# MODULATION OF INFLAMMASOME SIGNALING DURING CHRONIC BACTERIAL INFECTIONS

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## ABSTRACT

Inflammasome signaling during infections results in cell death and processing and secretion of cytokines from the IL-1 family, which facilitates control over an infection. *Pseudomonas aeruginosa* and *Salmonella* Typhimurium are opportunistic bacterial pathogens which may induce acute infections and activate various innate immune signaling pathways, including inflammasomes. However, under favourable conditions these pathogens may evade immune clearance resulting in the establishment of a chronic infection. In this study, I evaluated the modulation of host inflammasome signaling induced by *P. aeruginosa* and *S.* Typhimurium during chronic infections. I used a collection of *P. aeruginosa* clinical isolates obtained from the sputum of cystic fibrosis patients collected during stable and exacerbation periods of disease. I demonstrated that the majority of isolates displayed poor inflammasome signaling and only a small proportion of isolates retained their ability to induce inflammasome activation, which may be associated with pulmonary exacerbations in cystic fibrosis. Sequencing and bioinformatics revealed genetic variations within the type III and type VI secretion systems of *P. aeruginosa*. While an inactivation of the type III secretion system is expected to impair inflammasome signaling, my results indicate that the type VI secretion system inhibits inflammasome signaling in eukaryotic cells. Due to the lack of chronic animal models for *P. aeruginosa,* I utilized a murine model of chronic *S.* Typhimurium infection to assess the modulation of inflammasome signaling throughout the course of a chronic infection. I observed that *S.* Typhimurium isolated during the acute phase of infection displayed an increased potential to activate inflammasome signaling and

this ability progressively declined during the chronic phase of infection. This reduction in inflammasome activation was associated with reduced expression of bacterial virulence factors, such as flagella and the type III secretion system, and was dependent on the NLRP3 inflammasome. Overall, these results reveal that the expression of virulence factors is modulated during chronic bacterial infections, which results in a reduction of inflammasome activation leading to co-survival of the pathogen and host.

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| | |
|---|---|
| **ABX** | Antibiotic |
| **ADP** | Adenosine diphosphate |
| **ADPRT** | ADP-ribosyl transferase |
| **AIM2** | Absent in melanoma 2 |
| **ANOVA** | Analysis of variance |
| **ASC** | Apoptosis-associated speck-like protein containing a CARD |
| **ATP** | Adenosine triphosphate |
| **CARD** | Caspase activation and recruitment domain |
| **cAMP** | Cyclic adenosine monophosphate |
| **cDNA** | Complementary DNA |
| **cGMP** | Cyclic guanosine monophosphate |
| **Casp** | Caspase |
| **CCAC** | Canadian Council on Animal Care |
| **CF** | Cystic fibrosis |
| **CFTR** | Cystic fibrosis transmembrane conductance regulator |
| **CFU** | Colony-forming units |
| **CIITA** | MHC class II transcription activator |
| **DAMP** | Danger-associated molecular pattern |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **DSF** | Disulfiram (1-(Diethylthiocarbamoyldisulfanyl)-*N*,*N*-diethyl-methanethioamide) |
| **dsDNA** | Double-stranded DNA |
| **eDNA** | Extracellular DNA |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **Exo** | Exoenzyme |
| **FBS** | Fetal bovine serum |
| **FET** | Fisher's exact test |
| **γTuRC** | γ-tubulin ring complex |

| | |
|---|---|
| **GSDMD** | Gasdermin D |
| **Hcp** | Hemolysin-coregulated protein |
| **HET-E** | Heterokaryon E |
| **HRP** | Horseradish peroxidase |
| **IFN** | Interferon |
| **IL** | Interleukin |
| **LAMP** | Lysosomal associated membrane protein |
| **LB** | Lysogeny broth |
| **LPS** | Lipopolysaccharide |
| **LRR** | Leucine rich repeat |
| **MARCO** | Macrophage receptor with collagenous structure |
| **M-CSF** | Macrophages colony stimulating factor |
| **MLKL** | Mixed lineage kinase domain like pseudokinase |
| **MHC-II** | Major histocompatibility complex, class II |
| **MOI** | Multiplicity of infection |
| **mRNA** | Messenger RNA |
| **MYD88** | Myeloid differentiation primary response gene 88 |
| **NACHT** | NAIP, CIITA, HET-E and TP1 |
| **NAIP** | Neuronal apoptosis inhibitory protein |
| **NETs** | Neutrophil extracellular traps |
| **NLR** | NOD-like receptor |
| **NLRC4** | NLR family, CARD domain containing 4 |
| **NLRP1** | NLR family, pyrin domain containing 1 |
| **NLRP3** | NLR family, pyrin domain containing 3 |
| **NOD** | Nucleotide-binding oligomerization domain |
| **Nramp1** | Natural resistance-associated macrophage protein 1 |
| **PAMP** | Pathogen-associated molecular pattern |
| **PAPI** | *Pseudomonas aeruginosa* pathogenicity island |
| **PBS** | Phosphate buffered saline |

| | |
|---|---|
| **PMN** | Polymorphonuclear leukocyte |
| **PYD** | Pyrin domain |
| **PRR** | Pattern-recognition receptor |
| **PVDF** | Polyvinylidene fluoride |
| **qRT-PCR** | Quantitative real-time reverse transcription polymerase chain reaction |
| **RhoGAP** | Rho GTPase-activating protein |
| **RNA** | Ribonucleic acid |
| **RIPK** | Receptor interacting protein kinase |
| **ROS** | Reactive oxygen species |
| **RPMI** | Roswell Park Memorial Institute |
| **R8** | RPMI + 8% FBS |
| **SCV** | *Salmonella* containing vacuole |
| **SCV** | Small colony variants |
| **SDS** | Sodium dodecyl sulfate |
| **SDS-PAGE** | SDS-polyacrylamide gel electrophoresis |
| **SEM** | Standard error of the mean |
| **SPI** | *Salmonella* pathogenicity island |
| **sRNA** | Small RNA |
| **ST** | *Salmonella enteria* serovar Typhimurium |
| **TNF** | Tumor necrosis factor |
| **TRIF** | TIR-domain-containing adapter inducing interferon-β |
| **TLR** | Toll-like receptor |
| **TAP1** | Transporter associated with antigen processing 1 |
| **T2SS** | Type II secretion system |
| **T3SS** | Type III secretion system |
| **T6SS** | Type VI secretion system |
| **VgrG** | Valine-glycine repeat protein G |
| **WT** | Wild-type |

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# 1. INTRODUCTION

## 1.1 The immune response

The immune system confers a broad range of protection against foreign entities within the host by mediating the identification and elimination of pathogens through the action of immune cells. The immune system can be divided into two major branches: the innate and the adaptive immune systems. Innate immune cells express germline-encoded receptors that recognize and respond to a diverse range of pathogen-associated ligands, resulting in the activation of a rapid anti-microbial response and promoting the recruitment of additional immune cells to the site of infection. In contrast, the adaptive immune system confers long-lasting protection against specific pathogens through the selection of immune cell populations that respond to a specific pathogen-associated epitope. The interactions between the pathogen and host immune system may be manipulated by the pathogen to subdue the host immune response and promote pathogen persistence.

## 1.2 Innate immunity

The innate immune response is one of the first lines of defense against invading pathogens. The innate immune system utilizes germline-encoded pattern-recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMPs) and danger-associated

molecular patterns (DAMPs). These PRRs may be expressed in various cellular compartments where pathogens can be encountered, such as on the cell surface, within the cytosol, or within vesicles (1). PAMPs are only associated with foreign pathogens, however, DAMPs may be elicited in response to infection or cellular damage. PRR stimulation results in the activation of downstream signaling pathways, resulting in an inflammatory response to facilitate pathogen control (2).

Toll-like receptors (TLRs) are an important family of PRRs that detect pathogens. TLRs-1/2, -4, -5, and -6 play an important role in mediating host responses to bacterial pathogens through their recognition of bacterial lipoproteins, lipopolysaccharide (LPS), flagella, and zymosan, respectively (3, 4). TLRs are localized in the plasma membrane or on the endosomal membranes, where they facilitate the response to extracellular or intracellular PAMPs expressed by foreign pathogens. TLR engagement, such as recognition of bacterial LPS by TLR-4, leads to the activation of downstream signaling cascades mediated by common myeloid differentiation primary response gene 88 (MYD88) and TIR-domain-containing adapter inducing interferon-β (TRIF)-dependent pathways (5, 6). Ultimately, TLR activation results in the transcription of proinflammatory mediators including type-I interferons, IL-1 family cytokines, and NLRP3 (7). Secreted cytokines such as TNFα and IL-6 promote the recruitment of additional immune cells to the site of infection (2). TLR activation rapidly initiates a broad proinflammatory response and primes the immune system to respond against the invading pathogen.

Some bacterial pathogens possess the capability to secrete toxins into host cells, or to invade and proliferate intracellularly, thus evading extracellular TLR-mediated immune

surveillance. To effectively combat these pathogens, cells may also express intracellular PRRs such as nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). NLRs are typically localized to the cytosol, which facilitates their detection of intracellular pathogens and toxins (4). NLR activation results in the assembly of multimeric protein complexes known as inflammasomes, leading to the processing and secretion of IL-1β and IL-18 and the induction inflammatory cell death. Ultimately, engagement of PRRs results in the expression of cytokines, chemokines and host factors which recruit additional immune cells and prime the immune system to effectively respond against the invading pathogen.

### 1.3 Inflammasomes

Inflammasomes are multimeric innate immune signaling complexes that utilize PRRs from the NLR family to recognize pathogens. NLRs are expressed within the cytosol and survey the cell interior for PAMPs or DAMPs associated with infection or damage. NLR engagement results in receptor self-oligomerization and the recruitment of apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) and caspase-1 to assemble the inflammasome complex (8). Inflammasomes facilitate a robust immune response through the secretion of proinflammatory cytokines, such as IL-1β, and the induction of pyroptotic cell death.

The NLRP1, NLRP3, NLRC4, AIM2, and pyrin inflammasomes contribute to the antimicrobial response against pathogens (**Figure 1**) (9, 10). The NLRP1 and pyrin inflammasomes respond to bacterial toxins such as *Bacillus anthracis* lethal factor and *Clostridium difficile* toxin

B, respectively (11, 12). The AIM2 inflammasome responds to DNA from microbial, viral, or host origin (13–15). During bacterial infections, the NLRP3 and NLRC4 inflammasomes are key towards mediating host responses against invading pathogens.

The NLRP3 inflammasome is composed of NLRP3, ASC, and caspase-1. The expression of NLRP3 must be initiated by a priming step that is typically mediated by TLR engagement (16). Upon stimulation, NLRP3 self-oligomerizes through interactions between NACHT domains. Oligomerized NLRP3 then recruits ASC to the complex through interactions between common PYD domains. Multiple ASC monomers may also self-oligomerize through their PYD domains to form ASC filaments and subsequently ASC specks associated with the inflammasome complex (17). Caspase activation and recruitment domain (CARD) interactions facilitate the recruitment of pro-caspase-1 to the inflammasome complex where it undergoes proteolytic self-cleavage and maturation to active caspase-1 (18).

The NLRP3 inflammasome may respond to a diverse range of signals during microbial infection. NLRP3 is typically activated in response to DAMPs associated with cellular stress during infection such as ion flux or reactive oxygen species (ROS) (9). Many bacterial pathogens such as *Salmonella* and *Listeria* possess pore-forming toxins which create channels in the plasma membrane resulting in intracellular ion flux (19, 20). Changes in intracellular $K^+$ and $Ca^{2+}$ ion concentrations may activate NLRP3 through indirect, mitochondrial-mediated signaling pathways (21–23). NLRP3 may also indirectly respond to PAMPs such as bacterial flagellin, however, its activity is secondary and redundant to NLRC4 (24, 25).

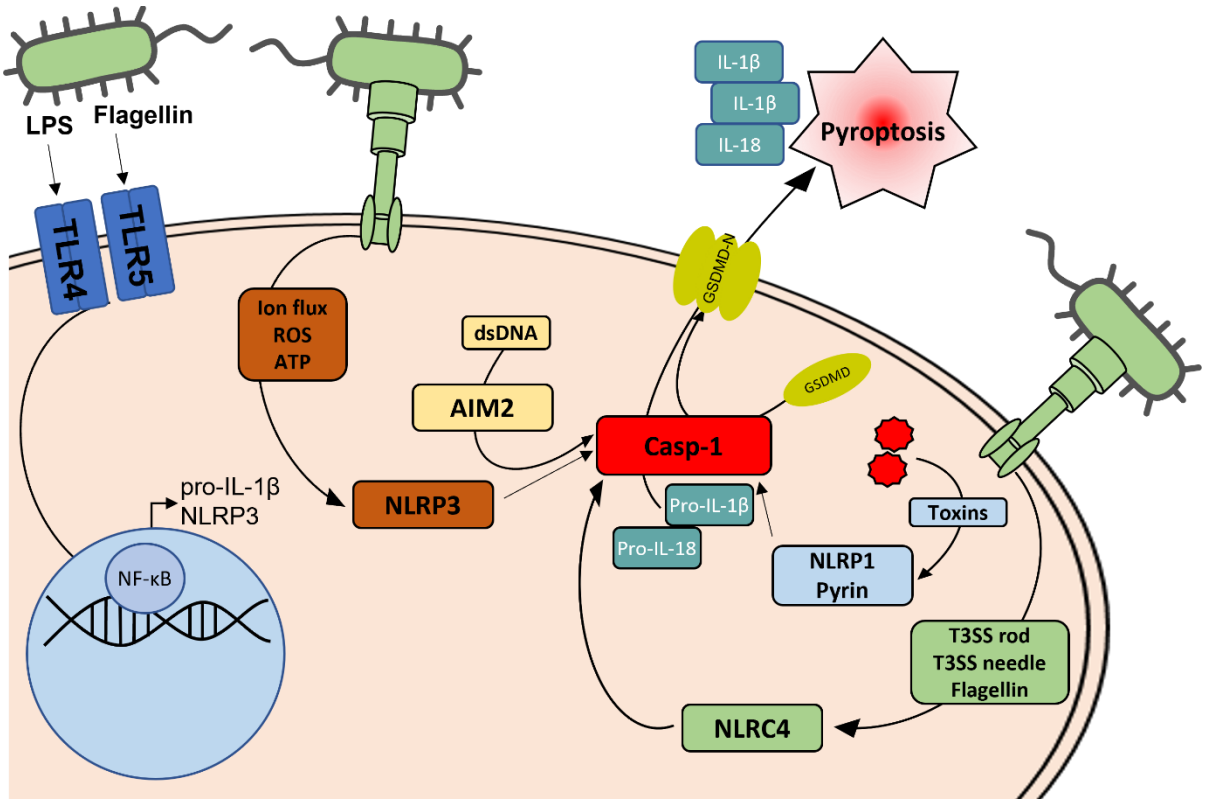


**Figure 1. Inflammasome activation during bacterial infections**

Inflammasome activation in response to PAMPs and DAMPs confers protection during bacterial infection. TLR engagement induces expression of pro-IL-1β and NLRP3. The NLRP1 and pyrin inflammasomes respond to bacterial toxins. The AIM2 inflammasome may be activated by dsDNA. NLRC4 is activated by bacterial flagellin and T3SS components. NLRP3 is activated in response to DAMPs such as ion flux and ROS. NLRP3 activation requires an initial priming step to induce NF-κB-dependent transcription of pro-IL-1β and NLRP3. Inflammasome engagement results in caspase-1 recruitment and proteolytic self-cleavage to its mature form. Active caspase-1 may process pro-IL-1β to its mature form and cleave gasdermin D to induce pyroptotic cell death.

The NLRC4 inflammasome is composed of neuronal apoptosis inhibitory protein (NAIP), NLRC4, ASC, and caspase-1. Unlike NLRP3, NLRC4 does not directly respond to ligands and instead employs NAIPs as upstream receptors. The murine NAIPs-5/6, 1, and 2 recognize bacterial flagellin, the T3SS inner rod protein, and the T3SS needle protein, respectively (26–28). Recognition of PAMPs by NAIPs results in NAIP-NLRC4 association, leading to a conformational change in NLRC4 which reveals an active catalytic site (29). Active NLRC4 may recruit and activate additional NLRC4 molecules, resulting in the assembly of a disk-like structure containing 9-11 NLRC4 molecule, but only 1 NAIP molecule (29). ASC and pro-caspase-1 may then be recruited to the NAIP-NLRC4 structure through interactions involving their PYD and CARD domains, respectively. Inflammasome scaffolding facilitates the initiation of a rapid and robust response.

Recognition of PAMPs or DAMPs by NLRs ultimately leads to inflammasome assembly and caspase-1 maturation. Active caspase-1 may proteolytically cleave pro-IL-1β and pro-IL-18 to their mature forms. Unlike the conventional cytokines that contain an N-terminal secretion signal which promotes their export out of the cell, the IL-1 cytokine family lacks this secretion signal and hence must be processed by inflammasome signaling for transport out of the cell (30). IL-1 family cytokines are highly pyrogenic and aid the host in eradicating the pathogen by promoting the recruitment of additional immune cells and activating the adaptive immune response (31–34). Caspase-1 may also cleave gasdermin D to initiate pyroptosis (35). The cleaved gasdermin D N-terminal domain assembles membrane pores to induce cell lysis, resulting in inflammatory cell death (36).

Inflammasomes may also be activated through non-canonical pathways independent of PRR-mediated pathogen recognition. Non-canonical inflammasome engagement results in the activation of caspase-11 in mice and caspases-4/5 in humans (37). The non-canonical inflammasome is typically activated in response to LPS within the host cytosol (38). Surprisingly, this recognition is independent of PRRs and is instead mediated by the direct sensing and binding of caspases to cytosolic LPS (38). Non-canonically activated caspase-11 may directly cleave gasdermin D to initiate pyroptosis and indirectly induce IL-1β secretion (37, 39). Non-canonical inflammasome activation may also induce NLRP3 activation, resulting in amplification of the inflammatory response (40, 41). Inflammasome activation elicits a rapid, protective inflammatory response against the invading pathogen.

### 1.4 Cell death

The induction of host cell death is a key process that occurs during bacterial infection (42). Cell death may be induced by the host as a protective mechanism, or by pathogens to facilitate their dissemination. Cell death may occur through apoptotic or necrotic mechanisms (**Figure 2**). Cells undergoing apoptotic death contain their intracellular contents within membrane-bound vesicles (blebs) which are phagocytosed by patrolling macrophages through efferocytosis (43). In contrast, cells undergoing necrotic death rupture and freely release their intracellular contents which are recognized as DAMPs by neighbouring cells. Apoptotic and necrotic cell death may both contribute to pathogen clearance and proliferation.

Apoptotic cell death is a form of programmed cell death that is mediated by caspases. Apoptosis may be triggered intrinsically through cellular stress, or extrinsically through the stimulation of membrane-bound death receptors (44). Intrinsic apoptosis is initiated by mitochondrial damage resulting in the release of cytochrome *c* and recruitment of caspase-9 to assemble the apoptosome complex. Extrinsic apoptosis is initiated by engagement of apoptotic receptors such as FasR (CD95) or TNFR1 which recruit caspases-8 and -10 to assemble the death-inducing signaling complex (DISC) (44). Ultimately, the initiator caspases-8, -9, and -10 may cleave and activate the executioner caspases-3 and -7 to induce cellular degradation and apoptotic bleb formation (43). Apoptotic cell death is regarded as noninflammatory since dying cells do not freely release their intracellular contents to the extracellular space.

Necrotic cell death is a form of unprogrammed cell death that occurs in response to extreme cellular damage. Necrotic cell death is characterized by loss of plasma membrane integrity, resulting in the release of intracellular contents to the extracellular space (45). Some pathogens may induce necrotic cell death through the action of their effector toxins; however, necrotic cell death may also occur through cell-mediated, regulated pathways. Regulated necrotic cell death, termed necroptosis, is initiated in response to caspase-8 inhibition during apoptotic stimulation (46). This results in the recruitment of receptor interacting protein kinase 1 (RIPK1), RIPK3 and mixed lineage kinase domain-like (MLKL) to assemble the necrosome complex (47). RIPK3-mediated phosphorylation of MLKL permits it to dissociate from the necrosome and permeabilize the plasma membrane, resulting in cell rupture (48).

Pyroptosis is a form of regulated necrotic cell death that is mediated by activated caspase-1/11. Recognition of PAMPs or DAMPs by NLRs results in the oligomerization of the inflammasome and consequent recruitment of caspase-1 to the inflammasome complex (9). Active caspase-1 may cleave gasdermin D to release its N-terminal domain which subsequently translocates to the plasma membrane to form a membrane-spanning pore, resulting in cellular permeabilization and rupture (49, 50). Pyroptotic cell death is highly proinflammatory due to the release of intracellular contents and proinflammatory cytokines such as IL-1β (51).

The induction of cell death in response to bacterial infection is usually protective. Destruction of infected tissues presents a strategy to eliminate bacterial niches conducive to pathogen proliferation. For example, cell death of macrophages infected by *Mycobacterium tuberculosis* eliminates its intracellular replication niche and the subsequent uptake of apoptotic blebs promotes phagosome-lysosome fusion in neighbouring phagocytes (52). In response, some pathogens may inhibit apoptosis as a means to promote their intracellular survival. Obligate intracellular pathogens such as *Rickettsia ricksetsii* induce enhanced host NF-κB signaling to prevent cell death (53). Conversely, some pathogens may actively induce host cell death to evade host defenses and promote dissemination. *Salmonella* Typhi may induce apoptosis within infected cells to generate apoptotic blebs containing *Salmonella* (54). These apoptotic blebs may be engulfed by incoming phagocytes, facilitating cell-to-cell transmission and systemic dissemination of the pathogen (55). Other pathogens such as *Pseudomonas* may

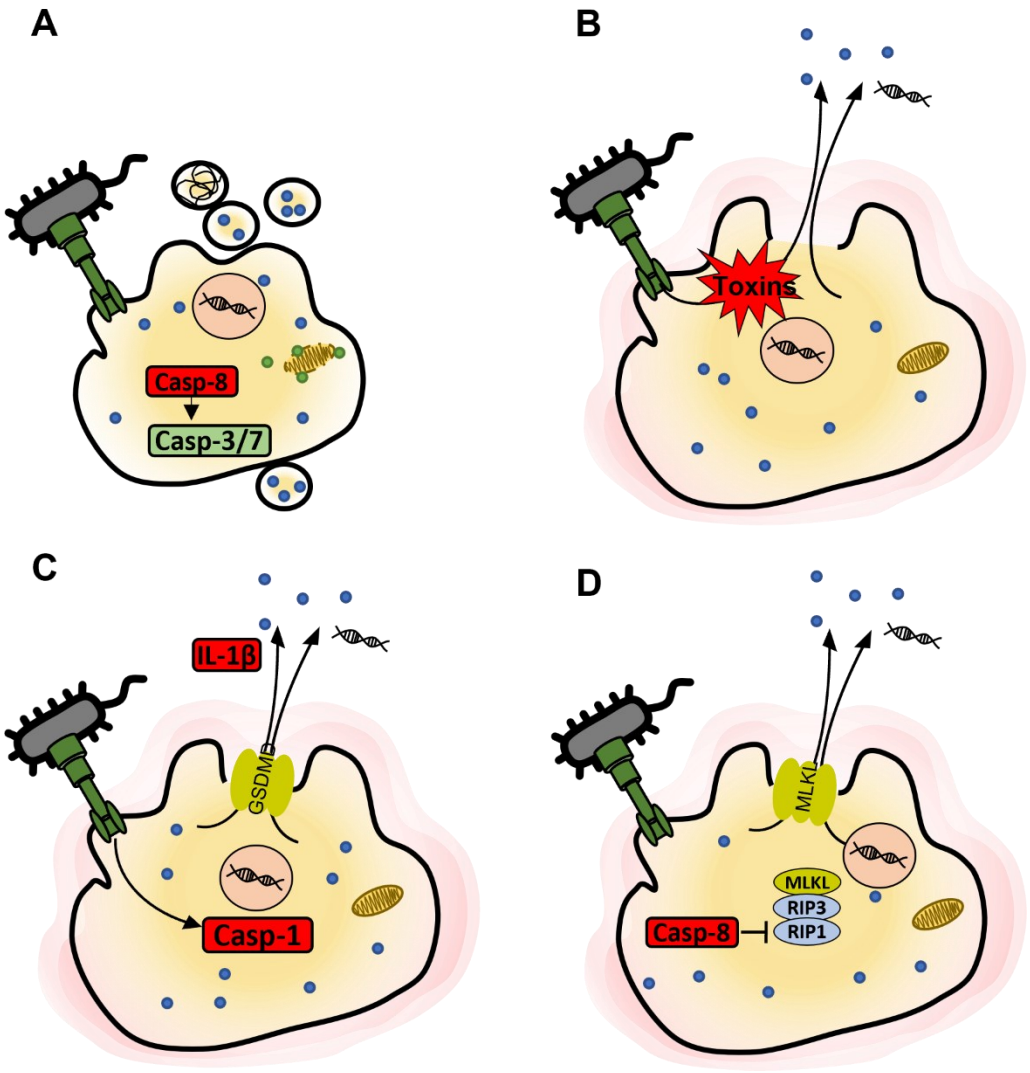


**Figure 2. Cell death during bacterial infection.**

Infected cells may undergo apoptotic or necrotic cell death. Apoptosis (**A**) is executed by caspases-3/7 and is characterized by the formation of membrane blebs which contain the contents of the dying cell. Necrosis (**B**) is characterized by a loss of membrane integrity and may be induced by bacterial toxins, resulting in release of the intracellular contents. Pyroptosis and necroptosis are regulated necrotic cell death pathways. Pyroptosis (**C**) occurs in response to inflammasome activation and is mediated by caspase-1. Necroptotic cell death (**D**) may be induced when caspase-8 is inhibited, preventing the induction of apoptotic cell death.

induce necrotic cell death to impair pathogen clearance. The *Pseudomonas* ExoU effector toxin directly degrades cellular membranes, resulting in lytic cell death (56, 57). Pyroptotic cell death facilitates pathogen clearance by releasing intracellular pathogens and proinflammatory mediators to promote pathogen clearance. Thus, host cell death pathways contribute to both pathogen destruction and persistence. Pathogens may exploit this duality to balance pathogen and host survival.

## 1.5 Host innate response to bacterial infection

Invading pathogens first encounter the innate immune system as they attempt to establish an infection. The innate immune system possesses many defenses to combat pathogens, including physical barriers, antimicrobial proteins, and circulating leukocytes. These components work together to protect the host against various pathogens, including bacteria.

The skin, gastrointestinal tract, and respiratory tracts are the primary interfaces between the host and its external environment. These epithelial layers serve as physical barriers against foreign microorganisms and are an important protective component against invading pathogens. Physical defenses such as mucosal linings and the acidity of the stomach are often sufficient to combat many pathogens, however, some pathogens such as *Salmonella* possess virulence mechanisms to overcome these initial defenses and gain access to the epithelial barrier (58, 59).

Invading pathogens may attempt to breach the epithelial barrier and gain access to normally sterile compartments. The epithelial cells composing this barrier express PRRs to initiate

an innate immune response against invading pathogens. PRRs such as TLRs and NLRs are ubiquitously expressed by mucosal epithelial cells within the respiratory and gastrointestinal tracts (60–62). Recognition of PAMPs such as bacterial LPS or flagellin initiates a proinflammatory response and recruitment of additional immune cells. The secretion of proinflammatory cytokines such as IL-1β, IL-18, TNFα, IL-7 and IL-8 promotes the infiltration of immune cells including monocytes, neutrophils, and macrophages to the site of infection (63–65).

Polymorphonuclear leukocytes (PMNs) and mononuclear phagocytes, including neutrophils and macrophages, are two key cell types involved in mediating the innate immune response against invading pathogens. These cells are highly phagocytic which permits them to engulf and eliminate pathogens they encounter. Neutrophils possess a variety of antimicrobial mechanisms, including superoxide, granules, and NETs. Neutrophil extracellular traps (NETs) are extracellular web-like structures that may be released to trap and neutralize extracellular bacteria (66). Neutrophils produce superoxide ($O_2^-$) through the action of the NADPH oxidase NOX-2 which is subsequently released into phagosomes (67, 68). Superoxide induces bacterial protein and DNA damage, resulting in irreparable cellular damage and death (69, 70). PMNs may also target bacteria-containing phagosomes with granules loaded with antimicrobial proteins such as defensins, elastases and peroxidases (71).

Macrophages play an important role in mediating the immune response against invading pathogens. Macrophages primarily eliminate bacteria through phagocytosis and the subsequent lysosomal degradation of the phagosomal contents. Stimulation of phagocytic receptors such as macrophage receptor with collagenous structure (MARCO) and FcγR induces phagocytosis (72,

73). Subsequently, phagosomes undergo a maturation process to become mature phagolysosomes. During maturation, the phagosomal interior is acidified to pH 4.5-5.5 (74). Lysosomes are simultaneously trafficked to the phagosome and phagosome-lysosome fusion delivers potent proteases that are activated by the acidic pH (75). Phagolysosomes efficiently degrade their intracellular contents to eliminate the pathogens within. Furthermore, activated macrophages may migrate to lymph nodes to induce activation of the adaptive immune system (76). Phagolysosomal degradation of pathogen-derived proteins generates antigenic peptides which may be loaded onto MHC class II molecules to further mediate adaptive immune responses (77, 78).

Innate immune cells such as macrophages and neutrophils function in concert to efficiently control and eliminate pathogens they may encounter. Despite these powerful tools, innate immune responses are unable to eliminate all pathogens, especially those which have evolved complex strategies to counteract the host immune system.

## 1.6 Bacterial virulence systems

Pathogenic bacteria possess a diverse range of virulence mechanisms to cause disease in hosts and promote bacterial persistence. These virulence mechanisms may be conserved across several different species or unique to specific pathogens. The ability of the host to identify and target these virulence mechanisms is key towards mediating responses against pathogenic

bacteria. Bacterial virulence mechanisms include, but are not limited to, lipopolysaccharides, motility apparatuses, toxin secretion systems, and biofilms (79).

Gram-negative bacteria are characterized by the presence of a second outer membrane composed of lipopolysaccharides. The LPS membrane functions to protect bacteria against extracellular toxins such as antibiotics or antimicrobial proteins (80, 81). LPS is the most abundant antigen expressed on Gram-negative bacteria and host recognition of LPS by PRRs is crucial for priming the immune response (82). Bacteria may modify their LPS composition over the course of an infection to reduce their immunogenicity and evade recognition (83).

Flagella are a bacterial organelle primarily utilized for motility and chemotaxis. Flagella are composed of three core structures: the basal body, a helical filament, and a hook which transfers torque from the basal body to the filament (84). The helical filament rotates, like an Archimedes screw, to generate motile propulsive force for the bacterium, known as swimming motility (85). Flagellar assembly is sequential and begins with assembly of the integral membrane components. The formation of the membrane-integral basal body facilitates the subsequent export and assembly of extracellular flagellar components. Extracellular components, such as the flagellar filament, are secreted through the export apparatus by a flagellum-specific type III secretion system (86). Failure to assemble proximal flagellar structures precludes the assembly of distal structures (84). Bacteria utilize their flagella in concert with environmental sensors to move towards or away from environmental stimuli such as nutrients or toxins (87, 88). Although flagella are typically associated with chemotactic movement, there is a strong relationship

between bacterial motility and pathogenicity (89). Flagella play an important role in promoting bacterial adhesion and invasion of target cells host (89, 90).

Pili are another important virulence factor that assist pathogenic bacteria in attaching to host cells to initiate infections. Gram-negative bacteria may possess five distinct classes of pili: chaperone-usher pili, type IV pili, type IV secretion pili, type V pili, and curli fibres (91). Type IV pili are the most ubiquitous and are expressed on numerous bacteria including *Salmonella* and *Pseudomonas*. Type IV pili contribute to bacterial pathogenesis and are sequentially assembled through a conserved secretion system similar to the type II secretion system (T2SS) (92, 93). Unlike other pili structures, type IV pili may elongate and contract through the action of cytoplasmic ATPases to facilitate bacterial twitching motility (94). The expression of bacterial flagella and pili is necessary for efficient bacterial invasion and colonization of the host (95–98).

The type III secretion system (T3SS) is a major bacterial virulence apparatus expressed by many Gram-negative bacteria including *Salmonella* and *Pseudomonas*. The T3SS functions as an injectisome, allowing for the direct translocation of bacterial effector toxins into the cytoplasm of eukaryotic cells through a needle-like structure (**Figure 3 A**). The T3SS is composed of three core structures: a basal body spanning both bacterial membranes, a needle which translocates unfolded effector proteins, and a translocation pore in the target cell membrane (99). The T3SS shares high homology to flagella and it is believed that the T3SS apparatus evolved from flagella (100). Similar to flagellar assembly, T3SS assembly is sequential and begins with the formation of the membrane-integral basal body and export apparatus. The T3SS rod, needle, and tip proteins are exported through the basal body and polymerize

extracellularly to assemble the complete structure (99). T3SS effector toxins are secreted through the injectisome in an unfolded conformation through the assistance of chaperones. These chaperones release their associated toxins to the cytoplasm of the target cell, permitting them to acquire their tertiary structure and associated effector function (101). Like flagellar assembly, the assembly of proximal T3SS structures is essential for the subsequent assembly of distal structures (102).

The activity of the T3SS is regulated at both the level of expression and secretion (103). Expression of the T3SS is typically induced by bacterial sensing of environmental changes such as osmolarity or pH (104, 105). The *Pseudomonas* T3SS may be activated in response to low $Ca^{2+}$ whereas the *Salmonella* pathogenicity island (SPI)-I T3SS may be activated in response to low pH or nutrients (103, 104). Sensing of environmental stimuli activates transcription of structural and secreted T3SS elements and induces T3SS assembly (106). Secretion through the T3SS apparatus is independently regulated in response to contact between the bacteria and host cells (107). Host cell contact induces a conformational change in the T3SS, resulting in opening of the translocation pore to permit toxin secretion (108). The effector toxins secreted through the T3SS may serve a broad range of functions, including promoting bacterial invasion, modifying host cellular processes to promote bacterial survival, or inducing host cell death to evade immune clearance (109, 110).

The type VI secretion system (T6SS) is a relatively recently described secretion system possessed by approximately 25% of Gram-negative bacteria (111–113). The T6SS is structurally similar to the bacteriophage tube and spike apparatus and may have been evolutionarily acquired

from phages (114, 115). The T6SS primarily functions as an antimicrobial system by targeting competing bacterial populations (**Figure 3 B**) (116). *Pseudomonas* may utilize its T6SSs to secrete Tse-family lytic enzymes, which degrade the peptidoglycan cell walls of rival bacterial populations (117, 118). However, some studies have demonstrated that the T6SS may also translocate effector proteins into eukaryotic cells to modulate host cellular processes (119, 120). For example, *Citrobacter freundii* may induce inflammasome activation through NLRP3-mediated recognition of the T6SS protein Hcp-2 (121). Although relatively little is known about T6SS interactions with eukaryotic hosts, it is likely that T6SS mediated intoxication and inflammation contributes to bacterial pathogenesis within hosts.

Bacteria may form biofilms during infections. Biofilms are multicellular microbial communities encased within an extracellular matrix secreted by the bacteria themselves (122). Biofilms are associated with bacterial persistence and it has been estimated that 80% of chronic bacterial infections involve biofilms (123). The biofilm extracellular matrix is typically composed of a polysaccharide biopolymer and additional proteins and/or DNA (124). The presence of a biofilm creates heterogeneous microbial populations since bacterial gene expression is dependent on extracellular conditions. The extracellular matrix creates a gradient, altering the spatial exposure of bacteria to extracellular nutrients, enzymes, or antibiotics. As a result, bacterial populations near the surface of a biofilm may be aerobic and resistant to antibiotics whereas populations embedded deep within a biofilm may be anaerobic and susceptible to antibiotics (125). Biofilms increase the resistance of the whole bacterial population against host

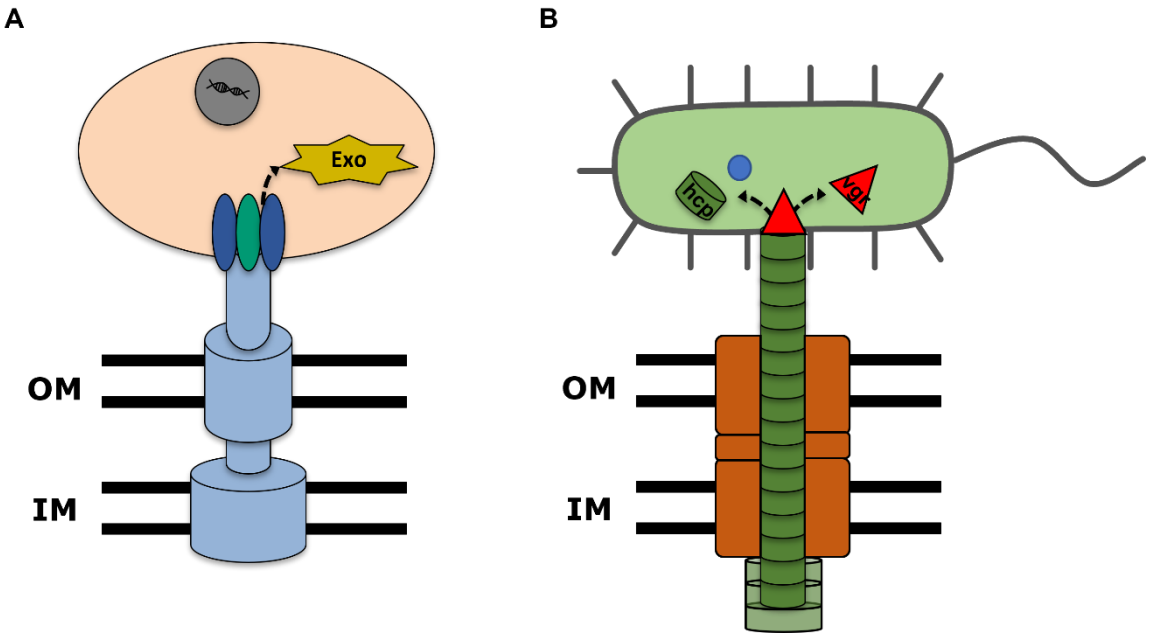


**Figure 3. Type III and VI secretion systems.**

The type III and type VI secretion systems permit pathogenic bacteria to intoxicate eukaryotic and prokaryotic cells. Both secretion systems span the bacterial inner membrane (IM) and outer membrane (OM). The T3SS (**A**) forms a translocation pore in eukaryotic cell membranes, which permits the secretion of bacterial effector toxins directly into the host cell cytoplasm. T3SS effector toxins may modify host processes and induce cell death. The T6SS (**B**) functions like a needle and injects effector toxins into other prokaryotes, however, recent studies have demonstrated that the T6SS may also target eukaryotic cells. T6SS effector toxins primarily function to kill competing bacterial populations.

immune-mediated clearance and antibiotic therapy (122). *P. aeruginosa* biofilm populations were observed to be resistant against macrophage and neutrophil phagocytosis (126). Furthermore, these biofilms resisted antibiotic penetration and possessed secreted enzymes that could inactivate biocides (127). The formation of biofilms during bacterial infections is a predictor for poorer clinical outcomes (123, 128). Pathogenic bacteria deploy their virulence mechanisms in tandem to promote efficient colonization and persistence within hosts.

### 1.7 *Pseudomonas aeruginosa*

*Pseudomonas* are Gram-negative, aerobic, flagellated bacilli. There are over 140 species of *Pseudomonas*, however, *Pseudomonas aeruginosa* and *Pseudomonas maltophilia* account for approximately 80% of clinical infections (129). *P. aeruginosa* is of particular interest due to its high prevalence in human disease (130, 131). *P. aeruginosa* is ubiquitous throughout the environment and is commonly found in soil and water (129, 132). Opportunistic *P. aeruginosa* infection typically results in acute infections that are rapidly resolved as most strains causing acute disease are susceptible to clearance by phagocytes and antibiotic therapy (133, 134). However, *P. aeruginosa* possesses a variety of virulence mechanisms, which permit it to evade clearance by immune cells and resist antibiotic therapy. *P. aeruginosa* has been estimated to be responsible for 10-15% of nosocomial infections worldwide and there has been a significant increase in the prevalence of multidrug-resistant strains (135, 136).

The T3SS of *P. aeruginosa* is its primary virulence apparatus (**Figure 4**). The T3SS permits *P. aeruginosa* to secrete bacterial effector toxins directly into host cells to induce cell death. The *P. aeruginosa* T3SS contains approximately 40 genes encoding regulatory, structural, and effector components (137, 138). T3SS assembly is sequential and the formation of a functional T3SS requires the basal body, needle, and translocation pore (102). *P. aeruginosa* may secrete the effector toxins exoenzyme S (ExoS), ExoT, ExoU and ExoY through its T3SS to mediate its pathogenesis.

The ExoS and ExoT effector toxins share high homology and both possess Rho GTPase activating protein (RhoGAP) and ADP-ribosyltransferase (ADPRT) activity (139, 140). Activation of ExoS and ExoT requires interaction with the host protein factor activating exoenzyme S (FAS) (141, 142). ExoS and ExoT function to induce host cell death and modify host cellular processes such as actin cytoskeletal dynamics (143). ExoU is the most potent T3SS effector toxin secreted by *P. aeruginosa* and it possesses phospholipase $A_2$ (PLA2) enzymatic activity (56). The PLA2 activity of ExoU permits it to directly degrade the plasma membrane of intoxicated cells to induce rapid, lytic cell death (57). Furthermore, ExoU-associated PLA2 activity enhances the production of arachidonic acid and prostaglandin E2 (PGE2), two important lipid mediators of inflammation (144). Infection by ExoU expressing *P. aeruginosa* strains has been linked to negative patient clinical outcomes and high mortality (145, 146). The ExoY effector toxin possesses nucleotidyl cyclase activity and promotes microtubule degradation (147, 148). Interestingly, infection by ExoY expressing *P. aeruginosa* strains has been associated with reduced cytotoxicity and improved patient clinical outcomes (145, 149). The expression of T3SS effector toxins varies

within *P. aeruginosa* strains. In a study examining clinical *P. aeruginosa* isolates, all isolates were observed to encode *exoT*, 72% encoded *exoS*, 28% encoded *exoU*, and 89% encoded *exoY* (150). The presence of ExoS and ExoU is almost mutually exclusive and the majority of *P. aeruginosa* strains only encode *exoS* (151).

The *P. aeruginosa* genome also encodes three type VI secretion systems (H1- to H3-T6SS). The T6SSs primarily target competing bacterial populations and have been demonstrated to contribute towards *P. aeruginosa* survival in heterogeneous infections (**Figure 3 B**) (117, 118, 152). Recent research has demonstrated that *P. aeruginosa* T6SSs may also target eukaryotic cells to modulate host signaling pathways (120). The H1-T6SS has not been shown to act on eukaryotic cells, however, the H2- and H3-T6SSs have been demonstrated to promote *P. aeruginosa* internalization within non-phagocytic cells (153). The H2- and H3-T6SS effectors PldA, PldB, and VgrG2b can induce host cytoskeletal remodelling, which promotes bacterial invasion (154, 155). *P. aeruginosa* T6SS expression is controlled by the GacS/GacA two-component regulatory system (**Figure 5**) (153).

*P. aeruginosa* may form biofilms composed of the exopolysaccharides alginate, Pel, and/or Psl (156). Biofilm formation is initiated by bacterial sensing of flagellar- or pili-mediated surface attachment to host cells (157). Attachment induces secretion of biofilm exopolysaccharides, extracellular DNA (eDNA), and additional secreted proteins including pili (158). These components cross-link to reinforce the extracellular structure and may also neutralize host anti-microbial responses and antibiotics (126, 159, 160). *P. aeruginosa* biofilms

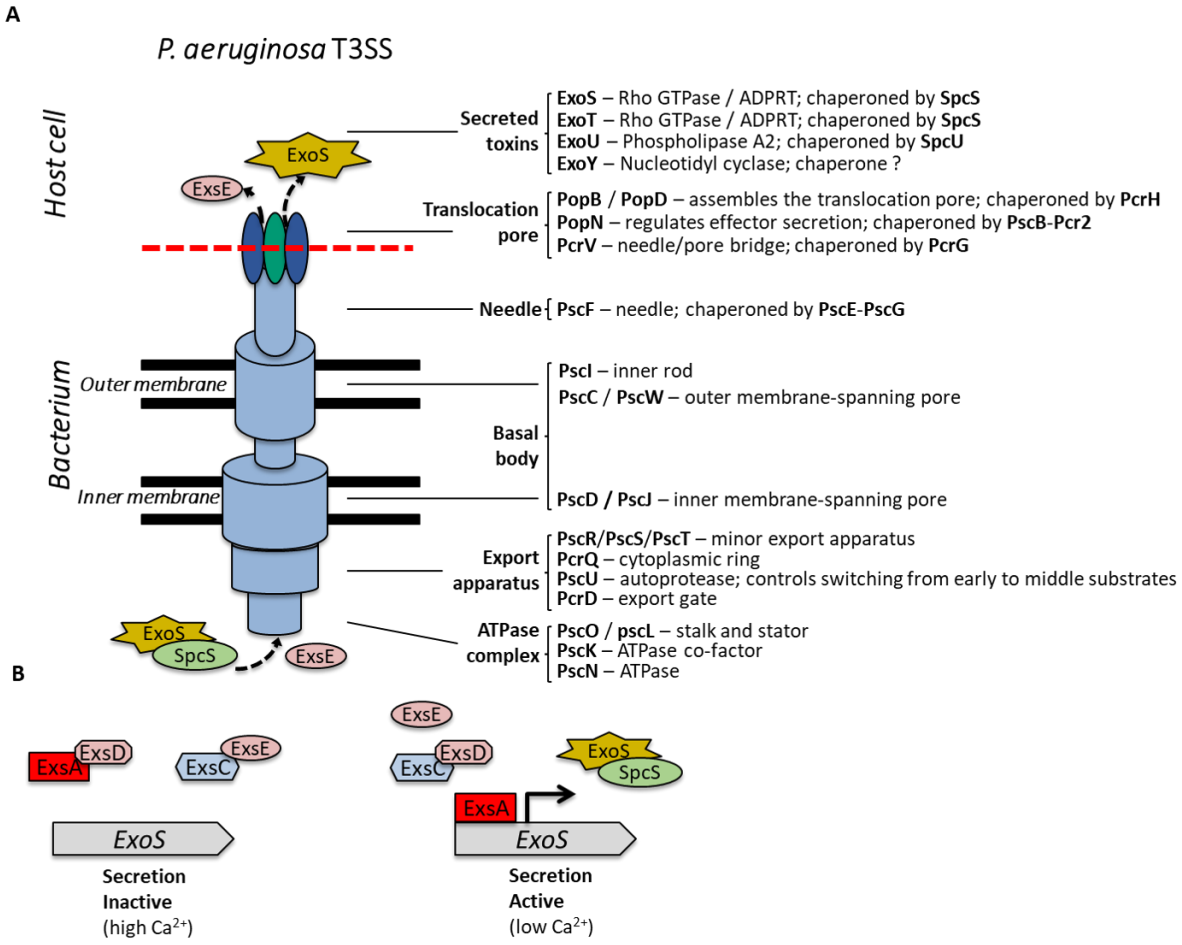


**Figure 4. The *P. aeruginosa* type III secretion system.**

The T3SS of *P. aeruginosa* permits intoxication of eukaryotic host cells. The T3SS (**A**) is composed of the ATPase complex, export apparatus, basal body, needle, and translocation pore. T3SS assembly (**B**) is initiated by sensing environmental stimuli, such as low $Ca^{2+}$, which induces transcription of T3SS genes by the transcriptional regulator ExsA. The basal body, export apparatus, and ATPase complex are the first structures assembled. Subsequent structures are secreted and polymerize extracellularly to assemble the needle and translocation pore. Sensing of target cell contact induces a conformational change in the T3SS, resulting in opening of the translocation pore and toxin secretion. Toxins are maintained in an unfolded state within the bacterium by chaperones to prevent self-toxicity.

contain heterogeneous bacterial populations that may differentially regulate their expression of T3SS, T6SS, flagella, and/or antibiotic resistance mechanisms (158, 161). *P. aeruginosa* biofilms are frequently observed in chronically infected cystic fibrosis patients and are highly associated with increased resistance to clearance (162, 163).

PA14 and PAO1 are two commonly utilized laboratory reference strains of *P. aeruginosa*. PA14 is a highly virulent strain isolated from the blood of a burn patient in the 1970s (164). PAO1 is a moderately virulent strain isolated from an open wound in the 1950s (165). PA14 displays increased virulence due to the presence of two pathogenicity islands (*Pseudomonas aeruginosa* pathogenicity island (PAPI)-1 and PAPI-2) that are absent in PAO1 (166). The T3SS effector toxin ExoU is encoded on PAPI-2 and thus PA14 may express ExoT, ExoY and ExoU (167). In contrast, PAO1 may express ExoS, ExoT and ExoY, but not ExoU (168). Furthermore, PA14 possesses a mutation in the *ladS* gene (169). The activity of the LadS, RetS and GacS sensor kinases control the expression of *P. aeruginosa* virulence genes and regulate the transition from an acute to chronic infection phenotype (170). Under normal conditions, LadS activates the GacS/GacA signaling cascade to suppress cytotoxicity and promote biofilm formation (170, 171). The mutant LadS encoded by PA14 fails to activate GacS/GacA signaling, resulting in enhanced T3SS expression and repressed biofilm formation (169).

## 1.8 *Salmonella* Typhimurium

*Salmonella* are Gram-negative, rod-shaped, flagellated, facultative intracellular bacteria. *Salmonella bongori* primarily infects cold-blooded hosts whereas *Salmonella enterica* may infect both warm- and cold-blooded hosts (172). *Salmonella enterica* subsp. *enterica* is typically associated with disease in humans. *S. enterica* serovars may be divided into two main groups, typhoidal and non-typhoidal (173). Typhoidal serovars such as *Salmonella* Typhi and *Salmonella* Paratyphi are the causative agents of enteric fever, which remains endemic in the developing world (174, 175). Non-typhoidal *Salmonella* infection typically manifests as acute gastroenteritis, however, non-typhoidal *Salmonella* may also manifest as an invasive, systemic disease when other co-morbidities are present (176). Non-typhoidal *Salmonella* infections are typically caused by the serovars Typhimurium and Enteritidis (177). *Salmonella* infections have been estimated to be responsible for 1.3 billion cases of gastroenteritis and 3 million deaths worldwide (178).

*Salmonella* possesses a diverse range of virulence mechanisms which facilitate its ability to infect hosts and cause disease. *Salmonella* virulence genes are arranged in large cassettes containing a series of related genes and operons, termed pathogenicity islands. Pathogenicity islands are expressed at a specific time during infection to manifest a particular virulence phenotype. *Salmonella* possesses five pathogenicity islands that encode the genes necessary for bacterial invasion, growth and survival (179). The type III secretion systems of *Salmonella* are regarded as its most important virulence hubs. *Salmonella* possesses two unique T3SSs, encoded by *Salmonella* pathogenicity island (SPI)-I and -II. The SPI-I T3SS promotes *Salmonella* invasion in

epithelial cells and the SPI-II T3SS facilitates *Salmonella's* intracellular survival and replication in epithelial cells and macrophages (104, 180).

The SPI-I T3SS promotes *Salmonella* invasion into non-phagocytic cells, such as epithelial cells within the gastrointestinal tract. SPI-I T3SS expression is regulated through sensing of environmental changes including pH, osmolarity, and $Mg^{2+}$ concentration, which influence the expression of the transcriptional regulators HilA and InvF (104). HilA and InvF regulate the expression of SPI-I genes, including its effector toxins (181). SPI-I T3SS effectors such as SipA, SopB, SopD, and SopE2 induce actin cytoskeletal rearrangement within host cells to create membrane ruffles which engulf *Salmonella* into vesicles (104, 109). SopA and SopD may also stimulate host proinflammatory responses to further induce enteritis and promote immune cell infiltration to the site of infection (182, 183). Critically, SopB and SipA function to maintain phagosome stability and mediate modification of the phagosome to become a *Salmonella*-containing vacuole (SCV) (184, 185). *Salmonella* may also induce the apoptotic death of infected epithelial cells through the SPI-I effector toxin SipB (186). Recruited phagocytic cells, such as macrophages, can capture the released apoptotic blebs containing *Salmonella* and traffic their contents to systemic sites of infection (55).

The SPI-II T3SS promotes *Salmonella's* survival and replication within SCVs. SPI-II effectors maintain SCV stability and prevent phagolysosome maturation to create a niche conducive to bacterial replication. Expression of the SPI-II T3SS is induced within phagosomes in response to phagosomal acidification (179, 187). SPI-II T3SS effectors such as SifA and SopD2 impair the delivery of lysosomes and lysosomal enzymes to the SCV, thus protecting the bacteria against

phagolysosomal destruction (188, 189). *Salmonella* may also hijack host vesicular trafficking through the formation of tubular membrane extensions known as *Salmonella*-induced filaments (SIFs). SPI-II effectors including SifA, SopD2, SseF, SseG and SseJ contribute to formation and maintenance of the SIF network and maintain late SCV stability (190–193). The activities of the SPI-I and SPI-II T3SSs are essential towards *Salmonella's* ability to infect and persist within host cells.

Although *Salmonella* may persist within soil and water, its primary reservoir is carriage within infected living hosts such as livestock, reptiles, and humans (194, 195). Approximately 3-5% of humans infected by *Salmonella* Typhi become chronic carriers after the resolution of acute disease (196, 197). These chronic carriers represent a significant source of transmission as they shed infectious bacteria in their feces. Although chronic *Salmonella* infections may persist for decades, infected hosts are frequently asymptomatic (198). Hosts T cells may suppress the infection, but the immune system is unable to completely eradicate the bacteria (199). Latent *Salmonella* infections may become reactivated when the host experiences other immune challenges (200). Thus, the ability of *Salmonella* to persist within the host without inducing unnecessary inflammation and toxicity represents a strategy for mutual survival of the pathogen and host.

*Salmonella enterica* serovar Typhi infection is restricted to humans and *S.* Typhi is unable to infect other hosts (201). *Salmonella enterica* serovar Typhimurium typically induces acute gastroenteritis in humans, however, it induces typhoid-like systemic disease in mice (173). *Salmonella* Typhimurium strain SL1344 is a lab-adapted reference *Salmonella* Typhimurium

strain. The original strain (ST4/74) was isolated from an infected calf and subsequently engineered to generate the histidine auxotroph SL1344 (202, 203). SL1344 is naturally streptomycin resistant and expresses both SPI-I and SPI-II loci (204). *S.* Typhimurium may persist chronically in permissive mouse strains expressing the functional Nramp1 (*Slc11a1*) protein (205). Natural resistance-associated macrophage protein 1 (Nramp1) is expressed exclusively within phagocytes and functions to promote phagolysosomal fusion and acidification (206, 207). Some inbred mouse strains, including C57BL/6, possess a mutant *Slc11a1* allele that results in the expression of a dysfunctional Nramp1 protein and compromised control of intracellular pathogens (208, 209).

## 1.9 Chronic bacterial infections

Bacteria are ubiquitous throughout the environment and hosts are constantly exposed to potential pathogens. Healthy individuals are typically not susceptible to chronic bacterial infections and chronic infections are usually observed in immunocompromised individuals. The innate immune system is capable of initiating a rapid inflammatory response to control the infection while the adaptive immune system is primed against the pathogen, however, this response may be insufficient which permits establishment of a chronic infection. Over the course of a chronic infection, pathogens may modulate the expression of their virulence factors to balance pathogen persistence and excessive host cytotoxicity. Pathogens may also exploit deficiencies in host defenses to establish infection niches permissive to bacterial proliferation.

*Pseudomonas aeruginosa* and *Salmonella* Typhimurium are two bacterial pathogens that may initiate opportunistic chronic infections in susceptible hosts.

## 1.10 Chronic *P. aeruginosa* infections

Cystic fibrosis (CF) is a genetic disease caused by autosomal recessive mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (*CFTR*) (210). CF is the most common, life-limiting recessive genetic disorder in Caucasians and it affects 1 in 3000 North Americans (211). CFTR is a chloride ion channel that transports $Cl^-$ ions to the apical lumen (212). CFTR is highly expressed on mucosal epithelial cells and mutations of *CFTR* may result in the expression of a dysfunctional CFTR protein (213, 214). This frequently manifests with the production of abnormally thick mucous, especially within the lungs (215, 216). Furthermore, dysfunctional CFTR may impair phagolysosome acidification and bacterial clearance (217, 218). As a result, CF patients experience increased susceptibility to chronic airway bacterial infections and increased mortality.

CF patients may experience pulmonary exacerbations, which are acute clinical events characterized by increased mucous production, shortness of breath, and inflammation (219, 220). Although the true cause of pulmonary exacerbations remains unknown, some researchers have proposed a relationship to the bacterial populations within the CF lung (221, 222). Fluctuations in bacterial population dynamics through the acquisition of new infections or

reactivation of dormant populations may disrupt the established homeostatic state, resulting in increased host inflammasome activation (223–225).

CF patients may become infected by many bacteria over the course of their lifetime including *Burkholderia complex*, *Haemophilus influenzae*, and *Staphylococcus aureus* (226). However, the prevalence of these species declines over time and approximately 80% of adult CF patients are chronically infected by *P. aeruginosa* (227). Acquisition of chronic *P. aeruginosa* is a strong predictor of mortality and morbidity in CF patients (221).

The prevalence of *P. aeruginosa* in CF patients is attributed to its ability to adapt to its environment, including the CF lung (162, 228). *P. aeruginosa* infections in CF patients are opportunistically acquired from the environment (229). Although these strains may induce cytotoxicity and inflammation during acute infections, they rapidly acquire a chronic phenotype in CF patients. *P. aeruginosa* undergoes immense selective pressure in the CF lung environment through exposure to host immune surveillance, antibiotics, and other bacteria (230, 231). Strains that are unable to compete and resist killing are eliminated whereas strains that adapt to the environment may persist. *P. aeruginosa* undergoes multiple adaptations to promote chronicity including the downregulation of virulence factors such as T3SS and flagella, and the upregulation of survival mechanisms such as biofilm formation and antibiotic resistance mechanisms (232, 233). Chronic *P. aeruginosa* infections in CF are associated with a loss of T3SS expression, increased biofilm formation, and enhanced antibiotic resistance (163, 234).

The GacS/RetS/LadS sensor kinases control *P. aeruginosa's* ability to switch between acute and chronic phenotypes during infection (**Figure 5**) (170, 235). These two-component systems inversely regulate the expression of acute virulence factors and chronic survival mechanisms. The GacS/GacA two-component system is the master regulator *P. aeruginosa* virulence and it reciprocally controls the expression of acute and chronic determinants (236, 237). GacS is regulated by the activities of the upstream sensor kinases RetS and LadS (170). RetS responds to cyclic-di-GMP and LadS responds to $Ca^{2+}$, respectively, to regulate GacS activation (170, 238). Activated GacS phosphorylates the global response regulator GacA, which induces the expression of the sRNAs RsmY and RsmZ, which in turn control the transcriptional regulator RsmA (239). RsmA binds to mRNA and inhibits its accessibility to ribosomes to control translation (240). During chronic infections in CF, *P. aeruginosa* may acquire loss-of-function mutations in the GacS/RetS/LadS regulatory system, which permanently direct bacteria towards the chronic infection phenotype (233, 241). Mutations in other regulatory systems, such as MucA*,* are also frequently observed in CF isolates (242). Loss of MucA's inhibitory control over AlgU results in alginate overproduction and a constitutive mucoid phenotype (243). Hypermutable *P. aeruginosa* strains are frequently observed in CF patients and their presence drives the selection of *P. aeruginosa* variants that are the most efficiently adapted to the CF lung environment (244, 245).

Although CF patients are usually colonized by only one or two genotypes of *P. aeruginosa*, multiple morphotypes are frequently observed in patient sputum samples

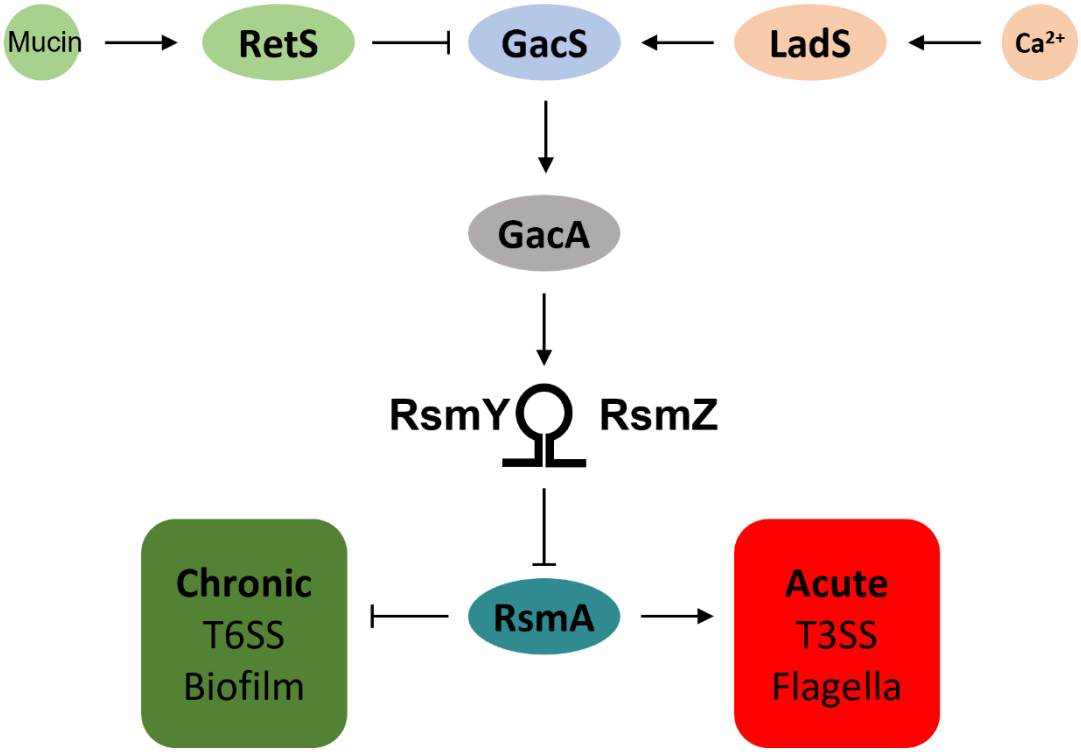


**Figure 5. *P. aeruginosa* regulation of acute and chronic infections.**

The GacS/GacA regulatory system controls the reversible transition from acute to chronic infections. Sensing of calcium by LadS induces GacS phosphorylation whereas sensing of mucin by RetS induces GacS dephosphorylation. Phosphorylation of GacA by GacS stimulates the production of the sRNAs RsmY and RsmZ, which bind and inhibit RsmA. The transcriptional regulator RsmA inhibits the translation of target mRNAs by blocking ribosome binding sites. PA14 possesses a mutation in LadS, resulting in a constitutive acute phenotype. Chronic *P. aeruginosa* isolates may possess dysregulation in GacS/GacA, resulting in permanent conversion to the chronic phenotype.

(246, 247). This phenomenon has previously been described as small-colony variants (SCVs) and it has been proposed that *P. aeruginosa* SCVs facilitate its survival and persistence within the cystic fibrosis lung environment (161, 248, 249). *P. aeruginosa* SCVs have been observed to differentially regulate their gene expression and most populations resembled a chronic infection phenotype (249, 250). However, small populations of *P. aeruginosa* were observed to retain an acute infection phenotype and were capable of inducing acute cytotoxicity (247, 249). Overall, mutations in *P. aeruginosa* during chronic infections frequently result in the permanent conversion to a chronic infection phenotype, which is associated with decreased bacterial virulence and increased bacterial persistence. However, some populations may retain their virulence mechanisms and become reactivated, resulting in host cytotoxicity and inflammation at later time intervals.

### 1.11 Chronic *Salmonella* infections

*Salmonella* infections typically induce acute gastroenteritis, however, susceptible individuals may become chronically infected (251). Approximately 3-5% of individuals infected by *Salmonella* become chronic carriers after the resolution of acute symptoms (196, 197). *Salmonella* primarily resides within chronically infected hosts and these chronic carriers are necessary to maintain *Salmonella's* continued survival (252). Interestingly, chronic carriers of *Salmonella* remain asymptomatic, suggesting that the host and bacteria co-exist in a homeostatic state.

*Salmonella* infections are usually acquired via the oral route through ingestion of contaminated food. During the early stages of infection, *Salmonella* expresses virulence factors such as the SPI-I T3SS and flagella to establish the infection niche. Flagella facilitate bacterial adhesion to host cells and SPI-I T3SS effector toxins promote *Salmonella* internalization by epithelial cells within the gastrointestinal tract (109, 253). Under normal conditions, host phagosomes are rapidly acidified and fuse with lysosomes to deliver lysosomal proteases which degrade the phagosomal contents (254). Some intracellular pathogens such as *Listeria* may lyse the phagosomal membrane and escape to the cytoplasm, however, virulence factors such as flagella and T3SS may be readily recognized by host NLRC4 inflammasomes in this compartment (19, 24). Thus, *Salmonella* employs an alternate survival strategy to evade host antimicrobial defenses.

*Salmonella* hijacks the host phagosome maturation pathway to establish an intracellular niche within known as the *Salmonella* containing vacuole (SCV). During the early stages of infection, SPI-I T3SS effectors function to establish the SCV. Effectors such as SipA promote actin cytoskeletal remodelling to generate membrane ruffles whereas effectors such as SopB and SopD facilitate closing of the phagocytic cup (255). The early SCV resembles a normal phagosome and carries markers such as Rab5 and EEA1 (256, 257). Bacterial sensing of phagosome acidification rapidly induces expression of the SPI-II T3SS and SCV maturation (258). SPI-II T3SS effectors such as SifA and SopD2 act to inhibit lysosomal trafficking and fusion to the SCV (188, 189). As the SCV matures, early phagosomal markers are lost and late endosomal markers such as Rab7 and LAMPs are acquired (257). The late SCV is characterized by the presence of *Salmonella*-induced

filaments (Sifs) radiating outwards from SCV, which permit *Salmonella* to redirect host vesicular trafficking to supply the SCV with nutrients (259). The same SPI-II effectors associated with SCV maturation are also associated with Sif biogenesis. SifA is indispensable towards Sif formation whereas effectors such as SseF and SseG play an important role in anchoring the SCV near the Golgi apparatus (193, 260). Through the action of the SPI-I and SPI-II T3SSs, *Salmonella* creates an intracellular niche within the SCV conducive to replication where it is protected from host immune clearance.

*Salmonella's* ability to reside within the SCV also impairs activation of the host adaptive immune response. Lysosomal degradation of phagosomal contents generates antigenic peptides that may be loaded on MHC-II molecules to elicit a T cell response (261). In addition to inhibiting phagosome-lysosome fusion, *Salmonella* may also hijack host vesicular trafficking to block transport of MHC-II molecules to the SCV (262). Furthermore, intracellular *Salmonella* within dendritic cells was observed to suppress MHC-II-dependent antigen presentation, resulting in decreased T cell activation after infection (263, 264). This suppression was observed to be dependent on the activity of SPI-II effector proteins which inhibited intracellular loading of peptides onto MHC-II (265). As a result, the activation of an effective adaptive immune response against *Salmonella* is markedly impaired, facilitating the chronic persistence of the pathogen (264).

During infections, *Salmonella* may induce host cell death through both apoptotic and necrotic pathways (54, 266–268). Phagocytes recruited to the site of infection such as macrophages may capture apoptotic blebs containing the contents of the dead cells,

perpetuating the cycle of cell-to-cell spread and facilitating systemic dissemination of *Salmonella* (55). Although the most common sites of infection are the ileum, liver, and spleen, *Salmonella* may also enter the gallbladder where it can chronically persist (269). Within the gallbladder, *Salmonella* resides in a latent state and hosts remain asymptomatic, even though the gallbladder is not an immune privileged site. Thus, *Salmonella* must modulate the expression of its virulence factors during chronic infections to evade host immune surveillance.

# 2. RATIONALE, HYPOTHESIS, AND OBJECTIVES

## 2.1 Rationale

Chronic bacterial pathogens may reside within infected hosts for extended durations. *Pseudomonas aeruginosa* and *Salmonella* Typhimurium are opportunistic pathogens that may cause chronic infections in susceptible hosts. Both *P. aeruginosa* and *S.* Typhimurium may persist within chronically infected hosts for significant durations of time, yet hosts do not succumb to these infections.

Previous research from our lab has demonstrated that clinical isolates of *P. aeruginosa* from chronically infected cystic fibrosis patients displayed poor motility and failed to induce inflammasome activation (270). However, other studies have reported that CF patients experience significant *P. aeruginosa*-induced airway inflammation, resulting in increased secretion of proinflammatory mediators including IL-1β (226, 271, 272). Furthermore, other researchers have demonstrated a relationship between CF lung-resident *P. aeruginosa* and host inflammation since acute pulmonary exacerbations may be suppressed by antibiotic treatment (273–275). Hence, there are inconsistencies between our prior work and the clinical picture in patients. Huus et al. examined a limited number of clinical isolates, which may not be representative of the significant population diversity within the CF lung microbiome. This highlights the need to comprehensively assess the heterogeneity of *P. aeruginosa* within the CF lung and its relationship to bacterial virulence and host inflammation.

Murine *in vivo* infection models may be utilized to experimentally study bacterial adaptation strategies over the course of a chronic infection. Unfortunately, murine models of cystic fibrosis and chronic *P. aeruginosa* infection do not accurately represent the normal progression of disease as significant attenuation of bacterial virulence is necessary to maintain host survival (276, 277). Therefore, alternate models of chronic infection may be explored to assess bacterial adaptation during chronic infections.

*Salmonella* Typhimurium is a highly virulent bacterial pathogen which may induce chronic infections detectable for over one year following the resolution of acute symptoms (252). *S.* Typhimurium and *P. aeruginosa* are both highly virulent, however, *S.* Typhimurium primarily induces inflammasome-dependent cytotoxicity and inflammation whereas *P. aeruginosa* may induce inflammasome -dependent and -independent toxicity. Host recognition of bacterial PAMPs promotes a proinflammatory response to facilitate pathogen clearance, however, chronic *Salmonella* carriers remain asymptomatic, indicating an impairment in the ability of the host to respond against the pathogen. During this time, host responses to other pathogens are not impaired (278). This suggests that *Salmonella* modulates the expression of its virulence factors to control inflammasome-mediated cytotoxicity and inflammation during chronic infection. Mouse strains such as 129X1/SvJ express a functional Nramp1 protein and are permissive of chronic *S.* Typhimurium infection (205). Thus, chronic *S.* Typhimurium infection of mice may be utilized to examine the *in vivo* modulation of pathogen-induced inflammasome signaling in various anatomical compartments throughout the course of a chronic infection.

## 2.2 Hypothesis

I hypothesize that bacterial pathogens modulate the expression of their virulence factors during chronic infections to achieve a balance between overt pathogen-induced toxicity and host-mediated immune clearance, thus promoting the mutual survival of the pathogen and host. However, some populations of bacteria may differentially express their virulence systems, including T3SS and T6SS, to modulate host cell death and toxicity during chronic infections.

## 2.3 Objectives

The objective of this study was to characterize bacterial adaptation strategies during chronic infections and the relationship to host cytotoxicity and inflammation. My first project focused on chronic *P. aeruginosa* infections in cystic fibrosis. The objectives of this project were to 1) assess the relationship between *P. aeruginosa* phenotype and genotype towards inducing host cytotoxicity and inflammation; 2) characterize differential inflammasome activation induced by heterogeneous populations of *P. aeruginosa* within the cystic fibrosis lung. My second project focused on examining bacterial adaptation during chronic infection *in vivo*. The objectives of this project were to 1) assess the modulation of *S.* Typhimurium virulence factor expression over the course of a chronic murine infection; 2) determine the mechanisms through which *S.* Typhimurium evades host inflammasomes during chronic infection.

# 3. MATERIALS AND METHODS

## 3.1 Mice Strains

All mice were maintained at the University of Ottawa Animal Care and Veterinary Services facility in accordance with the Canadian Council on Animal Care (CCAC) guidelines.

C57BL/6J (JAX stock #000664), 129X1/SvJ (JAX stock #000691), and NLRP3-deficient (JAX stock #021302) mice were purchased from The Jackson Laboratory. B6.Caspase-1,11$^{-/-}$ mice were provided by Dr. Richard Flavell (Yale University). B6.RipK3$^{-/-}$ mice were provided by Vishva Dixit (Genentech). NLRC4$^{-/-}$ mice were provided by Dr. Willie June Brickey (University of North Carolina at Chapel Hill). B6.Caspase-1,11$^{-/-}$RipK3$^{-/-}$ mice were generated by Dr. Bojan Shutinoski (University of Ottawa) by crossing B6.Caspase-1,11$^{-/-}$ mice with B6.RipK3$^{-/-}$.

## 3.2 Bacterial Strains and Culture

A complete list of bacterial deletion and mutant strains may be found in **Table 1**. All strains were stored at -80$^{o}$C in lysogeny broth (LB, BD Biosciences) media with 15% glycerol. Prior to usage, strains were streaked on LB agar plates and incubated overnight at 37$^{o}$C. Single colonies were isolated and expanded in liquid LB media at 37$^{o}$C with shaking (200rpm).

**Table 1. Bacterial strains utilized in this study.**

| Strain | Description | Source |
|---|---|---|
| PA14 | *Pseudomonas aeruginosa* UCBPP-PA14 wild-type | (279) |
| PAO1 | *Pseudomonas aeruginosa* PAO1 wild-type | (280) |
| ST-WT | *Salmonella* Typhimurium SL1344 wild-type | (281) |
| PA14 *exsA*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *exsA* | (279) |
| PA14 *pscC*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *pscC* | (279) |
| PA14 *pscD*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *pscD* | (279) |
| PA14 *pscI*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *pscI* | (279) |
| PA14 *pscJ*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *pscJ* | (279) |
| PA14 *pscK*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *pscK* | (279) |
| PA14 *pscL*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *pscL* | (279) |
| PA14 *pscR*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *pscR* | (279) |
| PA14 *pscT*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *pscT* | (279) |
| PA14 *pscU*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *pscU* | (279) |
| PA14 *pcrD*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *pcrD* | (279) |
| PA14 *popB*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *popB* | (279) |
| PA14 *popD*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *popD* | (279) |
| PA14 *spcS*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *spcS* | (279) |
| PA14 *spcU*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *spcU* | (279) |
| PA14 *exoT*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *exoT* | (279) |
| PA14 *exoU*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *exoU* | (279) |
| PA14 *exsC*::Tn | UCBPP-PA14 with MAR2xT7 transposon insertion in *exsC* | (279) |
| PW3725 | PAO1 with ISphoA/hah transposon insertion in *vgrG2a* | (280) |
| PW3726 | PAO1 with ISphoA/hah transposon insertion in *vgrG2a* | (280) |
| ST-Δ*invA* | SL1344 with *invA* deletion | (282) |
| ST-Δ*ssaR* | SL1344 with *ssaR* deletion | (281) |
| ST-Δ*fliF* | SL1344 with *fliF* deletion | (283) |

### 3.3 *P. aeruginosa* Isolates

All bacterial isolates were maintained as previously described. *Pseudomonas aeruginosa* isolates from environmental, acute clinical and chronic CF clinical sources were provided by Dr. Jeremy Dettman and Dr. Rees Kassen (University of Ottawa) (284).

Sputum samples were previously collected from cystic fibrosis patients enrolled in a two-year prospective observational study at The Ottawa Hospital, between June 2012 and April 2014 (study 20120292-91H) (285). Adult patients (>18 years old) with confirmed CF who were able to spontaneously produce sputum and had a history of chronic infection with *Pseudomonas aeruginosa* (>2 positive sputum cultures for *Pseudomonas* prior to study entry) were recruited to the study. Study participants provided sputum samples every 3 months during clinically stable periods, at the time of pulmonary exacerbation, and 30-60 days post-exacerbation. Patients were assessed using previously published clinical criteria to determine if they experienced a pulmonary exacerbation (286). Ethics approval for this study was obtained from the Ottawa Hospital Research Ethics Board in accordance with the amended Declaration of Helsinki and informed written consent was obtained from all subjects. Sputum samples were streaked on cetrimide agar (BD Biosciences), a *Pseudomonas*-selective growth medium, and incubated at room temperature for up to 48 hours. Individual colonies were isolated and expanded in LB broth to create glycerol stocks for storage at -80$^{o}$C. All subsequent experiments were performed using fresh cultures inoculated from the -80$^{o}$C stocks.

### 3.4 *In vivo* Infection Assay

*In vivo* infections were performed by injecting mice intravenously (*i.v.*) through the lateral tail vein with 100 µL of the indicated *S.* Typhimurium strain in isotonic saline. Mice were sacrificed at the indicated timepoints and the spleen, gallbladder, mesenteric lymph nodes, and inguinal lymph nodes were collected in cold PBS. The organs were homogenized to release the intracellular bacteria and the bacterial burden was determined by plating serial dilutions of the homogenates on LB agar plates supplemented with 50 µg/mL streptomycin (MilliporeSigma) or kanamycin (MilliporeSigma). Individual colonies were isolated and expanded in LB broth to create glycerol stocks for storage at -80$^{o}$C. All subsequent experiments were performed using fresh cultures inoculated from the -80$^{o}$C stocks.

### 3.5 Oral Gavage Infection

Oral gavage infections were performed following a previously described protocol (287). Mice were first gavaged with 20 mg of streptomycin in distilled water the day before infection. The following day, mice were gavaged with the desired dose of *S.* Typhimurium in isotonic saline. Mice were sacrificed at the indicated timepoints and the mesenteric lymph nodes were collected in cold PBS. The organs were homogenized to release the intracellular bacteria and the bacterial burden was determined by plating serial dilutions of the homogenates on LB agar plates supplemented with 50 µg/mL streptomycin.

### 3.6 Bone Marrow-Derived Macrophages

Primary murine bone marrow-derived macrophages (BMDMs) were generated *in vitro* following a previously described protocol (288). Mice of the indicated genotype were sacrificed and the coxae, femur and tibia were harvested. Bone marrow was flushed from the bones and resuspended in RPMI media (Gibco) supplemented with 8% fetal bovine serum (FBS, Gibco) and 50 mg/mL gentamicin (Gibco). Petri dishes were coated with macrophage colony stimulating factor (M-CSF, BioLegend) and bone marrow cells were cultured at a concentration of $1.5x10^6$ cells/mL in R8 media with 5 ng/mL M-CSF at 37$^oC$. Mature BMDMs were harvested for usage after seven days.

### 3.7 Inhibitors and Other Reagents

Inhibitors were reconstituted in dimethyl sulfoxide (DMSO). Control cells were treated with media containing comparable amounts of DMSO. The gasdermin D inhibitor disulfiram (DSF) was purchased from MilliporeSigma. Cells were pre-treated overnight with 100 ng/mL LPS (MilliporeSigma) where indicated.

### 3.8 *In vitro* Infection Assay

Mature BMDMs were harvested and seeded in 96-well plates at a density of $10^5$ cells per well in R8 media without antibiotics. The bacterial strains of interest were cultured in LB broth to mid-exponential growth phase or stationary phase. Bacterial density was approximated by

adjusting the cultures to $OD_{600}$=0.6. Bacteria were resuspended in R8 media without antibiotic and cells were infected at the indicated multiplicity of infection (MOI). Infected plates were centrifuged at 2500 rpm for 6 minutes to synchronize the bacterial uptake. *S.* Typhimurium infected cells were washed with warm PBS and incubated with R8 media containing 50 μg/mL gentamicin to remove extracellular bacteria. Cell viability was assessed at the indicated timepoints post-infection and cell supernatants were collected for cytokine quantification.

### 3.9 Cell Viability Assay

Cell viability was assessed by neutral red uptake assay. Following infection, cells were incubated with 0.33 mg/mL neutral red dye (MilliporeSigma) in R8 media at 37$^{o}$C until viable cells became visibly red. The cells were washed once with warm PBS to remove any free dye and the cells were lysed with a solubilization reagent containing 1% acetic acid in 50% ethanol. The absorbance of the solubilized dye was quantified by colorimetric analysis at 570-650nm using a FilterMax F5 plate reader (Molecular Devices).

### 3.10 Cytokine Quantification

Cell culture supernatants were collected following *in vitro* infections and stored at -80$^{o}$C prior to quantification. The expression of IL-1β (R&D Systems) and TNFα (BD Biosciences) were quantified by enzyme-linked immunosorbent assay (ELISA).

### 3.11 Western Blotting

Cell lysates were obtained by lysing cells in 1% SDS lysis buffer containing 1% β-mercaptoethanol (Gibco). The lysates were immediately boiled for 10 minutes to minimize protein degradation. Supernatants were diluted in SDS buffer and boiled for 10 minutes prior to loading. Equal quantities of sample were loaded and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories Inc.). Western blot analysis was performed using the following antibodies: rabbit anti-cleaved caspase-1 (89332S; Cell Signaling Technology), rabbit anti-gasdermin D (39754S; Cell Signaling Technology), rabbit anti-mouse cleaved caspase-8 (8592P; Cell Signaling Technology), mouse anti-RipK1 (610459; BD Biosciences), rabbit anti-RipK3 (2283; ProSci Inc, Poway, CA, USA), rabbit anti-phospho RipK3 (91702S; Cell Signaling Technology), rabbit anti-phospho MLKL (37333S; Cell Signaling Technology), rabbit anti-caspase-1 p10 (SC-514; Santa Cruz Biotechnology), mouse anti-caspase-1 (sc-56036; Santa Cruz Biotechnology), mouse anti-β-actin (SC-81178; Santa Cruz Biotechnology), goat anti-mouse IgG HRP (172-1011; Bio-Rad Laboratories Inc), and goat anti-rabbit IgG HRP (A6154; MilliporeSigma). The blots were subsequently detected by chemiluminescence with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo-Fisher Scientific Inc.) on a ChemiDoc MP imager (Bio-Rad Laboratories Inc.). Relative expression of proteins was quantified using Fiji software by comparing the expression of the protein of interest against the expression of β-actin.

### 3.12 Bacterial Swimming Motility Assay

Expression of bacterial flagella was assessed by swimming motility assay following a previously described protocol (289). Swim plates (LB broth with 0.3% w/v agar) were inoculated below the surface of the agar with a single colony of bacteria. Inoculated plates were incubated overnight at room temperature and the diameter of the swimming motility halo was visualized.

### 3.13 qRT-PCR

The bacterial strains of interest were grown to mid-exponential growth phase in LB broth and total RNA was extracted using a Qiagen RNeasy kit, according to the manufacturer's protocol (Qiagen). cDNA synthesis was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Inc.) according to the manufacturer's instructions and samples were stored at -20$^{o}$C until used. Quantitative real-time PCR was performed using the Bio-Rad CFX384 Touch Real-Time PCR System (Bio-Rad Laboratories Inc.) in conjunction with SYBR Green PCR Master Mix (Applied Biosystems, Thermo-Fisher Scientific Inc.) using the following thermal cycling parameters: initial activation at 95$^{o}$C for 2 minutes, followed by 40 cycles of denaturation at 95$^{o}$C for 5 seconds, and annealing/extension at 60$^{o}$C for 30 seconds. Analysis was performed in Bio-Rad CFX Manager™ software. On completion of the PCR amplification, a melt curve analysis was performed to confirm the presence of a single amplicon. Relative expression levels of the genes of interest were calculated using *dnaK*, *16s rRNA*, *rpoD* and *rspM* as reference genes. Primers used for gene expression are listed in **Table 2.**

**Table 2. qRT-PCR primers utilized in this study.**

| | | |
|---|---|---|
| *fliF* | FWD | AAGCCATTCTGTCGCCTATC |
| | REV | GCTCTTCCGTCTGCTCTTTAT |
| *flgB* | FWD | CCGCAGTGGATCTGCTTTAT |
| | REV | TTGAGACTGTTATCCGCAAACT |
| *fljB* | FWD | TGACGCTACCGATGCTAATG |
| | REV | TCCTGTCGCTTCATCGTAATC |
| *invA* | FWD | AGCGTACTGGAAAGGGAAAG |
| | REV | CACCGAAATACCGCCAATAAAG |
| *invJ* | FWD | GGATGAGGTTGGCGGTTTAT |
| | REV | TACGAAAGCATCGCCATAGTC |
| *prgH* | FWD | ACAGCAGGCGTTACCTTATTC |
| | REV | AATTGACGGGCTCTGAGTATTT |
| *prgJ* | FWD | GGCGGTCAATATCAGGTCTATG |
| | REV | GGTCCTCAATCCTGTTGGTAAT |
| *ssaR* | FWD | CGTCATGGGAACTTCTTTCCT |
| | REV | AAGGCCATACAGTGCGATATT |
| *ssaH* | FWD | GCGTTAACCATAGCCTGATTTC |
| | REV | CCAACAATAATGCCAGACATACC |
| *dnaK* | FWD | CGCTTCCAGGACGAAGAAGT |
| | REV | GGCGCCATTTTCTGACCTTT |
| *16s rRNA* | FWD | AGATGGGATTAGCTTGTTGGTGA |
| | REV | GTAACGTCAATGCTGCGGTTA |
| *rpoD* | FWD | GGTCTGACCATCGAACAGGTG |
| | REV | ATCAGACCGATGTTGCCTTC |
| *rspM* | FWD | CGTTGGTCTTGGTACGCTGA |
| | REV | GTGATCTGCGCCGTGAAATC |

### 3.14 Bioinformatic Analyses

*P. aeruginosa* isolates from environmental, acute clinical, and chronic CF patients were sequenced in a previous study by Dr. Jeremy Dettman (284). Draft genome sequences are available under GenBank accessions AWYJ00000000 to AWZG00000000 (284).

Bioinformatics analysis was performed on these sequences in collaboration with Katie Noah and Dr. Alex Wong (Carleton University, Ottawa). Genomes were BLASTed against the reference strain UCBPP-PA14 using an e-value cutoff of 0.001 and BLASTN 2.7.1 (290). Genes which did not have an ortholog in UCBPP-PA14 were referenced against PAO1. T-Coffee was used to translate to amino acid sequences and amino acid alignment using default settings with accurate alignment mode (291). Strains were classified by the source of the isolate as CF or non-CF (acute clinical and environmental) and Fisher's exact test was performed using the Python package *scikit-learn* to identify significant SNPs between the CF and non-CF groups. Manhattan plots were created using R with the package *qqman* (292). Genes containing SNPs were classified according to their association with T3SS, T6SS, motility, biofilm formation and antibiotic resistance, or biosynthesis. The number of genes containing SNPs with a $p$-value < 0.05 were enumerated to determine the number of mutations in each category. The relative frequency of mutation was determined by calculating the proportion of genes containing SNPs with a $p$-value < 0.05 against genes without significant SNPs.

### 3.15 Statistical Analysis

Statistical analysis was performed using Prism 9 software (GraphPad Software, San Diego, CA, USA). Student's *t*-test, one-way ANOVA with Tukey's post-hoc test or two-way ANOVA with Tukey's post-hoc test were used to confirm significance of results. *P*-values $<0.05$ were considered statistically significant.

# 4. RESULTS

## 4.1 *P. aeruginosa isolates from chronically infected cystic fibrosis patients modulate inflammasome signaling*

### 4.1.1 *P. aeruginosa* induces inflammasome-dependent and -independent cell death

Inflammasome activation and the associated cell death and release of IL-1β is an important protective mechanism against bacterial pathogens. I evaluated the extent of inflammatory cell death and IL-1β release *in vitro* following *Pseudomonas aeruginosa* infection of murine bone marrow-derived macrophages (BMDMs). Infection of wild-type BMDMs by the *P. aeruginosa* reference strains PA14 and PAO1 demonstrated that both strains induced cell death and IL-1β release, however, the kinetics of the two strains were vastly different (**Figure 6**). I observed that PA14 induced rapid cell death as early as 30 minutes post-infection (**Figure 6 A**). PA14 possess the ExoU toxin, which permits it to induce rapid cell death through the phospholipase $A_2$ activity of the toxin (56). In contrast, PAO1, an ExoU-deficient strain, did not induce appreciable cell death until 2 hours post-infection (**Figure 6 B**). PA14 infected BMDMs secreted lower quantities of IL-1β, likely due to the rapid induction cell death prior to inflammasome activation. In comparison, PAO1 induced higher quantities of IL-1β secretion at both 1- and 2- hours post-infection.

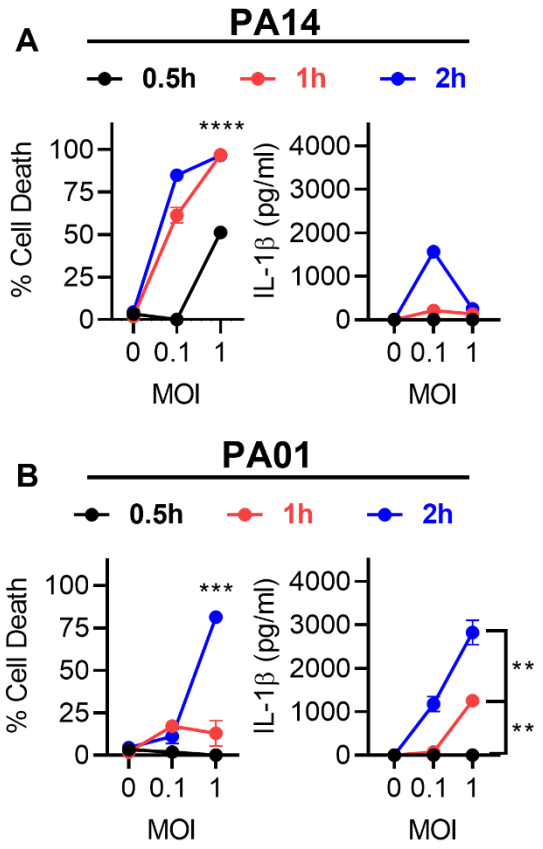


**Figure 6. PA14 induces potent cell death of macrophages relative to PAO1.**

BMDMs were differentiated from wild-type C57BL/6J mice and infected with PA14 (**A**) or PAO1 (**B**) at the indicated MOIs. At 0.5-, 1-, and 2- hours post-infection, cell death and IL-1β secretion were assessed by neutral red uptake and ELISA, respectively. Values represent mean ± SEM of triplicate cultures. Results are representative of three separate experiments. Mean values were compared by one-way ANOVA with post-hoc Tukey's multiple comparison test (***P* < 0.01; ****P* < 0.001; *****P* < 0.0001).

Caspases 1 and 11 are activated in response to inflammasome stimulation by NLR ligands. I next infected caspase-1/11$^{-/-}$ and caspase-11$^{-/-}$ BMDMs with *P. aeruginosa* to validate whether the cell death and IL-1β secretion observed was inflammasome dependent. Both PA14 and PAO1 induced cell death in wild-type BMDMs at 3 hours post-infection. However, PA14 also induced cell death in caspase-1/11$^{-/-}$ and caspase-11$^{-/-}$ BMDMs (**Figure 7 A**). In contrast, PAO1-induced cell death was dependent on the activity of caspase-1 only (**Figure 7 B**). Both PA14 and PAO1 induced IL-1β secretion at 3 hours post-infection in wild-type BMDMs. The induction of IL-1β secretion after PA14 and PAO1 infection was dependent on the activity of caspase-1, and caspase-11 was not observed to significantly contribute to inflammasome activation at 3 hours post-infection. Pre-treatment of BMDMs with disulfiram (DSF), a gasdermin D inhibitor, verified that the IL-1β release observed after PA14 and PAO1 infection was inflammasome-dependent. DSF pre-treatment inhibited the release of IL-1β in response to PAO1 and PA14 infection, however, PA14-induced cell death was not inhibited by DSF (**Figure 7 C, D**). These results indicate that PA14 may induce rapid, inflammasome-independent cell death and inflammasome-dependent IL-1β release whereas PAO1 induced-cell death and IL-1β secretion is dependent on inflammasome activation.

Caspase-1 is activated in response to inflammasome recognition of PAMPs or DAMPs. The NLRC4 inflammasome may recognize *P. aeruginosa* PAMPs such as flagella and the T3SS needle proteins whereas the NLRP3 inflammasome may respond to DAMPs associated with cellular stress during infection (293–295). In response to PA14 infection, NLRP3$^{-/-}$ or NLRC4$^{-/-}$ BMDMs did not show any impact on cell death or IL-1β release, likely due to the ability of PA14 to induce

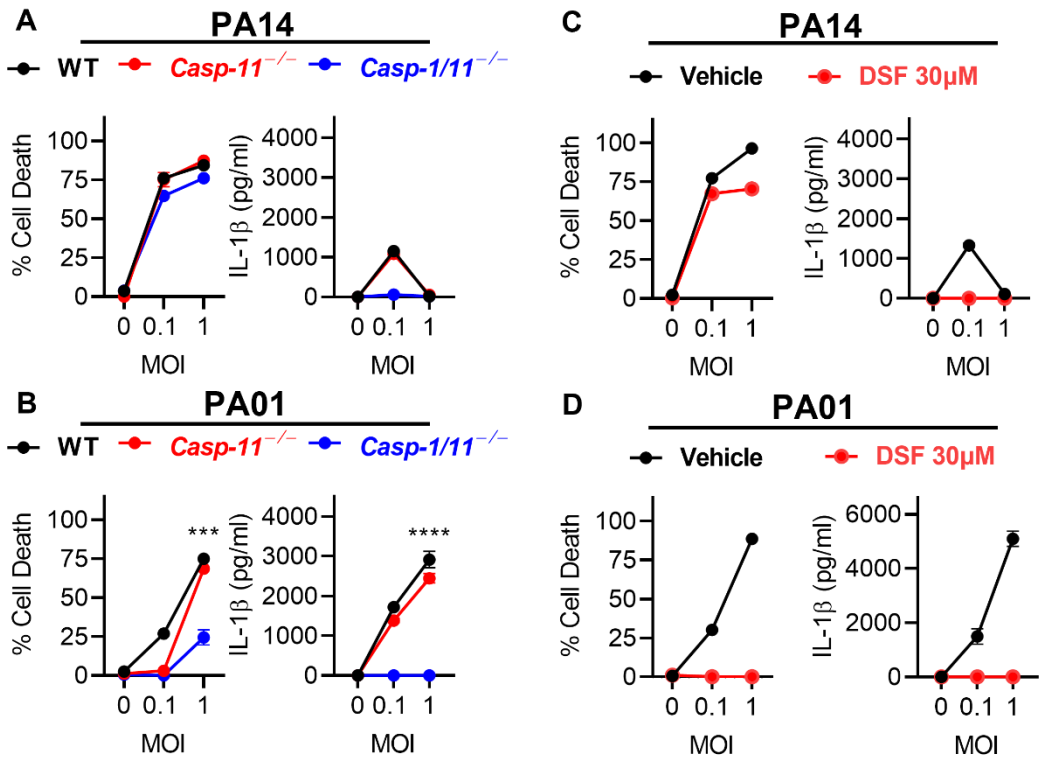


**Figure 7. PA14 induces inflammasome independent cell death.**

BMDMs were differentiated from C57BL/6 mice of wild-type, caspase-11$^{-/-}$, and caspase-1/11$^{-/-}$ backgrounds and infected with the indicated MOI of PA14 (**A**, **C**) or PAO1 (**B**, **D**). Where indicated, cells were pre-treated with 30μM DSF or vehicle (DMSO) for 1 hour prior to infection (**C**, **D**). At 3 hours (**A-D**) post-infection, cell death and IL-1β secretion were quantified by neutral red uptake and ELISA, respectively. Values represent mean ± SEM of triplicate cultures. Results are representative of three separate experiments. Mean values were compared by one-way ANOVA with post-hoc Tukey's multiple comparison test (**$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

inflammasome-independent cell death (**Figure 8 A, B**). Infection of NLRP3$^{-/-}$ BMDMs revealed that the NLRP3 inflammasome partially contributed to PAO1-induced IL-1β release, however, it did not significantly contribute to the induction of cell death (**Figure 8 B**). In contrast, NLRC4$^{-/-}$ BMDMs were significantly protected against PAO1-induced cell death and IL-1β release (**Figure 8 D**). Together, these findings demonstrate that *P. aeruginosa* strains may induce both inflammasome-dependent and -independent cell death and IL-1β secretion. For strains of *P. aeruginosa* that induce inflammasome signaling (e.g. PAO1), the NLRC4 and NLRP3 inflammasome platforms may both participate in the release of IL-1β whereas cell death is dependent on the NLRC4 inflammasome. The ability of *P. aeruginosa* to induce inflammasome-independent death was dependent on expression of the T3SS effector toxin ExoU.

### 4.1.2 The complete *P. aeruginosa* T3SS apparatus is necessary to induce cell death and IL-1β release

The T3SS is the primary virulence apparatus of *P. aeruginosa* and its expression is dependent on the transcriptional activator ExsA (137). PA14 mutants harbouring a transposon insertion in *exsA* failed to induce significant cell death or IL-1β release after infection, indicating that expression of the T3SS is necessary to induce cell death and IL-1β release (**Figure 9**). Since PA14 induces inflammasome independent cell death, these results indicate that T3SS expression is required for the induction of cell death. Furthermore, release of IL-1β occurs through

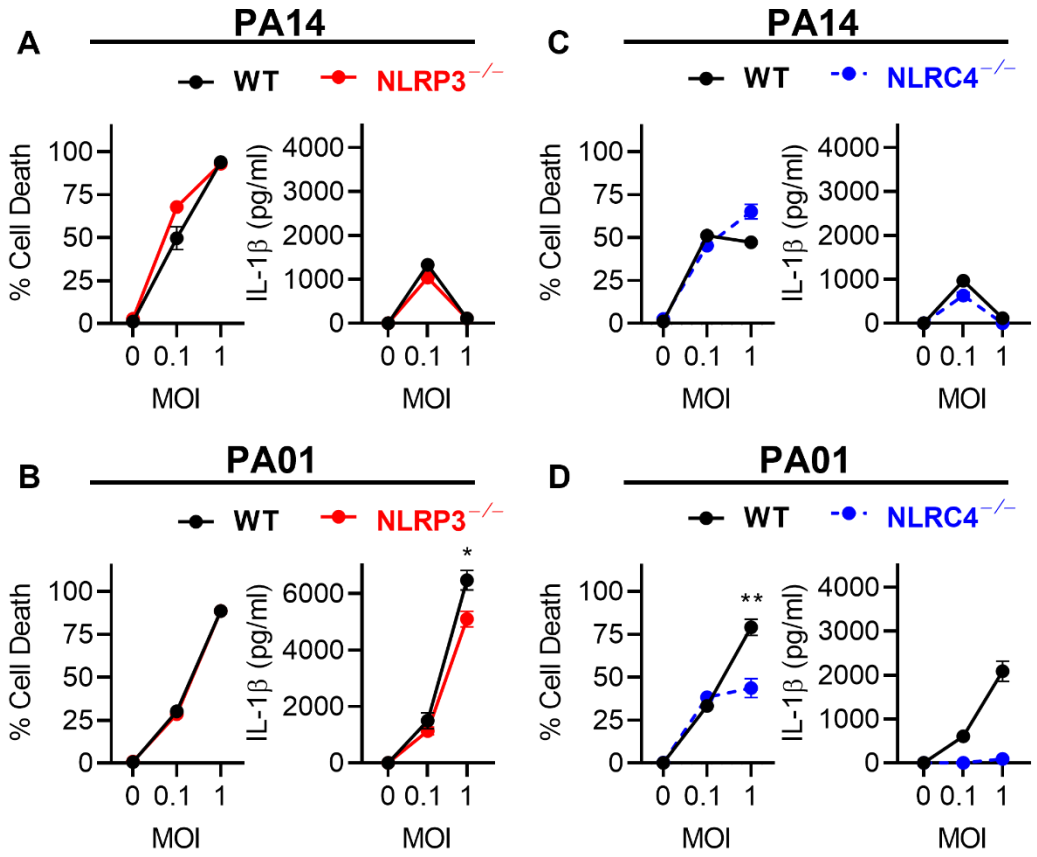


**Figure 8. *P. aeruginosa* induces NLRC4-dependent IL-1β secretion.**

BMDMs were differentiated from mice of wild-type, NLRP3$^{-/-}$, and NLRC4$^{-/-}$ backgrounds and infected with the indicated MOI of PA14 (**A**, **C**) or PAO1 (**B**, **D**) for 3 hours. Cell death and IL-1β secretion were quantified by neutral red uptake and ELISA, respectively. Values represent mean ± SEM of triplicate cultures. Results are representative of three separate experiments. Mean values were compared by Student's *t*-test (***P* < 0.01; ****P* < 0.001; *****P* < 0.0001).

inflammasome signaling and this was compromised with the ExsA mutant, which suggests that inflammasome signaling is also dependent on T3SS. Therefore, I sought to evaluate which specific components of the *P. aeruginosa* T3SS were essential towards inducing inflammasome activation.

I utilized PA14 mutants harbouring transposon insertions within T3SS genes to assess the impact of 18 individual T3SS components on host inflammasome activation (279). Infection of BMDMs confirmed that the induction of cell death by PA14 was not dependent on caspase-1 but required the expression of a functional T3SS (**Figure 10**). Mutations in genes encoding components of the ATPase complex (e.g. *pscL*), export apparatus (e.g. *pscT*), basal body (e.g. *pscJ*), or translocon (e.g. *popB*) resulted in complete abrogation of *Pseudomonas*-induced cell death after infection (**Figure 10 A**). Mutations in these genes likely impaired the sequential assembly of the T3SS apparatus, resulting in a T3SS-deficienct phenotype. Surprisingly, I observed that mutation of PscI, the T3SS inner-rod protein did not protect against *P. aeruginosa*-induced cell death. The rod and needle complex are an essential component of the T3SS injectisome, which suggests other structural proteins may compensate for the function of PscI in its absence. Mutations in genes encoding chaperones for exotoxin secretion (e.g. *spcU*) induced a reduced magnitude of cell death, however, these mutations did not fully rescue against *P. aeruginosa*-induced cell death. Mutations in genes encoding exotoxins (e.g. *exoT*) did not appear to impact *P. aeruginosa*-induced cell death, likely since multiple exotoxins may be simultaneously secreted. Inactivation of the highly potent ExoU toxin abrogated the ability of PA14 to induce caspase-1/11-independent cell death (**Figure 10 A, B**). In addition to caspase-1/11, other mediators of host

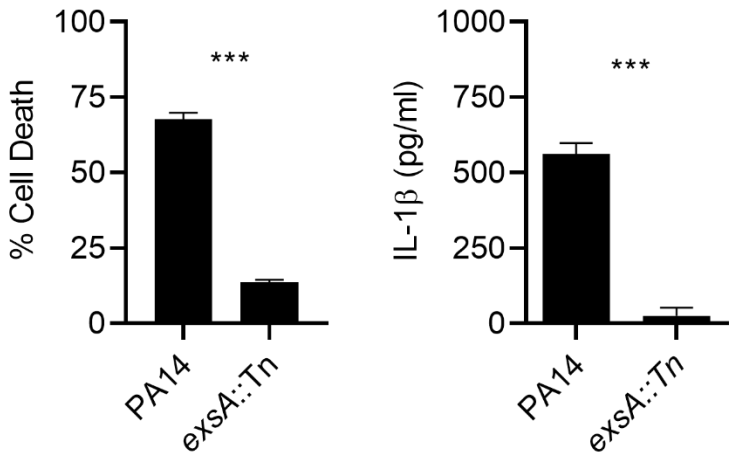


**Figure 9. Expression of the *P. aeruginosa* T3SS is necessary to induce inflammasome activation.**

Wild-type BMDMs were infected with PA14 or PA14 *exsA*::tn at 10 MOI for 3 hours. Cell death and IL-1β secretion were assessed by neutral red uptake and ELISA, respectively. Values represent mean ± SEM of triplicate cultures. Results are representative of three separate experiments. Mean values were compared by Student's *t*-test (***$P < 0.001$).

inflammatory cell death, such as RIPK3, were not observed to contribute towards PA14-induced cell death (**Figure 10 C, D**). These findings demonstrate that the assembly of a functional *P. aeruginosa* T3SS is necessary to induce inflammasome-independent cell death and release of inflammasome-dependent IL-1β. Secreted proteins such as chaperones and toxins are necessary to induce maximal cell death.

### 4.1.3 The majority of *P. aeruginosa* isolates from chronically infected cystic fibrosis patients display an impaired ability to induce cell death

I next sought to compare the extent of cell death induced by *P. aeruginosa* isolates from environmental, acute non-CF clinical, and chronic CF clinical sources. I tested *P. aeruginosa* isolates that were previously isolated from CF and non-CF sources and sequenced to facilitate the identification of the relationship between the observed phenotype and genotype (284).

Infection of wild-type BMDMs revealed distinct heterogeneity between the environmental, acute clinical, and CF chronic clinical isolates (**Figure 11 A, B**). *P. aeruginosa* isolates from environmental sources were observed to induce varying degrees of cell death and IL-1β secretion. The extent of cell death and IL-1β induced by the environmental isolates varied, indicating high heterogeneity within the isolate set. Isolates from acute non-CF clinical sources were observed to induce potent cell death and IL-1β secretion. Acute clinical isolates induced cell death and IL-1β comparable to PA14, a highly virulent *P. aeruginosa* strain (**Figure 11 A**). Overall, isolates from chronically infected cystic fibrosis patients did not induce appreciable cytotoxicity

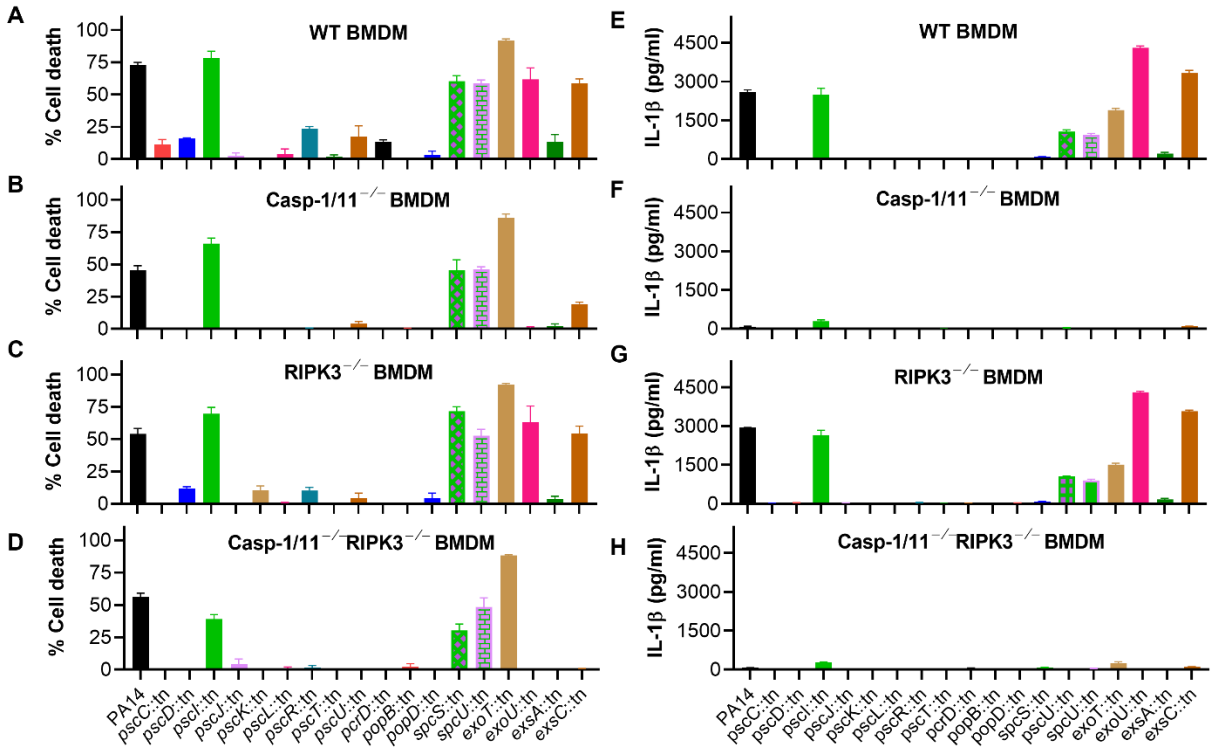


**Figure 10. Expression of the complete *P. aeruginosa* T3SS is necessary to induce inflammasome activation.**

BMDMs were differentiated from mice of C57BL/6 mice of wild-type (**A**), caspase-1/11$^{-/-}$ (**B**), RIPK3$^{-/-}$ (**C**), or caspase-1/11$^{-/-}$ RIPK3$^{-/-}$ (**D**) backgrounds and infected with 0.1 MOI of PA14 wild-type or the indicated PA14 transposon mutant. After 3 hours, cell death and IL-1β secretion were quantified by neutral red uptake and ELISA, respectively. Values represent mean ± SEM of triplicate cultures. Results are representative of three separate experiments.

and IL-1β release in comparison to environmental and acute clinical isolates (**Figure 11 A**). The majority (81%) of chronic CF clinical isolates did not induce cell death and half of the isolates did not induce significant IL-1β secretion, suggesting an impairment in their ability to activate inflammasome signaling (**Figure 11 C, D, I**). However, these isolates readily induced TNFα secretion, indicating that they were not capable of completely evading immune surveillance (**Figure 11 B, E, I**). Impairment in the induction of cell death and IL-1β by chronic CF isolates was not absolute as a significant proportion of chronic CF isolates retained their ability to induce cell death (19%) and IL-1β secretion (50%) (**Figure 11 F-H**). In contrast, most non-CF isolates assayed readily induced cell death (84%) and IL-1β secretion (72%) (**Figure 11 J**).

### 4.1.4 *Pseudomonas* isolates from chronically infected cystic fibrosis patients possess an increased frequency of genetic variation in virulence-related genes

I next sought to identify if there was a relationship between the *Pseudomonas* genotype and the previously observed cell death phenotypes (**Figure 11**). Since these isolates were sequenced, we performed bioinformatics analyses to identify genetic differences within CF isolates that may contribute to the reduced cell death observed. Bioinformatic analyses was performed in collaboration with Katie Noah and Dr. Alex Wong (Carleton University, Ottawa) using previously sequenced genomes (284). The sequenced genomes were aligned and annotated against UCBPP-PA14 or PAO1. Fisher's exact-test (FET) analysis was performed to

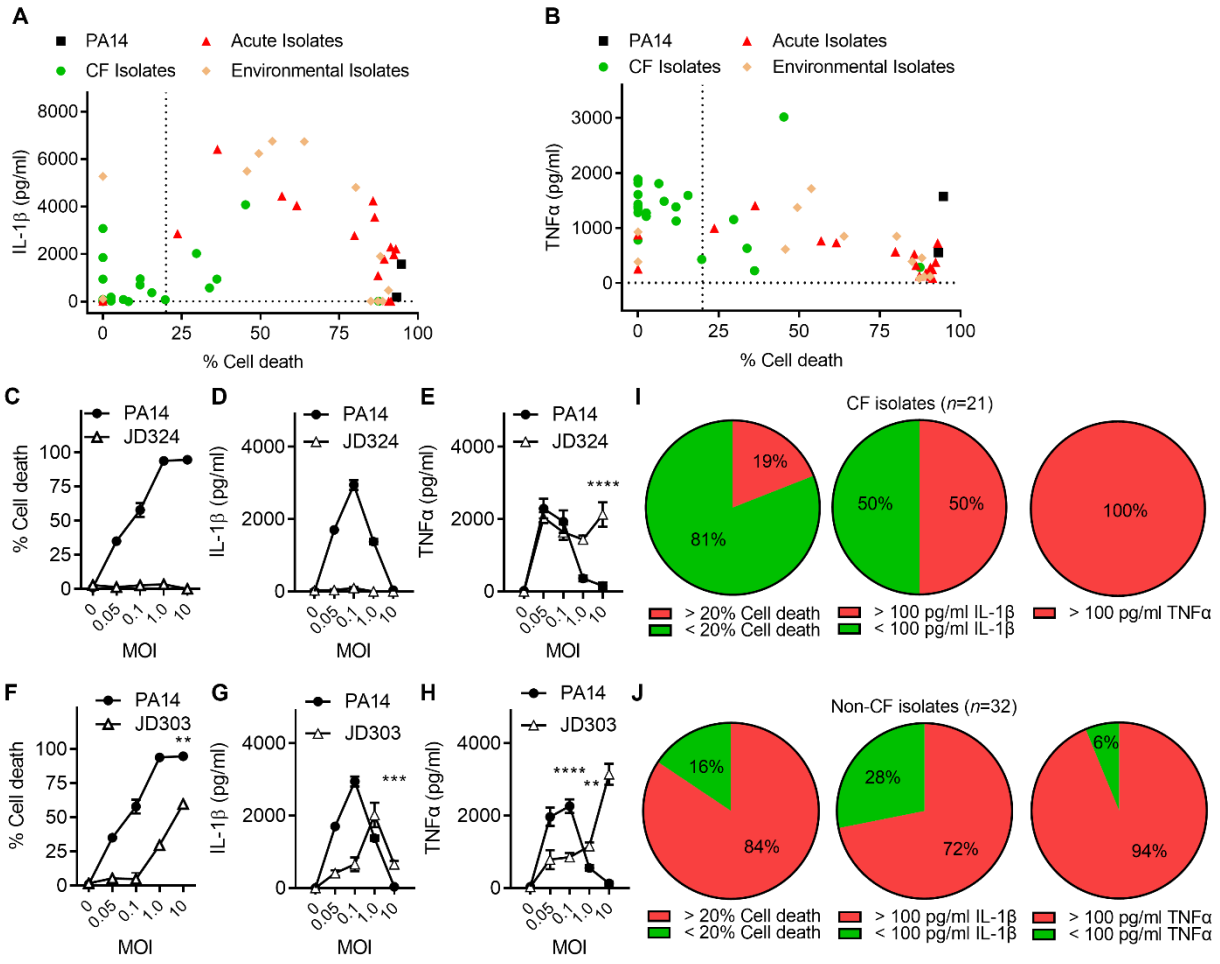


**Figure 11. *P. aeruginosa* isolates from chronically infected cystic fibrosis patients display reduced cytotoxicity.**

BMDMs were differentiated from wild-type C57BL/6J mice and infected with the indicated *P. aeruginosa* isolates from chronic CF clinical, acute non-CF clinical, or environmental sources. At 3 hours post-infection, cell death was quantified by neutral red uptake (**C**, **F**) and IL-1β (**D**, **G**) and TNFα secretion (**E**, **H**) were quantified by ELISA. Panels **C-E** are representative of chronic CF clinical isolates that do not induce cell death. Panels **F-H** are representative of chronic CF clinical isolates that retain their ability to induce cell death. Cell death was plotted against IL-1β (**A**) and TNFα (**B**) secretion at 1 MOI. The proportion of isolates capable of inducing > 20% cell death, > 100 pg/mL IL-1β or > 100 pg/mL TNFα at 1 MOI was quantified from CF (**I**) and non-CF (**J**) isolates. Values represent mean ± SEM of triplicate cultures. Results are representative of three separate experiments. Mean values were compared by two-way ANOVA with post-hoc Tukey's multiple comparison test (***P* < 0.01; ****P* < 0.001; *****P* < 0.0001).

identify significantly different genes between isolates from cystic fibrosis or non-cystic fibrosis origin (**Figure 12 A**).

Genes identified as being significantly different between the two groups ($p < 0.05$) were broadly categorized according to their association with motility, biosynthesis, antibiotic resistance and biofilm formation, T3SS, or T6SS. A complete table of genes examined is shown in **Table 3**. I observed a high frequency of genetic variation in genes associated with biofilm formation and antibiotic resistance (**Figure 12 B, C**). Genes associated with bacterial motility and biosynthesis possessed a lower frequency of variation. *P. aeruginosa* efficiently adapts to its environment and variation within these genes likely contribute to the pathogen's survival within the CF lung microbiome. Consistent with the cell death and IL-1β secretion results, I also observed an increased frequency of variation in T3SS-associated genes (**Figure 12 D**). Surprisingly,the highest frequency of variation occurred within T6SS-associated genes (**Figure 12 B, C**). All T6SS-associated variations were observed within PA14_44890 (*hcpA*). These findings demonstrate that *P. aeruginosa* isolates from chronically infected cystic fibrosis patients accrue significant genetic variations in virulence and survival-related genes, which contribute to the observed phenotype.

### 4.1.5 Modulation of cell death and IL-1β secretion by *P. aeruginosa* isolates during clinical exacerbations

The commonly accepted lab practice for isolating bacterial colonies is the selection of single colonies after overnight incubation on nutrient agar (296, 297). However, the selection of

a single colony may not be representative of the diversity within the whole bacterial population, especially in a highly heterogeneous environment such as the CF lung. Therefore, I evaluated a large collection of *P. aeruginosa* isolates from CF patient whole sputum to assess the relationship between isolate diversity, cell death, and IL-1β secretion. To determine whether the phenotype changes during exacerbation, I utilized sputum samples collected from the same patient during stable and exacerbation periods of disease. Sputum samples were collected as part of a previous study (285). Sputum samples were streaked on cetrimide agar to selectively isolate *Pseudomonas*. I tested 54 stable isolates and 135 exacerbation isolates using sputum samples from 3 CF patients (**Figure 13 A**). I infected wild-type BMDMs with these novel *P. aeruginosa* isolates to assess their ability to induce cell death and IL-1β secretion. *In vitro* infection of BMDMs revealed that the majority of *P. aeruginosa* isolates failed to induce significant cytotoxicity or IL-1β release after infection (**Figure 13 B**). Interestingly, I observed a significant difference between the ability of stable and exacerbation isolates to induce cell death (**Figure 13 C**). Only 6% of all stable isolates induced over 20% cell death and this increased to 15% within exacerbation isolates (**Figure 13 D**). Most notably, exacerbation isolates from patient 014 induced significant cell death in comparison to stable isolates (**Figure 13 E**). Exacerbation isolates from patients 003 and 009 did not induce a significant difference in cell death. I did not observe a difference in the overall ability of stable and exacerbation isolates to induce IL-1β following infection (**Figure 13 F**). In comparison to stable isolates, I observed that exacerbation isolates from patient 009 induced significantly higher levels of IL-1β secretion (**Figure 13 G**). Together, these findings demonstrate that the ability of *P. aeruginosa* to induce cytotoxicity and IL-1β secretion is elevated during cystic

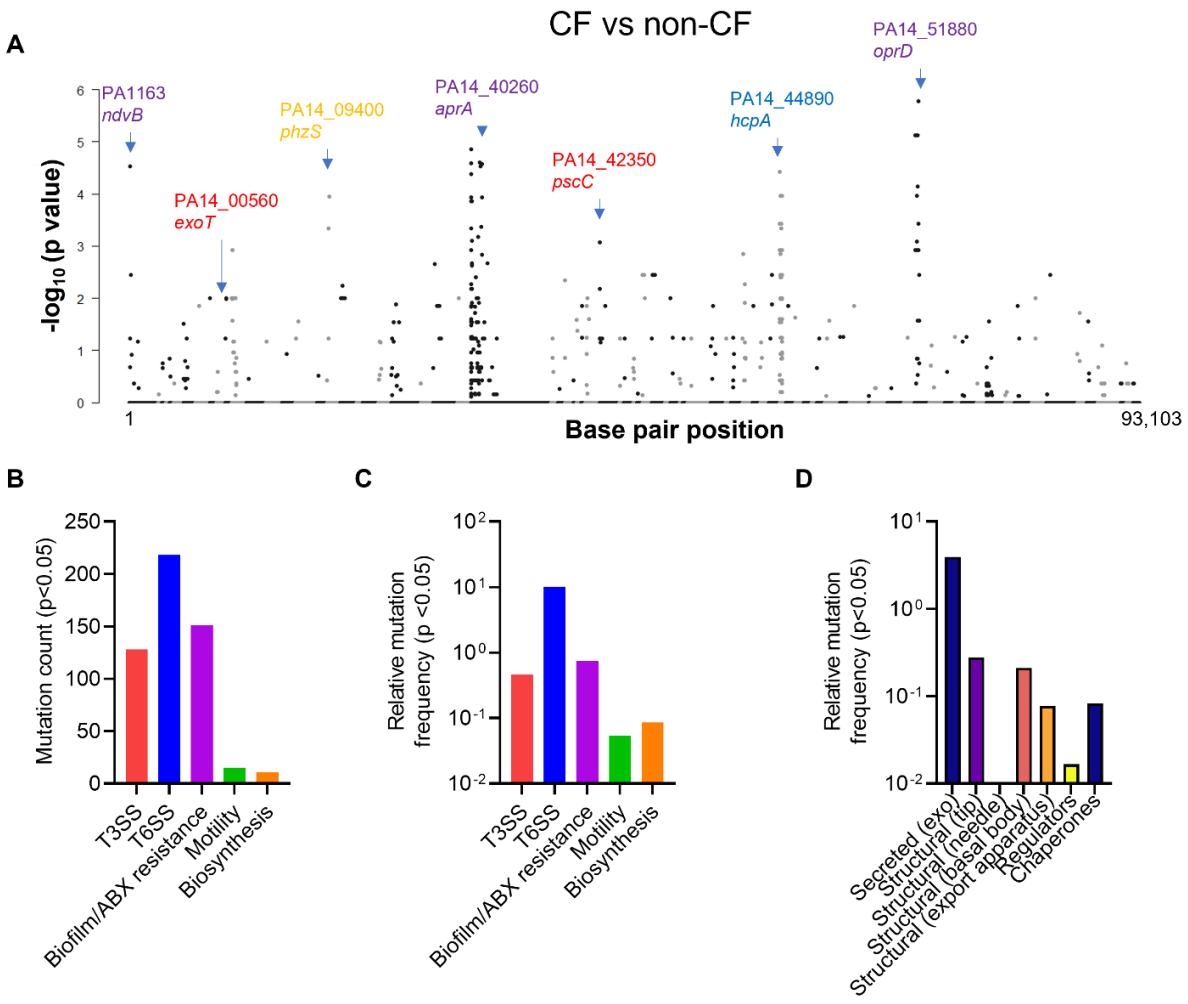


**Figure 12. *P. aeruginosa* isolates from chronically infected cystic fibrosis patients possess increased frequency of mutation in virulence-related genes.**

*P. aeruginosa* isolates from chronic CF clinical, acute non-CF clinical, or environmental sources were previously sequenced (284). Genomes were aligned and annotated against UCBPP-PA14 or PAO1. Annotated genomes of chronic CF clinical and non-CF sources (acute non-CF clinical and environmental) sources were compared by Fisher's exact test (FET) to identify statistically significant differences between the CF and non-CF groups. The results of Fisher's exact test are visualized as a Manhattan plot, depicting genomic position on the x-axis and -$\log_{10}$(*p*-value) on the y-axis (**A**). The absolute number of mutations and relative frequency of mutations within the indicated category with a *p*-value < 0.05 are depicted, respectively (**B, C**) The relative frequency of mutations with T3SS-associated genes with a *p*-value < 0.05 is depicted (**D**).

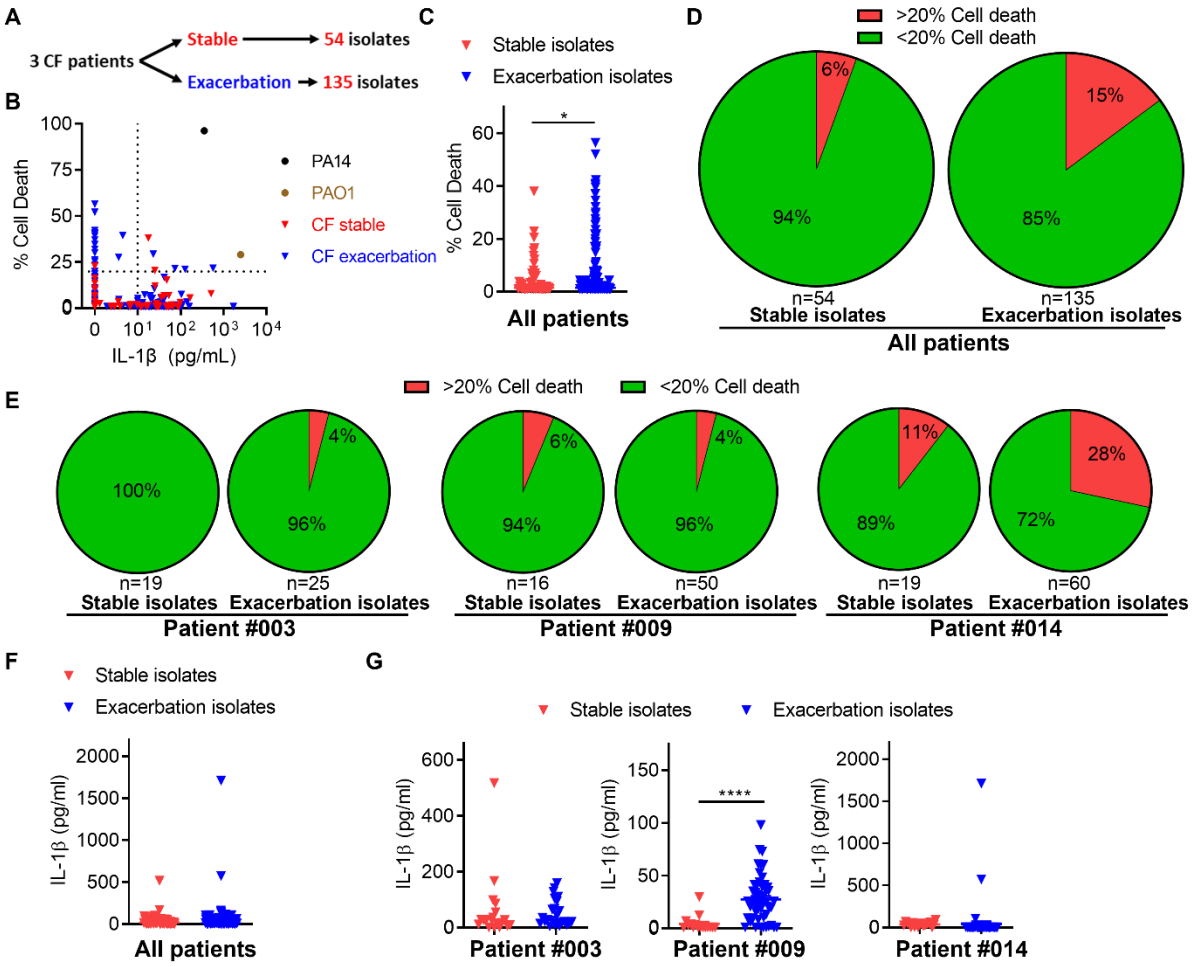


**Figure 13. *P. aeruginosa* isolates from cystic fibrosis pulmonary exacerbation sputum possess increased virulence.**

Sputum samples were collected from chronically infected cystic fibrosis patients during periods of stable disease and during pulmonary exacerbations. Sputum samples were cultured on cetrimide agar to select for *Pseudomonas aeruginosa* (**A**) and individual colonies were isolated and expanded in LB broth to create -80°C glycerol stocks which were utilized for the subsequent experiments. BMDMs were differentiated from wild-type C57BL/6J mice and infected with 10 MOI of PA14, PAO1, or the indicated *P. aeruginosa* isolates for 3 hours. Cell death was quantified by neutral red uptake (**B, C**) and IL-1β secretion was quantified by ELISA (**b, f, g**). The proportion of stable and exacerbation isolates capable of inducing >20% cell death was quantified within all isolates (**d**) or within the individual patients (**e**). Values represent mean ± SEM of triplicate cultures. Results are representative of three separate experiments. Mean values were compared by Welch's *t*-test (*$P < 0.05$; ****$P < 0.0001$).

fibrosis clinical exacerbations and this may be associated with increased host inflammation and poorer clinical outcomes.

### 4.1.6 *P. aeruginosa* T6SS inhibits host inflammasome signaling

I previously identified mutations in the T6SS component *hcpA* to significantly contribute to the chronic CF isolate phenotype (**Figure 12**). Thus, I next sought to evaluate if the T6SS may modulate host inflammasome signaling during infection. I elected to use *P. aeruginosa* transposon mutants on a PAO1 background to better appreciate the modulation of host inflammasome signaling observed since PAO1 induces inflammasome-dependent cell death and IL-1β secretion (**Figure 7**). HcpA oligomerizes to form the needle structure of the *P. aeruginosa* H2-T6SS, however, a transposon mutant of this protein was not available. Thus, I pursued alternate mutants deficient in H2-T6SS secretion to assess its contribution towards inflammasome activation. Strains PW3725 and PW3726 harbour a transposon insertion in PA1511, which encodes the VgrG2a tip protein of the H2-T6SS. Infection of wild-type BMDMs by PW3726 revealed that H2-T6SS deficiency significantly enhanced *P. aeruginosa*-induced cell death and IL-1β release, relative to wild-type PAO1 (**Figure 14 A, B**). This effect was observed at both 1 hour and 3 hours post-infection. Western blot analysis confirmed these findings. At both 1 hour and 3 hours post-infection, VgrG2a mutants elicited increased caspase-1 activation and increased gasdermin D cleavage in comparison to wild-type PAO1 (**Figure 14 C, D**). Together,

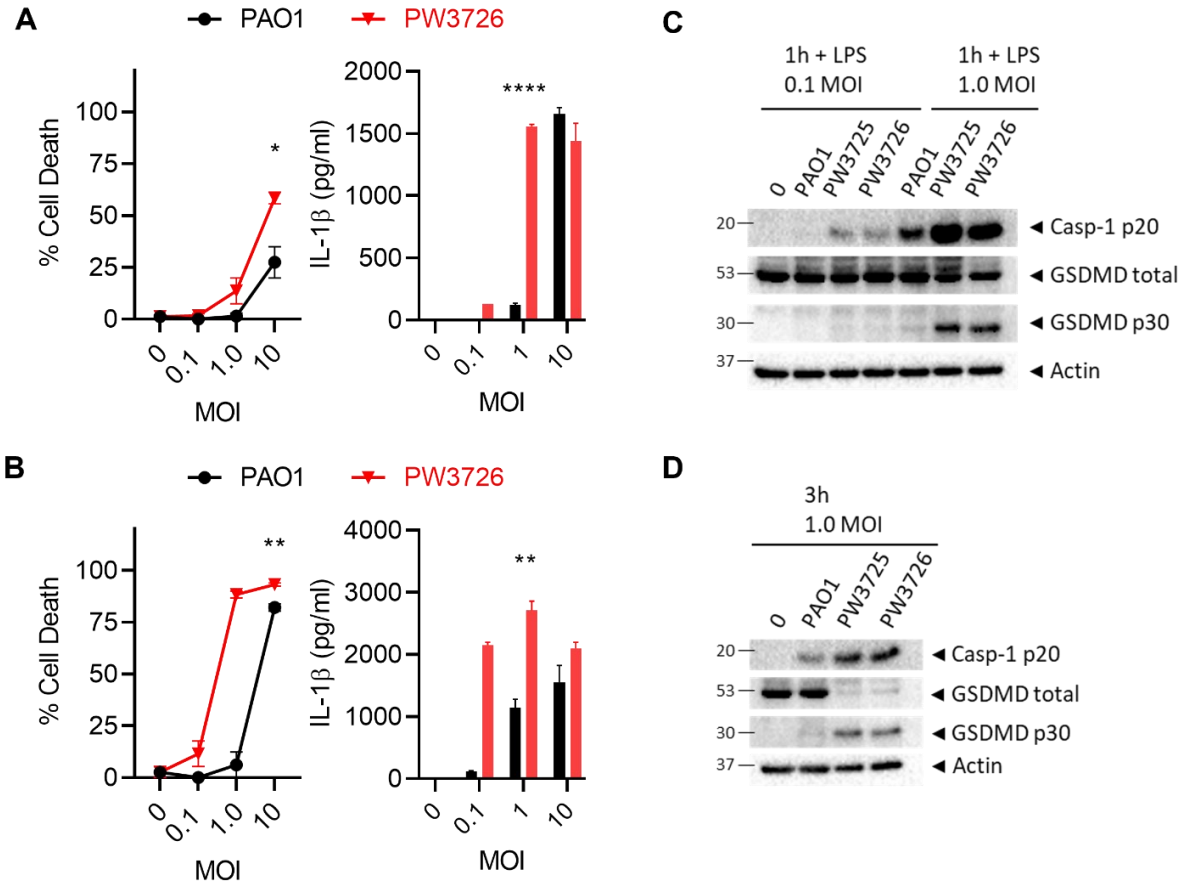


**Figure 14. *P. aeruginosa* T6SS inhibits inflammasome activation.**

BMDMs were differentiated from wild-type C57BL/6J mice and infected with wild-type PAO1, and PA01 with mutations in VgrG2a gene (PW3725 PA1151-H04::ISphoA/hah or PW3726 PA1151-B10::ISphoA/hah) for 1 hour (**A, C**) or 3 hours (**B, D**). Cells were pre-treated with 100 ng/mL LPS overnight (**A, C**). Cell death was quantified by neutral red uptake and IL-1β secretion was quantified by ELISA. Cell lysates were collected from infected cells and western blot analysis was performed to determine the relative expression of cleaved caspase-1, gasdermin D, and actin (**C, D**). Values represent mean ± SEM of triplicate cultures. Results are representative of three separate experiments. Mean values were compared by one-way Student's *t*-test (**P* < 0.05; ***P* < 0.01).

these results demonstrate that the *Pseudomonas* H2-T6SS can inhibit host inflammasome signaling during infection. Thus, it is conceivable that there will be an augmentation of inflammasome signaling in CF patients who possess an inactive *P. aeruginosa* T6SS.

### 4.1.7 Heterogeneous populations of *P. aeruginosa* induce differential inflammasome activation

Bacterial clinical isolates are typically generated by selecting single colonies after overnight culture, resulting in the expansion of a single clonal population (296, 297). To my surprise, I observed that some *P. aeruginosa* clinical isolates gave rise to multiple, morphologically unique colonies after extended culture (**Figure 15 A**). Thus, I sought to evaluate if these alternate small, slow-growing *P. aeruginosa* colonies possessed a differential ability to induce inflammasome activation in comparison to the larger, fast-growing colonies.

The appearance of morphologically unique colonies was observed in 5 of 21 chronic CF isolates, however, no environmental or acute non-CF isolates gave rise to multiple colony morphologies. Morphologically unique colonies were isolated after 48 hours of incubation at room temperature (**Figure 15 B, E**). Infection of wild-type BMDMs revealed that the small slow-growing colonies possessed a differential ability to induce inflammasome activation in comparison to the original isolate or the large fast-growing colonies. In comparison to the original isolate, some subisolates such as JD332-1 and JD332-2 induced increased IL-1β and cell death, respectively (**Figure 15 C, D**). In contrast, other subisolates such as JD333-2 induced less cell death

and higher IL-1β secretion, relative to the fast-growing isolate (**Figure 15 F, G**). These results demonstrate that *P. aeruginosa* isolates from chronically infected cystic fibrosis patients possess a high degree of heterogeneity and some bacterial sub-populations may retain their virulence and become reactivated to modulate host cytotoxicity and inflammation.

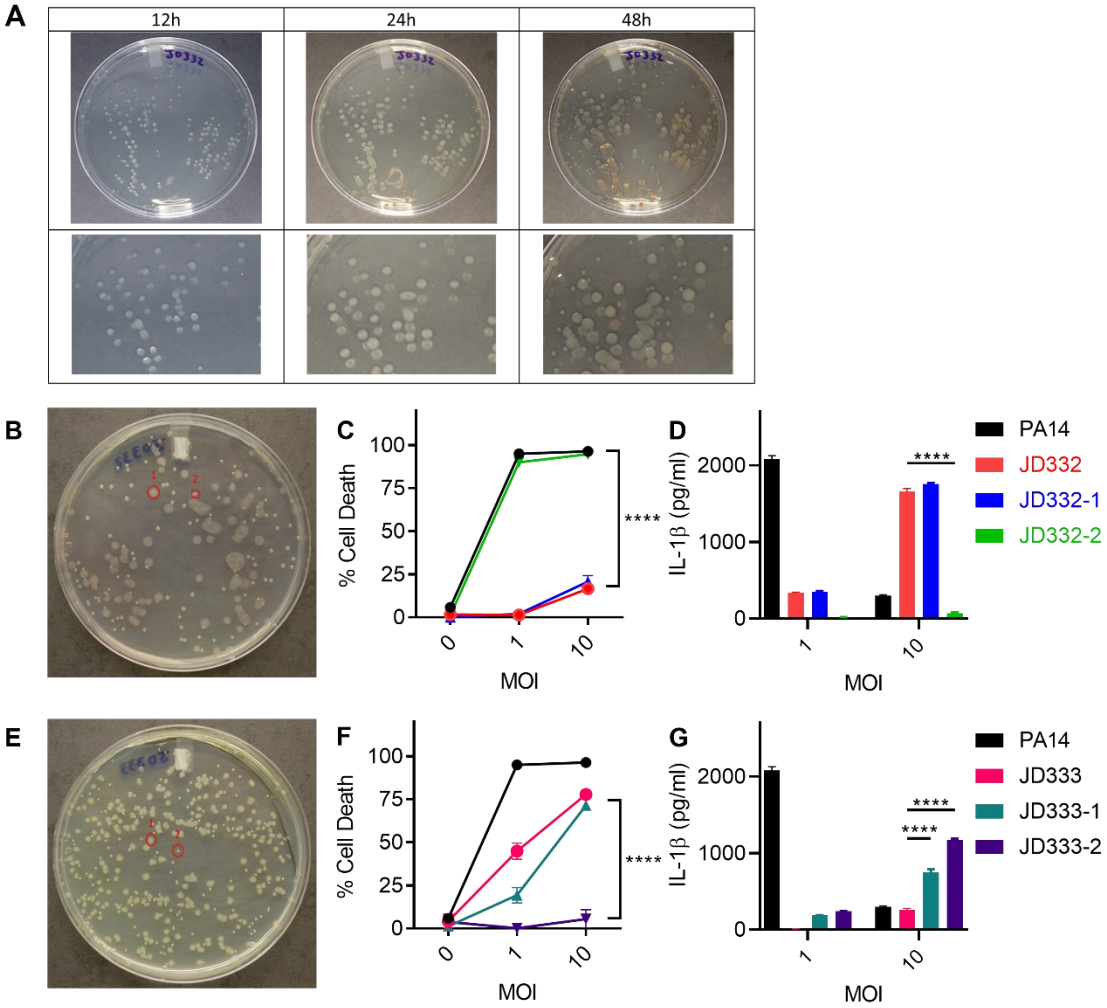


**Figure 15. Heterogeneous populations of *P. aeruginosa* induce differential inflammasome activation.**

*P. aeruginosa* clinical isolates were cultured for up to 48 hours and unique single colonies were selected according to colony morphology (**A, B, E**). Colonies were expanded in LB broth create -80°C glycerol stocks that were utilized for the subsequent experiments. BMDMs were differentiated from wild-type C57BL/6J mice and infected with PA14 or the indicated *P. aeruginosa* isolates for 3 hours. Cell death was quantified by neutral red uptake (**C, F**) and IL-1β secretion was quantified by ELISA (**D, G**). Values represent mean ± SEM of triplicate cultures. Results are representative of three separate experiments. Mean values were compared by one-way ANOVA with post-hoc Tukey's multiple comparison test (****$P < 0.0001$).

## 4.2 NLRP3 inflammasome signaling is dynamically modulated during chronic *Salmonella* Typhimurium infection of mice

### 4.2.1 *S.* Typhimurium induces inflammasome dependent cell death and IL-1β secretion

Murine models of chronic *P. aeruginosa* infection do not accurately represent the normal progression of disease (276, 277). Similar to *P. aeruginosa*¸ *S.* Typhimurium is also an opportunistic bacterial pathogen that induces acute infection which may transition to chronic disease in susceptible hosts. Both pathogens evade host immune clearance, resulting in chronic persistence. Thus, I utilized a murine *S.* Typhimurium chronic infection model to evaluate the modulation of cell death pathways throughout the course of a chronic infection *in vivo*.

Cell death of infected macrophages and the associated release of IL-1β functions as an important protective mechanism against intracellular pathogens. Therefore, I sought to evaluate the induction of inflammatory cell death and IL-1β release following *S.* Typhimurium infection. I infected wild-type BMDMs with *S.* Typhimurium SL-1344 (ST-WT), or *S.* Typhimurium strains deficient in SPI-I T3SS (ST-Δ*invA*), SPI-II T3SS (ST-Δ*ssaR*), or flagella (ST-Δ*fliF*) (**Figure 16**). I observed that the induction of inflammasome dependent cell death required the expression of the SPI-I T3SS and flagellin (**Figure 16 A**). SPI-I T3SS and flagella-deficient strains failed to induce remarkable cell death or IL-1β secretion following infection (**Figure 16 B, C**). Expression of the SPI-II T3SS was not necessary to induce cell death and IL-1β release, however, its activity contributed to maximal inflammasome activation (**Figure 16 D**). In accordance with these results,

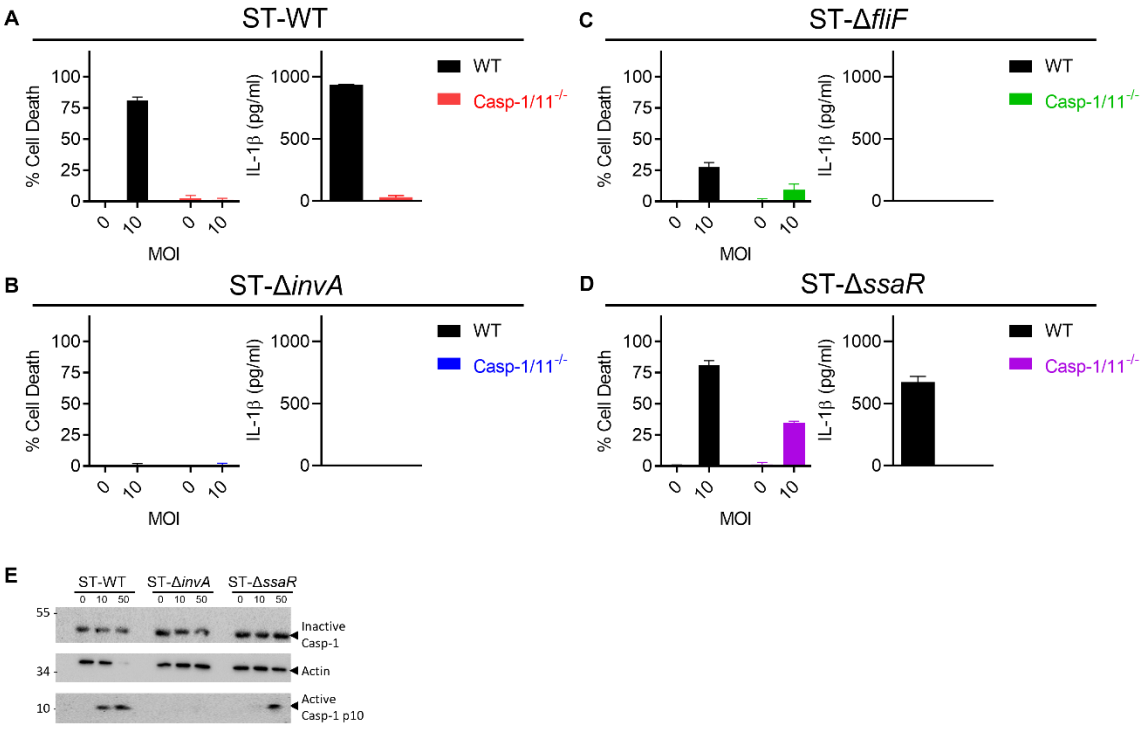


**Figure 16. *S.* Typhimurium-induced inflammasome signaling is dependent on SPI-I and flagella**

BMDMs were differentiated from wild-type C57BL/6J mice and infected with ST-WT (**A**), ST-Δ*invA* (**B**), ST-Δ*fliF* (**C**), or ST-Δ*ssaR* (**D**) for 3 hours. Cell death was quantified by neutral red uptake and IL-1β secretion at 10 MOI was quantified by ELISA. Caspase-1 processing (**E**) was assessed by Western blotting. Values represent mean ± SEM of triplicate cultures. Results are representative of three separate experiments. Mean values were compared by one-way ANOVA with post-hoc Tukey's multiple comparison test (**P* < 0.05; ****P* < 0.001).

*Cai et al. (2021). Isolates of Salmonella* Typhimurium *circumvent NLRP3 inflammasome recognition in macrophages during the chronic phase of infection. J. Biol. Chem. 298:1 doi:10.1016/j.jbc.2021.101461.*

cleaved caspase-1 was detected in WT BMDMs in response to both ST-WT and ST-Δ*ssaR* infection, but not in response to ST-Δ*invA* (**Figure 16 E**). These results demonstrate that the SPI-I T3SS, SPI-II T3SS, and flagella contribute towards *S.* Typhimurium-induced inflammasome activation.

The expression of bacterial virulence factors is dependent on bacterial growth phase. Logarithmic phase cultures of *Salmonella* express virulence factors such as SPI-I and flagella whereas stationary phase *Salmonella* cultures express SPI-II and downregulate flagellar expression (298). Thus, logarithmic and stationary phase cultures of *Salmonella* mimic the SPI-I and SPI-II-inducing conditions observed during the early and late stages of *Salmonella* infection (24, 299). I next evaluated the contributions of the NLRC4 and NLRP3 inflammasomes during the early and late stages of *S.* Typhimurium infection using representative logarithmic and stationary phase cultures (**Figure 17**). Logarithmic phase *S.* Typhimurium primarily induced NLRC4-dependent cell death (**Figure 17 A**). NLRP3 was observed to contribute towards cell death, however, its ablation did not completely protect cells against logarithmic phase *S.* Typhimurium (**Figure 17 C**). In contrast, IL-1β secretion was dependent on both NLRC4 and NLRP3. Both NLRC4$^{-/-}$ and NLRP3$^{-/-}$ BMDMs exhibited reduced IL-1β secretion in response to logarithmic phase *S.* Typhimurium (**Figure 17 A, C**). Stationary phase cultures displayed an increased dependence on the NLRP3 inflammasome. I observed that NLRC4$^{-/-}$ BMDMs underwent significant cell death in response to infection by stationary phase *S.* Typhimurium whereas NLRP3$^{-/-}$ BMDMs were significantly protected (**Figure 17 B, D**). Stationary phase *S.* Typhimurium induced IL-1β secretion in NLRC4$^{-/-}$, but not NLRP3$^{-/-}$ BMDMs. Furthermore, I observed activation of the NLRP3

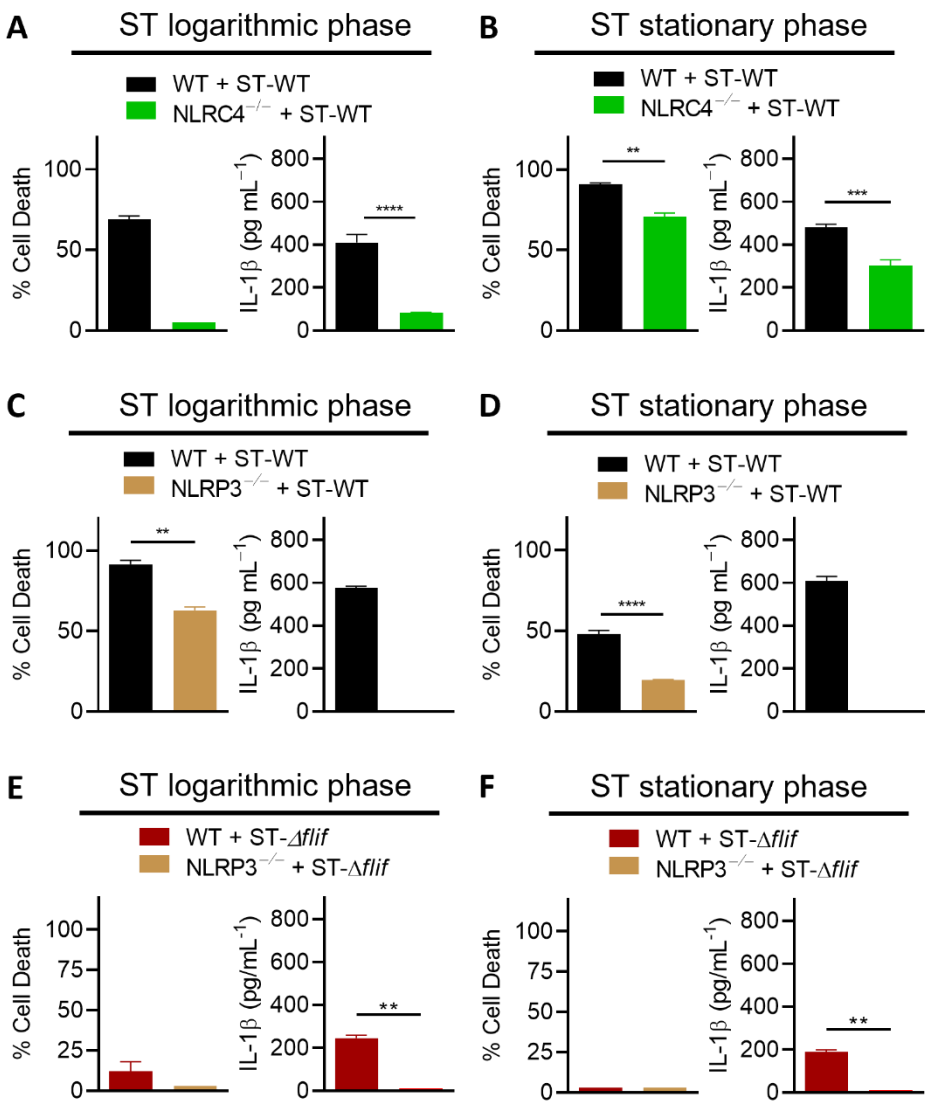


**Figure 17. *S.* Typhimurium induces NLRC4 and NLRP3-dependent inflammasome activation.**

BMDMs were differentiated from mice of the indicated genotypes and infected with 0.1 or 50 MOI of ST-WT cultured to logarithmic (**A, C, E**) or stationary (**B, D, F**) phases of growth for 3 hours or 24 hours, respectively. Cell death and IL-1β were quantified by neutral red uptake and ELISA. Values represent mean ± SEM. Mean values were compared by Student's *t*-test (**P < 0.01; ***P < 0.001; ****P < 0.0001). BMDMs in panels **A-D** were generated from 2 mice from each genotype. *Cai et al. (2021). Isolates of Salmonella* Typhimurium *circumvent NLRP3 inflammasome recognition in macrophages during the chronic phase of infection. J. Biol. Chem. 298:1 doi:10.1016/j.jbc.2021.101461.*

inflammasome by logarithmic and stationary phase *S.* Typhimurium to be dependent on the expression of flagella (**Figure 17 E, F**). Together, these findings demonstrate that the *S.* Typhimurium SPI-I T3SS and flagella contribute towards NLRC4 and NLRP3 inflammasome activation during infection.

### 4.2.2 *S.* Typhimurium-induced inflammasome signaling is dynamically modulated over the course of a chronic infection

I next sought to evaluate how inflammasome signaling is modulated over the course of a chronic *S.* Typhimurium infection. C57BL/6J mice carry an inactivating mutation in the *Nramp1* gene and hence rapidly succumb to *S.* Typhimurium infection; however, 129X1/SvJ mice expresses a functional Nramp1 protein and are permissive of chronic *S.* Typhimurium infection (205, 208). Thus, I utilized the 129X1/SvJ murine chronic infection model to evaluate if *S.* Typhimurium-induced inflammasome signaling is modulated over the course of a chronic infection.

129X1/SvJ mice were infected with *S.* Typhimurium and mice were sacrificed at 7-, 21-, and 120- days post-infection. *S.* Typhimurium was isolated from the spleen, gallbladder, mesenteric lymph nodes, and inguinal lymph nodes of infected mice and utilized to infect murine BMDMs *in vitro* to assess the ability of the chronic isolates to induce cell death and IL-1β production. I observed that *S.* Typhimurium-induced inflammatory cell death of BMDMs was dynamically modulated over the course of the infection (**Figure 18**). During the early stages of

infection (7 days post-infection), *S.* Typhimurium isolates induced increased cell death, relative to the original *S.* Typhimurium inoculum. This early enhancement was most apparent in isolates from the spleen and mesenteric lymph nodes (**Figure 18 A, C**). At the peak of infection (21 days post-infection), I observed a two-fold increase in the ability of isolates from all organs to induce cell death (**Figure 18 A-D**). During the late chronic stage of infection (120 days post-infection), I observed that isolates from all organs possessed a reduced ability to induce cell death, relative to the original inoculum. Isolates from the mesenteric and inguinal lymph nodes displayed a two to threefold reduction in their ability to induce cell death (**Figure 18 C, D**). Calculating the LD50 for cell death of infected macrophages revealed that during the early stages of infection, a reduced number of bacteria were required to induce 50% cell death of macrophages relative to the initial inoculum, whereas at the late chronic stage, an increased number of bacteria were required to induce 50% cell death (**Figure 18 E, F**). These results demonstrate that the ability of *S.* Typhimurium to induce host cell death is dynamically modulated over the course of a chronic infection.

### 4.2.3 Chronic *S.* Typhimurium isolates induce impaired inflammasome activation

I previously observed that the induction of macrophage cell death and the associated IL-1β release following *S.* Typhimurium infection was dependent on caspase-1/11 (**Figure 16**). I next sought to assess if the modulation of host cell death observed in **Figure 18** was dependent on changes in *S.* Typhimurium-induced inflammasome activation. I infected wild-type and caspase-

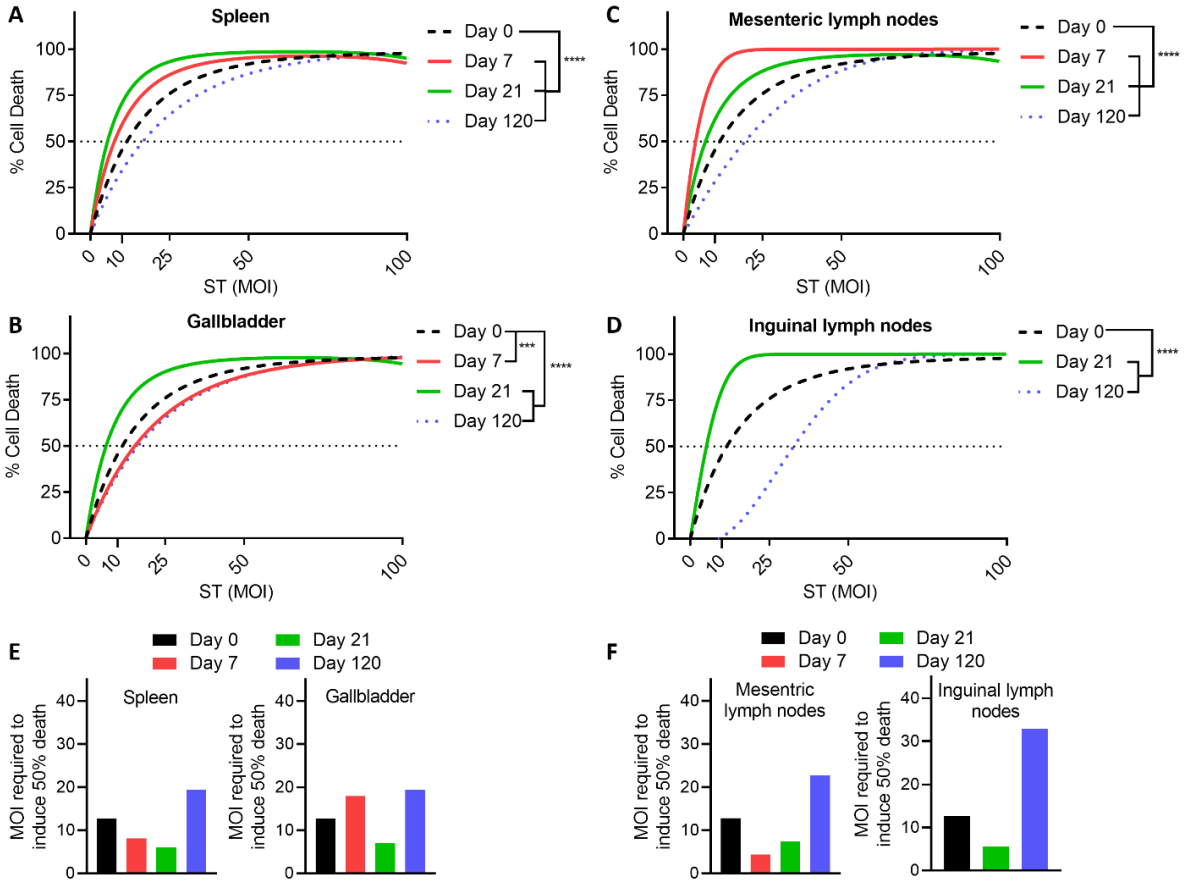
A
Spleen
% Cell Death
ST (MOI)
Day 0
Day 7
Day 21
Day 120
****
B
Gallbladder
***
****
C
Mesenteric lymph nodes
D
Inguinal lymph nodes
E
Spleen
Gallbladder
MOI required to induce 50% death
F
Mesentric lymph nodes
Inguinal lymph nodes

**Figure 18. Inflammasome signaling is dynamically modulated during chronic *S.* Typhimurium infection.**

129X1/SvJ mice were infected intravenously with $4x10^4$ CFU ST-WT and the mice were sacrificed at 7-, 21-, and 120-days post-infection. The spleen, gallbladder, mesenteric lymph nodes, and inguinal lymph nodes were excised, and serial dilutions were plated on LB agar to isolate single ST colonies. The ST isolates and ST-WT were expanded in LB broth and subsequently utilized to infect BMMs *in vitro* for 3 hours. Cell death post-infection (**A-D**) was determined by neutral red uptake assay and is depicted using a linear quadratic survival curve fit. The MOI necessary to induce 50% cell death (**E, F**) was determined by $LD_{50}$ regression in GraphPad Prism 9. Results represent the mean of 9 mice at day 0, 18 mice at day 7 and 21, and 12 mice at day 120. Experiments were repeated three times. Mean values were compared by one-way ANOVA (**A**-**D**) with post-hoc Tukey's multiple comparison test (***$P < 0.001$; ****$P < 0.0001$).
*Cai et al. (2021). Isolates of Salmonella* Typhimurium *circumvent NLRP3 inflammasome recognition in macrophages during the chronic phase of infection. J. Biol. Chem. 298:1 doi:10.1016/j.jbc.2021.101461.*

1/11$^{-/-}$ BMDMs with *S.* Typhimurium isolates from the spleen and gallbladder (**Figure 19**). I observed that all chronic isolates induced reduced IL-1β secretion relative to the original *S.* Typhimurium inoculum (**Figure 19 A, B, D, E**). The release of IL-1β was dependent on the activity of caspase-1/11 (**Figure 19 C, F**). Interestingly, I observed that the ability of chronic isolates to induce caspase-1/11-dependent death progressively declined over time (**Figure 19 G-I**). Together, these results show that *S.* Typhimurium modulates its ability to induce inflammasome activation over the course of a chronic infection.

### 4.2.4 Chronic *S.* Typhimurium isolates induce inflammasome-independent cell death

*Salmonella* may induce apoptotic and necroptotic cell death during infection (186, 267). Since I observed that chronic *S.* Typhimurium isolates may induce inflammasome-independent cell death, I next assessed if alternate cell death pathways were activated (**Figure 20**). In accordance with my previous results, chronic *S.* Typhimurium isolates induced reduced activation of caspase-1 and gasdermin D within infected BMDMs (**Figure 20 B-E**). I did not observe any significant activation of apoptotic or necroptotic cell death pathways in infected wild-type BMDMs (**Figure 20 F-I**). Interestingly, I observed that these pathways were only activated in the absence of caspase-1/11. In caspase-1/11$^{-/-}$ BMDMs, chronic *S.* Typhimurium isolates induced increased caspase-8 activation and increased MLKL phosphorylation, indicating activation of apoptotic and necroptotic cell death pathways, respectively (**Figure 20 G, I**). Thus, the processing of caspase-1/11 precludes the activation of other cell death pathways.

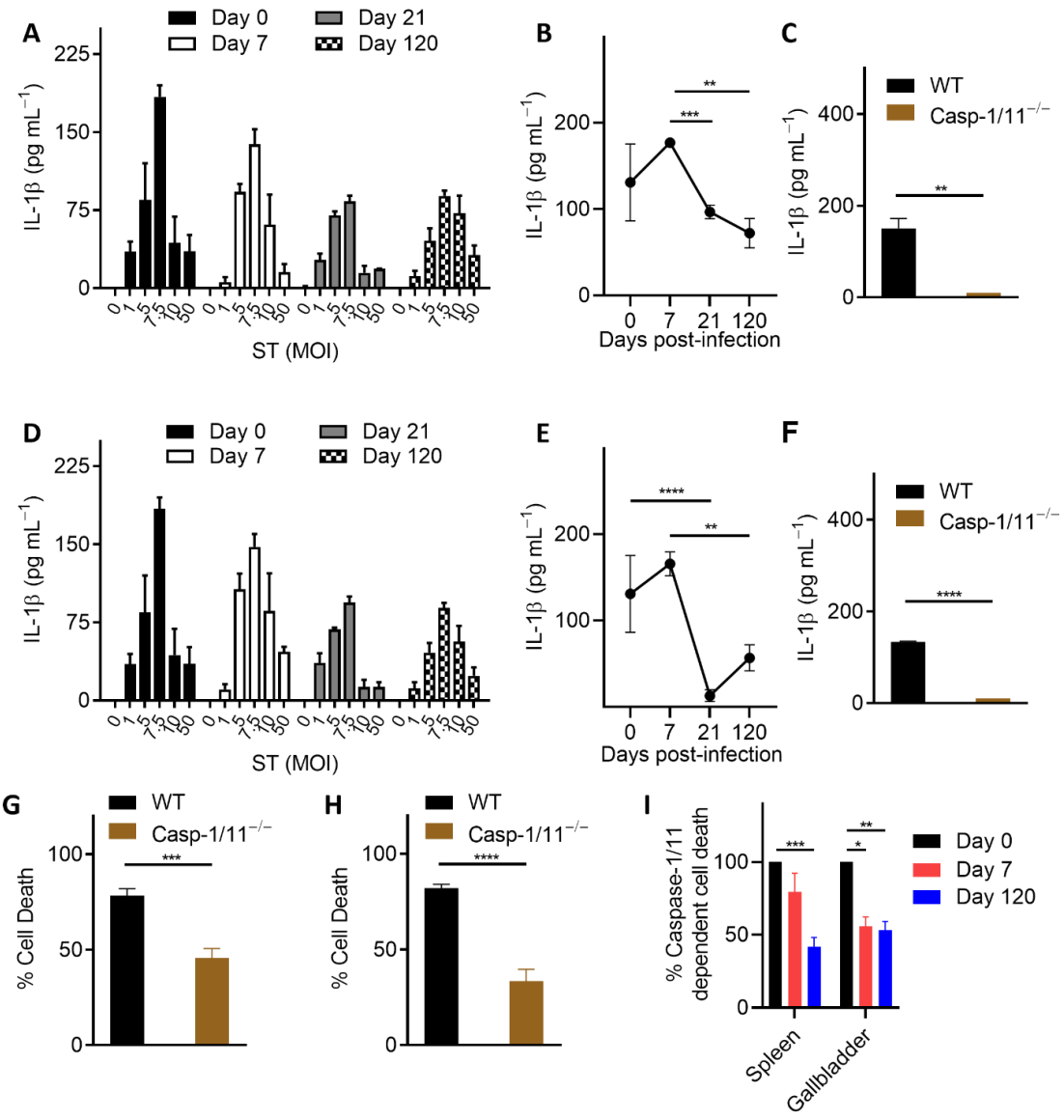
A
Day 0
Day 7
Day 21
Day 120
IL-1β (pg mL−1)
225
150
75
0
0 1 5 7.5 10 50
ST (MOI)
B
IL-1β (pg mL−1)
200
100
0
**
***
0 7 21 120
Days post-infection
C
WT
Casp-1/11−/−
IL-1β (pg mL−1)
400
200
0
**
D
Day 0
Day 7
Day 21
Day 120
IL-1β (pg mL−1)
225
150
75
0
0 1 5 7.5 10 50
ST (MOI)
E
IL-1β (pg mL−1)
200
100
****
**
0 7 21 120
Days post-infection
F
WT
Casp-1/11−/−
IL-1β (pg mL−1)
400
200
0
****
G
WT
Casp-1/11−/−
% Cell Death
100
50
0
***
H
WT
Casp-1/11−/−
% Cell Death
100
50
0
****
I
% Caspase-1/11 dependent cell death
100
50
0
***
**
*
Day 0
Day 7
Day 120
Spleen
Gallbladder

**Figure 19. Chronic *S.* Typhimurium isolates induce reduced inflammasome activation.**

Wild-type BMDMs were infected for 3 hours with ST-WT or isolates from the spleen (**A-C, G, I**) or gallbladder (**D-F, H, I**) and IL-1β secretion was quantified by ELISA. Panels **C** and **G** depict the day 120 spleen isolate and panels **F** and **H** depict the day 120 gallbladder isolate. Values represent mean ± SEM. Results in panels **A** and **C** represent data pooled from five separate experiments. Results in panels **E** and **H** are representative of three separate experiments at 1 MOI. The number of mice used at each time point was the same as mentioned in Figure 18. Mean values were compared by Student's *t*-test (***P* < 0.01; ****P* < 0.001; *****P* < 0.0001).

*Cai et al. (2021). Isolates of Salmonella* Typhimurium *circumvent NLRP3 inflammasome recognition in macrophages during the chronic phase of infection. J. Biol. Chem. 298:1 doi:10.1016/j.jbc.2021.101461.*

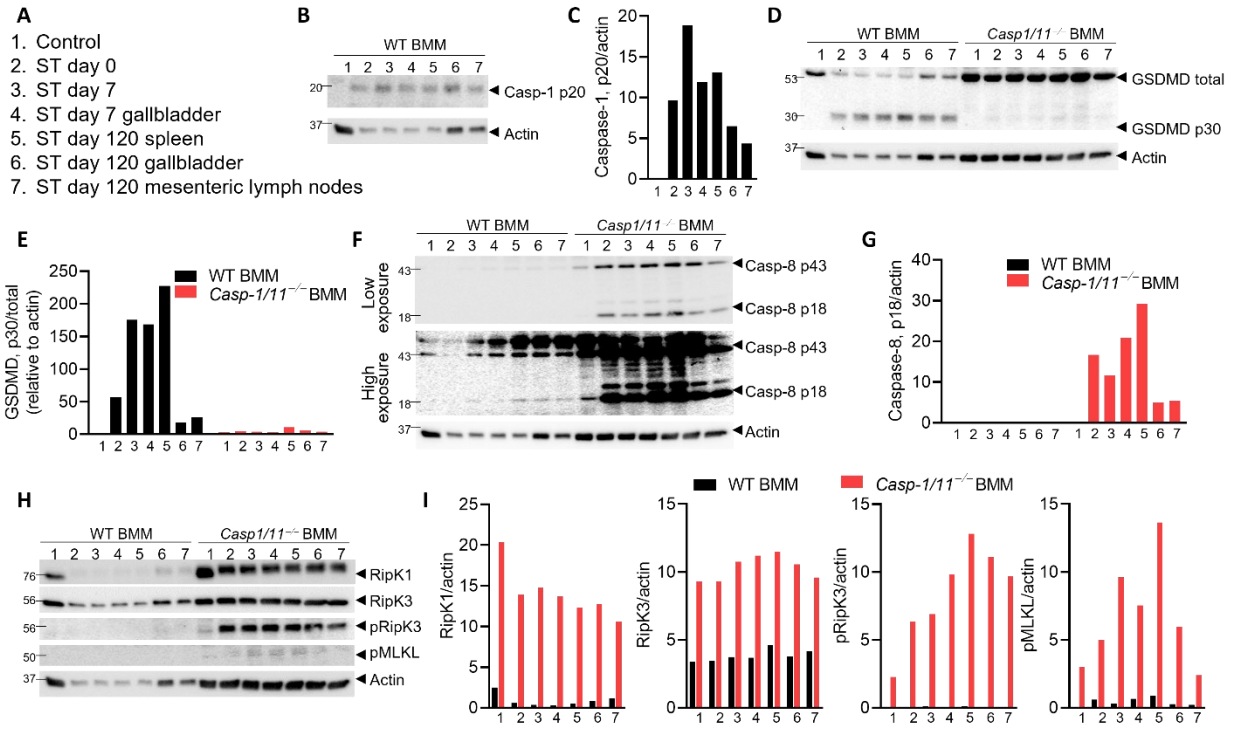


**Figure 20. Modulation of inflammasome signaling following infection with chronic *S.* Typhimurium isolates.**

BMDMs were generated from wild-type or caspase-1/11$^{-/-}$ C57BL/6 mice and infected with 10 MOI of the indicated bacterial isolates (**A**). Cell lysates were collected after 1.5 hours and Western blot analysis was performed to determine the expression of cleaved caspase-1, gasdermin D, cleaved caspase-8, RipK1, RipK3, pRipK3, pMLKL, and actin (**B**, **D**, **F**, **H**). Densitometric analysis was performed in Fiji to determine protein expression relative to actin (**C**, **E**, **G**, **I**).

*Cai et al. (2021). Isolates of Salmonella* Typhimurium *circumvent NLRP3 inflammasome recognition in macrophages during the chronic phase of infection. J. Biol. Chem. 298:1 doi:10.1016/j.jbc.2021.101461.*

### 4.2.5 Modulation of inflammasome signaling is organ-specific and NLRP3-dependent

As described earlier, my results indicate that the NLRC4 and NLRP3 inflammasomes are activated in response to *S.* Typhimurium infection, which mediate cell death and IL-1β release (**Figure 17**). I next sought to assess if the modulation in inflammasome signaling during the chronic phase of infection targeted the NLRC4 or NLRP3 inflammasomes (**Figure 21)**. Infection of NLRC4$^{-/-}$ and NLRP3$^{-/-}$ BMDMs revealed that chronic isolates displayed an impairment in their ability to induce NLRP3-dependent inflammasome signaling (**Figure 21 A**). This modulation was best appreciated by calculating the dependence on NLRC4 and NLRP3, relative to wild-type BMDMs (**Figure 21 B**). The ability of chronic isolates to induce NLRP3-dependent death progressively declined over time whereas NLRC4-dependent cell death remained unchanged. These findings demonstrate that NLRP3-mediated inflammasome activation is modulated during chronic *S.* Typhimurium infection.

### 4.2.6 *S.* Typhimurium T3SS expression is modulated during chronic infection

The SPI-I T3SS of *Salmonella* induces inflammasome activation resulting in cell death and IL-1β secretion whereas the SPI-II T3SS is necessary for intracellular proliferation (**Figure 16**). I next assessed if the expression of SPI-I and SPI-II T3SSs were modulated in chronic *S.* Typhimurium isolates. I performed qRT-PCR for various SPI-I and SPI-II T3SS associated genes (**Figure 22**). I observed that the expression of both SPI-I and SPI-II T3SS associated genes was significantly downregulated in chronic isolates (**Figure 22 A, B**). The progressive decline in SPI-I

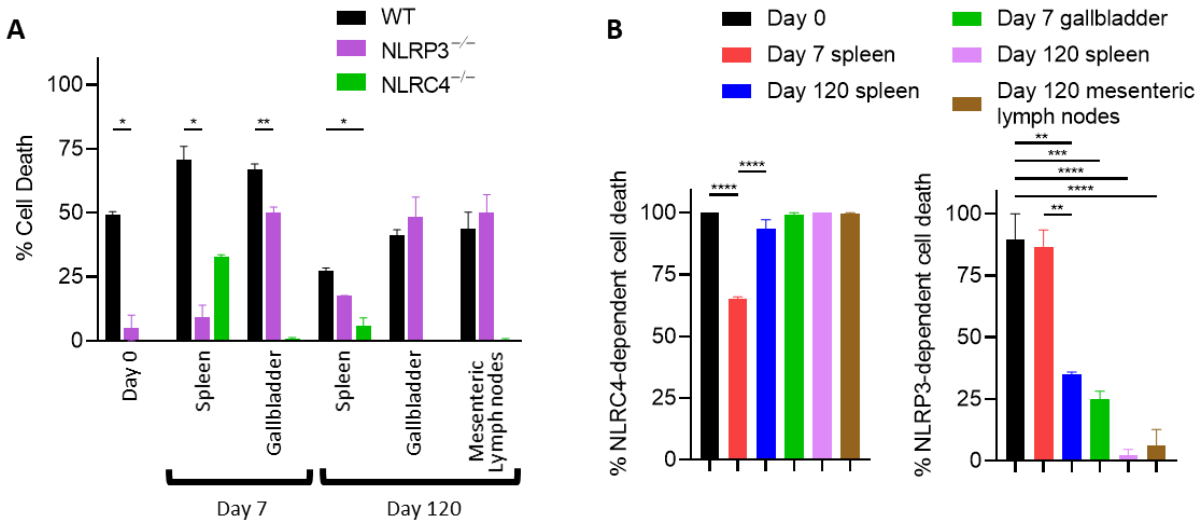


**Figure 21. Chronic *S.* Typhimurium isolates exhibit NLRP3-dependent, organ-specific modulation of inflammasome signaling.**

BMDMs were differentiated from mice of the indicated genotypes and infected with 1 MOI of ST-WT or the chronic ST isolates for 3 hours (**A**). Cell death was assessed by neutral red uptake assay. The percentage of NLRC4 or NLRP3-dependent cell death relative to wild-type was calculated (**B**). Values represent mean ± SEM of triplicate cultures. Results are representative of three separate experiments with BMDMs generated from two mice of each genotype. Mean values were compared by one-way ANOVA with post-hoc Tukey's multiple comparison test (**P* < 0.05; ***P* < 0.01; ****P* < 0.001; *****P* < 0.0001).

*Cai et al. (2021). Isolates of Salmonella* Typhimurium *circumvent NLRP3 inflammasome recognition in macrophages during the chronic phase of infection. J. Biol. Chem. 298:1 doi:10.1016/j.jbc.2021.101461.*

and SPI-II T3SS expression correlated with the decline in NLRP3-dependent cell death, but not NLRC4-dependent death (**Figure 22 C, D**). These results demonstrate that *S.* Typhimurium downregulates its expression of SPI-I and SPI-II T3SSs during the chronic phase of infection.

### 4.2.7 *S.* Typhimurium flagellar expression is modulated during chronic infection

Expression of *S.* Typhimurium's flagella is necessary to induce maximal cell death and IL-1β release following infection (**Figure 16**). Thus, I next assessed if the expression of flagella was modulated in chronic isolates (**Figure 23**). I performed swimming motility assays to characterize the flagellar-mediated motility of the *S.* Typhimurium isolates. Relative to ST-WT or early isolates, late chronic isolates exhibited reduced flagellar-mediated motility, reflected by a reduction in the diameter of their swimming motility halo (**Figure 23 A**). In accordance, I observed significantly reduced expression of *fliF* in chronic isolates. This reduction in *fliF* expression strongly correlated with the reduction in NLRP3-, but not NLRC4-dependent cell death (**Figure 23 C, D**). Together, these results demonstrate that changes in the expression of the SPI-I T3SS, SPI-II T3SS and flagella by chronic *S.* Typhimurium isolates modulates its ability to induce NLRP3-dependent inflammasome activation.

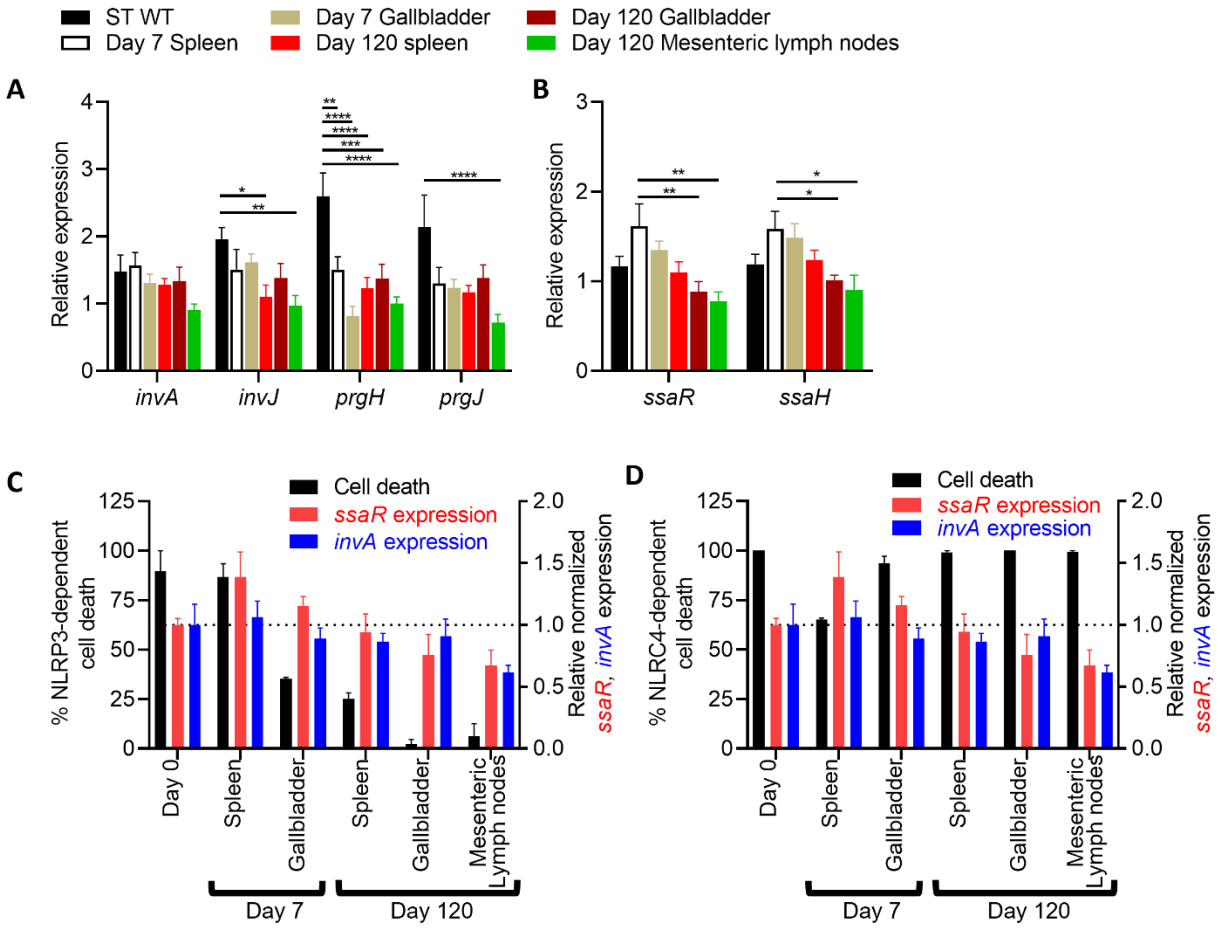


**Figure 22. *S.* Typhimurium T3SS expression is modulated during chronic infection.**

Relative expression of the SPI-I genes *invA, invJ, prgH, prgJ* (**A**) and SPI-II genes *ssaR* and *ssaH* (**B**) was determined by qRT-PCR. NLRP3 and NLRC4 -dependent induction of cell death by ST-WT or chronic ST isolates was compared against the expression of *ssaR* and *invA* (**C, D**). Mean values were compared by two-way ANOVA with post-hoc Tukey's multiple comparison test (**A, B**) (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

*Cai et al. (2021). Isolates of Salmonella* Typhimurium *circumvent NLRP3 inflammasome recognition in macrophages during the chronic phase of infection. J. Biol. Chem. 298:1 doi:10.1016/j.jbc.2021.101461.*

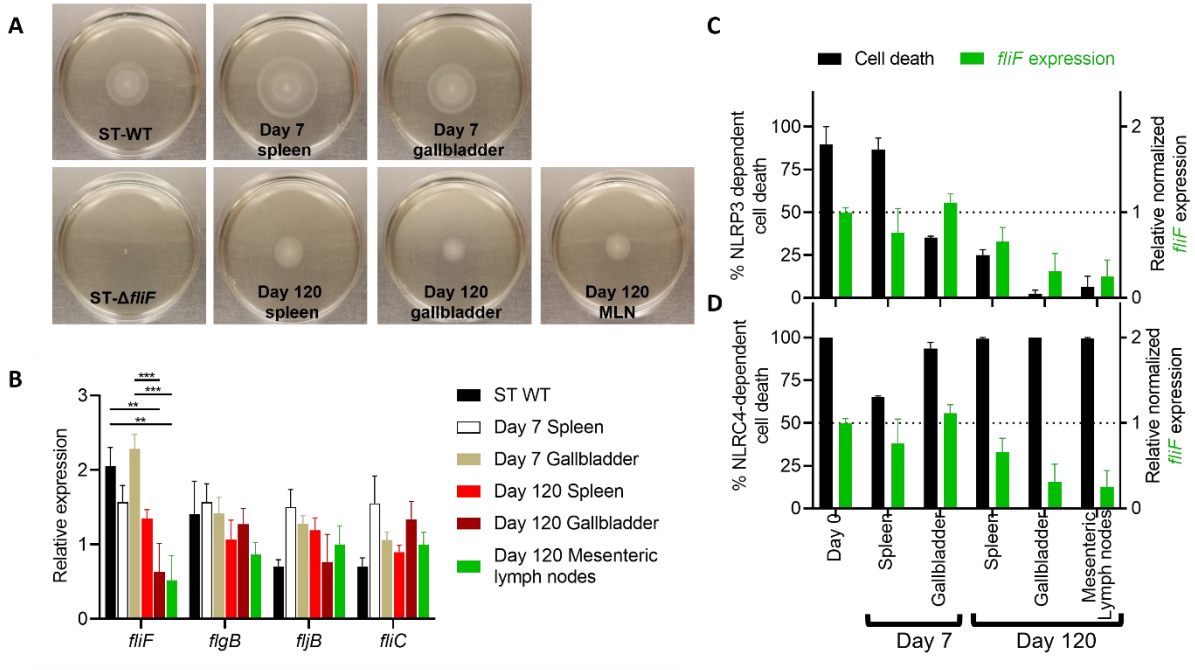


**Figure 23. *S.* Typhimurium flagellar expression is modulated during chronic infection.**

Swimming motility was assessed after overnight incubation of *S.* Typhimurium inoculums on swimming agar plates (**A**). Relative expression of the flagellar genes *fliF*, *flgB*, *fljB* and *fliC* was determined by qRT-PCR (**B**). NLRP3 and NLRC4 -dependent induction of cell death by ST-WT or chronic ST isolates was compared against the expression of *fliF* (**C, D**). Mean values were compared by two-way ANOVA with post-hoc Tukey's multiple comparison test (***P* < 0.01; ****P* < 0.001; *****P* < 0.0001).

*Cai et al. (2021). Isolates of Salmonella* Typhimurium *circumvent NLRP3 inflammasome recognition in macrophages during the chronic phase of infection. J. Biol. Chem. 298:1 doi:10.1016/j.jbc.2021.101461.*

### 4.2.8 SPI-I T3SS expression offers a competitive advantage to the bacterium during the acute phase, but not the chronic phase of infection

Expression of the SPI-I T3SS is necessary to initiate *S.* Typhimurium infection in epithelial cells, however, it is unknown if SPI-I expression contributes to chronic *S.* Typhimurium persistence *in vivo*. Therefore, I sought to assess if the presence of a functional SPI-I T3SS conferred a fitness advantage to *S.* Typhimurium during chronic infections. I infected 129X1/SvJ mice with an inoculum containing equal proportions of ST-WT and ST-Δ*invA* (SPI-I T3SS deficient) and sacrificed the mice at 6-, 30-, and 90- days post-infection. I exploited the differential antibiotic sensitivity of ST-WT and ST-Δ*invA* to enumerate the relative numbers of each strain.

I observed a reduction in the number of ST-Δ*invA* present relative to ST-WT within the spleen and gallbladder at 6 and 30 days post-infection (**Figure 24 A, B**). To better appreciate this difference, I calculated the ratio of ST-WT to ST-Δ*invA* present within the same mouse (**Figure 24 C, D**). This revealed that the expression of SPI-I conferred a significant survival advantage during the early stages of infection, however, this advantage was lost at day 90. The expression of SPI-I did not confer a significant survival advantage to *S.* Typhimurium populations found within the mesenteric or inguinal lymph nodes (**Figure 24 E, F**). These results support my previous findings that the expression of SPI-I is reduced during chronic infection.

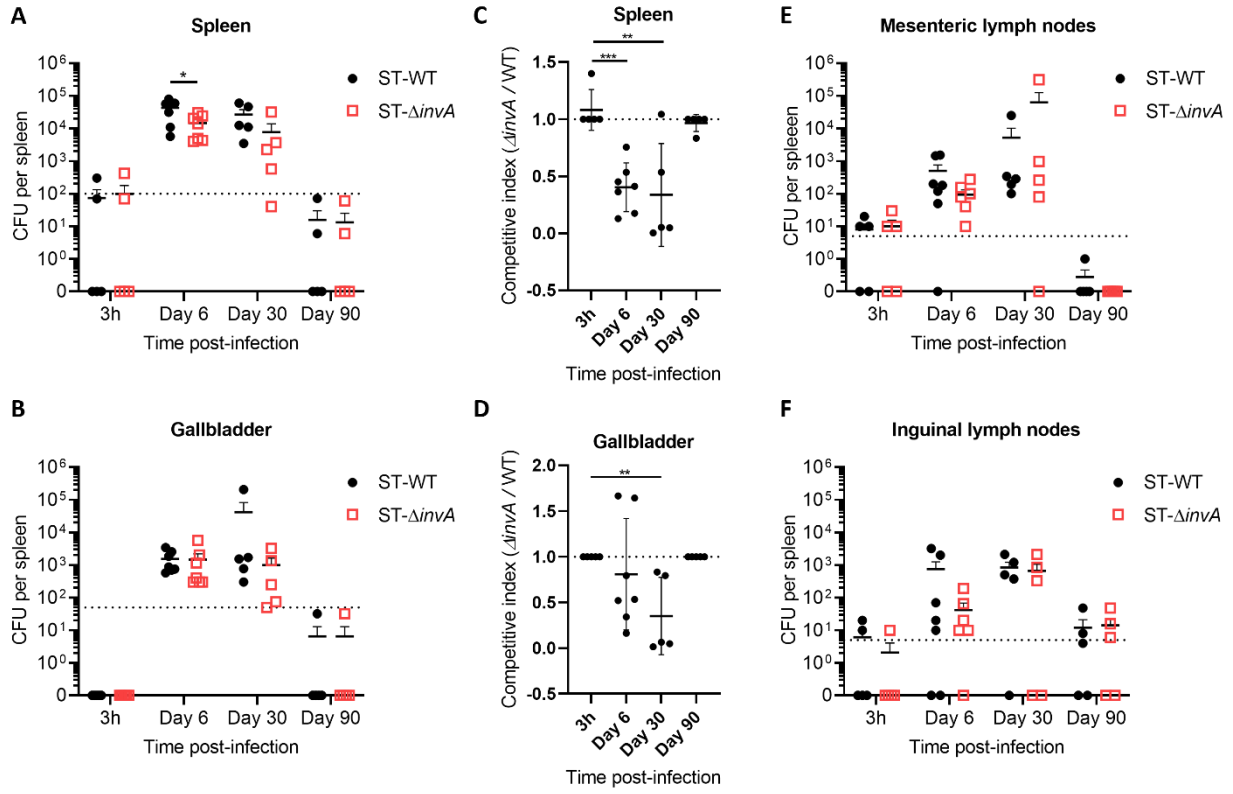


**Figure 24. SPI-I expression does not confer a competitive advantage to *S.* Typhimurium during chronic infection.**

129X1/SvJ mice were co-infected intravenously with an inoculum containing 1:1 CFU ST-WT and CFU ST-Δ*invA* ($10^4$ CFU total) and sacrificed at the indicated timepoints. Bacterial burden in the spleen (**A**), gallbladder (**B**), mesenteric lymph nodes (**E**), and inguinal lymph nodes (**F**) was determined by plating serial dilutions of the homogenates on LB agar plates supplemented with 50 µg/mL of streptomycin or kanamycin to isolate ST-WT or ST-Δ*invA*, respectively. The competitive index was calculated from the ratio of ST-WT and ST-Δ*invA* present within the spleen (**C**) and gallbladder (**D**). A competitive index of 1 indicates an equal proportion of ST-WT and ST-Δ*invA*. Results represent the mean of 5 mice ± SEM at 3h, day 30 and day 90 and mean of 7 mice ± SEM at day 6. Mean values were compared by Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) .

*Cai et al. (2021). Isolates of Salmonella* Typhimurium *circumvent NLRP3 inflammasome recognition in macrophages during the chronic phase of infection. J. Biol. Chem. 298:1 doi:10.1016/j.jbc.2021.101461.*

### 4.2.9 Chronic *S.* Typhimurium isolates exhibit reduced infectivity through the oral route

My previous findings indicated that chronic *S.* Typhimurium isolates express reduced levels of virulence factors such as flagella and SPI-I T3SS, resulting in impaired inflammasome activation. I next sought to evaluate if the *S.* Typhimurium chronic isolates also exhibited an impaired ability to infect new hosts through the oral route. In contrast to intravenous infection, which readily induces systemic dissemination, infection through the oral route is dependent on the activity of the SPI-I T3SS to promote disruption of the gut epithelial barrier and *S.* Typhimurium internalization (300). Oral infection of C57BL/6J mice revealed that chronic *S.* Typhimurium isolates possessed reduced infectivity (**Figure 25**). Relative to ST-WT, infection by the day 120 mesenteric lymph node isolate resulted in significantly reduced bacterial burden. These findings demonstrate that the chronic *S.* Typhimurium isolates possess impaired infectivity due to their reduced expression of flagella, SPI-I T3SS, and SPI-II T3SS.

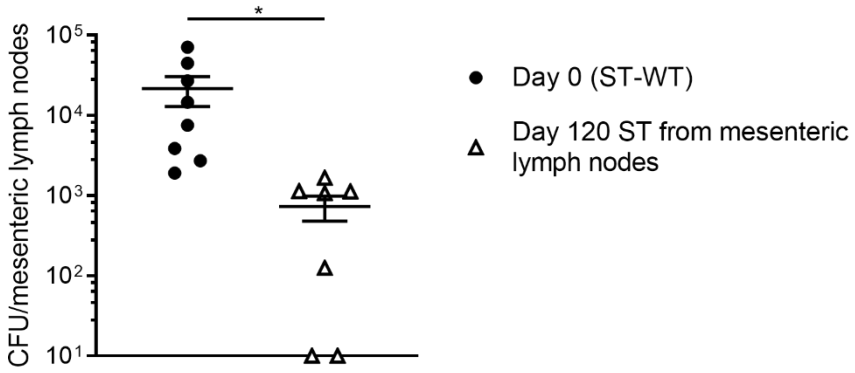


**Figure 25. Chronic *S.* Typhimurium isolates display attenuated virulence upon oral infection.**

C57BL/6J mice were infected orally with $1x10^8$ CFU of ST-WT or the day 120 isolate from the mesenteric lymph node. Mice were sacrificed at 5 days post-infection and the mesenteric lymph nodes were excised and homogenized. Serial dilutions of the homogenate were plated on LB agar to determine the bacterial burden. Results are representative of three experiments. Results represent the mean ± SEM of 7 mice at each timepoint. Mean values were compared by Student's *t*-test (**P* < 0.05).

*Cai et al. (2021). Isolates of Salmonella* Typhimurium *circumvent NLRP3 inflammasome recognition in macrophages during the chronic phase of infection. J. Biol. Chem. 298:1 doi:10.1016/j.jbc.2021.101461.*

# 5. DISCUSSION

The innate immune system plays a critical role in mediating host responses against invading pathogens. Innate immune cells rapidly recognize and respond to foreign entities and initiate a broad inflammatory response to control the infection. Some pathogens can evade immune recognition or modulate host immune responses to impede their clearance, resulting in establishment of a chronic infection. Although these pathogens are normally immunogenic, chronically infected hosts frequently remain asymptomatic. Persistent activation of the inflammatory response during a chronic infection would result in significant toxicity to both the host and pathogen. Therefore, bacterial pathogenicity and host immune responses are modulated during chronic infections to promote co-survival of the pathogen and host.

In this thesis, I have revealed adaptations acquired by the bacterial pathogens *Pseudomonas aeruginosa* and *Salmonella* Typhimurium during chronic infections which facilitate their ability to evade host innate immune recognition and promote bacterial persistence.

## 5.1 *P. aeruginosa* cytotoxicity and adaptations during chronic infections in cystic fibrosis

*Pseudomonas aeruginosa* is a bacterial pathogen that commonly induces disease in humans (129, 130). *P. aeruginosa* is ubiquitous throughout the environment and hosts may be frequently exposed to contaminated food, water, or surfaces (132). Healthy individuals are typically not susceptible to *P. aeruginosa* infection and opportunistic infections are usually observed in individuals with co-morbidities (129). Host co-morbidities that may compromise the

immune response, such as tissue injury, co-infection, or chronic disease significantly increase the risk of *P. aeruginosa* infection (131, 164, 165, 227). Acute *P. aeruginosa* infections are typically rapidly resolved through the actions of the host innate immune system and antibiotic therapy (133, 134). However, co-morbidities such as cystic fibrosis result in significant impairment of the host immune response and the creation of niches conducive to bacterial persistence (215, 216). Mutation of CFTR results in impaired chloride ion transport, which commonly manifests with the hyperproduction of abnormally thick mucous within the respiratory tract (212–214). This is further accompanied by defects in the ability of host innate immune cells to efficiently eradicate pathogens they may encounter (217, 218). As a result, CF patients exhibit high susceptibility to respiratory infections. CF patients may become infected by many different pathogens, however, the majority of adult CF patients are chronically infected by *P. aeruginosa* (227).

In the first part of this thesis, I have examined the ability of *P. aeruginosa* to induce host cytotoxicity and inflammation following infection. Furthermore, I explored the ability of chronic *P. aeruginosa* isolates to adapt to the CF lung environment and the relationship between *P. aeruginosa* infection and host inflammation in CF.

The ability of *P. aeruginosa* to induce host cytotoxicity is attributed to its type III secretion system. The T3SS permits *P. aeruginosa* to secrete effector toxins directly into the cytoplasm of target eukaryotic cells (103). In this study, I have demonstrated that expression of the complete *P. aeruginosa* T3SS apparatus is necessary to induce cytotoxicity and inflammation *in vitro*. T3SS assembly is sequential and begins with assembly of bacterial membrane-integral components, followed by assembly of the secreted structures (99, 102). I have demonstrated in **Figure 10** that

mutations which impair the assembly of proximal structures, such as PA14 *pscJ*::Tn and PA14 *pscL*::Tn, resulted in an impairment of the ability to assemble distal structures and therefore a functional T3SS. Interestingly, I observed that mutation of PscI, the T3SS inner-rod protein, did not protect against *P. aeruginosa*-induced cytotoxicity (**Figure 10**). Other studies have demonstrated that overexpression of PscF, the T3SS outer-rod protein, may compensate for PscI deficiency (301). Further characterization of PscI and PscF redundancy with deletion mutants, rather than transposon insertion mutants, may be necessary to confirm my findings. PA14 may secrete the T3SS effector toxins ExoT, ExoY and ExoU (167). The ADPRT and PLA2 enzymatic activities of ExoT and ExoU significantly contribute towards *P. aeruginosa*-induced cell death (57, 110). I observed that secretion of the full complement of effectors was not necessary to induce cytotoxicity and intoxication by a single effector was sufficient to induce cell death and IL-1β secretion (**Figure 10**).

Inflammasome activation in response to *P. aeruginosa* infection is protective and results in the induction of pyroptotic cell death and IL-1β secretion (9, 10). Inflammasome recognition of *P. aeruginosa* is primarily mediated by the NLRC4 inflammasome (**Figure 8 C, D**). The NLRC4 inflammasome may respond to the T3SS proteins PscI and PscF through NAIP1 and NAIP2-mediated ligand recognition, respectively (27, 28, 293). The redundancy in NLRC4 recognition of PscI and PscF mirrors their functional redundancy in T3SS secretion (**Figure 10**). I observed a minor contribution of NLRP3 towards the release of IL-1β in response to PAO1 infection (**Figure 8 B**). Opening of the T3SS translocation pore induces intracellular ionic flux that may activate NLRP3, however, the relative contribution of NLRP3 is secondary to that of NLRC4 (293).

PA14 may induce inflammasome-independent cell death through the action of ExoU (**Figure 7 A**; **Figure 10 B**). The ExoU effector toxin is a potent intracellular phospholipase that may directly degrade host plasma membranes to induce lytic cell death (56, 145). The ExoU deficient strain PA14 *exoU*::Tn failed to induce inflammasome-independent cell death **(Figure 10 A, B**). Furthermore, I observed that PA14 *exoU*::Tn infection also induced elevated IL-1β release (**Figure 10 A**). Previous studies have proposed that ExoU may inhibit caspase-1 due to reduced IL-1β release following infection by ExoU expressing strains (302, 303). However, my findings reveal that the potency of ExoU permits it to induce rapid cell death prior to inflammasome activation (**Figure 6**). Efficient inflammasome activation requires up to 40 minutes, however, I observed PA14 to induce significant cytotoxicity as early as 30 minutes post-infection (304). This may explain why PA14 infected BMDMs secreted reduced IL-1β levels in comparison to ExoU deficient strains such as PAO1 or PA14 *exoU*::Tn. Recent research has demonstrated that ExoU may in-fact activate caspase-1 rather than inhibit it. The PLA2 enzymatic activity of ExoU generates proinflammatory lipids and ROS, which can induce NLRP3-dependent inflammasome activation (144, 305). Further characterization of ExoU hydrolysis products is necessary to confirm this. The role of the ExoY effector toxin remains elusive. Some studies have implicated ExoY in acute cytotoxicity whereas other studies have proposed that ExoY expression is protective and improves patient outcomes (149, 306). Further research into the effect of ExoY's nucleotidyl cyclase activity is necessary to determine its contribution towards *P. aeruginosa* pathogenesis.

*P. aeruginosa's* ability to chronically persist within the CF lung is attributed to its ability to adapt to its environment (162, 163). Respiratory infections in CF patients are opportunistically

acquired through environmental exposure to pathogens that subsequently undergo rapid adaptation towards a chronic phenotype (229). The CF lung environment drives both phenotypic and genetic changes that promote bacterial resistance and survival. Under these conditions, *P. aeruginosa* undergoes numerous adaptations, including the downregulation of virulence systems such as T3SS and the upregulation of antibiotic tolerance mechanisms (162, 228, 233). The ability of *P. aeruginosa* to hypermutate contributes to the acquisition of these adaptations (244, 245). Conversely, the acquisition of a hypermutable phenotype is attributed to biocide-induced DNA damage, thus presenting a mechanism for bacterial resistance (307). Previous studies have demonstrated that exposure to the CF lung environment and antibiotics both induce convergent and parallel evolution to optimize pathogen fitness (241, 308). In accordance with these studies, I observed that the majority of *P. aeruginosa* isolates from chronically infected CF patients failed to induce inflammasome activation, indicating an adaptation to downregulate the expression of their virulence factors (**Figure 11**, **Figure 13**). The T3SS is the primary mediator of *P. aeruginosa* cytotoxicity and inflammasome activation. I observed a high frequency of genetic variation in T3SS-associated genes in chronic CF isolates, including *exoT*, *pscD*, and *popB/D* (**Figure 12, Table 3**). My previous findings demonstrated that mutations within these genes would impair the ability of *P. aeruginosa* to induce T3SS-mediated inflammasome activation (**Figure 10**). However, my bioinformatic analyses was limited by the number of samples (21 CF, 32 non-CF). Further analysis of a larger sample size would be necessary to draw conclusions regarding the contributions of individual mutations towards the chronic CF isolate phenotype.

The type VI secretion system is a bacterial secretion system that primarily targets competing bacteria within heterogeneous populations (112, 116, 118). However, some researchers have shown that T6SS effectors may also modulate eukaryotic host processes (119–121, 309). The T6SS is controlled through the GacS/GacA regulatory cascade and upregulation of T6SS expression is associated with the chronic *P. aeruginosa* phenotype (**Figure 5**) (170, 171, 310). In my study, I identified genetic variation within T6SS-associated genes to significantly contribute to the chronic CF *P. aeruginosa* isolate phenotype (**Figure 12**). This finding was validated in **Figure 14** where I demonstrated that the presence of the *P. aeruginosa* H2-T6SS significantly inhibited caspase-1 activation, IL-1β secretion, and gasdermin D cleavage. Together, these results suggest that inflammasome activation is impaired in the presence of H2-T6SS. Bioinformatic analyses revealed that all genetic variations within T6SS-associated genes were observed within *hcpA*. This may be a consequence of linkage (311, 312). HcpA self-oligomerizes to assemble the needle structure of the H2-T6SS (313). HcpA shares 100% amino acid homology with HcpB and HcpC, thus, HcpB/C may compensate for the absence of HcpA (314). The H2-T6SS mutant I examined, PW3726 (PA1151-B10::ISphoA/hah), harboured a transposon insertion in the H2-T6SS tip protein VgrG2a. *vgrG2a* is encoded downstream of *hcpA* within the PA1512 operon and upstream mutations within *hcpA* may disrupt the expression of *vgrG2a* (313). T6SS VgrG proteins have been proposed to possess enzymatic activity and therefore may function to modulate host cellular processes during infection (315). Sana et al. have demonstrated that the H2-T6SS effector protein VgrG2b is secreted in a VgrG2a-dependent manner and that VgrG2b induces γTuRC-dependent cytoskeletal remodelling to promote bacterial internalization (155).

Once internalized, *P. aeruginosa* may escape phagosomal killing and persist intracellularly (316, 317). Intracellular *P. aeruginosa* has been associated with recurrent chronic urinary tract infections and intracellular infections may serve as chronic reservoirs (316, 318). The ability of *P. aeruginosa* T6SSs to target eukaryotic cells remains relatively unknown and presents a novel avenue for future research to better understand how pathogens may modulate host inflammasome activation. Further studies regarding the ability of *P. aeruginosa* to reside intracellularly during chronic infections may reveal novel strategies for combatting chronic infections.

Pulmonary exacerbations in CF are associated with increased inflammation and poor clinical outcomes (219, 220). Previous studies from our lab did not identify a relationship between *P. aeruginosa* CF clinical isolates and increased cytotoxicity or inflammation during exacerbation (270). Other studies have revealed that approximately 10-20% of CF isolates retain their virulence and induce cytotoxicity *in vitro* (249, 319). In this study, I observed that approximately 10-20% of chronic CF isolates induced cytotoxicity (**Figure 11, Figure 13**). This was accompanied by the release of IL-1β, indicative of inflammasome activation. All CF isolates were observed to induce significant TNFα secretion. CF patients have been reported to have elevated TNFα levels within the respiratory tract and TNFα has been implicated in exacerbating CF pathologies (320, 321).

In this study, I further pursued the relationship between CF pulmonary exacerbations and inflammation induced by *P. aeruginosa*. I generated a novel collection of 189 *P. aeruginosa* isolates from CF patient sputum collected during the stable and exacerbation periods of disease (**Figure 13 A**). Stable and exacerbation sputum samples were longitudinally collected from the

same patients, which permitted me to characterize changes between stable and exacerbation periods of disease. Through this isolate collection, I identified a significant difference between the ability of stable and exacerbation isolates to induce cell death and IL-1β secretion. Relative to stable isolates, exacerbation isolates induced increased cell death and IL-1β secretion (**Figure 13**). Previous studies have proposed that fluctuations in bacterial population dynamics may trigger pulmonary exacerbations (221, 222). In response to infection by competing bacterial strains, lung-resident bacteria may modulate their T6SS activity (117, 118). My findings in **Figure 14** demonstrate that the *P. aeruginosa* T6SS may influence host inflammasome activation, thus presenting a novel relationship between chronic *P. aeruginosa* populations within the CF lung and pulmonary exacerbations (**Figure 26**).

The commonly accepted lab practice for isolating bacterial colonies is the selection of single colonies after overnight incubation on nutrient agar (296, 297). In my study, I observed that overnight incubation of streaked samples was not representative of the whole bacterial population. I observed that morphologically unique, slow-growing colonies appeared in chronic CF isolates after 24 and 48 hours of incubation (**Figure 15**). Similar colonies were not observed in non-CF (acute or environmental) isolates. Previous studies have reported the appearance of similar persister populations or small colony variants, however, their role remains unclear. Some researchers have demonstrated that SCVs possess reduced immunogenicity and upregulate antibiotic tolerance mechanisms (224, 322). Conversely, other studies have reported that SCVs induce enhanced cytotoxicity and inflammation (249, 323). In my study, I observed both phenotypes. Some SCV isolates, such as those from sample JD332, displayed increased

cytotoxicity relative to the fast-growing isolate (**Figure 15 B-D**). In contrast, other SCV isolates, such as those from sample JD333, possessed reduced cytotoxicity. This phenomenon has previously been reported as bacterial phase variation, a phenomenon which permits reversible variation of gene expression (324, 325). *Pseudomonas* phase variation produces genetic diversity within varying environments, thus increasing adaptation and facilitating expansion within the niche (326, 327). Further genetic analysis is necessary to validate if these diverse populations originate from clonally related phase variants or unique *P. aeruginosa* lineages. In addition, future research should be considerate of the diverse growth kinetics of clinical isolates to allow for proper selection of isolates that are representative of the whole population.

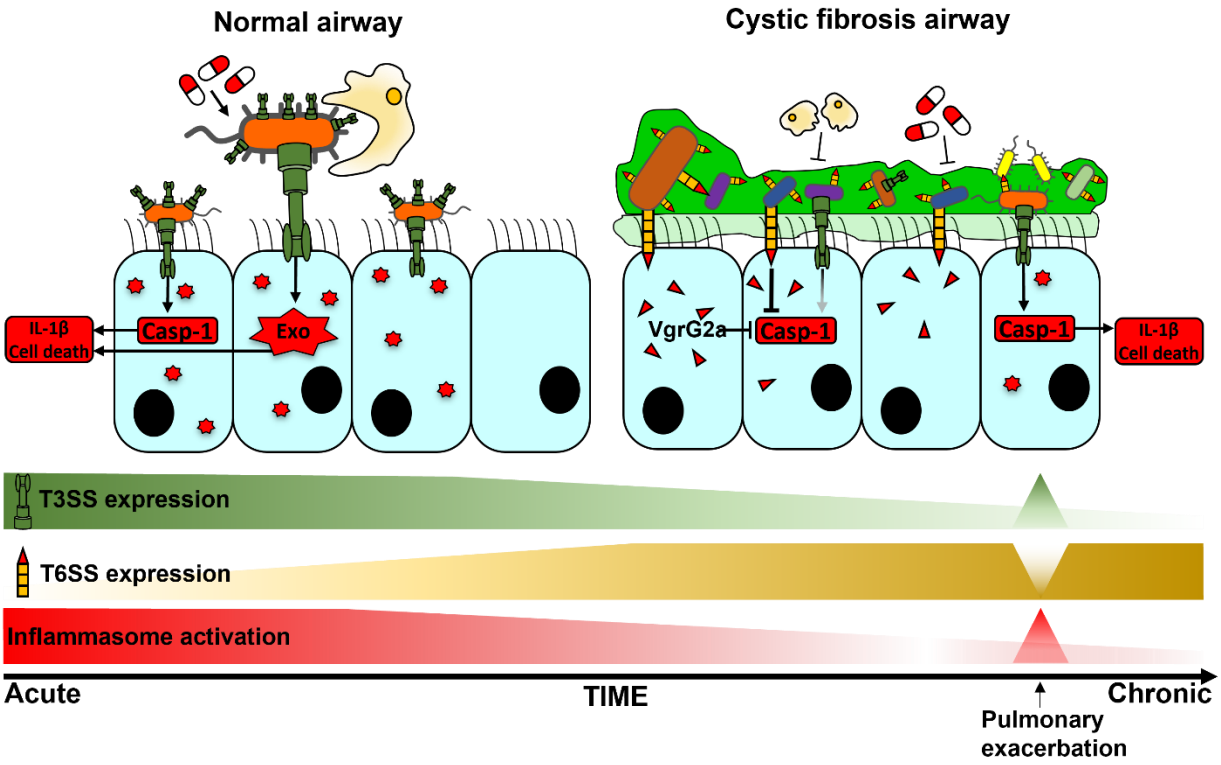


**Figure 26. Progressive modulation of inflammasome activation during chronic *P. aeruginosa* infection.**

During acute infections, *P. aeruginosa* induces potent cell death and inflammation. The expression of T3SS and flagella promote inflammasome activation and T3SS exotoxins induce rapid cell death. Acute infections are typically resolved through clearance by host phagocytes and antibiotic therapy. CFTR mutations result in the production of abnormally viscous mucous which promotes bacterial colonization of the respiratory tract. Over time, *P. aeruginosa* undergoes phenotypic and genotypic diversification to promote its persistence. T6SS expression, biofilm production and antibiotic resistance increase during chronic infection. The expression of T3SS and flagella progressively declines resulting in impaired inflammasome activation. The T6SS effector VgrG2a may further inhibit inflammasome activation. Changes in bacterial population composition may modulate the expression of *P. aeruginosa* virulence factors or reactivate persistent virulent populations, resulting in inflammation and pulmonary exacerbation.

## 5.2 Modulation of *S.* Typhimurium-induced NLRP3 inflammasome activation during chronic carriage

*Salmonella* Typhimurium is a bacterial pathogen that commonly induces disease in humans (176–178). *S.* Typhimurium infection typically induces acute gastroenteritis, however, a small proportion of infected individuals fail to completely eradicate the pathogen after the resolution of acute disease and instead become chronic carriers (196, 197, 251). Chronic carriers remain asymptomatic and are essential for *S.* Typhimurium*'s* survival since they serve as both a reservoir and vector for transmission (195, 198).

In the second part of this thesis, I have revealed the ability of *S.* Typhimurium to modulate host inflammasome activation during chronic infection. Furthermore, I explored the ability of *S.* Typhimurium to modulate the expression of its virulence factors during chronic infection as a mechanism to regulate its pathogenicity.

*Salmonella* is an intracellular pathogen that resides within host phagosomes. *Salmonella* hijacks the host phagosomal maturation pathway to form the *Salmonella*-containing vacuole, an intracellular niche conducive to its survival. Expression of the SPI-I T3SS is necessary to infect non-phagocytic cells and establish the SCV whereas expression of the SPI-II T3SS is necessary to maintain SCV stability and inhibit phagosome maturation (104, 179, 180). *Salmonella* virulence factors are recognized as PAMPs and expression of the SPI-I T3SS, SPI-II T3SS, and flagella are necessary to induce maximal inflammasome activation following *Salmonella* infection (**Figure 16**). NLRP3 and NLRC4 are two key inflammasomes activated in response to *Salmonella* infection

(24, 266). In this study, I employed logarithmic and stationary phase cultures of *S.* Typhimurium to mimic the SPI-I and SPI-II-inducing conditions during the early and late stages of *Salmonella* infection (**Figure 17**) (24, 299). I observed that NLRC4$^{-/-}$ BMDMs were significantly protected against pyroptotic cell death and IL-1β release in response to infection by logarithmic phase *S.* Typhimurium. NLRC4 responds to the *Salmonella* SPI-I T3SS rod and needle proteins and SPI-I T3SS expression is rapidly downregulated after the initial phase of infection (28, 104, 294). Broz et al. have proposed that flagellin may be secreted through the SPI-II T3SS to induce NLRC4 activation, however, I observed the contribution of NLRC4 to be minimal during SPI-II inducing conditions (24). Recent studies in human macrophages have demonstrated that flagellin may activate NLRP3 (25). My results in murine macrophages support this finding as I observed NLRP3$^{-/-}$ BMDMs to be significantly protected against IL-1β release in response to logarithmic and stationary phase *S.* Typhimurium. Furthermore, I observed that flagella-deficient *S.* Typhimurium (ST-Δ*fliF*) failed to induce inflammasome activation in NLRP3$^{-/-}$ BMDMs under both SPI-I and SPI-II T3SS-inducing conditions. Together, these findings demonstrate that both NLRC4 and NLRP3 contribute to protection against *S.* Typhimurium, however, NLRP3 plays a key role in mediating the response to flagella. Further research is necessary to elucidate the mechanism through which flagella induces indirect NLRP3 activation.

*Salmonella* resides intracellularly within macrophage phagosomes. The expression of functional Nramp1 is critical to control intra-phagosomal pathogens through the restriction of iron availability to the pathogen (205, 207). Nramp1-deficient mice, such as C57BL/6, rapidly develop sepsis and succumb to infection (208, 328). Mice expressing functional Nramp1, such as

129X1/SvJ, may control the infection and are permissive of chronic *S.* Typhimurium carriage (205, 328). Although these mice do not succumb to the infection, they are also unable to completely eradicate it. Intracellular pathogens such as *S.* Typhimurium*, Mycobacterium tuberculosis,* and *Listeria monocytogenes* actively manipulate host processes to evade immune clearance and chronically persist. *S.* Typhimurium and *M. tuberculosis* inhibit phagolysosome maturation whereas *L. monocytogenes* escapes the phagosome to prevent destruction (19, 329, 330). However, phagosomal pathogens may further evade adaptive immune responses. *S.* Typhimurium and *M. tuberculosis* may also undermine host antigen presentation, resulting in an impaired T cell response (262, 264, 331). Therefore, control of phagosomal pathogens such as *S.* Typhimurium remains reliant on the function of innate immune cells.

Inflammasome activation is protective during acute infections, however, persistent inflammasome activation during chronic infections may become toxic. To better appreciate the contribution of innate immune signaling during *S.* Typhimurium infection, I examined the ability of *S.* Typhimurium to modulate host inflammasome signaling over the course of a chronic infection. I utilized the previously established 129X1/SvJ chronic *S.* Typhimurium infection model to examine *S.* Typhimurium isolates at up to 120 days post-infection (**Figure 18**). I observed that the ability of *S.* Typhimurium to induce inflammasome activation was enhanced during the early periods of infection and this progressively declined over time (**Figure 19**). This enhancement corroborates with the established practice of passaging *S.* Typhimurium through mice to potentiate its virulence (332, 333). Accordingly, I observed an upregulation of virulence factors such as SPI-I and flagella during the early stages of infection (**Figure 22, Figure 23**). The SPI-I T3SS

and flagella promote bacterial invasion and establishment of infection, however, their continued expression would be detrimental to host survival and therefore *S.* Typhimurium*'s* survival. Thus, an evolution towards reduced inflammasome signaling induced by the bacterium represents a strategy of mutual benefit.

My results demonstrated that late chronic isolates downregulated their expression of the SPI-I T3SS, SPI-II T3SS, and flagella, relative to ST-WT (**Figure 22, Figure 23**). Previous studies have reported that following the initial phase of infection, SPI-I T3SS expression is downregulated and SPI-II T3SS expression is upregulated to promote *S.* Typhimurium*'s* intracellular survival (104, 334). However, these studies have only examined SPI-II T3SS expression in the context of acute infection and not chronic infection. My results demonstrated that SPI-II T3SS expression was elevated during the early phase of infection (7 days post-infection), however, this upregulation was transient and expression of the SPI-II T3SS was subsequently downregulated during the late chronic phase of infection (120 days post-infection) (**Figure 22**). The downregulation of virulence factors significantly impaired the ability of late chronic *S.* Typhimurium isolates to induce inflammasome-dependent cell death and IL-1β release (**Figure 19**). *Salmonella's* persistence within the host is dependent on host cell death to facilitate cell-to-cell transmission (55, 335). I observed that chronic *S.* Typhimurium isolates failed to induce pyroptotic cell death, suggesting a reliance on alternate cell death pathways to promote its persistence. Surprisingly, I found that these isolates possessed an enhanced ability to induce inflammasome-independent cell death. I observed activation of caspase-8 and MLKL, indicative of apoptotic and necroptotic cell death, respectively (**Figure 20**). *Salmonella* effector toxins may induce apoptosis and *Salmonella*-

induced type I IFN may induce necroptosis (186, 268). However, the activation of these alternate cell death pathways is secondary to inflammasome activation and the induction of pyroptosis precludes other forms of cell death (**Figure 27**) (267). Further research into the activation of alternate cell death pathways during chronic infection may present additional therapeutic opportunities to combat persistent chronic bacterial infections.

I observed that the downregulation of *S.* Typhimurium flagellar expression strongly correlated with a decrease in NLRP3-dependent cell death, but not NLRC4-dependent cell death (**Figure 23**). In accordance with my findings in **Figure 17**, chronic isolates displayed an increased dependence on NLRP3, which was likely mediated by flagellin. Previous studies have reported that *S.* Typhimurium*'s* modulation of flagellar expression is organ-specific and flagella are downregulated following traversal of the intestinal epithelial barrier (336, 337). In my study, the expression of *fliF* was downregulated in all chronic isolates (**Figure 23**). FliF assembles the MS ring, a component of the flagellar basal body, and its expression is indispensable towards initiating flagellar assembly (338, 339). FliC, the flagellin subunit, may be secreted through the SPI-I and SPI-II T3SSs to induce host inflammasome activation (24, 340, 341). The expression of *fliC* was not observed to be significantly modulated in my study. In addition, I observed that the expression of SPI-I and SPI-II T3SSs was reduced but not completely abrogated. Thus, it is possible for FliC to be secreted through the T3SSs to induce host inflammasome modulation in the absence of functional flagellar expression. Further research is necessary to validate if flagellar secretion through T3SSs is occurring in the chronic *S.* Typhimurium isolates described in this thesis.

Expression of flagella and the SPI-I T3SS by *Salmonella* is necessary to invade epithelial cells and initiate infections through the oral route (97, 300). I observed that chronic *S.* Typhimurium isolates were impaired in their ability to infect new hosts through the oral route (**Figure 25**). This demonstrated that the function of SPI-I was impaired in the late chronic isolates of *S.* Typhimurium. Previous studies have shown that the presence of a small SPI-I-positive population was sufficient to facilitate infection by neighbouring SPI-I-negative *S.* Typhimurium (342). I also observed that impairment of the SPI-I T3SS in chronic isolates did not impair the chronicity of the pathogen once the infection was established (**Figure 24**). SPI-I expression conferred a fitness advantage during the early stages of infection; however, this advantage was lost as the infection progressed. Future studies to elucidate *S.* Typhimurium dissemination *in vivo* (e.g. IVIS imaging) may reveal novel organ-specific contributions of *S.* Typhimurium virulence factors during chronic infection.

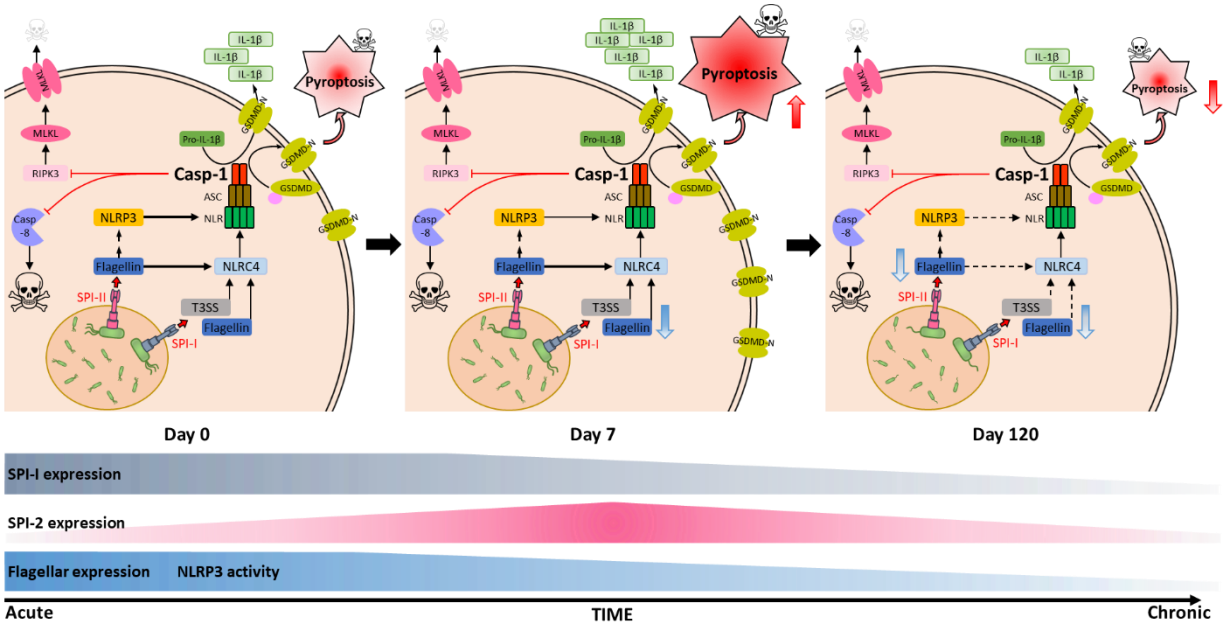


**Figure 27. Progressive modulation of cell death during the various stages of *S.* Typhimurium infection.**

Early cell death is dominated by caspase-1, which limits the participation of other pathways in promoting cell death. The expression of SPI-I and flagella is initially high, which promotes inflammasome activation. The expression of SPI-I and flagella progressively declines over the course of infection. The expression of SPI-II genes is elevated during the acute phase and later declines during the chronic phase of infection. The expression of flagella undergoes the greatest decrease, which correlates with the progressive decline in NLRP3-dependent cell death. NLRC4-dependent cell death is not affected since it may be activated by toxins and flagellin secreted through the SPI-I and SPI-II T3SSs.

*Cai et al. (2021). Isolates of Salmonella* Typhimurium *circumvent NLRP3 inflammasome recognition in macrophages during the chronic phase of infection. J. Biol. Chem. 298:1 doi:10.1016/j.jbc.2021.101461.*

## 5.3 Concluding remarks

In this thesis, I have revealed novel insights into the mechanisms of bacterial evasion that result in modulation of host immune responses during chronic infections. Although the host may recognize these pathogens and initiate an immune response, the response is insufficient to eradicate the pathogen. Persistent activation of the host inflammatory response would result in significant toxicity to the host and therefore be detrimental to pathogen survival. Therefore, modulation of the host inflammatory response as a result of reduced expression of virulence genes is mutually beneficial for co-survival of the pathogen and host.

The main findings of my thesis include:

1) *P. aeruginosa* strains induce inflammasome-dependent and -independent cell death and inflammasome-dependent IL-1β release. Inflammasome activation is primarily mediated by the NLRC4 inflammasome and inflammasome-independent cell death is dependent on T3SS effector toxin ExoU.
2) The majority of *P. aeruginosa* isolates from chronically infected cystic fibrosis patients demonstrate an impairment in their ability to induce cell death and possess an increased frequency of genetic variation in virulence-related genes, including the T3SS and T6SS. However, a small proportion of isolates retain their virulence factor expression and may become reactivated to induce host cell death and inflammation during pulmonary exacerbations.

3) The *P. aeruginosa* H2-T6SS inhibits host inflammasome signaling and modulation of T6SS expression may contribute to host inflammation.
4) *S.* Typhimurium-induced cell death is primarily dependent on NLRC4 and IL-1β release is dependent on both NLRC4 and NLRP3. Expression of flagella is necessary to activate NLRP3 inflammasome signaling at both the early and late stages of infection.
5) *S.* Typhimurium-induced inflammasome signaling is dynamically modulated throughout the course of chronic infection. Inflammasome signaling is upregulated shortly after infection and subsequently downregulated during the chronic phase of infection.
6) Inflammasome modulation during chronic infection is organ-specific and dependent on flagellar-mediated activation of NLRP3.
7) *S.* Typhimurium SPI-I T3SS expression is necessary to establish infections, however, its expression does not confer a competitive advantage during chronic infection.

Overall, in this thesis, I have demonstrated that chronic bacterial infections result in the modulation of host inflammasome signaling to achieve a balance between pathogen control and overt host toxicity. A better understanding of host-pathogen interactions during chronic infections may reveal novel avenues for therapeutics to combat the increasing prevalence of antibiotic resistant microbes.

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# 7. APPENDIX

**Table 3. Mutations identified by FET analysis of CF vs. non-CF *P. aeruginosa* isolates.**

FET was performed to compare *Pseudomonas* isolates from CF and non-CF sources. Genes containing SNPs were classified into categories according to their association with T3SS, T6SS, motility, biofilm formation and antibiotic resistance, or biosynthesis. The number of genes containing SNPs with a *p*-value < 0.05 are enumerated in column three. The relative frequency of mutation within a gene was determined by calculating the proportion of genes containing SNPs with a *p*-value < 0.05 against genes without significant SNPs.

| Gene | Function | Mutations $p < 0.05$ | Mutations total | Proportion $p < 0.05$ |
|---|---|---|---|---|
| PA1163 | abx/biofilm | 2 | 2611 | 0.076599 |
| PA14_00560 | T3SS | 102 | 1335 | 7.640449 |
| PA14_00640 | biosynthesis | 0 | 1834 | 0 |
| PA14_01020 | T6SS | 0 | 1498 | 0 |
| PA14_05190 | motility | 0 | 1150 | 0 |
| PA14_05360 | motility | 1 | 2050 | 0.04878 |
| PA14_05380 | motility | 0 | 877 | 0 |
| PA14_09400 | biosynthesis | 2 | 1211 | 0.165153 |
| PA14_14330 | T3SS | 0 | 352 | 0 |
| PA14_16250 | biosynthesis | 5 | 1498 | 0.333778 |
| PA14_19130 | abx/biofilm | 0 | 607 | 0 |
| PA14_22620 | biosynthesis | 0 | 1394 | 0 |
| PA14_36345 | T3SS | 0 | 1247 | 0 |
| PA14_37730 | biosynthesis | 3 | 2645 | 0.113422 |
| PA14_40230 | abx/biofilm | 0 | 1189 | 0 |
| PA14_40240 | abx/biofilm | 3 | 2177 | 0.137804 |
| PA14_40250 | abx/biofilm | 1 | 1279 | 0.078186 |

| | | | | |
|---|---|---|---|---|
| PA14_40260 | abx/biofilm | 117 | 7541 | 1.551518 |
| PA14_42250 | T3SS | 0 | 646 | 0 |
| PA14_42260 | T3SS | 0 | 622 | 0 |
| PA14_42270 | T3SS | 1 | 749 | 0.133511 |
| PA14_42280 | T3SS | 0 | 340 | 0 |
| PA14_42290 | T3SS | 2 | 434 | 0.460829 |
| PA14_42300 | T3SS | 1 | 349 | 0.286533 |
| PA14_42340 | T3SS | 3 | 1300 | 0.230769 |
| PA14_42350 | T3SS | 3 | 1805 | 0.166205 |
| PA14_42360 | T3SS | 0 | 424 | 0 |
| PA14_42380 | T3SS | 0 | 832 | 0 |
| PA14_42400 | T3SS | 0 | 415 | 0 |
| PA14_42430 | T3SS | 0 | 439 | 0 |
| PA14_42440 | T3SS | 4 | 889 | 0.449944 |
| PA14_42450 | T3SS | 2 | 1174 | 0.170358 |
| PA14_42460 | T3SS | 0 | 508 | 0 |
| PA14_42470 | T3SS | 3 | 887 | 0.338219 |
| PA14_42480 | T3SS | 0 | 298 | 0 |
| PA14_42490 | T3SS | 0 | 436 | 0 |
| PA14_42500 | T3SS | 0 | 2122 | 0 |
| PA14_42510 | T3SS | 0 | 331 | 0 |
| PA14_42520 | T3SS | 0 | 367 | 0 |
| PA14_42530 | T3SS | 0 | 373 | 0 |
| PA14_42570 | T3SS | 1 | 1325 | 0.075472 |
| PA14_42580 | T3SS | 0 | 478 | 0 |
| PA14_42600 | T3SS | 0 | 1121 | 0 |
| PA14_42610 | T3SS | 3 | 931 | 0.322234 |

| | | | | |
|---|---|---|---|---|
| PA14_42620 | T3SS | 1 | 655 | 0.152672 |
| PA14_42640 | T3SS | 0 | 790 | 0 |
| PA14_42660 | T3SS | 2 | 1051 | 0.190295 |
| PA14_44890 | T6SS | 218 | 664 | 32.83133 |
| PA14_45540 | motility | 1 | 892 | 0.112108 |
| PA14_45560 | motility | 1 | 742 | 0.134771 |
| PA14_45630 | motility | 0 | 746 | 0 |
| PA14_45790 | motility | 0 | 475 | 0 |
| PA14_45960 | abx/biofilm | 0 | 722 | 0 |
| PA14_48060 | biosynthesis | 1 | 1441 | 0.069396 |
| PA14_50110 | motility | 0 | 808 | 0 |
| PA14_50140 | motility | 1 | 1798 | 0.055617 |
| PA14_50160 | motility | 0 | 331 | 0 |
| PA14_50270 | motility | 0 | 1426 | 0 |
| PA14_50290 | motility | 0 | 1468 | 0 |
| PA14_51340 | abx/biofilm | 0 | 1000 | 0 |
| PA14_51880 | abx/biofilm | 26 | 1335 | 1.947566 |
| PA14_54390 | abx/biofilm | 0 | 1426 | 0 |
| PA14_54410 | abx/biofilm | 0 | 952 | 0 |
| PA14_54420 | abx/biofilm | 0 | 587 | 0 |
| PA14_54500 | biosynthesis | 0 | 1384 | 0 |
| PA14_58550 | biosynthesis | 0 | 901 | 0 |
| PA14_58750 | motility | 1 | 1717 | 0.058241 |
| PA14_58770 | motility | 0 | 875 | 0 |
| PA14_60250 | motility | 1 | 1594 | 0.062735 |
| PA14_60260 | motility | 1 | 1339 | 0.074683 |
| PA14_60290 | motility | | 826 | 0 |

| | | | | |
|---|---|---|---|---|
| PA14_60300 | motility | 2 | 589 | 0.339559 |
| PA14_64230 | T3SS | 1 | 2830 | 0.035336 |
| PA14_65450 | motility | 1 | 853 | 0.117233 |
| PA14_66540 | abx/biofilm | 1 | 982 | 0.101833 |
| PA14_66620 | motility | 0 | 2139 | 0 |
| PA14_66630 | motility | 0 | 526 | 0 |
| PA14_66640 | motility | 0 | 625 | 0 |
| PA14_66650 | motility | 0 | 598 | 0 |
| PA4527 | motility | 1 | 1128 | 0.088652 |
| PA4551 | motility | 3 | 559 | 0.536673 |

# 8. CONTRIBUTION OF COLLABORATORS

Bioinformatic analyses was performed in collaboration with Katie Noah and Dr. Alex Wong (Carleton University, Canada). I provided sequences and Katie performed alignment, annotation, and FET to identify SNPs within the sequences. I subsequently analyzed the FET results to identify genes that possessed significant SNPs and the frequency of SNPs.