIs. (**A**) 3 h postinfection, cell viability via neutral red and TNF and IL-1β in the supernatant via ELISAs were measured. (**B**) Infections were also performed using an MOI of 1, and cell viability and cytokines were measured at 1-h intervals for up to 3 h (**B**). Mean ± SD of experimental triplicates from one experiment are shown.

4.2 Role of *P. aeruginosa* ExoU in inducing IL-1β expression in macrophages and bronchial epithelial cells

Throughout this work exploring how host cell death can interfere with cytokine expression, I noticed certain trends in IL-1β expression between the reference strains PA14 and PAO1. As previously stated, PA14 is described to be more virulent than PAO1 and one of the genetic differences between the two strains that could account for this difference is the presence of ExoU in PA14 (*263*) ExoU has also been previously reported to inhibit caspase-1 and subsequent IL-1β production (*158*). Our lab has also observed these differences in induced cell death and IL-1β expression between PA14 and PAO1 when using murine bone marrow-derived macrophages (BMDMs) (*262*). However, preceding figures exploring how host cell death could impact cytokine expression has demonstrated that while PAO1 could induce greater expression of IL-1β compared with PA14 in THP-1 macrophages at MOIs of 1 or greater, PA14 would be able to induce greater IL-1β expression at an MOI of 0.1, which isn't commonly used in the literature. As a result, I continued to further investigate this finding in a series of experiments outlined below.

4.2.1 Differences in induced cell death and cytokine expression between PA14 and PAO1 infections of THP-1 macrophages appear to be driven by ExoU

While ExoU is one of the key differences between PA14 and PAO1, there are other reported genetic differences which could account for trends in induced host cell death that other have previously described (*238*). To evaluate how much of a role ExoU could play when comparing the two reference strains, the PA14 transposon mutant PA14 *exoU*::tn was used to determine its role in inducing rapid cell death and the subsequent impact on cytokine production, including IL-1β. Measuring the extent of THP-1 macrophage death at various MOIs for 3 h or at 1-h intervals, both PAO1 and PA14 *exoU*::tn induced cell death and IL-1β expression more slowly

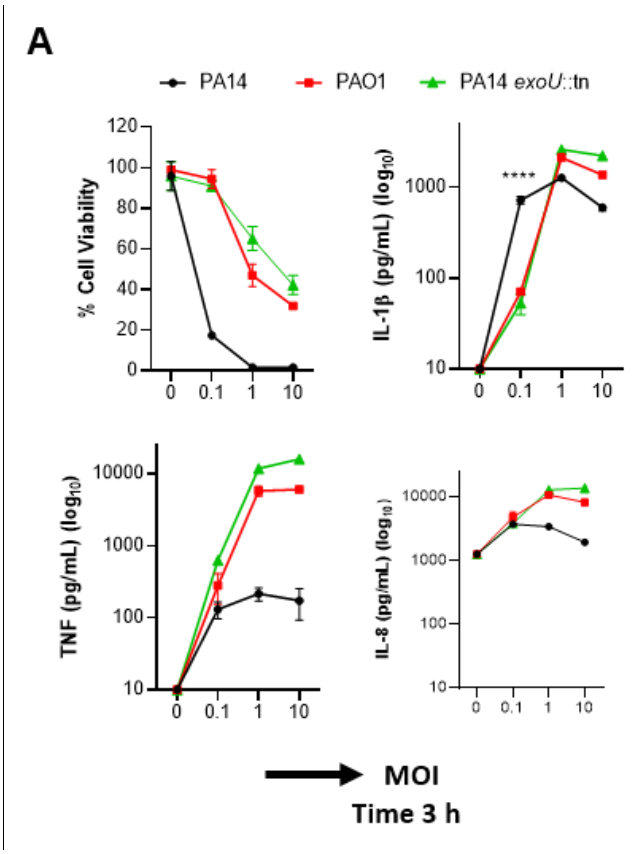
than WT PA14 (**Fig. 29**). WT PA14 was also found to be able to induce greater levels of IL-1β expression at a relatively low MOI of 0.1 and as early as 1 h postinfection at an MOI of 10 (**Fig. 29**). This suggests that ExoU-expressing WT PA14 could possibly induce IL-1β expression early over the course of an infection or under conditions when host cell death is not induced very rapidly. This positive effect of ExoU on IL-1β expression gets masked when cells are infected with higher MOIs or when cytokine expression is measured at later time intervals, in which case the ExoU expression by WT PA14 results in more cell death. Consequently, the expression of IL-1β is compromised by increased cell death.

4.2.2 Observed trends of cell death and IL-1β secretion are observed when using ExoU-expressing PAO1

To further validate the observed trends in cytokine expression induced by ExoU, I acquired ExoU-expressing PAO1 as well as relevant controls courtesy of Drs. Priebe and Pier from Boston Children's Hospital and Brigham and Women's Hospital. In their previously described work (*266*), PAO1 was transformed with the plasmid pUCP19exoUspcU, which contained the *exoU* gene and the gene of its associated chaperone protein *spcU*. THP-1 macrophages were infected with WT PAO1, PAO1 with the pUCP19 plasmid (i.e., PAO1+Vector), and ExoU-expressing PAO1 (i.e., PAO1+ExoU) at various MOIs for 3 h. Concurrent infections with either WT PA14 or PA14 *exoU*::tn were also completed for relative comparison in trends. As observed with WT PA14, PAO1+ExoU induced more cell death and IL-1β expression at an MOI of 0.1 compared with the other PAO1 controls (**Fig. 30 A, B**). The level of cell death between WT PA14 and PAO1+ExoU, as enumerated via neutral red assay and visualized via Hoechst/PI staining was comparable (**Fig. 30 C**). However, for higher MOIs, both WT PAO1 and PAO1 with the pUCP19 plasmid induced

greater IL-1β, TNF, and IL-8 expression, as previously seen with infections with PA14 *exoU*::tn in comparison to those with WT PA14 (**Fig. 30 A, B**).

Given how there are a number of cell types in the lung environment, I also wanted to see if ExoU would be able to induce IL-1β expression in bronchial epithelial cells. Therefore, NuLi-1 cells were also infected with the PAO1+ExoU and the two controls as well as WT PA14 and PA14 *exoU*::tn for 6 h. Once again, WT PA14 and PAO1+ExoU were able to induce higher levels of cell death and IL-1β expression across all MOIs compared with PA14 *exoU*::tn or the two control PAO1 strains (**Fig. 31 A, B**). IL-8 expression was also higher upon infections with WT PA14 and PAO1+ExoU, but this was reduced at an MOI of 100 which correlated with substantial increase in IL-1β expression, implying that ExoU may be partly responsible for the inverse correlation between cell death and cytokine expression (**Fig. 31 A, B**). As with infections with THP-1 macrophages, the level of cell death that was visualized using Hoechst/PI staining were comparable between infections with WT PA14 and PAO1+ExoU. Taken together, this further suggests that ExoU promotes IL-1β expression during infections at lower MOIs for the THP-1 macrophages and for infections with the NuLi-1 cells even when using different *P. aeruginosa* strains.


A
PA14
PAO1
PA14 exoU::tn
% Cell Viability
IL-1β (pg/mL) (log10)
TNF (pg/mL) (log10)
IL-8 (pg/mL) (log10)
****
MOI
Time 3 h

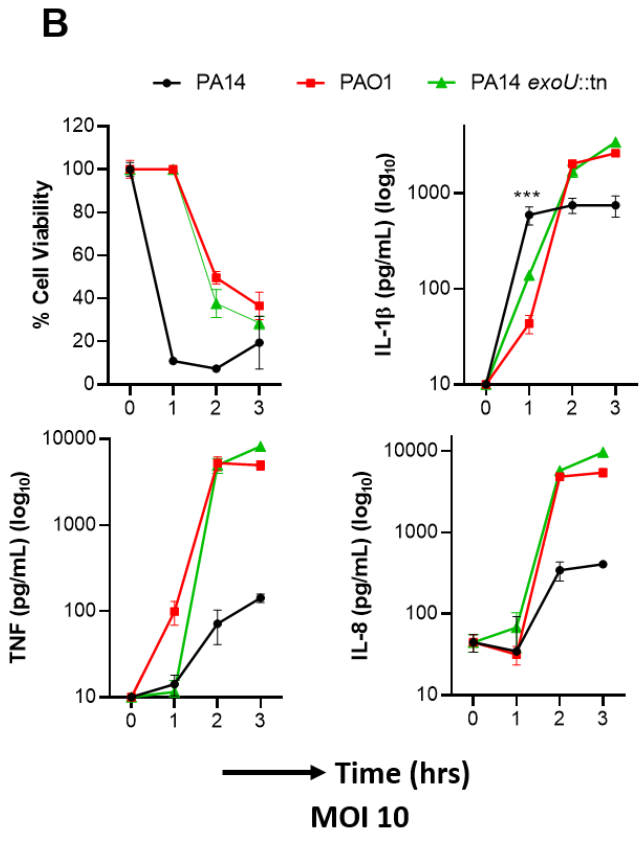

B
PA14
PAO1
PA14 exoU::tn
% Cell Viability
IL-1β (pg/mL) (log10)
TNF (pg/mL) (log10)
IL-8 (pg/mL) (log10)
***
Time (hrs)
MOI 10

Fig. 29. PA14 *exoU*::tn and PAO1 induce similar levels of cell death and cytokine expression which differ from infections with PA14. THP-1 macrophages (seeded at 100,000 cells/well) were infected with WT PA14, PA14 *exoU*::tn, and PAO1 either at (**A**) various MOIs for 3 h or (**B**) for 1-h intervals for up to 3 h (MOI 10). Cell viability was evaluated by neutral red assay. Cytokine production was measured in cell supernatants by ELISA. Mean ± SD of triplicates from a representative from 3 experiments are shown. One-way ANOVAs were conducted to compare mean values at 0.1 MOI and 1 h. (***$P < 0.001$, ****$P < 0.0001$).

Macrophages (THP-1)

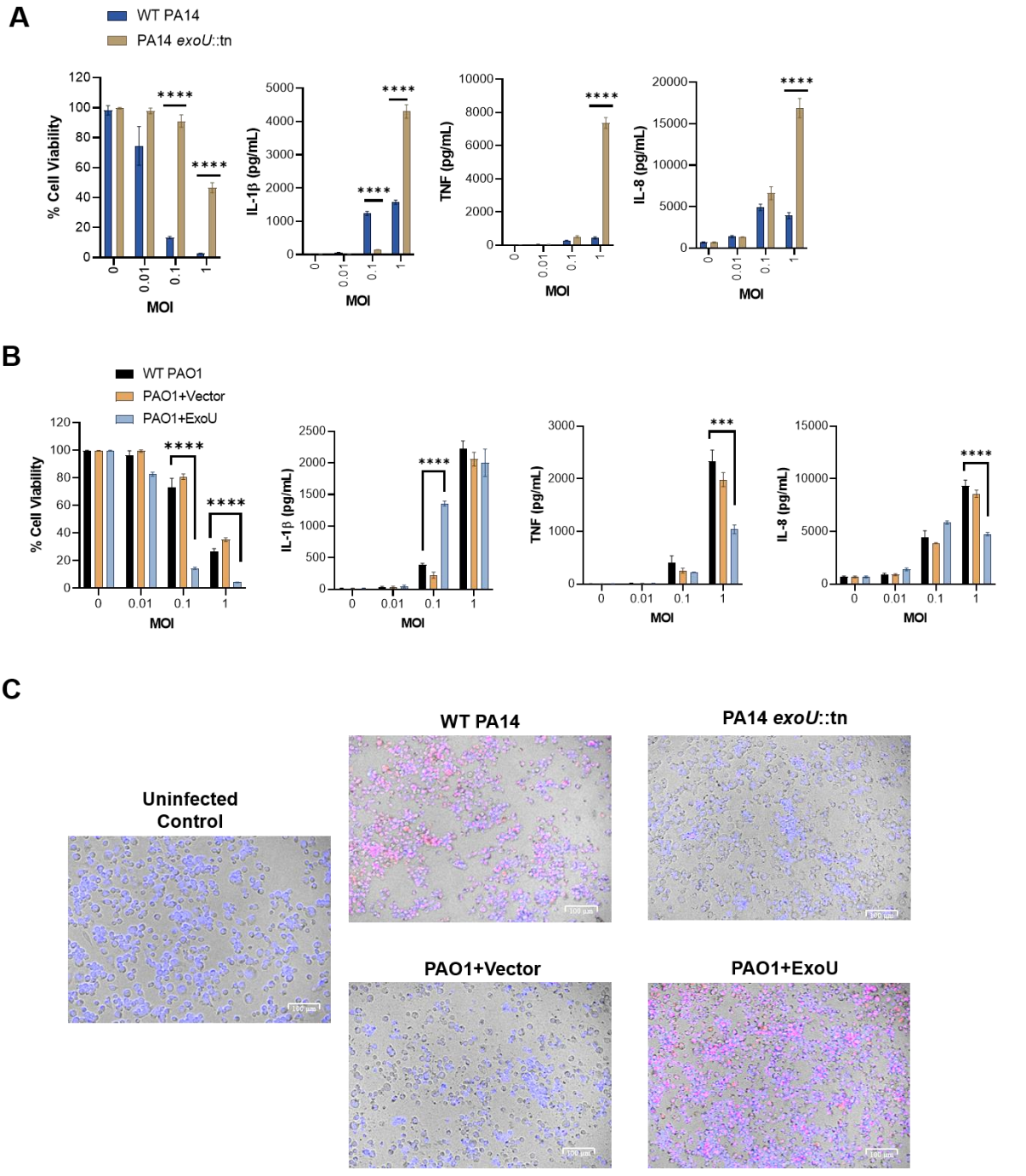
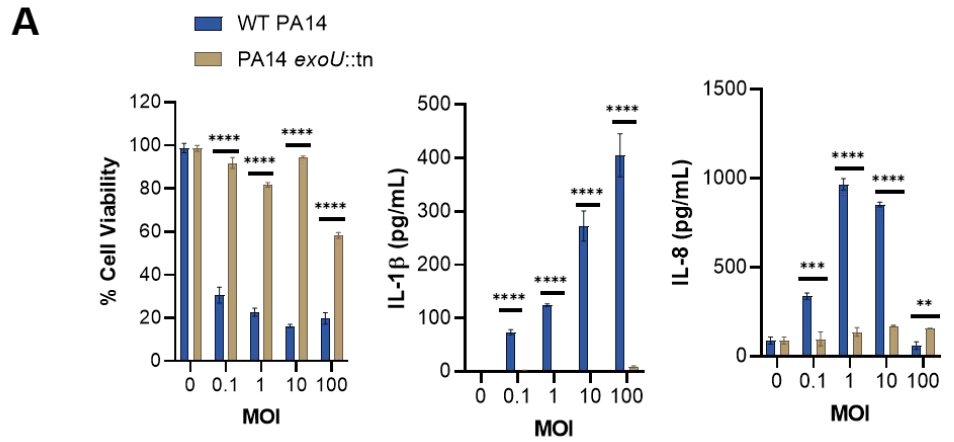
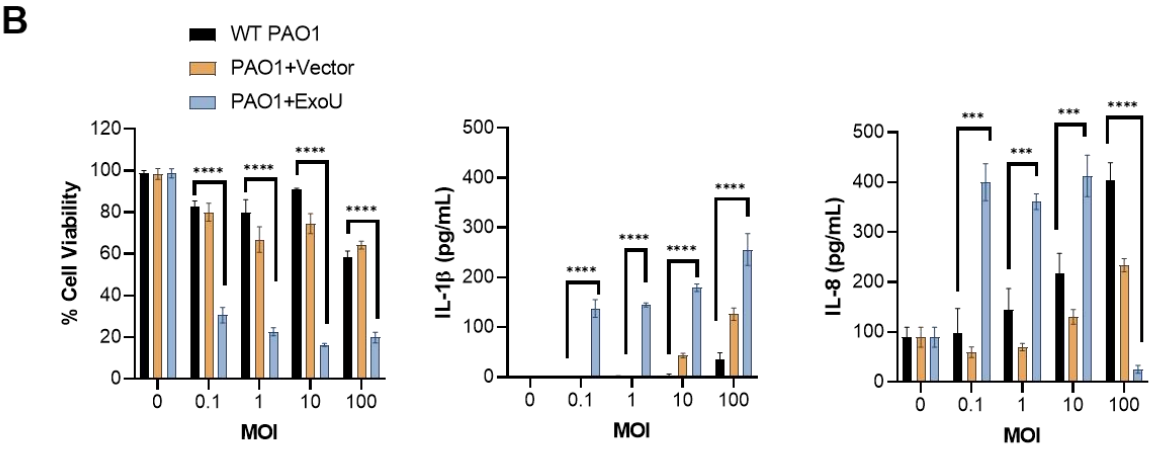


Fig. 30. ExoU-expressing PAO1 is able to induce IL-1β expression in THP-1 macrophages. THP-1 macrophages where seeded at 100,000 cells/well in a 96-well plate and infected with (**A**) WT PA14 and PA14 *exoU*::tn or (**B**) with WT PAO1, PAO1+pUCP19 plasmid (i.e., PAO1+Vector), and PAO1+pUCP19exoUspcU (i.e., PAO1+ExoU) at various MOIs for 3 h. Cell viability was evaluated using neutral red uptake assay and cytokine production was measured in cell supernatants by ELISA. Mean ± SD of triplicates are shown from a representative experiment from 3 independent experiments. (**A**) Student's t-tests and (**B**) one-way ANOVAs followed by Dunnett's multiple comparisons tests were conducted to compare means. THP-1 macrophages were seeded at 500,000 cells/well in a 24-well plate and infected with WT PA14, PA14 *exoU*::tn, PAO1+Vector, and PAO1+ExoU at an MOI of 1. (**C**) At 2 h postinfection, cells were stained with Hoechst/PI to distinguish live (blue) from dead (pink) cells. Data shown are a representative from 3 experiments. (***$P < 0.001$, ****$P < 0.0001$).


A
WT PA14
PA14 exoU::tn
% Cell Viability
IL-1β (pg/mL)
IL-8 (pg/mL)
MOI
0
0.1
1
10
100


B
WT PAO1
PAO1+Vector
PAO1+ExoU
% Cell Viability
IL-1β (pg/mL)
IL-8 (pg/mL)
MOI

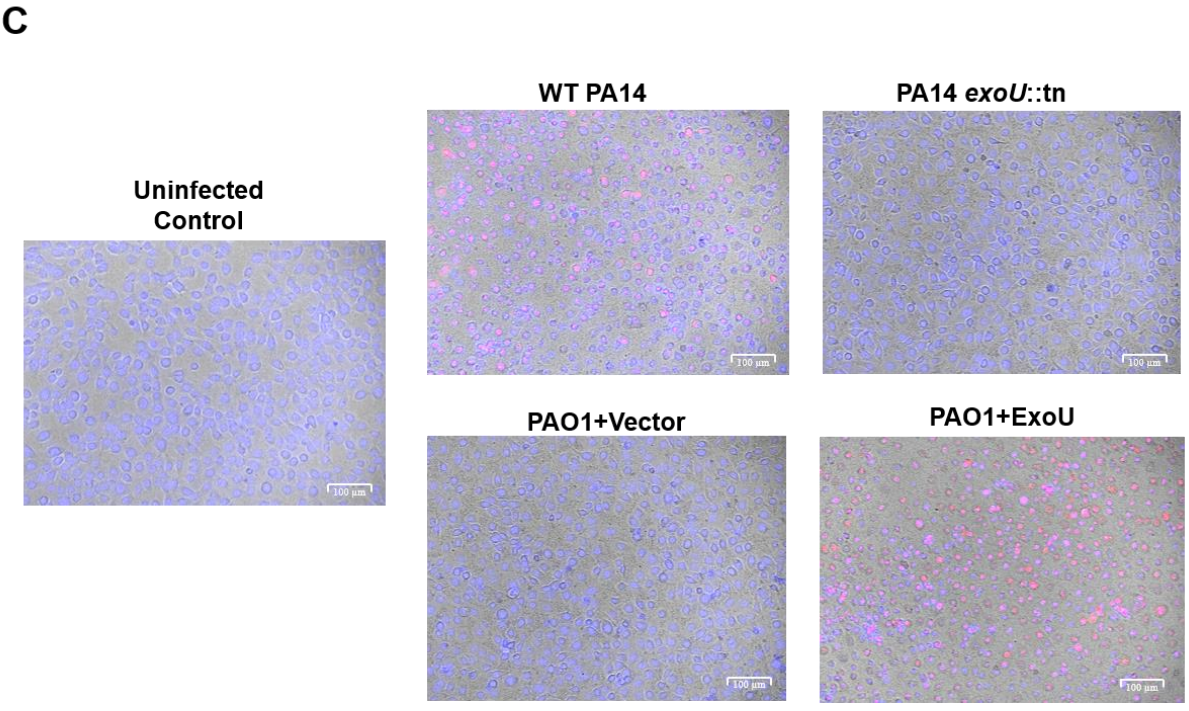

C
Uninfected Control
WT PA14
PA14 exoU::tn
PAO1+Vector
PAO1+ExoU

Fig. 31. ExoU-expressing PAO1 and WT PA14 are able to induce IL-1β expression in NuLi-1 cells. Nuli-1 cells (seeded at 30,000 cells/well in a 96-well plate) were infected with (**A**) WT PA14 and PA14 *exoU*::tn or (**B**) with WT PAO1, PAO1+Vector, and PAO1+ExoU at various MOIs for 6 h. Cell death was evaluated using neutral red uptake assay and cytokine production was measured in cell supernatants by ELISA. Mean ± SD of triplicates are shown. (**A**) Student's t-tests and (**B**) one-way ANOVAs followed by Dunnett's multiple comparisons tests were conducted to compare means. NuLi-1 cells were seeded at 150,000 cells/well in a 24-well plate and infected with WT PA14, PA14 *exoU*::tn, PAO1+Vector, and PAO1+ExoU at an MOI of 10. (**C**) At 2 h postinfection, cells were stained with Hoechst/PI to distinguish live (blue) from dead (pink) cells. Data shown are a representative from 3 experiments. (***P* < 0.01, ****P* < 0.001, *****P* < 0.0001).

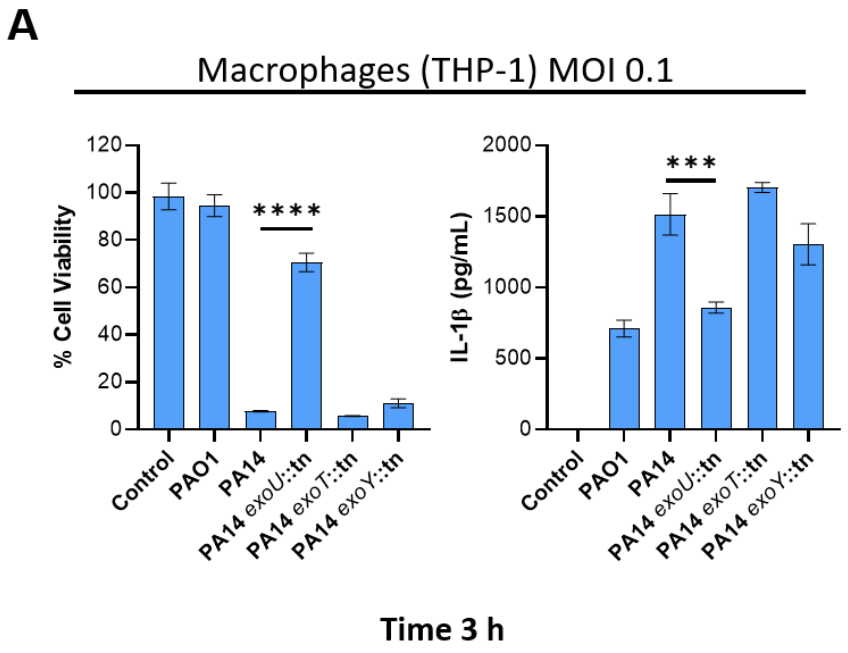
4.2.3 Observed trends of cell death and IL-1β secretion for THP-1 and NuLi-1 cells when infected with PA14 are dominated by ExoU over other P. aeruginosa *effector proteins*

THP-1 macrophages were infected with WT PA14 or PA14 transposon mutants for various T3SS effector proteins (i.e., ExoU, ExoT, and ExoY) for 3 h. It was found that infections with PA14 *exoU*::tn at an MOI of 0.1 induced less cell death and IL-1β expression for THP-1 macrophages compared with WT PA14 and other mutants, which induced similar levels of cell death and IL-1β expression (**Fig. 32 A**). However, at an MOI of 1, the trends for infections with PA14 *exoU*::tn changed in that less cell death was still induced but more IL-1β expression was observed (**Fig. 32 B**), suggesting that increased cell death by ExoU may compromise IL-1β expression. This once again confirmed the influence of relative bacterial burden on IL-1β expression upon exposure to the cytotoxin ExoU.

The full spectrum of T3SS effector proteins includes ExoS, ExoT, and ExoY in addition to ExoU (*184*). Not every strain of *P. aeruginosa* expresses all effector proteins, and the expression of ExoS and ExoU has been reported to be frequently mutually exclusive among *P. aeruginosa* strains (*158, 283*). To examine the role that ExoS may have in cell death and IL-1β secretion, as well as observe whether the other effector proteins could have a greater impact on these processes without the presence of ExoU, I used WT PAO1, which lacks ExoU, and its corresponding transposon mutants for its effector proteins. Interestingly, all of these cytotoxins were found to impact IL-1β expression (**Fig. 33**). There was also reduced cell death in THP-1 cells infected by the ExoS and ExoY mutants at an MOI of 1 compared with WT PAO1 (**Fig. 33 A**). As well, measured IL-1β secretion in the supernatant at an MOI of 1 for all transposon mutants was less compared with WT PAO1, suggesting that each T3SS effector protein could possibly play a role in inducing cytokine expression (**Fig. 33 B**).Therefore, when contrasting the results between the

PA14 and PAO1 transposon mutants, it is conceivable that there is redundancy in the induction of cell death and IL-1β expression by these toxins. As well, ExoU may play a dominant role when it is expressed, as is the case for PA14.

The role of these toxins was also evaluated in the bronchial epithelial cells NuLi-1 and CuFi-1, an immortalized HBE cell line derived from a donor who was homozygous for the ΔF508 CFTR mutation. In contrast to ExoU, other cytotoxins did not show any impact on cell death or IL-1β secretion for both HBE cell lines (**Fig. 34 A, B**). Furthermore, induced cell death and IL-1β expression were found to be comparable regardless if the cells were homozygous for WT CFTR (NuLi-1) or the ΔF508 mutation (CuFi-1). As previous work has shown that WT PAO1 is attenuated in the induction of host cell death and IL-1β secretion for epithelial cells, this finding solidifies the crucial role ExoU plays in stimulating these mechanisms compared with other effector proteins.


A
Macrophages (THP-1) MOI 0.1
% Cell Viability
IL-1β (pg/mL)
****
***
Control
PAO1
PA14
PA14 exoU::tn
PA14 exoT::tn
PA14 exoY::tn
Time 3 h

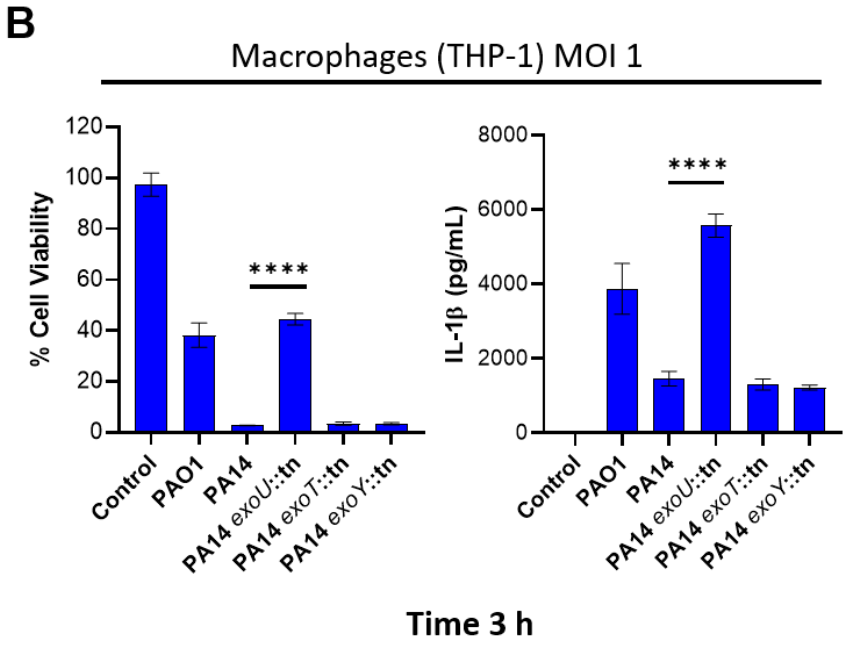

B
Macrophages (THP-1) MOI 1
% Cell Viability
IL-1β (pg/mL)
****
****
Control
PAO1
PA14
PA14 exoU::tn
PA14 exoT::tn
PA14 exoY::tn
Time 3 h

Fig. 32. Observed cell death and IL-1β secretion trends during PA14 infections of THP-1 macrophages are dependent on ExoU but not on other effector proteins. THP-1 macrophages were seeded at 100,000 cells/well and were infected with WT PAO1, WT PA14, PA14 *exoU*::tn, PA14 *exoT*::tn, and PA14 *exoY*::tn at (**A**) 0.1 MOI and (**B**) 1 MOI. At 3 h postinfection, cell death was evaluated by neutral red assay and IL-1β production was measured in cell supernatants by ELISA. Mean ± SD of triplicates from a representative from 3 independent experiments are shown. One-way ANOVAs followed by Dunnett's multiple comparisons tests were conducted to compare means. (***$P < 0.001$, ***$P < 0.0001$).

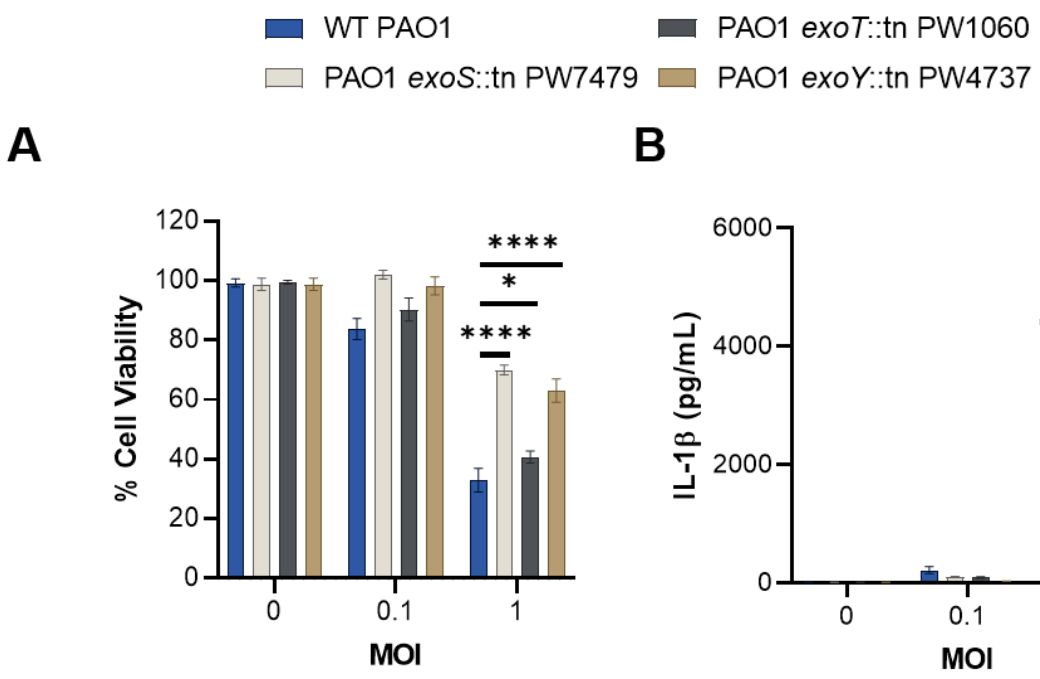
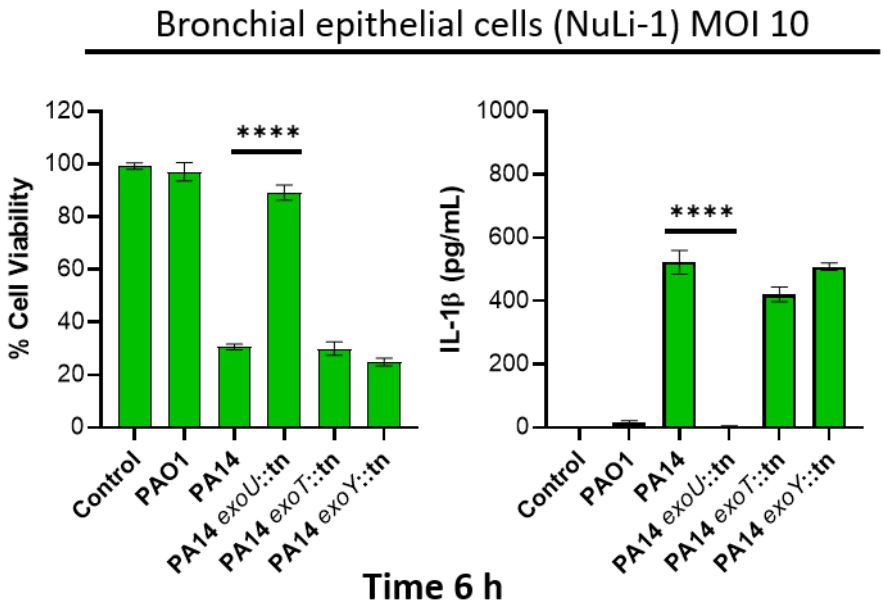


Fig. 33. ExoS, ExoT, and ExoY play a role in IL-1β secretion during PAO1 infections of THP-1 macrophages. THP-1 macrophages were seeded at 100,000 cells/well and infected with WT PAO1 and PAO1 transposon mutants for ExoS, ExoT, and ExoY at indicated MOIs. (**A**) 3 h postinfection, cell viability was evaluated by neutral red assay. (**B**) IL-1β secretion was measured in cell supernatants by ELISA. Mean ± SD of experimental triplicates from a representative experiment are shown. Specific strain names for isolates used are indicated in the figure. One-way ANOVAs followed by Dunnett's multiple comparisons tests were conducted to compare means. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ***$P < 0.0001$).

A


Bronchial epithelial cells (NuLi-1) MOI 10
% Cell Viability
IL-1β (pg/mL)
****
****
Control
PAO1
PA14
PA14 exoU::tn
PA14 exoT::tn
PA14 exoY::tn
Time 6 h

B

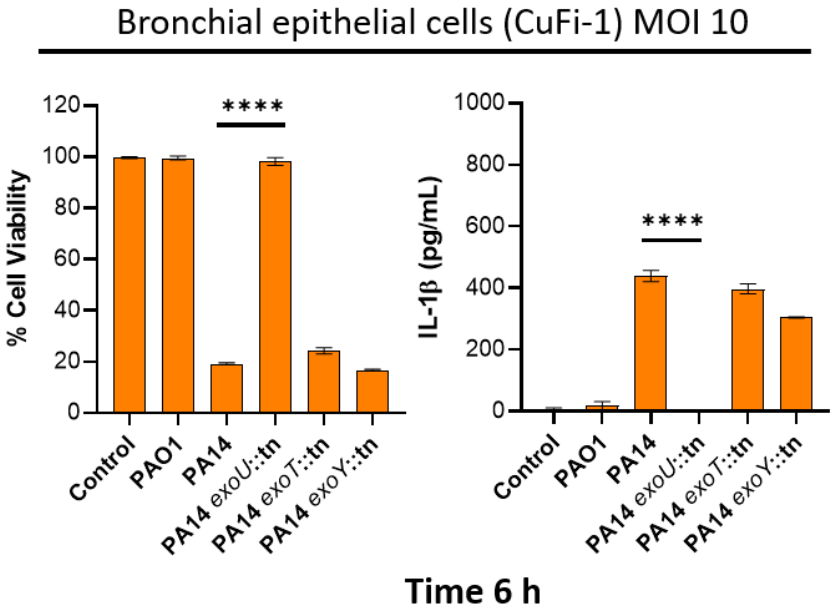

Bronchial epithelial cells (CuFi-1) MOI 10
% Cell Viability
IL-1β (pg/mL)
****
****
Control
PAO1
PA14
PA14 exoU::tn
PA14 exoT::tn
PA14 exoY::tn
Time 6 h

Fig. 34. Observed cell death and IL-1β secretion trends during PA14 infections of HBE cells are dependent on ExoU but not on other effector proteins. (**A**) NuLi-1 and (**B**) CuFi-1 cells (seeded at 30,000 cells/well) were infected with WT PAO1, WT PA14, PA14 *exoU*::tn, PA14 *exoT*::tn, and PA14 *exoY*::tn at an MOI of 10. At 6 h postinfection, cell death was evaluated by neutral red assay, and IL-1β production was measured in cell supernatants by ELISA. Mean ± SD of triplicates from a representative from 3 independent experiments are shown. One-way ANOVAs followed by Dunnett's multiple comparisons tests were conducted to compare means. (***$P <$ 0.0001).

4.2.4 ExoU induces relatively early IL-1β secretion and cell death in THP-1 cells and NuLi-1 cells

As previously shown, IL-1β secretion can occur more rapidly during infections with WT PA14 compared with infections with PA14 *exoU*::tn. To investigate this in greater depth, THP-1 cells were infected with either WT PA14 or PA14 *exoU*::tn at various MOIs, where cell death and IL-1β production were measured at different time points for up to 6 h. It was revealed that WT PA14 could induce cell death and IL-1β expression at relatively earlier time points and at lower MOIs when infecting THP-1 macrophages compared with infections with PA14 *exoU*::tn (**Fig. 35**). This suggests that ExoU does play a role in stimulating IL-1β maturation and secretion. At later time intervals of infection with higher MOIs, the expression of IL-1β, IL-8, IL-6, and IL-10 was enhanced in comparison to WT PA14, and this correlated inversely with cell death (**Fig. 35**).

The impact of ExoU on NuLi-1 cells over time was also investigated. After infecting NuLi-1 cells with WT PA14 or PA14 *exoU*::tn at various MOIs and at different time points for up to 6 h, it was found that ExoU is necessary for *P. aeruginosa* to induce cell death and greater IL-1β, IL-8, and IL-6 secretion (**Fig. 36**). Since the mechanisms of cell death are induced at a much slower rate for NuLi-1 cells compared with THP-1 macrophages, ExoU did not have a negative impact on the expression of these cytokines a these MOIs (**Fig. 36**).

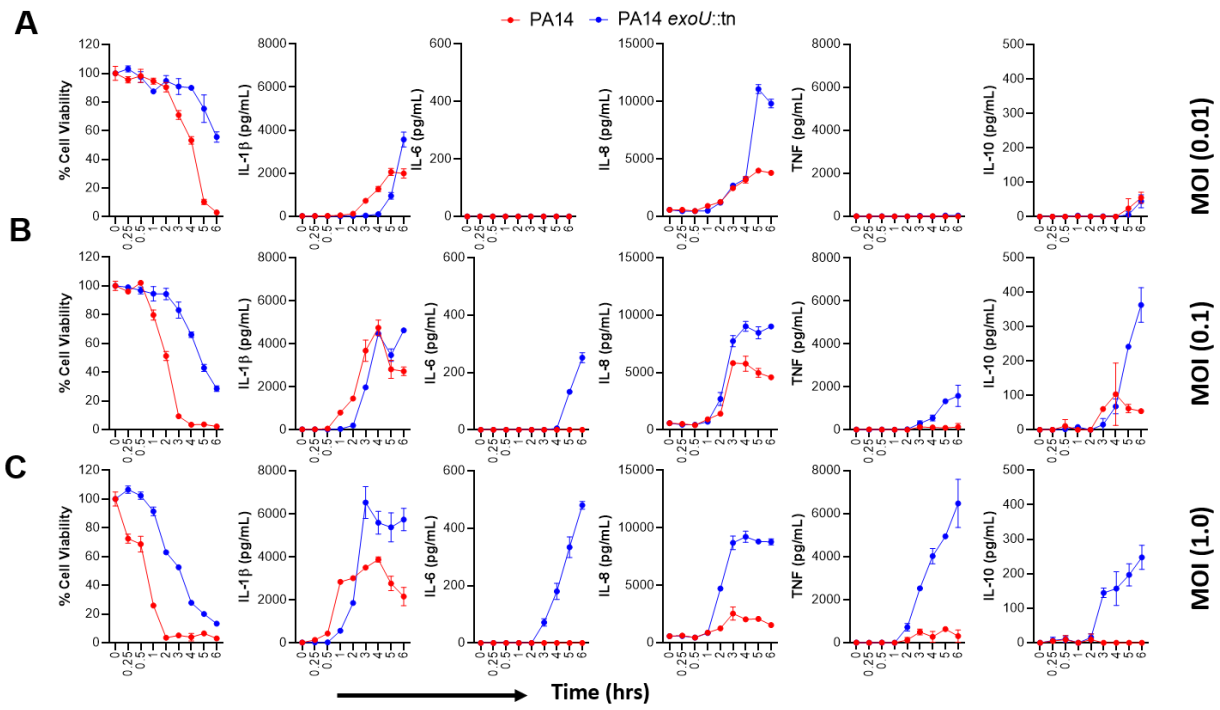


Fig. 35. ExoU induces relatively early IL-1β secretion and cell death in THP-1 cells. THP-1 macrophages (seeded at 100,000 cells/well) were infected with WT PA14 or PA14 *exoU*::tn at an MOI of (**A**) 0.01, (**B**), 0.1, or (**C**) 1 for up to 3 hours. Cell death was evaluated using neutral red assay and cytokine production was measured using ELISA at various indicated time points. Mean ± SD of triplicates from a representative of 3 independent experiments are shown.

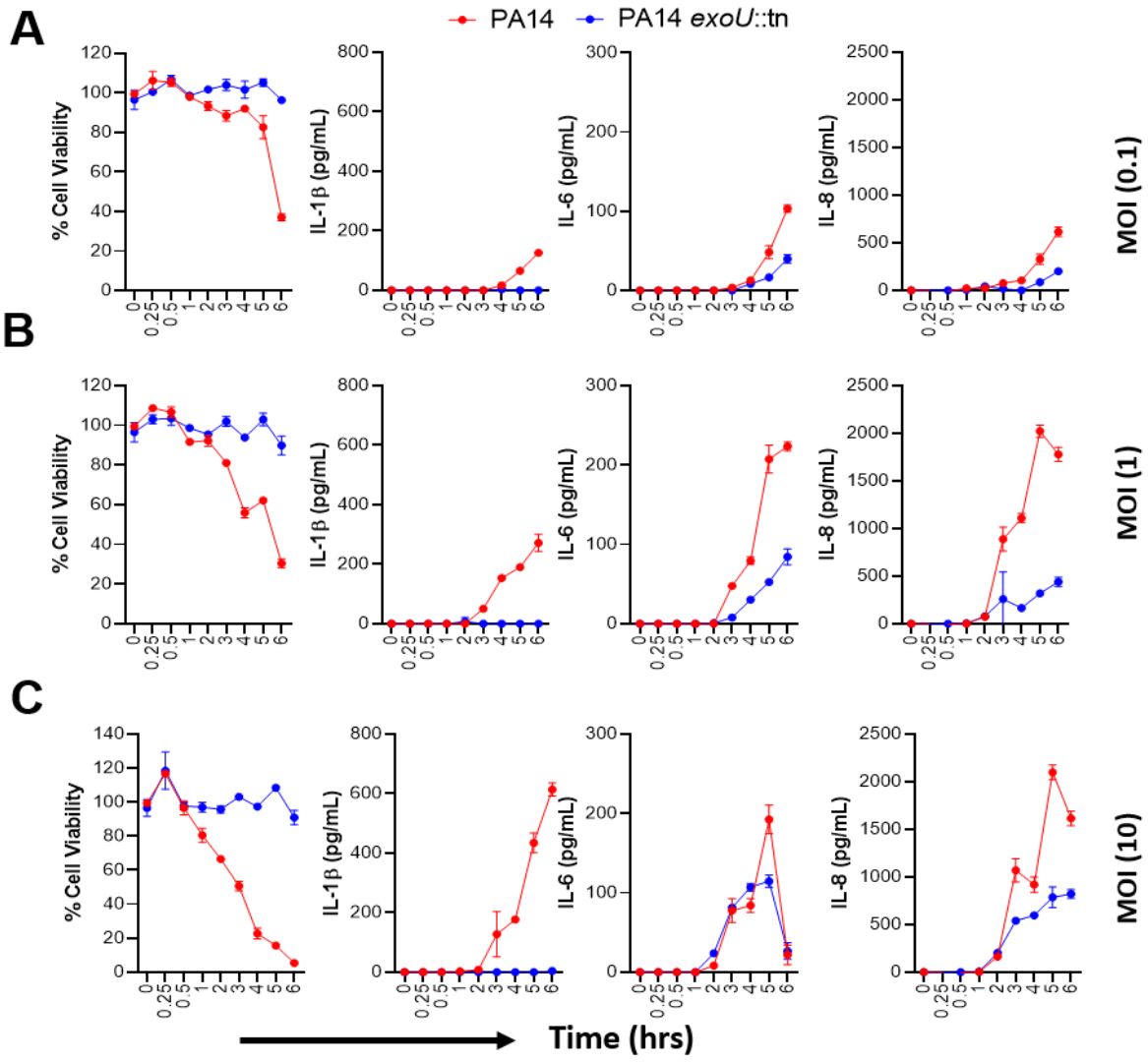


Fig. 36. ExoU induces greater levels of cell death and cytokine secretion in NuLi-1 cells. Nuli-1 cells (seeded at 30,000 cells/well) were infected with WT PA14 or PA14 *exoU*::tn at an MOI of (**A**) 0.1, (**B**), 1, or (**C**) 10 for up to 6 h. Cell death was evaluated using neutral red assay and cytokine production was measured using ELISA at various indicated time points. Mean ± SD of triplicates from a representative of 3 independent experiments are shown.

4.2.5 Caspase-1 and caspase-8 are activated upon infection with ExoU-expressing P. aeruginosa

IL-1β activation is dependent on both the canonical and non-canonical pathways, which involve any one of a number of caspases, including caspase-1 and caspase-8. To determine their involvement in host cell death and IL-1β expression in response to ExoU expression by *P. aeruginosa*, THP-1 macrophages were infected with our available laboratory strains at an MOI of 0.1: WT PA14, PA14 *exoU*::tn, PAO1+ pUCP19 plasmid, and PAO1+ExoU. Either a DMSO vehicle control, or 10μM each of Z-IETD-fmk (i.e., caspase-8 inhibitor), Ac-YVAD-cmk (i.e., caspase-1 inhibitor), or emricasan (i.e., pan-caspase inhibitor) were used to pre-treat the cells for 2 h prior to infection, and the treatments were continued over the course of the 3 h infection. It was observed that for all strains of *P. aeruginosa*, inhibition of the indicated caspases did not impact cell death (**Fig. 37**). That said, selective inhibition of caspase-8, caspase-1, and pan-caspase activation reduced IL-1β production (**Fig. 37**). Interestingly, pan-caspase inhibition was not able to reduce IL-1β production as extensively for ExoU-expressing *P. aeruginosa* strains (**Fig. 37 A, D**). Pan-caspase involvement in IL-1β production, but not cell death, in NuLi-1 cells upon being infected with WT PA14 was also observed (**Fig. 38**)**.** Therefore, for THP-1 macrophages, involvement of the aforementioned caspases in IL-1β expression, but not necessarily cell death, is consistent with our previous findings.

Given these observations, we sought to detect the activation of relevant caspases via immunoblotting. Secreted active caspase-1, -4, -5, and -8 were detected in the supernatants of WT PA14-infected THP-1 cells after 3 h at a relatively low MOI of 0.5 (**Fig. 39 A**). Activation of these caspases was compromised when cells were infected with the ExoU mutant of PA14. Expression of the non-activated versions of these enzymes (detected in cell lysates) was not modulated by ExoU. I also detected low levels of cleaved-gasdermin D in cell extracts during infections with

WT PA14 at an MOI of 0.1 which was reduced in cells infected with the ExoU mutant of PA14 (**Fig. 39 B**). These results indicate the presence of low level of pyroptotic cell death during WT PA14 infections. Similarly, upon infecting NuLi-1 cells and conducting immunoblotting in both supernatant and cell extract samples, WT PA14 was found to induce activation of caspase-1, -4, and -8 (**Fig. 40 A**) and cleavage of gasdermin D (**Fig. 40 B)**.

4.2.6 NLRP3 plays a minor role in IL-1β expression during P. aeruginosa *infection*

As previously described, NLRP3 may play a role during *P. aeruginosa* infections (*270, 277*), and I sought whether the impact of this adaptor protein is influenced by ExoU expression by the bacterium. Through the use of the NLRP3 inhibitor MCC950, it was found that NLRP3 inhibition slightly reduced IL-1β expression in THP-1 macrophages, but not NuLi-1 cells (**Fig. 41**). Any differences observed in IL-1β production between PA14 and PA14 *exoU*::tn was not found to be NLRP3-dependent (**Fig. 41**). These results suggest that NLRP3 also does not appear to play a role in cell death during *P. aeruginosa* infections for either THP-1 or NuLi-1 cells (**Fig. 41**).

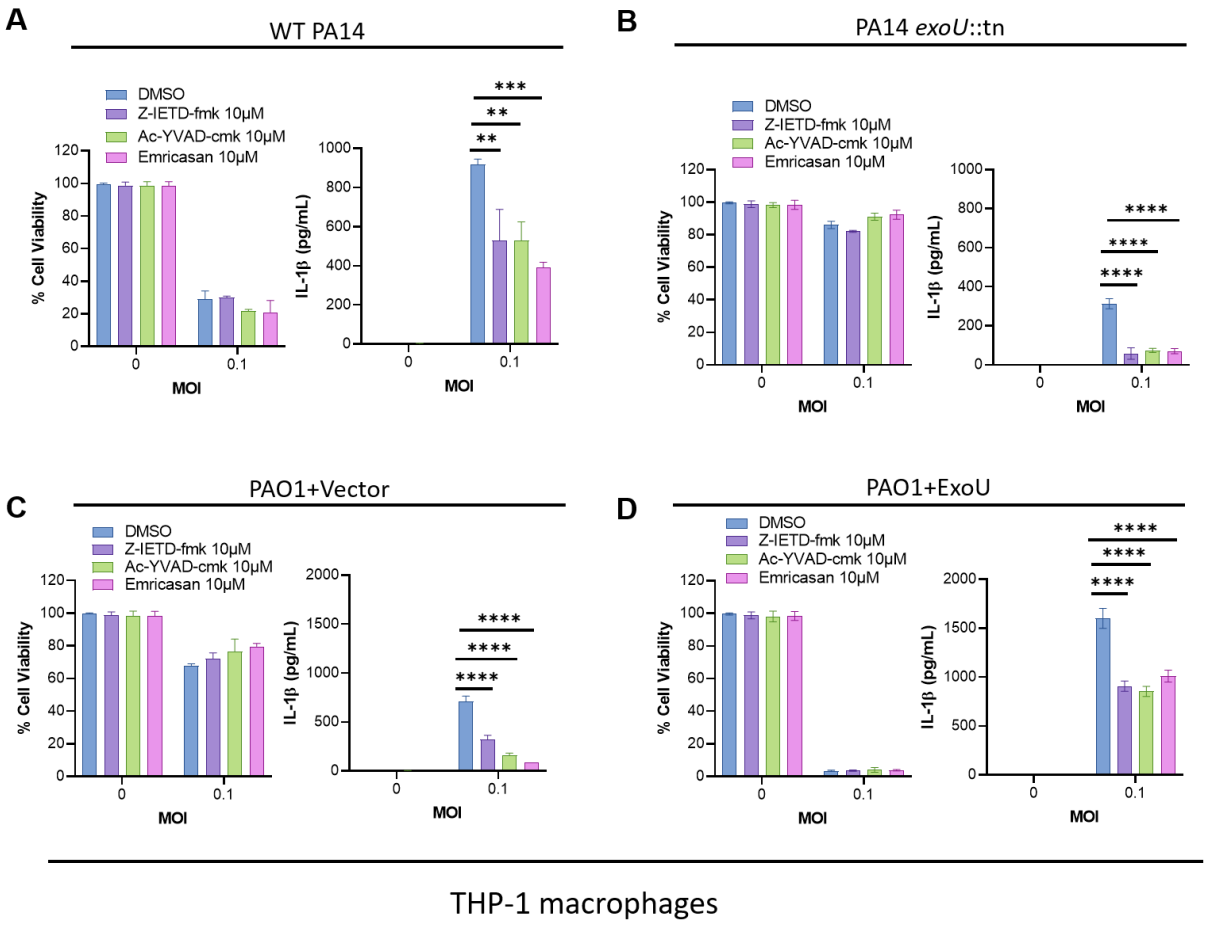


Fig. 37. Pan-caspase involvement in IL-1β expression, but not cell death, is observed in THP-1 macrophages. THP-1 macrophages were seeded at 100,000 cells/well in a 96-well plate. 2 h prior to infection, THP-1 macrophages were pre-treated with 10 μM of either Z-IETD-fmk, Ac-YVAD-cmk, or emricasan or a DMSO vehicle control. Treatments were continued over the course of the 3 h infection with either (**A**) WT PA14, (**B**) PA14 *exoU*::tn, (**C**) PAO1+Vector, or (**D**) PAO1+ExoU (MOI 0.1). At 3 h postinfection, cell viability was evaluated by neutral red assay, and IL-1β production was measured in cell supernatants by ELISA. One-way ANOVAs followed by Dunnett's multiple comparisons tests were conducted. Mean ± SD of experimental triplicates from a representative experiment are shown. (**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

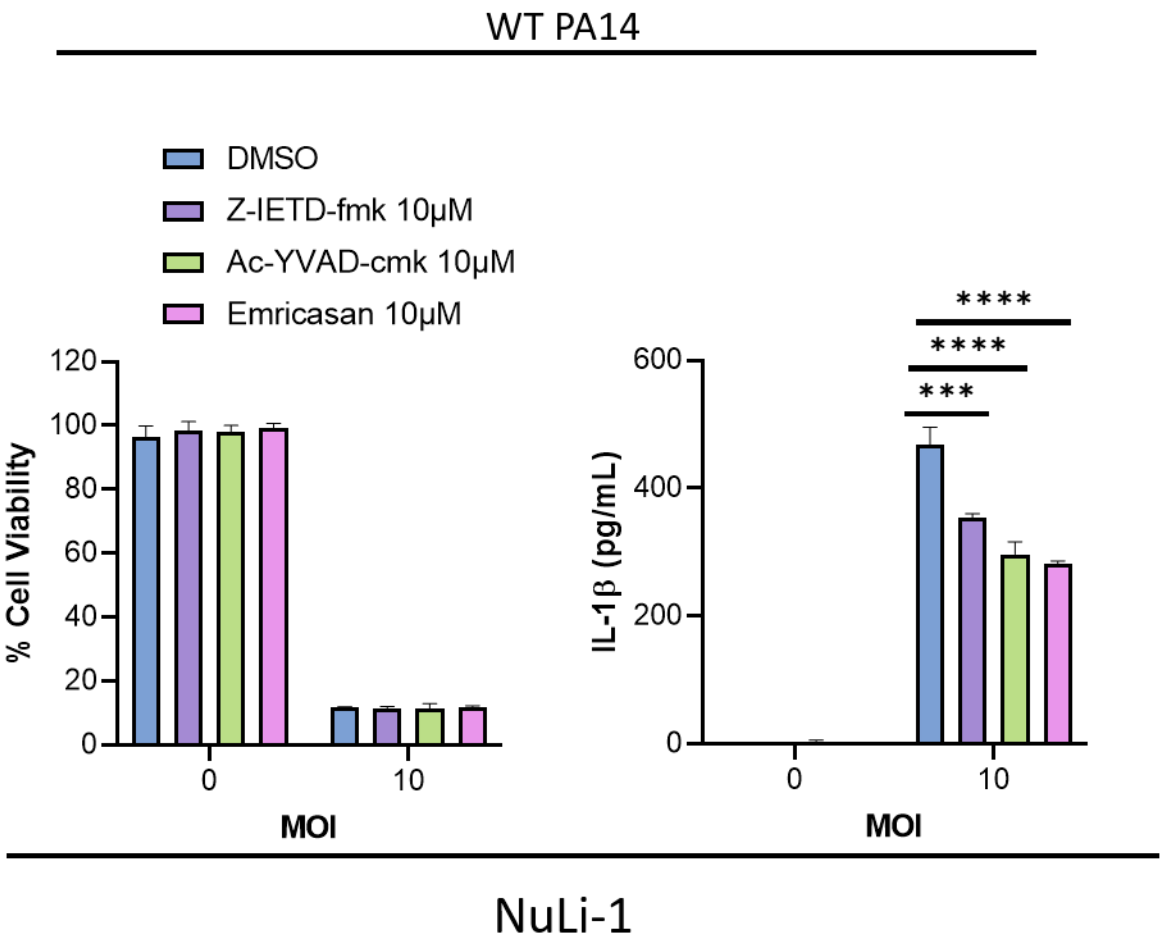


Fig. 38. Pan-caspase involvement in IL-1β expression, but not cell death, is observed in NuLi-1 cells. NuLi-1 cells were seeded at 30,000 cells/well in a 96-well plate. At 2 h prior to infection, NuLi-1 were pre-treated with 10 μM of either Z-IETD-fmk, Ac-YVAD-cmk, or emricasan or a DMSO vehicle control. Treatments were continued over the course of the 6 h infection with WT PA14 (MOI 10). At 6 h postinfection, cell viability was evaluated by neutral red assay, and IL-1β production was measured in cell supernatants by ELISA. One-way ANOVAs followed by Dunnett's multiple comparisons tests were conducted. Mean ± SD of experimental triplicates from a representative experiment are shown. (***$P < 0.001$, ****$P < 0.0001$).

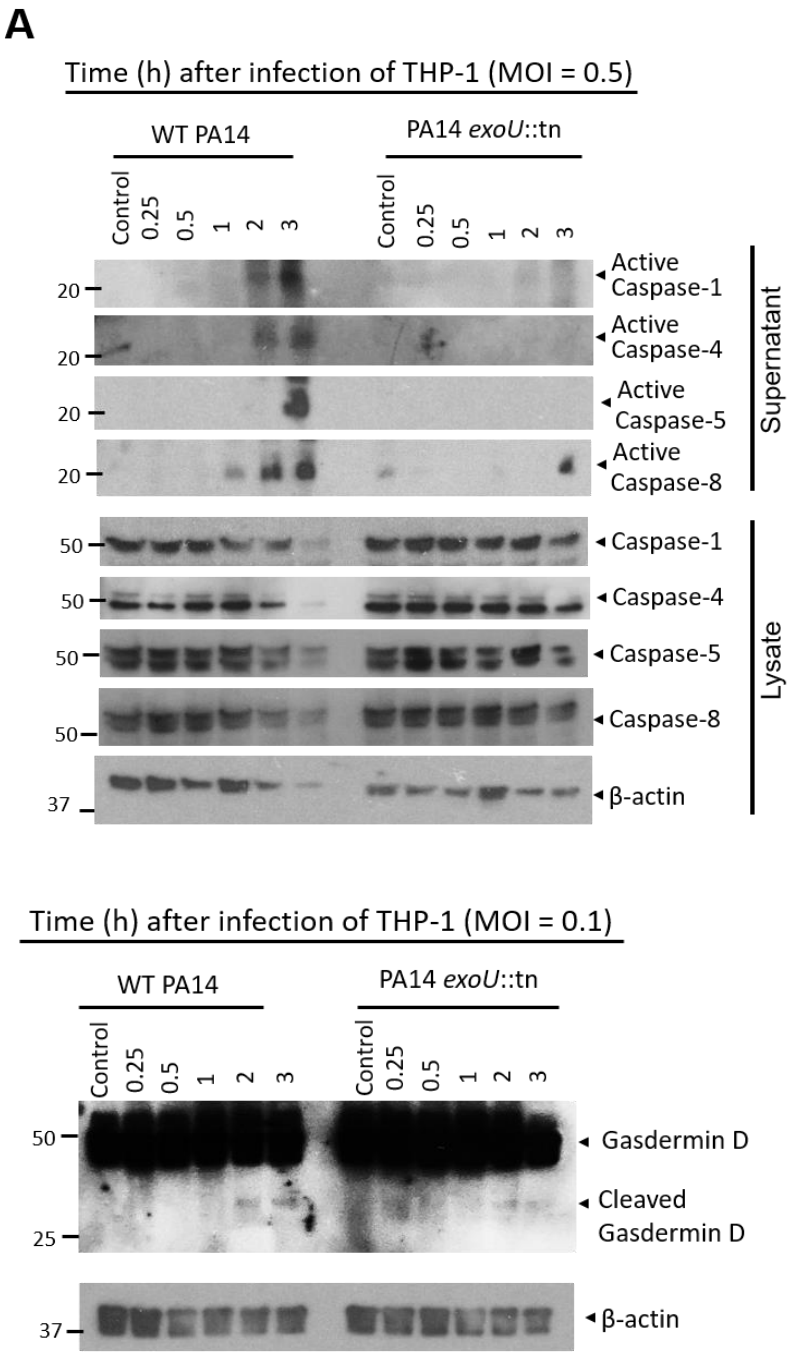

A
Time (h) after infection of THP-1 (MOI = 0.5)
WT PA14
PA14 exoU::tn
Control
0.25
0.5
1
2
3
Control
0.25
0.5
1
2
3
20
20
20
20
Active Caspase-1
Active Caspase-4
Active Caspase-5
Active Caspase-8
Supernatant
50
50
50
50
37
Caspase-1
Caspase-4
Caspase-5
Caspase-8
β-actin
Lysate
B
Time (h) after infection of THP-1 (MOI = 0.1)
WT PA14
PA14 exoU::tn
Control
0.25
0.5
1
2
3
Control
0.25
0.5
1
2
3
50
25
37
Gasdermin D
Cleaved Gasdermin D
β-actin

Fig. 39. ExoU-expressing PA14 is able to induce caspase-1, -4, -5, and -8 activation upon infecting THP-1 macrophages. THP-1 macrophages were seeded at 500,000 cells/well in a 24-well plate. The cells were infected with either WT PA14 or PA14 *exoU*::tn at the indicated time intervals for up to 3 h. (**A**) Infections were conducted at an MOI of 0.5, and immunoblotting was performed on cell supernatants or extracts using specific antibodies shown. (**B**) Infections were conducted at an MOI of 0.1, and immunoblotting was performed on cell extracts using the indicated antibodies. Shown is a representative panel from 3 experiments.

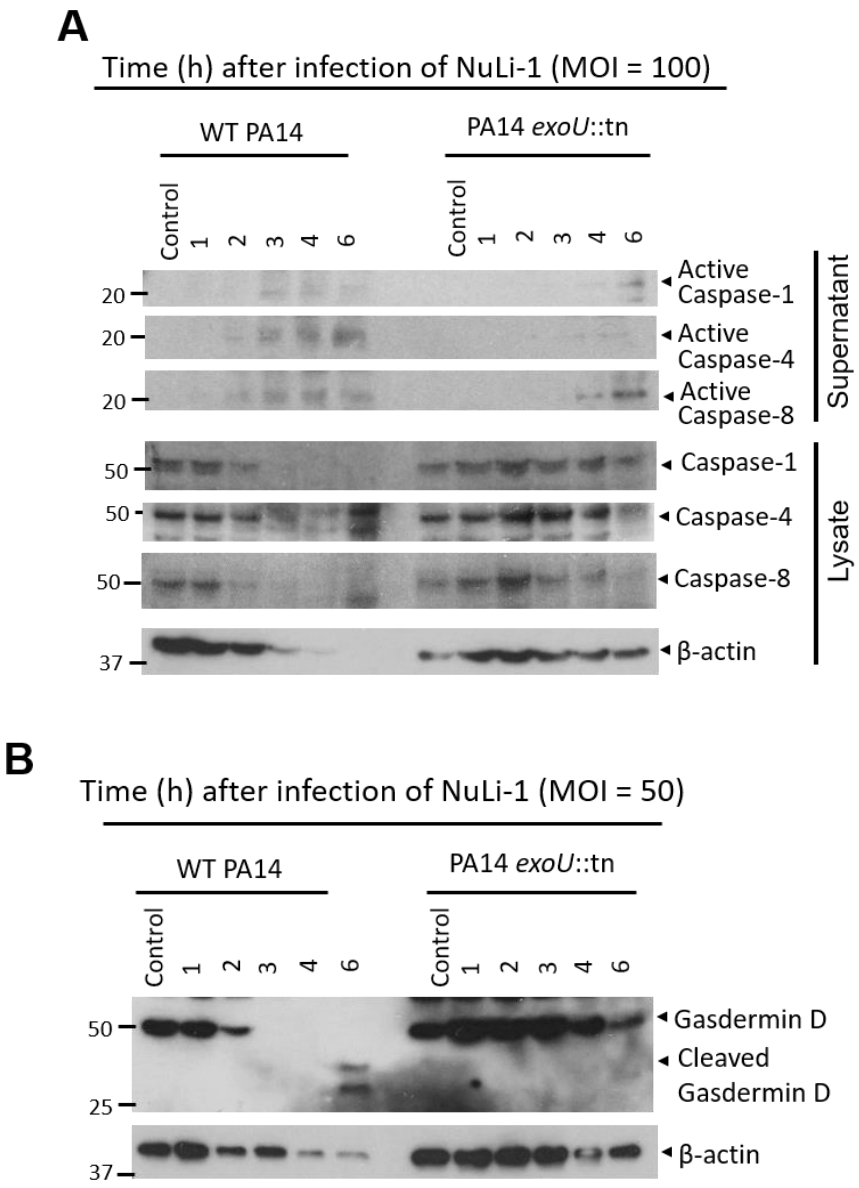


Fig. 40. ExoU-expressing PA14 is able to induce caspase-1, -4, and -8 activation upon infecting NuLi-1 bronchial epithelial cells. NuLi-1 cells were seeded at 150,000 cells/well in a 24-well plate. The cells were infected with either WT PA14 or PA14 *exoU*::tn at the indicated time intervals for up to 6 h. (**A**) Infections were conducted at an MOI of 100, and immunoblotting was performed on cell supernatants or extracts using specific antibodies shown. (**B**) Infections were conducted at an MOI of 50, and immunoblotting was performed on cell extracts using the indicated antibodies. Shown is a representative panel from 3 experiments.

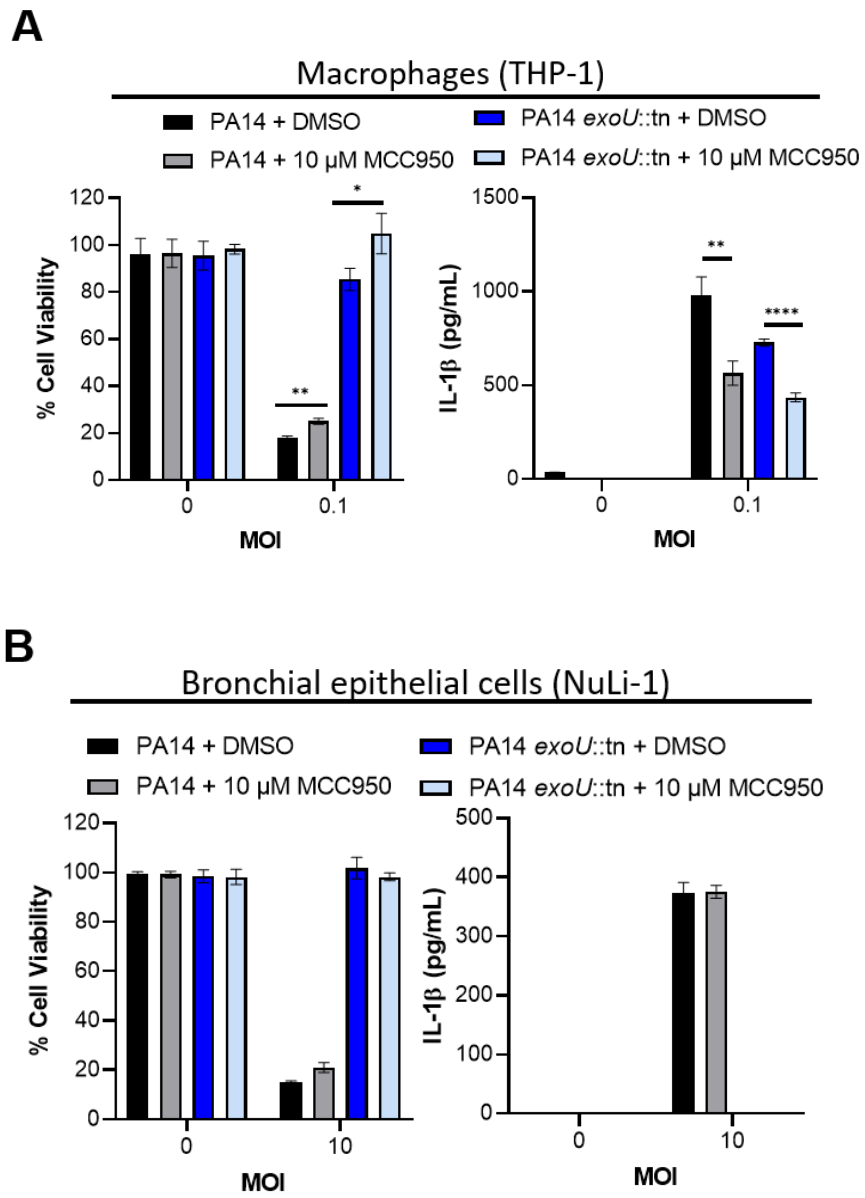

A
Macrophages (THP-1)
PA14 + DMSO
PA14 + 10 μM MCC950
PA14 exoU::tn + DMSO
PA14 exoU::tn + 10 μM MCC950
% Cell Viability
IL-1β (pg/mL)
MOI
B
Bronchial epithelial cells (NuLi-1)
PA14 + DMSO
PA14 + 10 μM MCC950
PA14 exoU::tn + DMSO
PA14 exoU::tn + 10 μM MCC950
% Cell Viability
IL-1β (pg/mL)
MOI

Fig. 41. NLRP3 contributes to IL-1β production, but not cell death, during infections with ExoU-expressing *P. aeruginosa* and non-ExoU-expressing *P. aeruginosa*. (**A**) THP-1 macrophages were seeded at 100,000 cells/well in a 96-well plate. The cells were infected with either WT PA14 or PA14 *exoU*::tn with a DMSO vehicle control or 10 μM MCC950 which was added at the start of the infection. At 3 h postinfection, cell death was evaluated by neutral red assay, and IL-1β production was measured in the supernatants by ELISA. (**B**) NuLi-1 cells were seeded at 30,000 cells/well in a 96-well plate and underwent the same treatment as well as infected with the same strains of *P. aeruginosa*. At 6 h postinfection, cell death was measured by neutral red assay and IL-1β production was measured by ELISA. Student's t-tests were conducted. Mean ± SD of triplicates of a representative experiment are shown. (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$).

4.2.7 ExoU promotes MAPK activation

Given differences in expression in TNF, IL-8, and IL-6 in infections with *P. aeruginosa* strains with and without ExoU expression, I wanted to determine if there were differences in induction in corresponding signalling pathways during these infections. Interestingly, I observed earlier, and at times greater, activation of proteins relevant to the MAPK pathway for THP-1 macrophages upon being infected with WT PA14 compared with PA14 *exoU*::tn (**Fig. 42**). Phosphorylation of p38 MAPK and its downstream kinase MK2 were significantly reduced in THP-1 cells infected with PA14 *exoU*::tn (**Fig. 42**). In contrast, the activation of NF-κB did not appear to be significantly modulated by ExoU (**Fig. 42**). Similar results were observed in bronchial epithelial cells (**Fig. 43**).

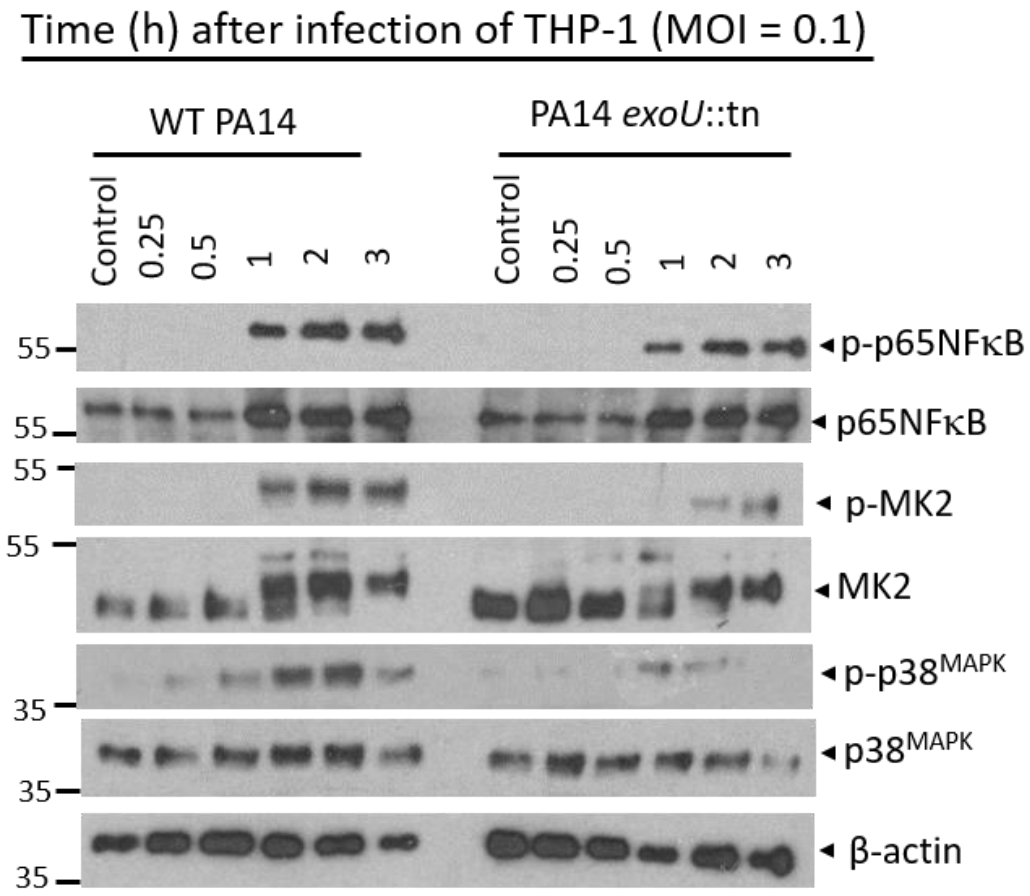


Fig. 42. ExoU-expressing PA14 is able to induce early activation of the MAPK pathway in THP-1 macrophages. THP-1 macrophages were seeded at 500,000 cells/well in a 24-well plate. Immunoblotting was conducted on lysates collected at the indicated time intervals for up to 3 h postinfection with either WT PA14 or PA14 *exoU*::tn (0.1 MOI). Shown is a representative panel from 2 independent experiments.

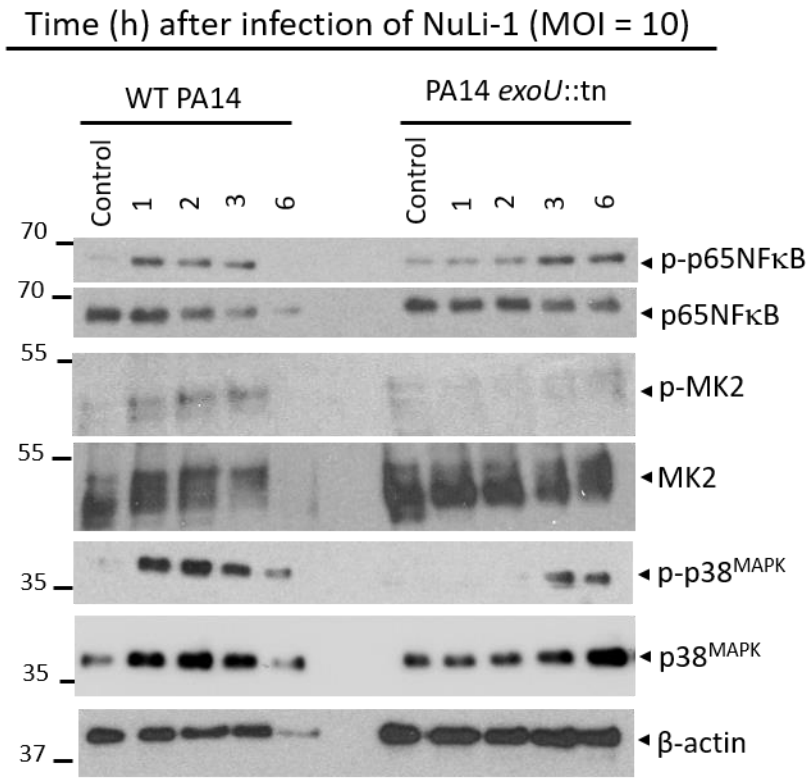


Fig. 43. ExoU-expressing PA14 is able to induce early activation of NF-κB and MAPK pathways in NuLi-1 cells. NuLi-1 cells were seeded at 150,000 cells/well in a 24-well plate. Immunoblotting was conducted on lysates collected at the indicated time intervals for up to 6 h postinfection with either WT PA14 or PA14 *exoU*::tn (10 MOI). Shown is a representative panel from 2 independent experiments.

5.0 DISCUSSION

The human respiratory system is one of the internally located organ systems that, along with the gastrointestinal system, is exposed to the outside environment, necessitating robust defense mechanisms and immune responses for host protection (*30, 284*). CF, however, is associated with structural and physiological changes to the respiratory system, resulting in patients' increased susceptibility to various pathogens and microbial infections (*240*). With time, and for reasons perhaps not entirely apparent, *P. aeruginosa* eventually dominates the lung environment and causes chronic infections in a large proportion of older CF patients (*241*, *251*). Therefore, *P. aeruginosa* is an insidious environmental organism that plagues the lung environment for this patient group, making it a pathogen of high significance for further study.

CF patients are prone to developing chronic infections. During this process, pathogens tend to adapt to the airway environment, of which *P. aeruginosa* is no exception (*98, 285*). It has been reported that evolved *P. aeruginosa* isolates from the lungs of CF patients often display a phenotype of reduced acute virulence that is characterized by poor motility and growth, loss of quorum sensing, and overproduction of alginate (*286, 287*). These adaptations have been proposed to decrease virulence and facilitate evasion of host immunity (*224*). Despite this decrease in acute virulence, CF patients with ongoing chronic *P. aeruginosa* respiratory infections are still reported to have elevated inflammatory cytokines found in their BALF and persistent respiratory disease (*14, 95, 257, 288*). This inflammation is further tied with bacterial burden since inflammatory markers are found to be reduced after antibiotic treatment (*98, 102, 289, 290*). Thus, these trends lend further evidence that *P. aeruginosa* at various stages of CF respiratory infections are able to induce significant inflammation.

5.1 *P. aeruginosa* virulence and proinflammatory properties in acute and chronic infections

5.1.1 P. aeruginosa *from early infections induces many pathways of programmed cell death*

P. aeruginosa has been reported to initiate various forms of programmed cell death, including apoptosis, necroptosis, and pyroptosis (*155*, *175*, *177*). This reported ability to stimulate different mechanisms of cell death is corroborated by the aforementioned results. Apoptosis is characterized as a non-inflammatory form of programmed cell death which is essential for homeostasis and cell cycling in multicellular organisms (*126*, *180*, *291*, *292*). Given how apoptotic cells are rapidly phagocytosed by efferocytosis, apoptosis results in minimal inflammation in extracellular spaces (*150–152*). Necrosis, however, is a form of inflammatory cell death, as it results in the release of intracellular contents such as DAMPs (*180*, *292*, *293*). In acute infection settings, necrosis confers advantages, but this processes can become detrimental if induced chronically due to sustained inflammatory cascades (*180*, *292*, *293*). As necrosis is defined by characteristic changes in cellular morphology (e.g., cell swelling and plasma membrane breakdown), there are indeed recognized mechanisms of necrotic programmed cell death within this category (*180*, *292*, *293*). Such mechanisms include pyroptosis and necroptosis. My observations indicate that *P. aeruginosa* induces necrotic cell death in host cells. Microscopy images with Hoescht and PI, namely the observed PI staining in dead cells, demonstrate that host membrane integrity is compromised in response to infections, indicating the occurrence of necrotic cell death (*294–296*). As well, my inhibitor work may demonstrate that a number of cell death pathways, including compensatory pathways, can be induced in response to *P. aeruginosa* infections. Inhibition of caspase-8 or RIP3 inhibitor, which would disrupt predominantly apoptotic and necroptotic cell death, respectively, did not alter host cell death in response to *P. aeruginosa* isolates from acute infections, suggesting redundant or compensatory mechanisms are involved.

5.1.2 Inverse relationship between host cell death and cytokine production during infections with chronic CF respiratory P. aeruginosa *isolates*

Work from **Section 4.1** seeks to explain how *P. aeruginosa* from chronic infections, despite being able to evade mechanisms of inflammatory cell death, can still drive inflammation and decline in lung function in CF. The investigations that followed have revealed an inverse relationship between host cell death and cytokine production in human macrophages and bronchial epithelial cells. This is a novel interpretation, as necrotic cell death by pyroptosis or necroptosis that can occur during infections are, by definition, inflammatory (*182, 297*). In acute infections, bacteria produce virulence factors that enable host immune cell recognition, thereby leading to bacterial clearance and inflammatory cell death. This inflammatory cell death would also serve to restrict the overall immune response and subsequent cascades. In the CF chronic infection context, however, I propose that necrotic cell death cannot occur because the virulence factors that would normally trigger these signalling cascades are not produced by CF-adapted *P. aeruginosa*. The subsequent immune response is exaggerated and uncontrolled, potentially resulting in respiratory pathology due to persistent cytokine production. This inverse relationship was evident when comparing isolates from patients' first *P. aeruginosa* respiratory culture compared with isolates identified in patients with a longstanding history of *P. aeruginosa* infections. In addition, the same trends were observed among a cohort of longitudinal isolates, confirming that *P. aeruginosa* adapts with time, as CF patients experience chronic infections, to evade promoting host cell death.

My data repeatedly demonstrated relationships between macrophage cell death, including pace and magnitude, and proinflammatory cytokine expression. Specifically, production of TNF, IL-6 and IL-8 was significantly reduced when we observed greater and more rapid macrophage cell death. In contrast, IL-1β was still secreted even when early host cell death was induced. These

observed cytokine trends are likely tied with induced host cell death compromising cytokine processing. It has been demonstrated that isolates from early and chronic infections do not necessarily induce different levels of transcription for the cytokines of interest in this study. THP-1 macrophage exposure to heat-killed bacteria, thereby restricting the induction of cell death, stimulated the same levels of IL-1β and IL-8 secretion between the early and chronic groups of isolates. Collectively, these findings are consistent with previous reports describing how the mechanisms behind IL-1β maturation and secretion differ from those of other cytokines. Cytokine secretion by monocytes involves the conventional endoplasmic reticulum (ER)-Golgi route, similar to other secreted proteins that undergo post-translational modifications prior to exocytosis (*298*, *299*). In contrast, monocytes must use a diverse set of pathways to secrete IL-1β, as the IL-1 cytokine family lacks the secretion signal to be trafficked through the ER-Golgi route. These alternate pathways, which may also be more rapid, include lysosome exocytosis, microvesicle shedding, and caspase-1-dependent cellular pore formation (*298*, *299*). Therefore, it may be that THP-1 macrophages can engage these relatively rapid pathways even when infected with early-infection *P. aeruginosa* isolates or otherwise virulent bacteria. However, under the same circumstances, the expression of other cytokines could be compromised due to insufficient time for traditional cytokine processing to occur. Conversely, since *P. aeruginosa* isolates from chronic infections do not induce rapid cell death, the macrophages would be able to complete all the necessary steps to complete post-translational modifications and ultimately secrete these cytokine proteins.

THP-1 macrophages, in response to *P. aeruginosa* infections, were shown to produce significant levels of the inflammatory cytokines and chemokine IL-1β, TNF, and IL-8, which is relevant in the context of CF. TNF and IL-1β play an important role in numerous inflammatory

pathways (*182, 300*), while IL-8 helps recruit and activate a variety of immune cells, including neutrophils (*301*). Interestingly, IL-1β expression, although reduced, was still detected in appreciable quantities when infecting THP-1 macrophages with chronic isolates. These findings may be reflective of clinical reports indicating that IL-1β, TNF, IL-8, and IL-6 have been found in the BALF of CF patients compared with non-CF controls and can be produced in response to bacterial LPS (*15, 99, 257, 289*). The one trend that may not correlate well clinically is that I also observed more IL-10 production in response to chronic *P. aeruginosa* isolates, whereas IL-10 is typically found in lower levels in samples from CF patients (*302*). In the clinical context, low levels of IL-10 is presumed to be indicative of the known dysregulation of proinflammatory pathways during respiratory infections in CF (*302, 303*). Furthermore, THP-1 macrophages do not appear to be a significant source of IL-6 production based on my observations. Therefore, it may be that both my chosen cell model type (i.e., THP-1 cells) and *in vitro* infection model may not accurately recapitulate the entire cytokine profile seen in CF patients. Both the observation of a single type of immune cell, in comparison to the variety which reside in or migrate to the lung environment, as well as the relatively short course of infection likely limits the ability to appreciate significant IL-10 and IL-6 levels. Given that the trends in IL-1β, TNF, and IL-8 are of relevance to CF and my findings correlated well with clinical reports, my proposed model likely has applicability for more rapidly secreted cytokines.

Studies of various cytokines known to be dysregulated in CF and their possible clinical implications have been conducted. It has been suggested that there are correlations between elevated cytokine levels in CF patient sputum and clinical parameters, such as declining lung function (*101, 113, 304*). Contradictory data on TNF and its impact on CFTR expression have been reported. It has been proposed that TNF is able to restore CFTR localization and function in

ΔF508 bronchial epithelial cells (*305*), while other observations suggest that TNF downregulates CFTR expression in murine smooth muscle cells (*306*) as well as induces increased airway epithelial apoptosis (*307*). Furthermore, *TNF* gene polymorphisms that are associated with increased TNF activity have been associated with higher levels of neutrophil elastase among CF patients (*308*). IL-8 was deemed as a marker that may be predictive of pulmonary re-exacerbation among CF patients (*258*) as well as a marker of bacterial colonization and lung damage severity (*113*).

It is also of interest to note that in experiments using heat-killed bacteria, there was evidence that heat-stable *P. aeruginosa* factors could still induce inflammatory mechanisms when interacting with host cells. Namely, chronic isolates during these experimental conditions still demonstrated a slightly enhanced ability to induce NF-κB activation as well as TNF production. One possible factor which could account for this difference in TNF expression in particular may be LPS. Changes in LPS structure in *P. aeruginosa* isolated from CF patients have been reported, including changes to the lipid A structure or loss of the O antigen (*309–312*). Interestingly, most known adaptations in LPS structure are affiliated with loss of immunogenicity, whereas any changes associated with inducing greater inflammatory responses, such as in lipid A, have been identified in relatively earlier CF infections for patients with severe respiratory diseases (*309, 312, 313*).

This described paradigm of extensive cell death limiting cytokine expression may apply broadly to diverse bacteria, provided that sufficient host cell death is induced to compromise cytokine processing. Most of my findings demonstrating extensive, rapid cell death was upon infecting cells with PA14, a strain of *P. aeruginosa* that is known to be highly virulent (*238*). Using clinical isolates, I observed that *P. aeruginosa* isolated from CF patients with early infections can

also limit cytokine expression despite their more moderate virulence compared with PA14. While these early-infection CF *P. aeruginosa* isolates did not induce macrophage cell death as quickly as PA14, the kinetics were still sufficiently rapid to limit proinflammatory cytokine expression. The same trends can be appreciated when observing THP-1 macrophage response to *P. aeruginosa* isolates not from CF patients (e.g., from wounds or the environment), among PA14 transposon mutants, and comparing PA14 to a laboratory reference strain of *S. aureus*. Regardless of the group of bacteria under study, for any strain that did not induce rapid host cell death compared with more virulent strains, they were able to elicit greater IL-8 and TNF production. Furthermore, I could appreciate this trend even when comparing different genus of bacteria. *S. aureus* is also a frequent colonizer of patients with CF in their respiratory tract and is often associated with early respiratory infections (*282*). When comparing a reference strain of *S. aureus* with the virulent WT PA14, I could still appreciate the ability for *S. aureus* to induce greater production of cytokines while not inducing as much host cell death as WT PA14 at certain MOIs. Therefore, this inverse relationship between the induction of host cell death and inflammatory activity, as outlined in **Fig. 44**, reaffirms how the definition of virulence factors should take into consideration both persistence of infection and induced host inflammation when discussing *P. aeruginosa*, CF, and chronic infections, as persistent inflammatory mechanisms in the absence of acute virulence factors can contribute to pathogenesis.

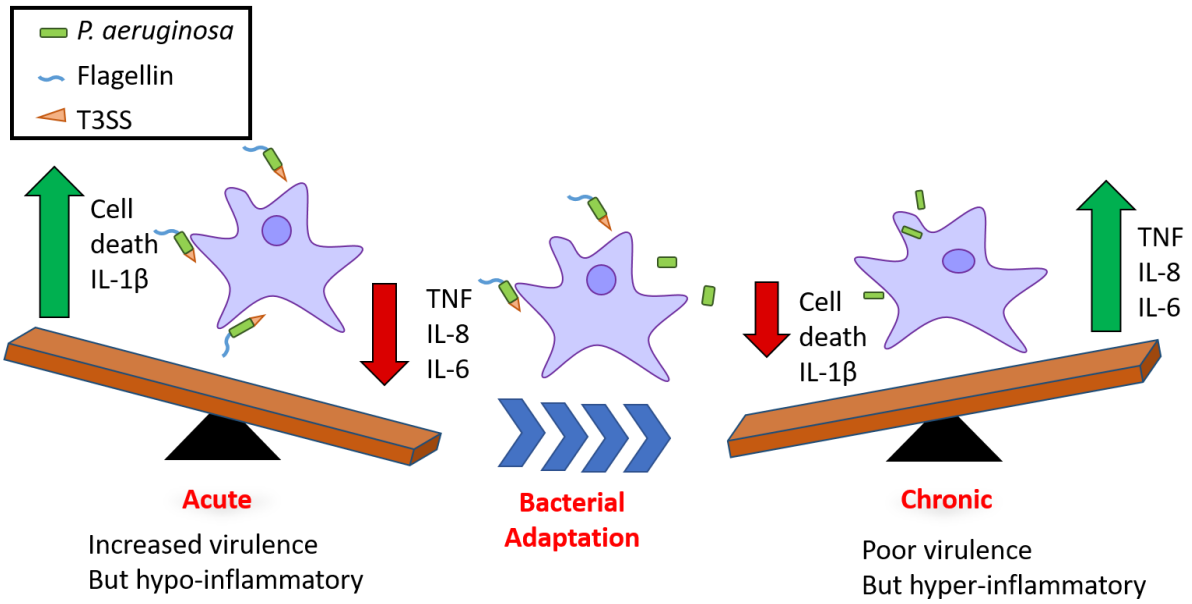


Fig. 44. Proposed model outlining *P. aeruginosa* virulence and proinflammatory properties. In acute infections, *P. aeruginosa* is able to induce high levels of host cell death, such as with macrophages, disrupting signalling pathways that would result in the expression of proinflammatory cytokines. However, as *P. aeruginosa* adapts in the CF lung environment during chronic infections, acute virulence factors are often downregulated. As a result, *P. aeruginosa* from chronic infections are unable to induce cell death of macrophages, allowing for the signalling pathways to proceed and contribute to a hyperinflammatory lung environment.

5.1.3 P. aeruginosa *virulence and impact on cell death and cytokine signalling in CF infections*

Over the course of developing chronic infection, *P. aeruginosa* has often been found to downregulate the expression of flagellin and T3SS components, which would otherwise be virulence factors recognizable by the immune system of a host (*224*, *228*). Consequently, their downregulation could aid in the bacterium's ability to evade host immune surveillance (*224*, *228*). *P. aeruginosa* flagellin is a PAMP that activates the NLRC4 inflammasome, resulting in pyroptosis and IL-1β secretion (*314–316*). This process appears to be dependent on the presence of functional flagellin (*315–317*). Similarly, *P. aeruginosa* T3SS components activate NLRC4, pyroptosis, and IL-1β secretion (*158*, *318*). Considering the frequent downregulation of these virulence factors (*102*, *256*, *262*, *286*, *287*), my results suggest that pyroptosis is relatively diminished during chronic infections, possibly facilitating persistence of *P. aeruginosa*. Using PA14 transposon mutants to infect THP-1 cells demonstrated that acute virulence factors could indeed play a role in the inverse relationship between cell death and cytokine expression. It was found that T3SS components, but not the flagellum structural component FliC, were required to induce cell death. This is consistent with the importance of the T3SS for injecting cytotoxins into host cells to cause host cell death (*319*).

As expected given the observed production of IL-1β production in this work, upon infecting THP-1 cells with clinical *P. aeruginosa* isolates, I found that multiple caspases were engaged. Observations of caspase-1 involvement were evident via immunoblotting as well as the use of the THP1-defCASP1 cell line. Interestingly, caspase-1 was found to play a predominant role in IL-1β production, but not cell death. However, use of the NLRP3 inhibitor MCC950, which is viewed as being upstream of caspase-1 activity (*320*, *321*), impacted IL-1β production extensively as well as had a greater impact on observed host cell death induced by early CF clinical

isolates of *P. aeruginosa*. This difference in the roles of caspase-1 and NLRP3 on cell death could be due to a number of factors. One limitation is that the THP1-defCASP1 cells have been validated to express 7-fold less active caspase-1 compared with WT THP-1, as opposed to being a complete knockout cell line. However, with the NLRP3 inhibitor, there could be a higher level of potency associated with this method of inhibition. Furthermore, caspase-8 is known to promote NLRP3 inflammasome signalling while in the presence of caspase-1 (*160*). In the absence of caspase-1, NLRP3 is also known to recruit caspase-8 to induce noncanonical pyroptosis (*160*), suggesting that there are different NLRP3-driven mechanisms which can stimulate host cell death.

I found that caspase-1, -5, and -8 were all poorly activated by chronic CF infection isolates of *P. aeruginosa*. Caspase-1 is viewed as having a role in the canonical inflammasome pathway, where it initiates pyroptosis by cleaving pro-IL-1β as well as GSDMD, thereby resulting in plasma membrane rupture and subsequent inflammatory cell death (*322*). However, non-canonical pathways that activate IL-1β have been observed. Murine caspase-11, the homolog of human caspase-5/-4, has been shown to mediate non-canonical inflammasome signalling in response to cytosolic bacterial LPS, which still results in IL-1β production (*159*, *323*, *324*). The precise mechanism involved upon LPS-activating human caspase-4 and -5, such as during *P. aeruginosa* infections, is still not precisely clear. Caspase-8, however, has multiple roles in promoting cell death by the extrinsic apoptotic pathway, the ripoptosome cell death signalling pathway, and the NLRP3 inflammasome pathway (*154*, *160*, *325–328*). Furthermore, caspase-8 is increasingly recognized to have a role in IL-1β activation and pyroptosis through cleaving GSDMD (*161*, *329–331*). Given the observed low activation of all these caspases, these mechanisms are likely diminished during chronic CF infection with *P. aeruginosa* compared with acute respiratory infections.

Alterations in cell signalling in the context of CFTR dysfunction have yielded contradicting results, but have nonetheless been essential to study due to the increased inflammation often seen in CF. It has been reported by other groups that there is an increase in baseline activation of NF-κB and p38 MAPK pathways in CF airway epithelial cells compared with WT CFTR cells as well as in response to *P. aeruginosa* infections (*332–334*). Elevated NF-κB and p38 MAPK would have obvious implications for increased cytokine expression, including the cytokines of interest that have been previously discussed (*116*, *124*, *136*, *138*, *139*). However, using $CFTR_{inh}$-172 to inhibit CFTR function did not reveal any noticeable differences in cell death and cytokine expression. Interestingly, this is reflective of a previous study which demonstrated no increase in IL-1β secretion upon treating THP-1 macrophages with $CFTR_{inh}$-172 (*270*). Furthermore, the use of other cellular or animal models to observe the impact of CFTR dysfunction on IL-1β secretion have yielded contradictory results (*270*, *335*, *336*). It may be that the degree and mechanism of CFTR dysfunction in CF varies from use of the CFTR inhibitor. Indeed, the mechanism of action for the CFTR inhibitor is via increasing the mean close time of the ion channel (*337*). In contrast, there are thousands of *CFTR* mutations that are known to cause different phenotypes of CF, although the ΔF508 mutation is easily the most common (*9*). Disease-causing *CFTR* mutations may result in compromised transcription, translation, protein trafficking, channel gating, chloride conductance, and CFTR stability (*9*). ΔF508 is classified in the "no CFTR trafficking" group (*9*), and this form of impairment varies from CFTR inhibition by $CFTR_{inh}$-172 and would perhaps entail misfolded CFTR protein free to engage with other proteins or cascades in the cytosol (*338–340*). It is, however, important to note that for the purposes of this work, even with some level of CFTR inhibition, the paradigm of rapid host cell death disrupting cytokine processing and secretion still applies when observing infections between PA14 and PAO1 for both THP-1

macrophages and bronchial epithelial cells. Therefore, there could still be important implications for the elevated inflammatory markers seen in this work in the context of CF patients.

5.2 *P. aeruginosa* induces IL-1β expression in human macrophages and bronchial epithelial cells

5.2.1 IL-1β expression during P. aeruginosa *infections and implications in respiratory infections*

In **Section 4.2**, I described a novel observation that ExoU is able to induce IL-1β secretion. ExoU is an effector protein secreted by the *P. aeruginosa* T3SS and is an identified acute virulence factor isolated among clinical isolates, including those found in CF (*168*, *221*, *341*, *342*). It has also been previously described to inhibit caspase-1 and subsequent IL-1β secretion in murine BMDMs (*158*). The precise nature of this inhibition, however, has not been elucidated, although it was noted that active caspase-1 was not detected via immunoblotting after conducting infections with the ExoU-expressing strain PA103 (*158*).

My data has repeatedly shown the ability of the ExoU-expressing WT PA14 or PAO1+ExoU to induce IL-1β expression. Indeed, at relatively lower MOIs or at earlier time points, I can appreciate how ExoU, even when expressed in different backgrounds of *P. aeruginosa*, can induce greater levels of IL-1β expression compared with counterparts that do not express ExoU. This discrepancy from other groups' findings may likely be due to host cell death kinetics, where any experimental condition that prolongs the occurrence of cell death will allow for ExoU-induced IL-1β processing and secretion to occur. In addition, ExoU-expressing strains of *P. aeruginosa* consistently promote IL-1β expression upon infecting NuLi-1 cells, indicating broader implications for how this cytotoxin can engage various cell types and cascades involved in innate immunity during respiratory infections. The consistent ability for ExoU to induce IL-1β in NuLi-1 cells is likely due to the slow rate at which NuLi-1 cells engage cell death mechanisms in contrast

to THP-1 cells. Throughout this work, it has been frequently demonstrated that 3 h is a sufficient amount of time for observable cell death to occur in THP-1 cells during *in vitro P. aeruginosa* infections. However for NuLi-1 cells, at least 6 h is required for even virulent bacteria (i.e., PA14) to reach an equivalent level of cell death as observed in THP-1 cells. This relative delay in cell death permits NuLi-1 cells to engage in cell signalling to release IL-1β, albeit at more modest levels.

The role of IL-1β during infections is somewhat contentious, and its impact on *P. aeruginosa* infections and in CF are no exception. Bronchial epithelial cells (*270*, *343*) and hematopoietic cells (*270*) are sources of IL-1β production, albeit it has been argued that hematopoietic cells would be the predominant source. My data corroborates these findings given that THP-1 macrophages are able to induce much higher levels of IL-1β secretion than NuLi-1 cells, although NuLi-1 cells are able to produce IL-1β in response to ExoU. In clinical observations and clinical reports on CF, evidence suggests that IL-1β generally plays a pathological role in CF. Enhanced IL-1β secretion has been measured in cells isolated from CF patients and in CF models (*335*, *344*, *345*), and IL-1β has been found in elevated levels in the BALF, serum, and sputum of CF patients (*14*, *15*, *98*, *99*, *257*, *270*, *288*). These elevated IL-1β levels in airway secretions are in turn correlated with clinically relevant frameworks measuring CF disease severity, such as FEV_1 and CF-ABLE score (*346*). IL-1β in the lung environment is also associated with mucus hypersecretion in both non-CF and CF human bronchial epithelial cells (*104*). The proposed mechanism was that the cytokine can induce MUC5B and MUC5AC protein secretion in airway mucus as well as mucus hyperconcentration, which could be attenuated through the use of an IL-1 receptor (IL-1R) antagonist (*104*).

Where controversy lies with regards to IL-1β is its role in general respiratory infections and whether its secretion drives pathology. This controversy is further heightened given the limited representative animal models that are available for study. Various CF mouse models fail to fully recapitulate spontaneous respiratory disease while under standard, non-sterile laboratory conditions (*347*), unlike humans with CF, where arguably early structural abnormalities can be detected in young patients (*348*). The discrepancies are also apparent for models of chronic *P. aeruginosa* infection, which are often spontaneous among CF patients as *P. aeruginosa* undergoes adaptations in the CF lung (*347*, *348*). In murine models, however, chronic infections are established via the surgical implantation of *P. aeruginosa*-embedded agar beads, often using non-mucoidal strains of *P. aeruginosa*, to mimic this phenomenon (*349*). Therefore, whether findings derived from animal models accurately model human disease may be challenging to discern. Despite this caveat, studies have shown that rapid IL-1 release is a part of the early innate immune response to *P. aeruginosa* infection, as shown in both cultured epithelial cells and through hematopoietic cell cultures (*270*, *343*, *350*). It has been suggested that moderate IL-1 secretion could stimulate processes that would be protective against bacterial or fungal infections (*351–353*). However, for *P. aeruginosa* pneumonia, studies demonstrate that IL-1β compromises *P. aeruginosa* clearance as well as stimulates mucus hypersecretion (*31*, *354*, *355*), and the secretion of other proinflammatory cytokines (*346*). IL-1R$^{-/-}$ mice have been reported to have fewer CFU of *P. aeruginosa* in the lungs compared with WT mice in an acute pneumonia model (*355*), while IL-1β neutralizing antibodies have been found to have a protective effect in mice against severe pneumonia (*356*). It has been demonstrated that that excessive IL-1β activation upon infection with *P. aeruginosa* correlates with increased CFU, inflammation, lung epithelium damage, and mortality in CFTR$^{-/-}$ mice (*356*).

It has been reported that for chronic infection models, however, moderate IL-1 signalling may play a protective role. Robust immune responses of IL-1R$^{-/-}$ mice to chronic infection with the PAO1 strain have been observed (*194, 357–359*). Similar results have also been reported using different strains of *P. aeruginosa*. Namely, Reiniger et al. found that upon using a chronic infection model, IL-1R$^{-/-}$ mice had enhanced *P. aeruginosa* lung colonization compared with WT mice (*336*). Whether this distinction observed in chronic infections in contrast to acute infections reflects human infections or is an artifact from controversial chronic murine infection models is perhaps a point of contention. Reiniger et al. argued, however, that these observed differences in findings compared with acute infection models was due to the difference in bacterial burden upon initiating the infection, where chronic infection models tend to use a smaller bacterial inoculum that is less likely to overwhelm the innate immune system (*336*, *360*). This interpretation on the importance of bacterial burden is interesting given my experimental conditions studying ExoU and the importance of using lower MOIs to elicit contrary findings.

Whether the level of IL-1β expression that can be induced by ExoU-expressing strains of *P. aeruginosa* can be deemed as excessive may be challenging to extrapolate through *in vitro* work. *P. aeruginosa* strains which express ExoU tend to be associated with acute respiratory infections rather than chronic ones (*361*). Stimulating acute respiratory infections in murine models is known to require relatively larger pathogen burdens (*343*, *360*), and my work demonstrates that this approach could induce rapid and extensive host cell death that could compromise many cellular inflammatory processes. The size of the inoculum that would be sufficient to cause acute infection in humans or that is regularly encountered in clinical contexts is, however, not a simple discussion and could depend on host factors. Nevertheless, ExoU-expressing strains have generally been

associated with poorer outcomes in human studies investigating pneumonia and sepsis, including a higher risk of mortality (*341*, *361–363*).

Using the inhibitor MCC950 showed modest NLRP3 involvement in IL-1β secretion in *P. aeruginosa* infections with laboratory strains for THP-1 macrophages, but no effect on NuLi-1 bronchial epithelial cells. ExoU may potentially activate the NLRP3 inflammasome due to its ability to disrupt the host plasma membrane (*174*, *175*). NLRP3 is also of interest because various CF studies suggest that the disease is associated with increased NLRP3-inflammasome activation (*356*, *364*, *365*). This enhanced NLRP3 activation, as observed in both murine and human cells (*274*), could be intrinsic to CFTR dysfunction due changes in mitochondrial calcium ion levels (*176*). Further evidence correlating CFTR function with NLRP3 activation can be found in studies using CFTR modulators *in vitro* and in human studies which show modulation of hyperinflammatory states after LPS/ATP stimulation in response to these treatments. Ivacaftor/lumacaftor or ivacaftor/tezacaftor combinations reduced IL-18 secretion and caspase-1 activation by PBMCs isolated from CF patients, while the latter drug combination reduced IL-1β secretion (*365*). Similarly, among human subjects, serum IL-18 and IL-1β decreased significantly upon use of the CFTR modulators (*365*). Reducing NLRP3 activation and subsequent IL-1β secretion has also been shown to improve infection severity in CF (*352*).

5.2.2 P. aeruginosa effector proteins and their impact on respiratory infections

ExoU, ExoT, ExoS, and ExoY are all effector proteins secreted by the *P. aeruginosa* T3SS apparatus, and their role in inducing inflammatory mechanisms has been investigated. One such study determined whether any T3SS effector proteins that are expressed by *P. aeruginosa* could confer communal advantages for neighbouring, less virulent strains which are simultaneously colonizing the host (*228*). It was found that ExoU was the one effector protein which rapidly

induced host cell death, thereby creating an immunocompromised state and allowing other strains to survive in the host (*228*). This proposed mechanism is consistent with work from other groups including time kinetic observations which demonstrated that a delay in exposure to ExoU could improve bacterial clearance in murine pneumonia models (*225, 350, 366*). My work has also repeatedly shown that ExoU rapidly induces extensive host cell death, thereby limiting cytokine expression and other immune cell functions.

Interestingly, it appears that ExoU can play a dominant role over other T3SS effector proteins, such as in the case with *in vitro* infections using PA14. However, *in vitro* infections using PAO1 and its transposon mutants for ExoS, ExoT, and ExoY, indicated that these effector proteins could play a role in inducing IL-1β expression, which would possibly counter previous reports. Of these effector proteins, ExoY is often viewed as a relatively minor *P. aeruginosa* effector protein which has been described in one study to delay NF-κB and caspase-1 activation through its adenylate cyclase activity, thus reducing IL-1β expression (*367*). Interestingly, ExoS has been found to negatively regulate IL-1β production via its ADP ribosyltransferase activity (*368*). It was also noted that despite ExoT sharing the same enzyme activity, increases in mature IL-1β upon using isolates without ExoT were not observed (*368*). Differences in whether these effector proteins stimulate or inhibit IL-1β production may be due to differences in choice of experimental model and conditions. Whereas I have consistently used THP-1 macrophages for this work, others have reported different findings using different cell lines, including epithelial cell lines, as well as different MOIs.

5.2.3 ExoU-induced signalling mechanisms during infections

It has been shown that *P. aeruginosa* is able to induce a multitude of inflammatory mechanisms, including pyroptosis. Observations from immunoblots and the use of inhibitors

reveal that WT PA14 is able to induce activation of caspase-1, -4, -5, and -8 among THP-1 macrophages to a greater extent than PA14 *exoU*::tn at lower MOIs. Correlating with observed IL-1β expression during infections with epithelial cells and WT PA14, I also found activation of caspase-1, -4, and -8 in the supernatant for NuLi-1 cells. These findings are corroborated with the detection of cleaved GSDMD in the cell extracts at earlier time points for WT PA14 infections of THP-1 macrophages and NuLi-1 cells compared with PA14 *exoU*::tn. All of the aforementioned caspases have been implicated in IL-1β maturation, and caspase-1, -4, and -5 are known initiator inflammatory caspases involved in pyroptosis in human cells (*322*). Caspase-8 is also an initiator caspase that regulates NLRP3 (*160*), although it predominantly plays a role in the apoptotic cascade (*322*). Therefore, the ability for *P. aeruginosa*, including those expressing ExoU, to activate these various caspases for both THP-1 macrophages and NuLi-1 cells suggests that complex inflammatory mechanisms can be engaged during *P. aeruginosa* infections.

Interestingly, use of the pan-caspase inhibitor emricasan did not entirely abolish IL-1β secretion during infections with ExoU-expressing *P. aeruginosa*. Given the observed potency of the inhibitor during infections with *P. aeruginosa* strains that do not express ExoU, it may be that there are indeed caspase-independent mechanisms of ExoU-induced IL-1β secretion. ExoU is a phospholipase A_2, and interestingly, calcium-dependent phospholipase A_2 may play a role in lysosome-mediated IL-1β processing and secretion (*369–371*), although its precise role has been disputed (*372*).

The detection of cleaved caspases in the supernatant in this work vastly differs from previously published reports in that there was no detection of cleaved bands for infections with ExoU-containing strains (*158*). This contrast in results is most likely due to differences in selected cell model and MOI as experimental parameters. Namely, I used a relatively lower MOI of 0.1 or

0.5 for immunoblotting with THP-1 macrophages for the work investigating ExoU induced-signalling in contrast to an MOI of 20 used by Sutterwala et al (*158*). Indeed, I observed that when MOI increases, more IL-1β is induced by bacteria lacking ExoU, which is due to reduced cell death and sustained secretion of cytokines. Thus, I propose that the inhibition of inflammasome signalling by ExoU may be an experimental artifact in part caused by using relatively high MOI. Precisely which bacterial burden relative to host cells most accurately recapitulate infections in CF is challenging to discern. Human studies have reported the level of bacterial burden in sputum or BALF, often ranging from 10^5 CFU/mL in acute infections (*373*) to as high as 10^7-10^8 CFU/mL during chronic infections (*257*, *374*, *375*). Bacterial burden of *P. aeruginosa* strains expressing ExoU may fall closer to the acute infection range, given how this effector protein tends to be downregulated over the course of adaptations during the development of chronic infections (*224*, *228*, *361*). The bacterial burden relative to the number of host cells, however, is a parameter that is not frequently reported. One study that directly measured bacterial load relative to host cell count in BALF samples reported a mean neutrophil:bacteria ratio of 36:1 among a cohort of CF children, with the ratio being reduced to 4.9:1 when analyzing a subset of patients with a higher overall bacterial burden (*373*). This analysis, however, does not account for the other types of immune or epithelial cells that encounter invasive pathogens as well as their viability during infections (*42*). Broadening laboratory conventions of bacterial burden relative to the number of host cells could thus be critical to recapitulate human respiratory infections.

Another critical difference between our findings and previous reports is that I primarily used THP-1 macrophages and NuLi-1 cells, whereas other groups have used murine BMDMs (*158*). It has been reported that PMA treatment to differentiate the THP-1 cells acts on protein kinase C-δ (PKCδ), which in turn induces pro-IL-1β production via the JNK MAPK pathway

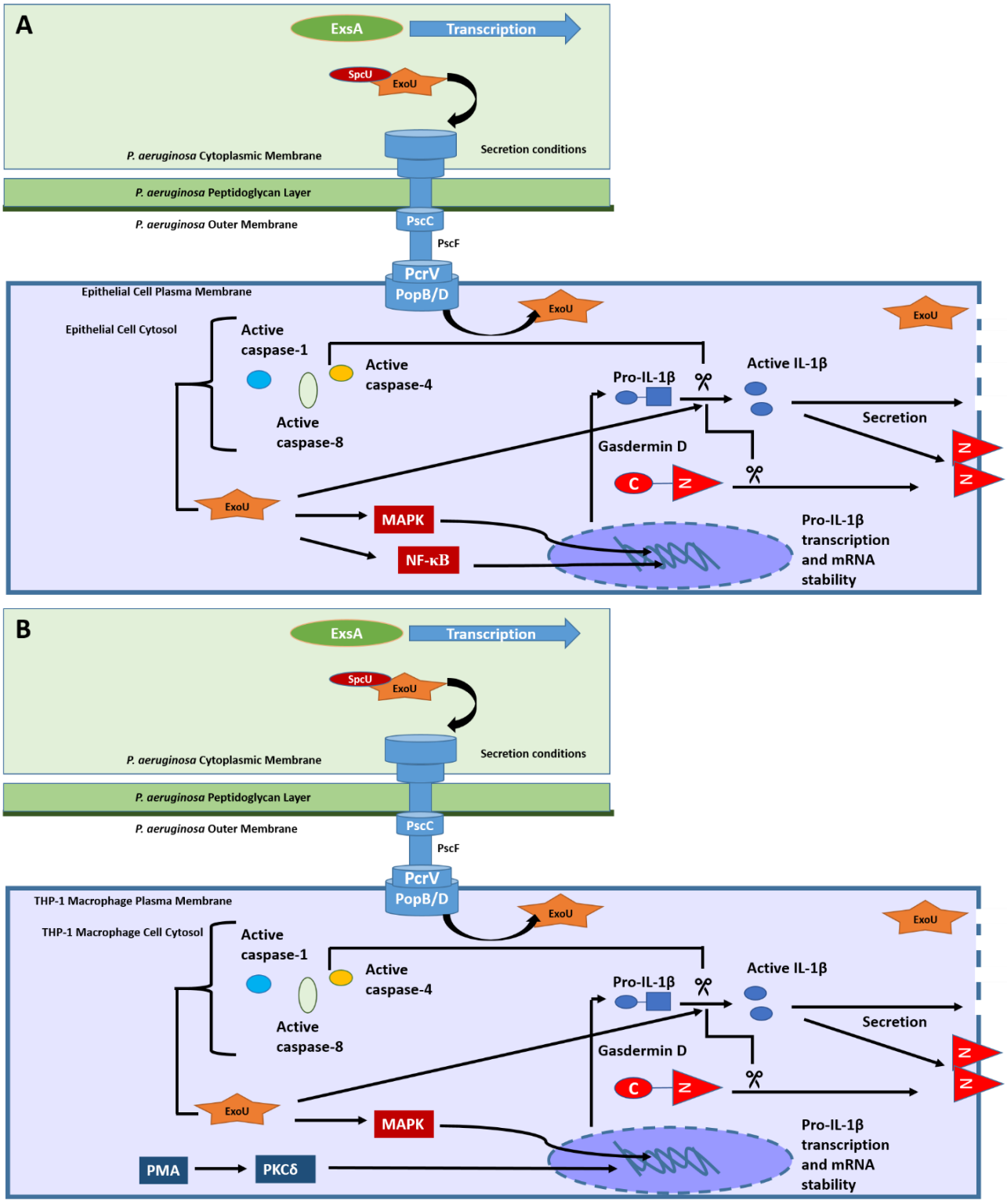
(*376*). This increase in available pro-IL-1β could aid in rapid cleavage and secretion of mature IL-1β in response to ExoU. It should also be noted, however, that caspase-1 activation and IL-1β expression was induced in NuLi-1 cells in response to ExoU, suggesting that priming mechanisms are unnecessary for epithelial cells.

I also observed that ExoU activates the MAPK pathway in THP-1 macrophages and NuLi-1 cells as well as the NF-κB pathway in NuLi-1 cells. This is consistent with previous reports investigating ExoU-induced signalling cascades among epithelial cells. In alveolar epithelial cells, ExoU was found to activate the canonical NF-κB pathway through platelet-activating factor, which contributes to a positive feedback loop, resulting in further NF-κB activation and subsequent enhanced IL-8 secretion (*377, 378*). Similarly, ExoU has been found to induce MAPK signalling in respiratory epithelial cells, once again resulting in increased IL-8 expression (*379*). Increased expression of IL-1β, IL-8, and IL-6 were all appreciated in NuLi-1 cells infected with *P. aeruginosa* expressing ExoU compared with strains not expressing the cytotoxin at lower MOIs. Therefore, provided that NuLi-1 bronchial epithelial cell death was not rapidly induced, cytokine expression could be positively correlated with cell death. The consequences of early MAPK pathway in THP-1 macrophages upon being infected with ExoU-expressing strains, however, may be inconsequential for cytokines other than IL-1β. While the activation is evident via immunoblotting, the rapidly induced cell death likely compromises any downstream cytokine maturation, namely TNF, IL-6, and IL-8.

The MAPK protein MK2 have been observed to have a role in IL-1β expression (*145*), as LPS and TLR4 signalling are able to activate the p38 MAPK pathway (*380*). MAPK signalling may also contribute to uncontrolled murine lung inflammation in response to LPS treatment via NLRP3 activation and IL-1β secretion, which was further validated *in vitro* using rat macrophages

(*381*). In addition, inhibition of MK2 was found to suppress IL-1β production in colorectal cancer models, and MK2 is known to play a role in increasing IL-1β mRNA stability (*145*, *146*). NF-κB appears to play a dual role, as it has been shown to both promote and inhibit IL-1β production. NF-κB activation plays a role in the production of NLRP3 (*382*, *383*) and pro-IL-1β (*270*, *384*, *385*). However, deletion of IKKβ in murine macrophages, essentially attenuating NF-κB activation, was shown to increase IL-1β secretion (*386*, *387*).

Taken together, this work adds to the growing evidence of different mechanisms involved during infections with *P. aeruginosa*, particularly in the presence of ExoU (**Fig. 45**). Observations in bronchial epithelial cells suggest that ExoU induces earlier activation of the NF-κB and p38 MAPK pathways, both of which are known to regulate transcription of the *IL-1β* gene and the latter of which results in downstream MK2 activation which can stabilize the pro-IL-1β transcript (*145*, *146*). Concurrently, ExoU was associated with activation of caspase-1, -4, and -8, which may play a role in cleaving pro-IL-1β and GSDMD cleavage. Both the GSDMD N-terminal domain and ExoU would play a role in plasma membrane disruption, allowing for the release of mature IL-1β and subsequent inflammatory cell death. In THP-1 macrophages, ExoU does not appear to enhance NF-κB activation, although it does appear to play a role in p38 MAPK activation. Unique to THP-1 cells is the nature of monocyte differentiation to the macrophage phenotype, where PMA treatment is known to act on PKCδ to induce *IL-1β* transcription (*376*). The role of ExoU in activating various caspases, cleaving GSDMD, and thereby promoting IL-1β secretion are otherwise similar to those in NuLi-1 epithelial cells.


A
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P. aeruginosa Outer Membrane
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PscF
PcrV
PopB/D
Epithelial Cell Plasma Membrane
ExoU
Epithelial Cell Cytosol
Active caspase-1
Active caspase-4
Active caspase-8
Pro-IL-1β
Active IL-1β
Secretion
Gasdermin D
C
N
MAPK
NF-κB
Pro-IL-1β transcription and mRNA stability
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P. aeruginosa Outer Membrane
PscC
PscF
PcrV
PopB/D
THP-1 Macrophage Plasma Membrane
ExoU
THP-1 Macrophage Cell Cytosol
Active caspase-1
Active caspase-4
Active caspase-8
Pro-IL-1β
Active IL-1β
Secretion
Gasdermin D
C
N
MAPK
PMA
PKCδ
Pro-IL-1β transcription and mRNA stability

Fig. 45. Proposed mechanism for *P. aeruginosa* ExoU-induced IL-1β secretion. *P. aeruginosa* ExoU may participate in the activation of multiple components which can contribute to IL-1β production and secretion. (**A**) In bronchial epithelial cells, ExoU induces activation of the NF-κB and p38 MAPK pathways, in turn activating transcription of IL-1β. p38 MAPK results in downstream activation of MK2, which is able to stabilize the pro-IL-1β transcript. ExoU is also able to activate caspase-1, -4, and -8, which all play a role in cleaving pro-IL-1β. ExoU was also associated with GSDMD cleavage, and both the N-terminal domain of GSDMD and ExoU would play a role in plasma membrane disruption, allowing for the release of mature IL-1β. (**B**) In THP-1 macrophages, PMA treatment induces IL-1β transcription as could ExoU-induced activation of p38 MAPK. Otherwise, the activation of various caspases, cleavage of GSDMD, and the possibility of the engagement of a caspase-independent mechanism, are not unlike with NuLi-1 epithelial cells.

5.3 Applications to clinical approaches in CF treatment

This doctoral work contributes to our understanding of inflammatory mechanisms behind commonly seen respiratory infections in cystic fibrosis. I have validated previous findings demonstrating that hematopoietic immune cells are the predominant source of inflammasome-mediated IL-1β production over airway epithelial cells in response to *P. aeruginosa* and that many complex, and in some ways redundant, inflammatory mechanisms are involved during these infections. Ultimately, this work could shed light on possible interventions that can be further explored to address the morbidity observed in CF. While we are in the advent of new CFTR modulators which have the potential to improve the quality of life for many CF patients (*9*), it is possible that some of the physiological changes and subsequent tissue damage that occurs early in life prior to modulator use can linger. Therefore, continuing to understand the underlying mechanisms behind respiratory infections and the general immune response in CF could be worthwhile to understand disease progression early in patients' lives as well as during possible chronic infections.

Immunomodulatory approaches to CF have already been explored, and the anti-IL-1R antagonist anakinra has been considered as a contender. *In vitro* infections with THP-1 and NuLi-1 cells demonstrate the possibility that cells involved in the innate immune response readily secrete IL-1β regardless of the bacteria virulence and the pace of host cell death. Following the logic that sustained IL-1β secretion can result in lung pathology, murine CF models and human CF epithelial cells treated with anakinra resulted in decreased neutrophil infiltration and attenuated *P. aeruginosa*-associated inflammation (*343*). Reports have also suggested that anakinra has a promising safety profile with no reported risk of superinfections or adverse events, which are

common concerns when considering immunomodulatory approaches (*388*). A clinical trial for efficacy among CF patients is now underway and anticipated to be concluded in 2022 (*389*).

The widespread adoption of CFTR modulator therapy is to be anticipated given the relatively recent Health Canada and FDA approval (*390*, *391*). It is therefore also important to consider how these therapies can impact hyperinflammation and infection seen in CF. The largest advancement in this field was the approval of the elexacaftor/tezacaftor/ivacaftor combination (Trikafta®), in which an estimate of 90% of all CF patients will be eligible to receive the treatment (*392*). One *in vitro* study of this combination therapy shows its potential to reduce apoptotic mechanisms among epithelial cells while also possibly promoting macrophage recruitment via changes in epithelial lipid metabolism (*244*, *392*, *393*), which may aid in respiratory structure integrity but also drive inflammation. However, dual therapy combination ivacaftor/tezacaftor showed a decline in patient serum IL-1β (*394*). Interestingly, human studies on the CF lung microbiome and how it changes in response to CFTR modulator therapy have demonstrated a mixed picture as to whether pathogenic bacteria are eradicated upon restoration of CFTR function, especially with regards to *P. aeruginosa* burden (*395*). It is important to note, however, that available data on this topic only includes dual or mono-CFTR modulator therapy as well as patients with a relatively less common CFTR mutation than ΔF508 (*395*). Clinical trends with the triple CFTR modulator therapy will be more apparent with time as more patients have access to this therapy, allowing us to understand the degree of immunomodulation that may occur, if any.

Given the *in vitro* nature of this work, there may be some limitations for its applicability to clinical contexts, and the findings described therein can be viewed as a preliminary step for future investigations. One limitation is that the use of immortalized cell lines to model human cells found in the lung environment may not precisely reflect typical *in vivo* conditions, especially THP-1 cells

compared with primary, circulating monocytes and alveolar macrophages. This model selection was chosen in order to conduct experiments using a large number of *P. aeruginosa* isolates of interest concurrently. However, I also validated the inverse relationship between induced host cell death and cytokine expression using human PBMCs differentiated into macrophages with a limited number of isolates, and the paradigm was found to still hold true under these experimental conditions. In addition, *in vivo* animal work to study CFTR infections is not without its own limitations. $CFTR^{-/-}$ mice, as previously described, do not establish chronic *P. aeruginosa* spontaneously, unlike CF patients (*347–349*). These challenges may be a consequence of inherent differences in the murine and human respiratory system, including CFTR expression (*347–349*). Chronic infections in humans also take years to establish, which is not a feasible timeline for murine models, especially for the $CFTR^{-/-}$ mice which often experience early mortality compared with WT mice due to gastrointestinal complications (*347–349*). Therefore, both *in vitro* and *in vivo* work have their inherent advantages and disadvantages, and it is simply necessary to consider the respective findings collectively and in relation to the clinical context.

Another limitation may be that the infection conditions used in this aforementioned work involved planktonically grown bacteria due to the ease in being able to estimate the bacterial burden relative to number of host cells for each experiment, which was essential for work investigating ExoU. Biofilm formation, however, is a hallmark of chronic *P. aeruginosa* infections (*396*). These complex structures are also known to impact bacterial growth and metabolism as well as virulence, where bacteria grown as biofilms are typically less virulent in order to evade immune recognition (*396*).

Finally, the obtained clinical isolates are from cultures derived from sputum or oropharyngeal swabs. In the latter mode of sample collection, the bacteria will reflect the upper

respiratory tracts of CF patients as opposed to bacteria found within the lung spaces. In sputum cultures as well, it may be argued that there is the risk of contamination of sputum samples with upper respiratory tract pathogens during sample collection. This is a typical limitation upon isolating respiratory cultures in CF studies to minimize patient discomfort and inconvenience, as an alternative approach entails conducting invasive bronchoalveolar lavages. It is also interesting to note, however, that previous reports suggest that sputum samples for the purpose of isolating common CF pathogens of interest, including *P. aeruginosa*, tend to accurately reflect BALF cultures (*397*). Another study also compared the composition of sputum and mouthwash samples from patients with CF and found little concordance between the two groups, suggesting contamination is perhaps not as significant of a concern (*398*). Therefore, while the aforementioned doctoral work involves caveats that should be considered, there is consistency in the application of described paradigms, especially compared to previous literature, that could still have importance in clinical contexts.

6.0 CONCLUSION

Overall, the work described in this thesis adds to our understanding of possible underlying inflammatory mechanisms that occur during *P. aeruginosa* respiratory infections found in CF. The work in general uses THP-1 macrophages and NuLi-1 cells to model different components of the innate immune system in the lung environment. First, I described observed differences in inflammatory mechanisms induced by *P. aeruginosa* isolates from CF patients experiencing early or chronic respiratory infections. In observing that chronic *P. aeruginosa* isolates still retain the ability to stimulate various inflammatory pathways by evading host cell death, a mechanism for how chronic infections can drive further respiratory decline is proposed. **This work, entitled "Impairment in inflammasome signalling by the chronic *Pseudomonas aeruginosa* isolates from cystic fibrosis patients results in an increase in inflammatory response" was published in March 2021 in *Cell Death and Disease*.**

As well, I describe how the cytotoxin ExoU is able to induce IL-1β production despite previous reports indicating that it inhibits caspase-1 activity and cytokine expression downstream. However, given the rapid and extensive host cell death induced by ExoU, I can appreciate how ExoU can exert immunosuppression and limited cytokine expression with time or at high bacterial loads. This work will impact our typical understanding of how ExoU, a not uncommon virulence factor seen among clinical isolates, induces inflammatory mechanisms, including pyroptosis.

It was originally hypothesized that I could identify bacterial factors which could cause sustained inflammation and possibly contribute to disease progression in respiratory infections in CF. Collectively, both **Aims** add further evidence that immunomodulatory approaches to CF treatment may be considered to attenuate the persistent engagement in inflammatory mechanisms that occur during infections. Given how *P. aeruginosa* can still engage the host immune system in

a number of ways, even during chronic infections, investigating the inhibition of relevant pathways and cytokine expression is warranted. The identification and discussion of novel insights into respiratory infections broadens treatment possibilities for a complex clinical problem to improve patient care.

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Table 8. List of individuals who have contributed materials and review of this work.

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Dr. Daniel Wolter	University of Washington	Identification of longitudinal clinical isolates from CF patients and manuscript review
Dr. Lucas Hoffman	University of Washington	Identification of longitudinal clinical isolates from CF patients and manuscript review
Dr. Gregory Priebe	Boston Children's Hospital	Provision of WT PAO1, PAO1+Vector, and PAO1+ExoU strains
Dr. Gerald Pier	Brigham and Women's Hospital	Provision of WT PAO1, PAO1+Vector, and PAO1+ExoU strains
Dr. Ardeshir Ariana	University of Ottawa	Isolation and provision of primary human macrophages from PBMCs

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Impairment in inflammasome signaling by the chronic Pseudomonas aeruginosa isolates from cystic fibrosis patients results in an increase in inflammatory response

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