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Identification of *Pseudomonas aeruginosa* ExoT amino acid residues necessary for its proteasomal dependent degradation in the host cytoplasm

by

Armando Josue Lemus-Hernandez

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences Program

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

[redacted]

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by
Armando Josue Lemus-Hernandez

DEDICATION:

I am thankful to God for giving me a wonderful wife and two marvelous children. I dedicate my work to them for they are the reason of my happiness.

ACKNOWLEDGEMENTS:

I would like to thank Dr. Joanne Engel for her mentorship and support during the course of my Ph.D. training. She has been an enormous asset to my development as a scientist and as a person. I would also like to thank the other members of my dissertation committee, Dr. Anita Sil and Dr. Jeffery Cox for their support and advice throughout my training. I thank all the Engel laboratory members for their technical advice and for teaching me many of the techniques that I have used in the projects I have pursued as a graduate student. I thank my wife and my parents for their immense support throughout my training. Without their help and encouragement, it would have been very difficult to accomplish everything that I have done.

Identification of *Pseudomonas aeruginosa* ExoT amino acid residues necessary for its proteasomal dependent degradation in the host cytoplasm

By: Armando Josue Lemus-Hernandez

ABSTRACT:

The opportunistic human pathogen *Pseudomonas aeruginosa* utilizes a type III secretion system to deliver virulence factors that disrupt host cell processes and host innate immunity. Four type III-secreted toxins have been identified in this bacterium: ExoS, ExoT, ExoU and ExoY. ExoT is a bifunctional enzyme whose N-terminus encodes a GTPase activating protein with activity towards Rho, Rac, and Cdc42 while the C-terminus encodes an ADP ribosyl transferase with activity towards Crk. ExoT inhibits bacterial internalization into host cells, disrupts the actin cytoskeleton, and blocks wound repair. We previously showed that ExoT is degraded in a proteasomal dependent manner and that *in vivo* resistance against this toxin is Cbl-b-dependent. Though Cbl-b is known to influence actin cytoskeleton and neutrophil infiltration into LPS treated host tissues, we show that Cbl-b is not necessary for phagocytosis of *P. aeruginosa* by macrophages or neutrophil infiltration into *P. aeruginosa* infected host tissues. In addition, we demostrate that ExoT lysine residues 49 and 74 control its proteasomal dependent degradation in the host cytoplasm. We further demonstrate by host cell rounding assays and an acute pneumonia mouse model that mutating these lysine residues to arginine increases the virulence of *P. aeruginosa*. Interestingly, mutating lysine 49 and 74 did not alter the virulence of *P. aeruginosa* in *cbl-b*$^{-/-}$ mice, which suggests that Cbl-b-mediated protection of the host against *P. aeruginosa* must depend on the targeting of these two lysine residues in the toxin. Although mutation of lysine 49 to arginine is able to increase toxin secretion rate into the medium, we found that mutations in both lysine 49 and 74 are necessary for increased levels of translocated toxin in the host cytoplasm. Collectively, these data demonstrate that ExoT lysine residues at positions 49 and 74 play a key role in toxin degradation by the host proteasome, which leads to an alteration of ExoT-mediated virulence of *P. aeruginosa*. Our findings also suggest that Cbl-b targets these residues through either ubiquitination or another yet unidentified molecular process. Experiments are underway to determine whether Cbl-b is able to polyubiquitinate the mutant toxin as it does on wild type ExoT.

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Chapter 1:

INTRODUCTION

Pseudomonas aeruginosa and human disease

The Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* is a leading cause of acute infections in immunocompromised individuals and hospitalized patients (Mandell, Bennett et al. 2010). In patients with Cystic Fibrosis, *P. aeruginosa* chronically colonizes pulmonary tissues, leading to severe lung damage and death (Mandell, Bennett et al. 2010). Although this organism rarely affects healthy individuals, those that have some level of epithelial cell barrier injury and/or are immunocompromised can develop pneumonia, bacteremia, catheter related infections, urinary tract infections and wound infections (Gaynes and Edwards 2005; Engel and Balachandran 2009; Hauser 2009).

P. aeruginosa infections also represent a serious health threat to patients in intensive care units (ICU) who require ventilators for assisted breathing (Gaynes and Edwards 2005; Fujitani, Sun et al.). Ventilator associated pneumonia caused by *P. aeruginosa* has an attributable mortality rate of up to 42.8%, despite proper antibiotic treatment (Fujitani, Sun et al. 2011). Patients who develop pneumonia in surgical ICUs have a 56% antimicrobial treatment failure rate when infected with this bacterium (Malangoni, Crafton et al. 1994). In addition, the decline in susceptibility to previously

effective antibiotics has been alarming. Although 89% of *P. aeruginosa* strains collected in one study were susceptible to ciprofloxacin in the period of 1990-1993, only 68% showed susceptibility by the year 2000 (Neuhauser, Weinstein et al. 2003). The financial cost of *P. aeruginosa* infection outbreaks also has an enormous impact on ICU patients and health care institutions, with an average increase of costs of 66% and an extra length in hospital stay of 70 more days compared to non-infected patients (Bou, Lorente et al. 2009; Fujitani, Sun et al.). Interestingly, other nosocomial infections in the ICU only caused an increase in hospital stay of 21 extra days compared to non-infected patients (Bou, Lorente et al. 2009; Fujitani, Sun et al.). This finding suggests that *P. aeruginosa* infections have a greater detrimental effect on the health of affected individuals compared to other infections acquired during an ICU stay, and that these infections tend to require greater attention and resources by the health care institution (Bou, Lorente et al. 2009). Together, these factors make *P. aeruginosa* infections a formidable health care problem, which requires significant attention by the health care establishment in order to minimize the impact that this highly virulent opportunistic pathogen has on vulnerable patients.

Association of the P. aeruginosa type III secretion system and bacterial virulence

Successful Gram-negative pathogens must subvert the killing mechanisms of environmental predators and host immune responses that target them for destruction. This constant battle for survival has driven the selection of bacterial macromolecular machines

that allow the pathogen to modulate, circumvent, or interfere with host cell processes (Engel and Balachandran 2009; Hauser 2009). *P. aeruginosa* encodes a large repertoire of cell associated and secreted virulence factors, which include, but are not limited to, type IV pili, pyocyanin, siderophores, hydrogen cyanide, rhamnolipids, alginate, exopolysaccharides, and type I, II, III, V, and VI secretion systems (Lau, Hassett et al. 2005; Engel and Balachandran 2009; Hauser 2009). The development of novel therapeutic treatments to combat human disease caused by *P. aeruginosa* requires an in depth understanding of the biology of these virulence factors and macromolecular structures.

One such structure is a needle-like complex known as the type III secretion system, which is derived from flagella and is evolutionarily conserved in Gram-negative pathogens (Hueck 1998; Hauser 2009). The type III secretion apparatus allows the bacterium to deliver bacterial effectors directly from the bacteria, through their inner and outer membrane and through the host cell membrane, into the host cell cytoplasm (Marlovits and Stebbins). Once translocated, these effectors have rapid access to a wide variety of intracellular targets, including proteins involved in cytoskeletal rearrangement, cell cycle progression, programmed cell death and endocytic trafficking (Engel and Balachandran 2009; Hauser 2009). A clear correlation between severity of disease in ventilator associated pneumonia caused by *P. aeruginosa* and activity of the type III secretion system has been established (Hauser, Cobb et al. 2002). The importance of this secretion system in *P. aeruginosa* pathogenesis is also evident in numerous animal studies (Hauser 2009).

Structural and regulatory components of the *P. aeruginosa* type III secretion system are encoded in 36 known genes located in five tandemly arranged operons, while the 4 secreted effectors and their chaperones are scattered throughout the genome (Hauser 2009). Based on the importance that this secretion apparatus plays in *P. aeruginosa* pathogenesis, it is critical to develop an in-depth understanding of its involvement in the virulence of this bacterium in order to develop new treatment strategies against *P. aeruginosa*. Indeed, recent studies have identified potential novel therapeutics molecules that inhibit Type III secretion in *P. aeruginosa*, as well as other pathogens that contain a type III secretion system such as *Yersinia pestis* (Aiello, Williams et al. ; Swietnicki, Carmany et al.) and *Chlamydia trachomatis* (Izore, Job et al. 2011).

P. aeruginosa type III secretion system effectors

Unlike other Gram-negative pathogens which secrete a large number of effectors through their type III secretion system, only four different effectors have been identified in *P. aeruginosa*: ExoY, ExoU, ExoS and ExoT (Engel and Balachandran 2009; Hauser 2009). Although most strains encode ExoT and ExoY, very few environmental or clinical isolates encode or produce all four effectors. In addition, most strains encode either ExoS or ExoU, but not both (Fleiszig, Wiener-Kronish et al. 1997).

ExoY is an adenylate cyclase whose activity requires a host co-factor, which has not yet been identified (Yahr, Vallis et al. 1998; Hauser 2009). Its translocation can lead to increased cAMP levels in the host cell, which can affect expression levels of host

genes dependent on cAMP (Ichikawa, English et al. 2005; Hauser 2009). ExoY can cause host cell rounding and delayed phagocytic activity in mammalian cells (Cowell, Evans et al. 2005[, and it has been shown to be toxic when expressed in yeast [Arnoldo, 2008 #4498). However, studies thus far have not clearly defined a key role for ExoY during *P. aeruginosa* infections.

ExoU has a patatin-like domain with phospholipase A_2 activity (Hauser 2009). Upon translocation, this effector is rapidly trafficked to the plasma membrane where it is able to interact with its substrates, which include phospholipids, lysophospholipids, and neutral lipids (Phillips, Six et al. 2003; Sato, Frank et al. 2003; Tamura, Ajayi et al. 2004). ExoU activity causes rapid loss of integrity of the plasma membrane in host cells, which leads to cell lysis and necrotic cell death (Finck-Barbancon, Goranson et al. 1997; Hauser, Kang et al. 1998; Sato and Frank 2004). Previous studies have suggested that ExoU may specifically target innate immune cells and epithelial barriers in the human host (Finck-Barbancon, Goranson et al. 1997; Diaz and Hauser 2010). This idea is consistent with the severe clinical outcomes observed in patients infected with strains that express this effector (Finck-Barbancon, Goranson et al. 1997; Hauser, Kang et al. 1998; Schulert, Feltman et al. 2003).

ExoS and ExoT are bifunctional virulence factors that share 76% protein sequence homology, and most likely arose by gene duplication (Barbieri and Sun 2004; Engel and Balachandran 2009). ExoT is encoded in almost all *P. aeruginosa* isolates, suggesting that it plays a key function in survival of *P. aeruginosa* in diverse hosts. ExoT has also been shown to contribute to *P. aeruginosa* virulence in several animal models of disease

(Garrity-Ryan, Kazmierczak et al. 2000; Shaver and Hauser 2004; Vance, Rietsch et al. 2005; Balachandran, Dragone et al. 2007). ExoS-mediated *P. aeruginosa* virulence has been shown to have greater detrimental effects on the host than ExoT (Shaver and Hauser 2004).

Both ExoS and ExoT contain an N-terminal secretion domain, a chaperone binding domain, a membrane localization domain (MLD), a GTPase activating protein (GAP) domain, and an ADP-ribosyl transferase (ADPRT) domain, which includes a co-factor binding region for 14-3-3 (Hauser 2009). The N-terminal GAP domains of these two cytotoxins have identical biological targets, while critical differences in the substrate binding regions of the C-terminal ADPRT domains allow these toxins to target distinct host cell substrates for ADP-ribosylation (Sun, Maresso et al. 2004). The GAP activity of ExoS and ExoT inhibits Rac1, RhoA, and Cdc42, which leads to alterations in the host cell actin cytoskeleton (Wurtele, Wolf et al. 2001; Henriksson, Sundin et al. 2002; Kazmierczak and Engel 2002; Krall, Sun et al. 2002). In cell-based assays, GAP activity is manifested by host cell rounding, disruption of cell-cell junctions, and inhibition of cell migration, cell division, and wound repair (Garrity-Ryan, Kazmierczak et al. 2000; Geiser, Kazmierczak et al. 2001; Kazmierczak and Engel 2002; Shafikhani and Engel 2006). Interestingly, in the context of *P. aeruginosa* virulence in mice, several studies have demonstrated that absence of GAP activity in both ExoS and ExoT does not statistically change the colonization of lung tissues (Garrity-Ryan, Shafikhani et al. 2004; Shaver and Hauser 2004). This finding suggests that the ADPRT domains of these two

toxins play an important role in colonization and persistence of bacteria in pulmonary tissues.

The ADPRT domain from ExoS targets a broad range of substrates, which include low molecular weight G proteins in the Ras, Rab and Rho families, as well as ExoS itself (Barbieri and Sun 2004; Sun, Maresso et al. 2004; Deng and Barbieri 2008). Previous studies have shown that ExoS ADPRT activity disrupts cytoskeletal rearrangement, tight junctions, endocytosis, gene expression, vesicular trafficking, and DNA synthesis (Hauser 2009). *In vivo* models of disease have also demonstrated an important role of ExoS ADPRT activity during infection (Shaver and Hauser 2004). The ADPRT domain of ExoT has a more limited set of targets, which include PGK-1, CrkI and CrkII (Sun, Maresso et al. 2004). Crk proteins are a family of host cell adaptor proteins found in focal adhesions that are important in eukaryotic signal transduction pathways that control cell motility and phagocytosis (Feller 2001; Garrity-Ryan, Shafikhani et al. 2004; Deng, Sun et al. 2005). ADP-ribosylation of Arg^{20} in the Crk protein hinders interactions between the SH_2 domain of Crk and its binding partners, disrupting focal adhesions (Sun and Barbieri 2003; Deng, Sun et al. 2005). By targeting the host adaptor proteins CrkI and CrkII, the ADPRT domain of ExoT inhibits integrin-mediated phagocytosis, cell proliferation and migration (Garrity-Ryan, Kazmierczak et al. 2000; Garrity-Ryan, Shafikhani et al. 2004; Shafikhani, Morales et al. 2008).

Regulation of the P. aeruginosa type III secretion system

Efficient modulation of host cell processes by type III secreted effectors requires exquisite coordination of temporal and spatial delivery of these effectors. Potential steps of regulation occur upon transcription, post-transcription, secretion, and translocation. Once type III secretion effectors are injected into the host cell, a new set of regulatory steps can affect their activity. These regulatory mechanisms include differential effector degradation, regulation of effector function, and intracellular localization.

Transcriptional regulation controls the expression of the components of the system. The *P. aeruginosa* type III secretion system is tightly regulated and is highly dependent on extracellular signals. Both host cell contact and low extracellular Ca^{++} concentrations are known to activate type III secretion system genes, while metabolic stress, high levels of Cu^{2+}, DNA damage, and low osmolarity repress its activation (Frank 1997; Vallis, Yahr et al. 1999; Yahr and Wolfgang 2006). Transcriptional induction of the type III secretion system in *P. aeruginosa* depends on different regulatory molecules, which include Vfr and its allosteric effector cAMP, and a regulatory circuit that includes the transcriptional activator ExsA, along with its partner switching molecules ExcC, ExsD, and ExsE. The mechanism by which Vfr and cAMP control the expression levels of type III secretion genes is not entirely understood, although it has been postulated that Vfr-cAMP may function in a common pathway with ExsA (Yahr and Wolfgang 2006). Type III secretion genes are also activated by the transcriptional regulator ExsA, a homolog of

Yersinia VirF (Hovey and Frank 1995; Hueck 1998; Brutinel, Vakulskas et al. 2008). However, under non-inducing conditions, ExsA is bound to its inhibitor ExsD (McCaw, Lykken et al. 2002). This interaction can be interrupted by ExsC, but this protein is normally bound to ExsE, which inhibits ExsC interactions with the ExsA-ExsD complex (Dasgupta, Lykken et al. 2004; Rietsch, Vallet-Gely et al. 2005; Urbanowski, Lykken et al. 2005). Under inducing conditions, ExsE is secreted through the Type III secretion needle complex into the extracellular environment or host cell, which leads to the depletion of ExsE levels in the bacterial cytoplasm. This relieves ExsC from inhibition by ExsE, and allows it to interrupt the ExsA-ExsD complex by binding ExsD (Urbanowski, Brutinel et al. 2007). Once ExsA is released from inhibition, it binds to promoter regions of type III secretion genes and increases their expression (Hovey and Frank 1995; Hueck 1998; Brutinel, Vakulskas et al. 2008; Hauser 2009).

Another important global regulatory mechanism that controls type III secretion transcription is the GacA/GacS pathway. This system activates expression of the regulatory RNA molecule *rsmZ*, a small regulatory RNA that binds to and inhibits RsmA (Heurlier, Williams et al. 2004). The RNA binding protein RsmA is involved gene expression at the post-transcriptional level and positively regulates type III secretion genes (Mulcahy, O'Callaghan et al. 2006; O'Grady, Mulcahy et al. 2006; Yahr and Wolfgang 2006). This system also utilizes two different sensors, RetS and LadS, which are thought to detect host cell carbohydrates (Laskowski, Osborn et al. 2004; Ventre, Goodman et al. 2006) . RetS induces type III secretion gene expression, while it inhibits biofilm production by inhibiting of expression of *rsmZ* (Goodman, Kulasekara et al.

2004; Laskowski, Osborn et al. 2004; Zolfaghar, Angus et al. 2005). LadS, on the other hand, performs the opposite activity of RetS and inhibits type III secretion gene expression (Ventre, Goodman et al. 2006).

The *P. aeruginosa* type III secretion system can also be regulated at the secretion level. Although not completely understood, this regulation is thought to be mainly dependent on binding of the effectors proteins to chaperones, which are adaptor proteins that maintain the effector molecules in an unfolded form in the bacterial cytoplasm, which may facilitate preservation of a favorable protein conformation for translocation until the effectors are injected into the host cell (Shen, Quenee et al. 2008; Hauser 2009). Chaperones are also thought to prevent premature interactions between the effector and other type III secretion system components (Hueck 1998; Shen, Quenee et al. 2008). Although the physiological stimulus for secretion is known to be host cell contact, the actual trigger is not completely understood. Recent studies have demonstrated that both albumin and casein can trigger secretion of type III secretion effectors, but the actual mechanism for this process remains elusive (Kim, Ahn et al. 2005). Laboratory conditions utilize depletion of Ca^{++} in the growth medium to induce type III secretion activity, but it is clear that lack of Ca^{++} is not sufficient to trigger the system since glutamate is also necessary for effector secretion. This suggests that the citric acid cycle also plays a key role in Type III secretion activity (Hauser 2009).

Once effectors are translocated into the host cell, their activity and function can be regulated in a multitude of ways. Degradation rate and localization of translocated effectors, as well as timing of delivery and post-translational modifications can affect the

modulation exerted by these proteins. Some bacteria, like *Salmonella spp.*, are known to differentially regulate type III secreted effectors by delivering them at different times and locations during the infection process. This is accomplished through the activation of two distinct Type III secretion systems. The SPI-1 type III secretion system translocates effectors across the host cell plasma membrane, while the SPI-2 system does it across the vacuolar membrane once the pathogen has been internalized. *Salmonella spp.* also control levels of effectors in the host tissues through differential degradation rates of these effectors. A particularly interesting example involves SopE and SptP; SopE is a guanine exchange factor (GEF) for small GTPases, while SptP has GTPase activing protein (GAP) activity (Hardt, Chen et al. 1998; Fu and Galan 1999; Stender, Friebel et al. 2000). Although similar amounts of SopE and SptP are translocated into the host cell during infection, their degradation rate differs considerably (Kubori and Galan 2003). SopE has a shorter half-life compared to SptP due to targeting of this effector to proteasomal degradation by an ubiquitin mediated process (Kubori and Galan 2003). This mechanism of temporal regulation appears to assist the pathogen by coordinately regulating the activity of two effectors with opposing activity on similar substrates. SopE activates Rho family GTPases, and causes significant changes on host actin cytoskeleton, which result in membrane ruffling and bacterial uptake. In contrast, the GAP activity of SptP inactivates Rho family GTPases, reversing the SopE-mediated changes in the host membrane and inhibiting further bacterial uptake (Hardt, Chen et al. 1998; Fu and Galan 1999; Stender, Friebel et al. 2000; Kubori and Galan 2003). This elegant mechanism of effector temporal regulation allows *Salmonella spp.* to coordinate activity of these

effectors so as to meet the pathogen's objectives at different points during the infection process, while translocating the virulence factors at the same time.

Ubiquitin mediated regulatory mechanisms in eukaryotic cells

The post-translational modification of proteins known as ubiquitination participates in a multitude of molecular networks in eukaryotic cells (Sun and Chen 2004; Gomez-Martin, Diaz-Zamudio et al. 2008; Finley 2009). These include pathways involving cell cycle progression, transcriptional regulation, and signal transduction among others (Angot, Vergunst et al. 2007). The ubiquitination process usually involves an ATP-dependent protein modification where a C-terminal glycine residue of an ubiquitin molecule is covalently linked to a side chain of a lysine residue in the target protein (Sun and Chen 2004; Newton, Matsumoto et al. 2008). This alteration can lead to significant changes in the function, localization, or stability of the substrate protein (Gomez-Martin, Diaz-Zamudio et al. 2008).

Three sets of proteins are responsible for carrying out the ubiquitination of target substrates. These include a single ubiquitin-activating enzyme (E1), a few ubiquitin-conjugating enzymes (E2), and many ubiquitin ligases (E3) (Sun and Chen 2004; Schmidt and Dikic 2005; Finley 2009). E3 ubiquitin ligases provide the specificity for ubiquitination targets, and can be classified into two broad groups: (a) Homologous to the E6 associated protein C terminus (HECT), and (b) Really Interesting New Gene (RING)

ubiquitin ligases (Gomez-Martin, Diaz-Zamudio et al. 2008). These groups differ mainly on the fact that RING ubiquitin ligases lack a catalytic domain. Unlike the HECT ubiquitin ligases, RING ligases do not participate directly on the transfer of the ubiquitin molecule, instead they facilitate the interactions of the E2-ubiquitin complex with the target protein (Gomez-Martin, Diaz-Zamudio et al. 2008).

The fate of the ubiquitinated protein depends on the molecular pattern in which the attached ubiquitin molecules are arranged by the ubiquitination machinery (Gomez-Martin, Diaz-Zamudio et al. 2008). This pattern can include the addition of (a) a single ubiquitin molecule at a particular site (monoubiquination), (b) a single ubiquitin molecules at multiple sites within the substrate, and (c) chains of multiple covalently bound ubiquitin molecules attached at a single site or multiple sites of the target protein (polyubiquination). Monoubiquitination of single or multiple sites of the target protein usually modifies protein activity by a proteasome-independent manner (Sun and Chen 2004). Formation of polyubiquitin chains requires the binding of one of the seven lysines residues in the ubiquitin molecule to the C-terminal glycine of another ubiquitin (Sun and Chen 2004; Newton, Matsumoto et al. 2008). The best known forms of polyubiquitination involve the conjugation of ubiquitin molecules to the residues at position K48 and K63 within another ubiquitin. K48 polyubiquitination pattern usually targets the protein substrate for proteasomal dependent degradation, whereas K63 linkage tends to modulate protein activity in a proteasome-independent manner (Sun and Chen 2004; Newton, Matsumoto et al. 2008).

Ubiquitination as a mechanism for regulating type III secreted effector activity

Recent studies have demonstrated that the ubiquitin-proteasome system plays a key role in host defense by targeting bacterial virulence factors that reach the host cytoplasm to proteasomal degradation (Kubori and Galan 2003; Balachandran, Dragone et al. 2007; Hentschke, Trulzsch et al. 2007). Some pathogens, on the other hand, have evolved the capacity to manipulate this protein modification process to their own advantage (Zhang, Higashide et al. 2005; Angot, Vergunst et al. 2007; Patel, Hueffer et al. 2009). For instance, *Salmonella spp.* have evolved the ability to exploit the host ubiquitin machinery in order to increase its survival within the cell. As previously described, *Salmonella spp.* utilize the ubiquitin proteasome system to carefully regulate SopE effector levels in the host cell, in order to manipulate host cytoskeleton rearrangement (Kubori and Galan 2003). This bacterium also appears to utilize the ubiquitin machinery to control the activity of SopA, whose ubiquitination is postulated to lead to bacterial escape from *Salmonella*-containing vacuoles into the host cytosol, where the bacterium can replicate more rapidly (Zhang, Higashide et al. 2005). Like *Salmonella* SopE and *P. aeruginosa* ExoT, stability of the type III secreted effector YopE from *Yersinia spp.* is also regulated in an ubiquitin-proteasome dependent manner in the host cytosol (Hentschke, Trulzsch et al. 2007). Interestingly, different serogroups of *Yersinia* carry unique YopE variants, which are not degraded at the same rate (Hentschke,

Trulzsch et al. 2007). Sequence analysis of these YopE variants demonstrated that two lysine residues at positions 62 and 75, appear to regulate YopE degradation (Hentschke, Trulzsch et al. 2007). Biochemical analysis demonstrated that these two lysine residues are ubiquitin acceptor sites, which destabilize the effector protein upon modification (Hentschke, Trulzsch et al. 2007). Nevertheless, a recent study showed that increased stability of YopE did not correlate with increase dissemination of the bacterium in an animal model (Gaus, Hentschke et al.). Since the GAP activity of YopE affects the delivery of Yops through the translocation pore into the host cell, these results suggest that *Yersinia* may utilize the ubiquitin proteasome system to regulate the post-translocation levels of YopE, which in turn may affect translocation of other Yops into the host (Gaus, Hentschke et al. ; Mejia, Bliska et al. 2008).

P. aeruginosa ExoU is also ubiquitinated upon translocation into the host cytoplasm (Stirling, Cuzick et al. 2006). Two ubiquitin molecules are added to lysine 178, but the degradation rate of the toxin is only modestly increased following this post-translational modification. Furthermore, ubiquitination at position 178 does not appear to be responsible for determining intracellular localization of ExoU, but it may be the result of trafficking of the toxin to the plasma membrane (Stirling, Cuzick et al. 2006). Thus, the biological significance of ExoU ubiquination remains to be elucidated.

Regulation of ExoT-mediated P. aeruginosa virulence by the host ubiquitin ligase Cbl-b

As previously described, ExoT has been shown to contribute to *P. aeruginosa* virulence in several animal models of disease (Garrity-Ryan, Kazmierczak et al. 2000; Shaver and Hauser 2004; Vance, Rietsch et al. 2005; Balachandran, Dragone et al. 2007). Our laboratory has also shown that ExoT is degraded in a proteasomal dependent manner in the host cytosol (Balachandran, Dragone et al. 2007). ExoT, Crk (the binding target of the ExoT (ADPRT) domain), and the E3 ubiquitin ligase Cbl-b, interact in a complex in the host cell cytoplasm. Cbl-b was shown to enhance the ubiquitination and proteasome-mediated degradation of ExoT (Balachandran, Dragone et al. 2007). This multidomain RING E3 ubiquitin ligase, which belongs to the Cbl family of proteins, has been shown to play an important role in the regulation of cell surface receptor activity, phosphatidylinositol 3-kinase signaling, and allergic and inflammatory reactions in mast cells (Schmidt and Dikic 2005; Gustin, Thien et al. 2006; Bachmaier, Toya et al. 2007; Oksvold, Dagger et al. 2008). Unlike *Salmonella* SopE downregulation, ExoT degradation appears to benefit the host instead of the invading pathogen, since depletion of Cbl-b increases the half-life of the toxin, and complete absence of Cbl-b increases the susceptibility of mice to colonization by ExoT producing *P. aeruginosa* in an intranasal infection pneumonia model (Balachandran, Dragone et al. 2007). Interestingly, although all the evidence points to Cbl-b mediated ubiquitination of ExoT as a requirement for

host protection, it is still unclear whether the ubiquitin ligase activity of Cbl-b is necessary for limiting ExoT mediated *P. aeruginosa* virulence *in vivo*. Indeed, a previous study has shown that ubiquitin ligase-independent activity of Cbl-b is sufficient for regulating antigen-induced cytokine gene expression, suggesting that other domains in this protein are important for its activity (Qu, Sada et al. 2004).

Conclusion

The Type III secretion of *P. aeruginosa* is an essential component of the virulence mechanisms of this organism, especially during acute infections. Studies of this apparatus and its effectors have already elucidated a large amount of information about *P. aeruginosa* strategies for colonization and persistence in host tissues. As the field moves forward, it is clear that further understanding of the virulence mechanisms of this apparatus and its effectors has the potential to further shed light into the pathogenesis of this organism and perhaps demonstrate possible ways to combat the detrimental effects of infections caused by *P. aeruginosa*.

CHAPTER 2

Identification of *Pseudomonas aeruginosa* ExoT amino acid residues necessary for its proteasomal dependent degradation in the host cytoplasm

INTRODUCTION

Pseudomonas aeruginosa is one of the most virulent opportunistic pathogens in humans (reviewed in (Mandell, Bennett et al. 2010)). Patients with some degree of immunocompromise and/or epithelial barrier injury, such as those with severe burns or surgical incisions, on mechanical ventilation, or receiving cytotoxic chemotherapy, are particularly vulnerable to acute infection. This Gram-negative bacterium is also the leading cause of chronic pulmonary infections and death in cystic fibrosis patients (Mandell, Bennett et al. 2010). Even with appropriate medical treatment, mortality remains high and antibiotic resistance is increasingly common (Malangoni, Crafton et al. 1994; Fujitani, Sun et al.).

Many evolutionary adaptations have allowed *P. aeruginosa* to manipulate host defenses, facilitating its pathogenesis in a susceptible environment (Lau, Hassett et al. 2005). One of these adaptations is the ability to deliver bacterial effectors directly into the cytoplasmic compartment of the host cell through a highly conserved needle-like macromolecular structure known as the type III secretion system (TTSS) (Hauser 2008). The discovery of a clear correlation between the presence of an active TTSS and severity of human infections suggests that this secretion apparatus and its components play a key role in the morbidity and mortality of *P. aeruginosa* infections (Roy-Burman, Savel et al. 2001; Hauser, Cobb et al. 2002).

Four type III-secreted toxins have been identified in this bacterium: ExoS, ExoT, ExoU and ExoY, although almost no isolates examined thus far encodes all four effectors (reviewed in (Engel and Balachandran 2009; Hauser 2009)). ExoS and ExoT are 76% identical and encode an N-terminal GTPase activating protein (GAP) domain and a C-terminal ADP ribosyltransferase (ADPRT) domain. The GAP domains of ExoS and ExoT both target the small GTPases Rho, Rac, and Cdc42 (Kazmierczak and Engel 2002; Barbieri and Sun 2004). In contrast, the substrate specificities of the ExoS and ExoT C-terminal ADPRT domains are distinct. The ExoS ADPRT domain targets cytoskeletal regulators, such as Ras, Ral, several Rabs, Cdc42, ERM family proteins, and ExoS itself (Barbieri and Sun 2004; Sun, Maresso et al. 2004). The ExoT ADPRT domain interacts with Crk family proteins and phosphoglycerate kinase-1 (Sun and Barbieri 2003; Deng, Sun et al. 2005). Together, ExoS and ExoT modulate many processes that involve the actin cytoskeleton. These include disruption of cell-cell and cell-substrate contacts that

are key to maintaining the integrity of the mucosal barrier, inhibition of wound repair by affecting cell migration and cell proliferation, disruption of signaling pathways, particularly those that regulate the innate immune response, and modulation of bacterial internalization by epithelial cells (reviewed in (Engel and Balachandran 2009)). ExoT is thought to be particularly important in the pathogenesis of *P. aeruginosa* infections, since almost all strains encode this type III secreted effector, and isogenic mutants lacking ExoT showed reduced dissemination and virulence, notably in the context of dissemination of bacteria to distant sites (Garrity-Ryan, Kazmierczak et al. 2000; Shaver and Hauser 2004; Lee, Smith et al. 2005; Vance, Rietsch et al. 2005).

Although the activity of many bacterial type III secreted effectors has been extensively studied, little is known about the regulation of these effectors after translocation into the host cell cytoplasm. There are many points at which regulation could be exerted, including post-translocation modification, localization and degradation. Indeed, recent studies have demonstrated that the ubiquitin-proteasome system plays an important role in host defense by targeting translocated bacterial virulence factors to proteasomal dependent degradation (Kubori and Galan 2003; Balachandran, Dragone et al. 2007; Hentschke, Trulzsch et al. 2007). Protein ubiquitination involves the action of three sets of proteins which include an ubiquitin-activating enzyme (E1), a few ubiquitin-conjugating enzymes (E2) and many ubiquitin ligases (E3) (Schmidt and Dikic 2005; Finley 2009). Target specificity is provided by the E3 ubiquitin ligases, which can be divided into two broad groups: (a) Homologous to the E6 associated protein C terminus (HECT), and (b) Really Interesting New Gene (RING) ubiquitin ligases (Gomez-Martin,

Diaz-Zamudio et al. 2008). These groups differ mainly on the fact that RING ubiquitin ligases lack a catalytic domain. Unlike the HECT ubiquitin ligases, RING ligases do not participate directly on the transfer of the ubiquitin molecule, but instead facilitate the interactions between the E2-ubiquitin complex and the target protein (Gomez-Martin, Diaz-Zamudio et al. 2008).

We have previously reported that Cbl-b, which binds to Crk, polyubiquinates ExoT, leading to its proteasome-dependent degradation (Balachandran, Dragone et al. 2007). Within 5 minutes of infection with *P. aeruginosa*, Cbl-b, not c-Cbl, is phosphorylated in the host cytoplasm. ExoT interacts with both Cbl-b and Crk, and its degradation is dependent on the ubiquitin ligase activity of Cbl-b, as overexpression of a RING-finger mutant of Cbl-b inhibits ExoT degradation and decreases its ubiquitination. Depletion of Cbl-b or of Crk also increases the half-life of ExoT, further confirming the involvement of this complex formation in the degradation of the toxin. ExoS degradation was shown to be much slower than that of ExoT, and a chimeric protein containing the ExoT GAP domain and the ExoS ADPRT domain was degraded with kinetics similar to ExoS, suggesting that the GAP domain of ExoT does not contain the determinants necessary for Cbl-b mediated degradation. We demonstrated the functional significance of these *in vitro* findings by showing that *cbl-b*$^{-/-}$ mice were more susceptible than wild type mice to lung colonization and dissemination by ExoT producing *P. aeruginosa.* In contrast, no difference in susceptibility was observed between wild type and *cbl-b*$^{-/-}$ mice upon infection with an isogenic mutant strain in which ExoT was deleted. Although Cbl-b has subsequently been shown to regulate inflammatory responses through the

downregulation of TLR4 signaling (Bachmaier, Toya et al. 2007), our *in vivo* animal results demonstrated that the virulence of a strain of *P. aeruginosa* in which ExoT was deleted was similar in wild type mice and *cbl-b*$^{-/-}$ mice. Thus, the effect of Cbl-b on *P. aeruginosa* virulence is ExoT specific, rather than a general influence on TLR-mediated inflammatory responses.

Although our results clearly demonstrate that Cbl-b-mediated ubiquination regulates ExoT stability, it is possible that other activities of Cbl-b could impact *P. aeruginosa* infection *in vivo*. For example, Cbl-b could also affect bacterial internalization, through its known influence on the actin cytoskeleton (Schmidt and Dikic 2005) or recruitment of neutrophils into the infected tissues. Indeed, Fcγ-receptor mediated phagocytosis in macrophages and neutrophil infiltration into LPS treated tissues is abnormal in the absence of Cbl-b (Bachmaier, Toya et al. 2007; Dale, Traum et al. 2009). In this study, we compared the internalization of *P. aeruginosa* or an isogenic mutant lacking ExoT in wild type and *cbl-b*$^{-/-}$ bone marrow derived macrophages, and found that Cbl-b did not affect bacterial uptake. Likewise, neutrophil infiltration into *P. aeruginosa* infected lung tissues was indistinguishable between wild type and *cbl-b*$^{-/-}$ mice. However, we identified two lysines in the N-terminus of ExoT that are crucial determinants of its Cbl-b dependent virulence *in vivo* and of its stability upon translocation into host cells. Together, these studies extend our understanding of how a host ubiquitin ligase mediates the effect of the type III secreted toxin.

RESULTS

Cbl-b is not required for phagocytosis of ExoT producing P. aeruginosa in bone marrow-derived macrophages (BMDMs)

To test whether Cbl-b plays a role in macrophage-mediated phagocytosis of *P. aeruginosa*, we infected wild type and *cbl-b*$^{-/-}$ BMDMs with *P. aeruginosa*. For these experiments, we used two isogenic mutants of PA103, both of which have a functional Type III secretion and translocation apparatus but that lack the potent cytotoxin ExoU: PA103ΔU, which contains an in-frame deletion in the type III secreted phospholipase ExoU, but produces and translocates ExoT; and PA103ΔUΔT, which contains a deletion in ExoT and does not produce any known type III secreted effectors. Standard aminoglycoside protection assays were performed to quantify invasion, in which the bacteria were co-cultivated with the macrophages for 2 hrs, extracellular bacteria were removed and killed by washing and addition of amikacin for 1 hr, and intracellular bacteria were enumerated upon host cell lysis. Consistent with our previously published results (Garrity-Ryan, Kazmierczak et al. 2000; Garrity-Ryan, Shafikhani et al. 2004), bacterial internalization was enhanced ~ 3-fold in the absence of ExoT (Fig. 1, $p < 0.05$; compare PA103ΔU to PA103ΔUΔT). Similar results were observed in *cbl-b*$^{-/-}$ BMDMs (Fig. 1). Importantly, the absolute levels of bacterial internalization were similar in wild

type and *cbl-b*$^{-/-}$ BMDMs. These results suggest that Cbl-b does not regulate *P. aeruginosa* internalization under the conditions of our experiments.

Cbl-b is not necessary for neutrophil infiltration into lung tissue after P. aeruginosa infection

Previous studies have demonstrated that neutrophil infiltration into LPS treated lungs is increased in the absence of Cbl-b, leading to increased inflammation in the infected tissues (Bachmaier, Toya et al. 2007). To test whether *cbl-b*$^{-/-}$ mice exhibit abnormal innate immune cell recruitment during *P. aeruginsa* infection, we quantified lung neutrophil recruitment by measuring myeloperoxidase activity in lung homogenates obtained from wild type and *cbl-b*$^{-/-}$ mice intranasally infected with PA103ΔUΔT expressing HA-tagged ExoT under control of its native promoter (PA103ΔUΔT::CTX-*exoT-HA*). In control experiments, we verified that the presence of the HA-tag did not affect ExoT virulence *in vivo* (data not shown). We found no statistical difference in bacterial lung colonization at 2 or 4 hours post infection (hpi) between wild type and *cbl-b*$^{-/-}$ mice (Fig. 2A). While neutrophil recruitment slightly increased between 2 and 4 hpi, no statistical significant difference was observed between wild type and *cbl-b*$^{-/-}$ mice at either time point (Fig. 2B). We conclude that Cbl-b is not required for lung neutrophil recruitment at early times post infection in the context of *P. aeruginosa* lung infection.

Lysine residues at position 49 and 74 in ExoT control its degradation rate in the host cytoplasm

Our results thus far suggest that Cbl-b-mediated degradation of ExoT may be important in controlling *P. aeruginosa* virulence *in vivo*. To further test this notion, we sought to identify the lysine residues in ExoT that control its degradation in the host cytoplasm. Our previous studies suggested that ADPRT activity of ExoT enhances its degradation in the host cell cytoplasm, whereas the GAP domain did not influence its degradation. Therefore, we initially focused on the lysine residues in the ADPRT domain (Balachandran, Dragone et al. 2007). All of the 7 lysine residues in the ADPRT domain were changed to an arginine (to preserve charge) and the mutated ExoT gene, including its native promoter, was C-terminally fused to an HA tag. All mutant constructs were integrated into the chromosome at the CTX site in PA103ΔUΔT (Fig 3A). The presence of the HA tag facilitated detection of the protein in host cell lysates. The half-life of these ExoT mutant toxins in the host cell cytoplasm was determined by a pulse-chase experiment (see materials and methods and (Balachandran, Dragone et al. 2007)). Briefly, the bacteria were co-cultivated with host cells for 1.5 hrs, removed by washing, and inhibitors of bacterial protein synthesis added. The steady state levels of ExoT in host cell lysates was determined by immunoblotting with an antibody to HA at various times after removal of the bacteria and addition of antibiotics. Figure 3B shows that ExoT-HA is degraded in a proteasomal dependent manner in L929 cells, albeit with a somewhat slower time course than what was observed in HeLa cells (Balachandran,

Dragone et al. 2007). Mutation of the 7 lysine residues in the ADPRT domain did not affect ExoT half-life (data not shown). We therefore changed the two remaining lysines outside of the GAP domain, lysine 49, located in the predicted chaperone binding domain and lysine 74, located in the region of the protein with homology to the membrane localization domain of ExoS, to arginine (Fig 3A). Mutation of both of these lysine residues in conjunction with mutations of all of the lysine residues in the ADPRT domain increased the half-life of translocated ExoT (ExoT(K49R, K74R+ADPRTΔlys)-HA; Fig 3C). We then tested the role of lysine 49 and 74 individually or in combination to determine whether they were necessary or sufficient to regulate ExoT degradation. As shown in Figure 3C, mutation of lysine 74 or lysine 49 had no effect on ExoT half-life, whereas mutation of both residues increased the stability of the protein, although not quite to the same degree as mutation of all 9 lysines. We thus conclude that lysines 49 and 74 are critical determinants of ExoT stability upon translocation into the host cell.

K49R mutation in ExoT is sufficient to enhance toxin secretion, but not translocation

We noted in our translocation assays that at early time points, the levels of ExoT(K49R, K74R)-HA were greater than wild type ExoT-HA. While this observation could reflect the decreased degradation of ExoT (K49R, K74R)-HA, we specifically tested whether production, secretion, or translocation of ExoT was affected. Overnight

cultures of bacteria were diluted into media that induces type III secretion (MinS-calcium), and cell associated and secreted ExoT-HA were measured hourly by immunoblot analysis. As shown in Figure 4A, the levels of cell associated toxin were similar between wild type ExoT-HA and ExoT (K49R, K74R)-HA. Surprisingly, however, the double lysine mutant was secreted at greater levels than the wild type protein. We repeated the experiment with the individual lysine mutants, ExoT (K49R)-HA and ExoT (K74R)-HA, and found that increased secretion was only dependent upon the K49R mutation (Fig. 4A). To test whether decreased degradation or increased secretion of the toxin was responsible for the higher levels of ExoT (K49R, K74R)-HA compared to wild type ExoT-HA in the host cytoplasm, we measured ExoT-HA levels at early time points after translocation. As shown in Figure 4B, only levels of ExoT (K49R, K74R)-HA differed significantly compared to wild type toxin levels. Thus, while altering lysine 49 increases ExoT secretion, both lysines must be altered to increase its levels in the host cells. We thus conclude that it is unlikely that the enhanced secretion of ExoT (K49R, K74R)-HA is sufficient to explain its decreased degradation rate in host cells.

ExoT(K49R, K74R)-HA *induces more rapid and sustained changes in host cell morphology*

ExoT is a potent cytotoxin that leads to extensive inhibition of cytoskeletal rearrangement in host cells (Garrity-Ryan, Shafikhani et al. 2004). To determine whether

the lysine mutations affected ExoT activity or had physiological consequences at the cellular level, we assessed the effect of wild type ExoT-HA or ExoT (K49R, K74R)-HA on host cell morphology by light microscopy. *P. aeruginosa* expressing ExoT (K49R, K74R)-HA caused a more rapid increase in cell rounding compared to a strain expressing wild type ExoT-HA (Fig. 5A), although at later time points, cell rounding and detachment were observed for both strains (data not shown).

Previous studies have demonstrated that the cytoskeletal changes induced by the GAP activity of ExoT are rapid and reversible whereas those induced by ADPRT ribosylation of Crk occur over a slower time course and are not reversible (Garrity-Ryan, Shafikhani et al. 2004; Sundin, Hallberg et al. 2004; Shafikhani and Engel 2006). We compared the rate of recovery of L929 cells infected with *P. aeruginosa* expressing the wild type ExoT-HA or the ExoT (K49R, K74R)-HA mutant toxin. As shown in Figure 5B, host cells infected with *P. aeruginosa* expressing wild type ExoT-HA demonstrated normal cell morphology at 72hrs after removal of bacteria, whereas cells infected with *P. aeruginosa* expressing ExoT(K49R, K74R)-HA were still rounded. Together, these results suggest that the ExoT (K49R, K74R)-HA protein retains its cell rounding activity and causes prolonged changes in the host cell cytoskeleton, at least in part due to its decreased degradation compared to wild type toxin. Our results indirectly imply that mutation of lysines 49 and 74 do not affect the ADPRT or GAP activity of ExoT towards Crk,

Expression of ExoT(K49R, K74R)-HA *leads to increased colonization of the lung in wild type, but not cbl-b$^{-/-}$ mice*

Our results predict that *P. aeruginosa* expressing ExoT (K49R, K74R)-HA should exhibit increased virulence *in vivo*. Moreover, if the lysines at position 49 and 74 are targets of Cbl-b-mediated polyubiquitination followed by proteosome-mediated degradation, then the enhanced virulence of ExoT(K49R, K74R)-HA should be Cbl-b independent. To test this hypothesis, wild-type or *cbl-b*$^{-/-}$ mice were infected with *P. aeruginosa* expressing ExoT-HA or ExoT (K49R, K74R)-HA, and at 18 hpi, lung colonization was quantified. *P. aeruginosa* expressing ExoT(K49R, K74R)-HA demonstrated ~10-fold more colonization compared to infection with bacteria expressing wild type ExoT-HA (Fig 6A, $p < 0.05$). In contrast, in *cbl-b*$^{-/-}$ mice, there was no difference in the lung colonization between the two strains (Fig 6B). Together, these results demonstrate that lysine 49 and 74 play a key role in ExoT mediated virulence *in vivo* and that their role is dependent upon Cbl-b. The simplest interpretation of these results is that ExoT(K49R, K74R)-HA is insensitive to Cbl-b-mediated ubiquitination and subsequent degradation *in vivo*.

DISCUSSION

Successful pathogenesis involves the finely tuned interplay between microbes and their hosts. The pathogen must obtain nutrients, establish a protected niche, evade the host immune system, proliferate, and disseminate. Premature death of the host can be deleterious to the pathogen. An emerging theme in host-pathogen interactions is regulation of secreted effectors by host ubiquination. We have previously shown that the levels of translocated *P. aeruginosa* type III secreted effector ExoT, which uses failsafe and redundant mechanisms to disrupt the actin cytoskeleton, inhibit host cell division and apoptosis, and to inhibit wound healing, is regulated by ubquination. The ADPRT domain of ExoT targets the adaptor protein Crk, which in turn recruits Cbl-b, leading to ExoT polyubiquination and proteosome-mediated degradation. Cbl-b mediated degradation of ExoT appeared to be important *in vivo* because *cbl-b*$^{-/-}$ mice were more sensitive than wild type mice to infection by ExoT-producing *P. aeruginosa*, whereas no difference was observed between *cbl-b*$^{-/-}$ mice and wild type mice upon infection with an isogenic ExoT mutant. While these studies were consistent with the notion that Cbl-b mediated ubiquination of ExoT and subsequent destruction were important *in vivo*, we now demonstrate that Cbl-b is not necessary for bacterial uptake into BMDMs or for neutrophil infiltration into *P. aeruginosa* infected lung tissue. Furthermore, we identify two lysine residues in ExoT that are required for proteosome-mediated degradation in cell based assays, and that also regulate susceptibility of mice to acute lung infections in a

Cbl-b dependent manner. Since the innate immune cell functions tested in this study are intact in the absence of Cbl-b, together these studies are consistent with the model that Cbl-b limits ExoT mediated *P. aeruginosa* virulence by targeting the toxin for degradation.

Our results are distinct from a recent report that Cbl-b regulates TLR4-mediated acute inflammatory responses induced by sepsis (Bachmaier, Toya et al. 2007). Although that study demonstrated that genetic deletion of Cbl-b increased sepsis-induced inflammation and mortality upon polymicrobial infection, our results demonstrate that absence of Cbl-b does not render the host more susceptible to *P. aeruginosa* infection. Rather, our results were ExoT-specific.

We initially expected the lysine residues in the ADPRT domain of ExoT to be the targets of ubiquitination, since eliminating the catalytic activity of the ADPRT domain increases its half-life. Surprisingly, two lysines in the N-terminus, preceding the GAP and ADRPT domains, appeared to be critical. Lysine 49 is located in the predicted chaperone binding domain. Although mutation from lysine to arginine would not be expected to affect chaperone binding, we found that altering lysine 49 actually increased type III-mediated secretion of the protein in the media in the absence of host cells. However, we did not find evidence that altering only lysine 49 significantly affected translocation levels of the toxin or subsequent proteasome-mediated degradation of ExoT. Lysine 74 is located in a region with homology to the membrane localization domain of ExoS. Whether this domain plays a role in ExoT function is not known. We predict that lysines 49 and 74 are targets of Cbl-b mediated ubiquitination, and experiments are ongoing to

test this hypothesis. Although lysines 49 and 74 are also found in the N-terminus of ExoS, ExoS is not subject to Cbl-b mediated degradation, likely because its ADPRT domain does not recruit Crk and hence not Cbl-b.

Interestingly, *Yersinia* YopE from serogroup O9, a type III secreted effector with similar GAP activity towards small GTPases as *P. aeruginosa* ExoS and ExoT, evades proteasomal dependent degradation and causes greater cytotoxic effects at the cellular level compared to YopE from *Yersinia* serogroup O8, which undergoes ubiquitin mediated degradation (Hentschke, Trulzsch et al. 2007). Upon amino acid sequence analysis, it was determined that, unlike YopE-O8, YopE from serogroup O9 did not contain two lysine residues at position 62 and 75 (Hentschke, Trulzsch et al. 2007). These residues serve as polyubiquitination acceptor sites in YopE-O8, and were subsequently shown to destabilize the effector protein in the host cell cytoplasm (Hentschke, Trulzsch et al. 2007). Interestingly, a recent study showed that greater stability of YopE in cultured cells did not correlate with increased dissemination in mouse infection model (Gaus, Hentschke et al.). This finding could be explained by the ability of YopE to regulate delivery of other Yops into the host (Mejia, Bliska et al. 2008). *Yersinia* may utilize the ubiquitin proteasome system to downregulate this effector, so as to allow increased delivery of other virulence factors into the host tissues. These reports provide further evidence that changes in lysine residues can affect translocated toxin stability and activity, and show the importance of proteasomal dependent degradation in host defense against type III secreted factors.

Another example of orchestrating the activies of type III secreted effectors involves *Salmonella* SptP and SopE. Although similar amounts of SopE and SptP are translocated into the host cell during infection, their degradation rate differs considerably (Kubori and Galan 2003). SopE has a shorter half-life compared to SptP due to targeting of this effector to proteasomal degradation by an ubiquitin mediated process (Kubori and Galan 2003). This mechanism of temporal regulation appears to assist the pathogen by coordinately regulating the activity of two effectors with opposing activity on similar substrates. SopE causes significant changes on host actin cytoskeleton, which result in membrane ruffling and bacterial uptake by activating Rho family GTPases. In contrast, SptP inactivates Rho family GTPases, which leads to inhibition of bacterial uptake (Hardt, Chen et al. 1998; Fu and Galan 1999; Stender, Friebel et al. 2000; Kubori and Galan 2003). This elegant mechanism of effector temporal regulation allows *Salmonella spp.* to closely coordinate the activity of these effectors during the infection process.

Using a variety of bioinformatic analyses, we were not able to identify any common motifs surrounding the lysine residues targeted by ubiquitination in ExoT, SopE, and YopE. Whether ubiquitination of ExoT affects the ability of *P. aeruginosa* to deliver additional type III secreted toxins through the translocation pore remains to be determined, as our studies were all carried out in a strain that lacks additional secreted effectors.

Collectively, the results of this study demonstrate that ExoT lysine residues at position 49 and 74 play a key role in toxin degradation by the host proteasome, which leads to alteration of ExoT-mediated virulence of *P. aeruginosa*, and that Cbl-b may

target these residues through either ubiquitination or another yet unidentified molecular process. Experiments are underway to determine whether Cbl-b is able to polyubiquitinate the mutant toxin as it does on wild type ExoT.

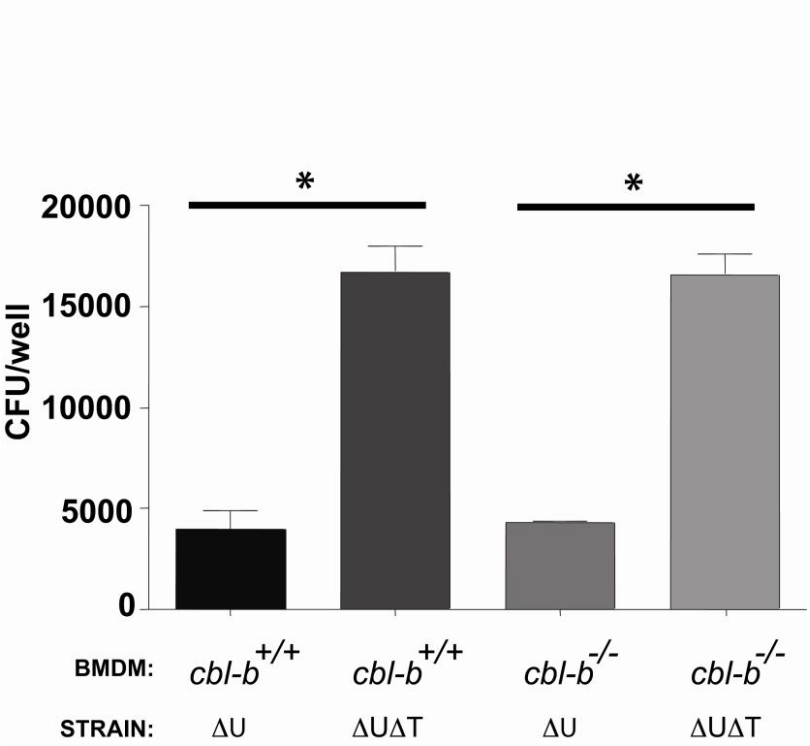
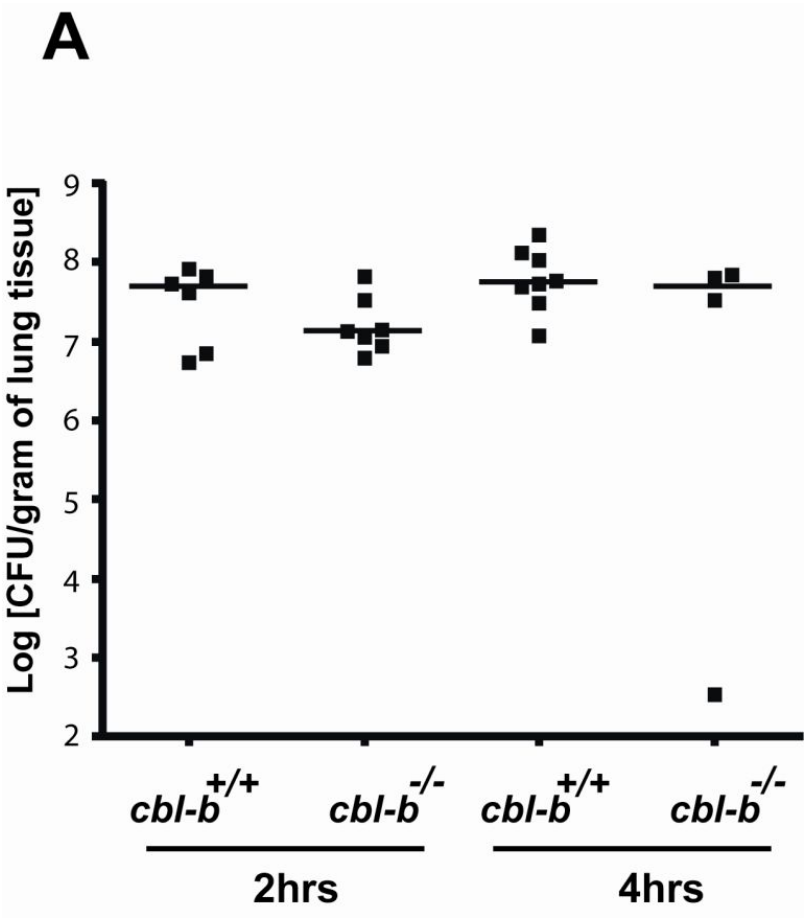


Figure 1. Phagocytosis of *P. aeruginosa* is similar in *cbl-b*$^{+/+}$ and *cbl-b*$^{-/-}$ BMDMs. BMDMs were infected with PA103ΔU or PA103ΔUΔT for 2 hrs. Bacteria were removed by washing and new medium with antibiotics was added for 1 hr to kill the remaining extracellular bacteria. BMDMs were lysed, and released bacteria were enumerated by plating. Phagocytosis assays were done in triplicate and error bars indicate standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA with Tukey post test. * $p < 0.05$.


A
Log [CFU/gram of lung tissue]
9
8
7
6
5
4
3
2
cbl-b+/+
cbl-b-/-
cbl-b+/+
cbl-b-/-
2hrs
4hrs

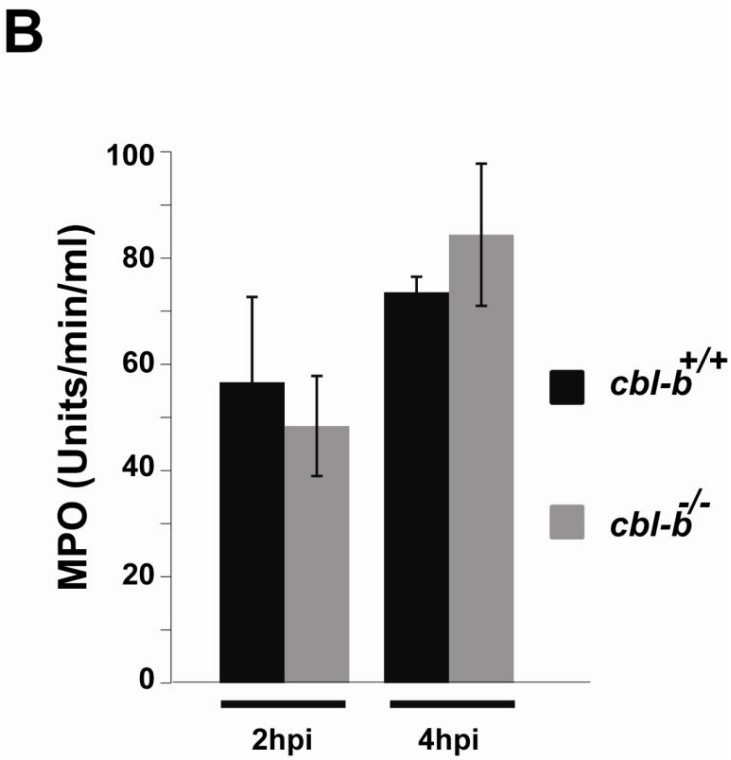

B
MPO (Units/min/ml)
100
80
60
40
20
0
2hpi
4hpi
cbl-b+/+
cbl-b-/-

Figure 2. Early neutrophil infiltration into infected lung tissue is not affected by the absence of Cbl-b. C57BL/6 (*cbl-b*$^{+/+}$) and C57BL/6 (*cbl-b*$^{-/-}$) mice were infected intranasally with PA103ΔU::CTX-*exoT-HA* at 5x10^{6} CFU/mouse and the lungs were harvested at 2 hpi and 4 hpi. **(A)** CFUs were measured from the left lung of each mouse. The medians from each condition were not statistically different ($P > 0.05$, 2-tailed Mann-Whitney *U* test). **(B)** Neutrophil migration, as assessed by the myeloperoxidase (MPO) assay, was measured in the right lung from each mouse . Error bars indicate SEM. ($P > 0.05$, one-way ANOVA)

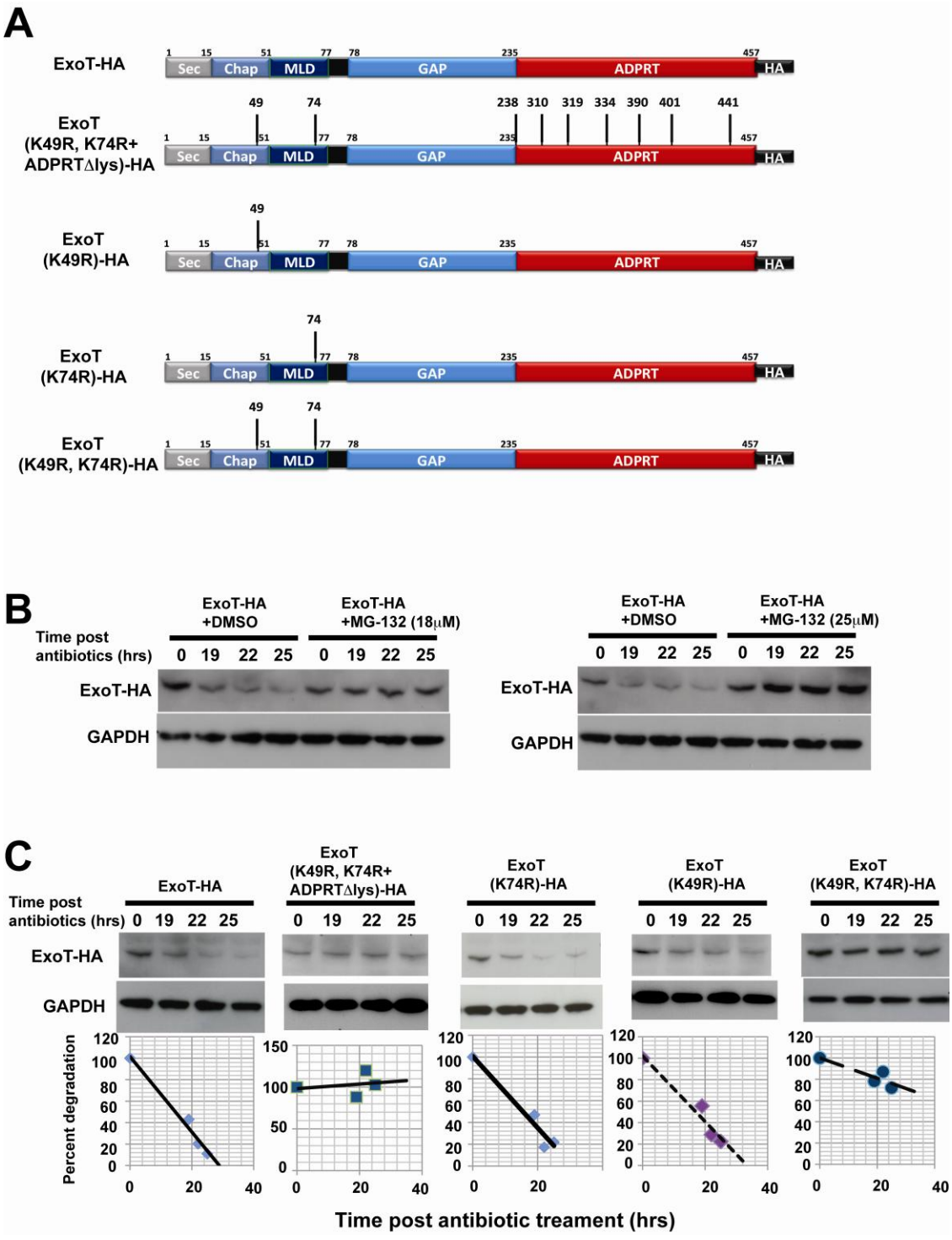
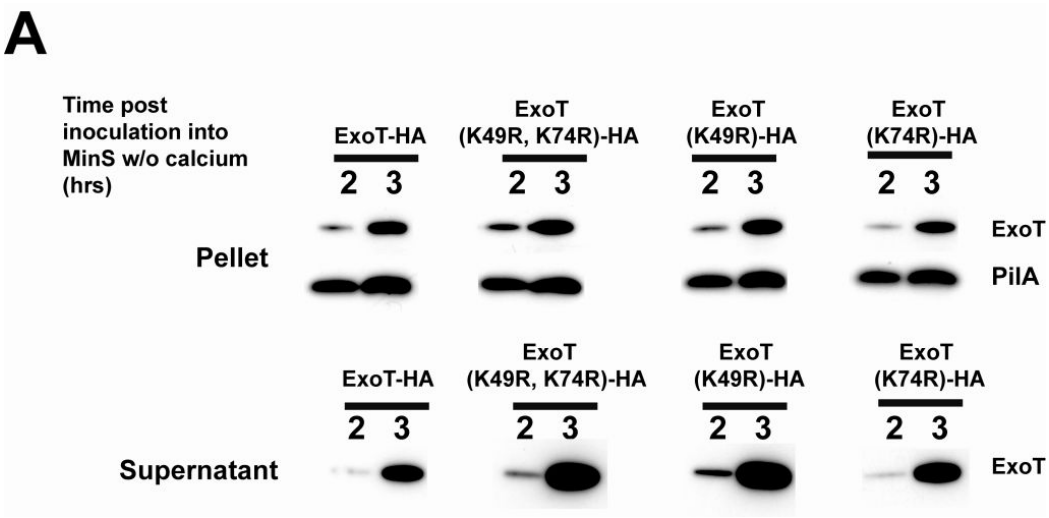

A
ExoT-HA
Sec
Chap
MLD
GAP
ADPRT
HA
ExoT (K49R, K74R+ ADPRTΔlys)-HA
ExoT (K49R)-HA
ExoT (K74R)-HA
ExoT (K49R, K74R)-HA
B
ExoT-HA +DMSO
ExoT-HA +MG-132 (18μM)
ExoT-HA +MG-132 (25μM)
Time post antibiotics (hrs)
0 19 22 25
ExoT-HA
GAPDH
C
ExoT (K49R, K74R+ ADPRTΔlys)-HA
ExoT (K74R)-HA
ExoT (K49R)-HA
ExoT (K49R, K74R)-HA
Percent degradation
Time post antibiotic treament (hrs)

Figure 3. K49 and K74 residues in ExoT are critical determinants for proteasomal-dependent degradation of the toxin in the host cytoplasm. (A) Schematic representation of ExoT and ExoT lysine mutants created in this study. The indicated lysine residues were changed to arginine. All constructs were C-terminally fused to HA and were integrated into the PA103ΔUΔT chromosome at the *CTX* integration site and are under the control of the native *exoT* promoter. (B) **ExoT is degraded in a proteasomal-dependent manner in L929 cells**. L929 were co-cultivated with PA103ΔUΔT::CTX *exoT-HA* (MOI=200) for 1.5 hrs. Bacteria were removed by washing, and new medium with antibiotics was added. At the indicated times, cytoplasmic extracts were prepared and translocated ExoT levels were determined by immunoblotting with antibody to HA. To control for loading efficiency, the blots were probed with anti-GAPDH mAbs. Where indicated, the proteasomal inhibitor MG-132 (18μM or 25μM final concentration) was present throughout the assay to inhibit proteasomal dependent degradation. (C) **Lysine residues at position 49 and 74 control the stability of translocated ExoT**. The levels of translocated ExoT were determined as in (3B). **(D)** The rate of degradation of ExoT was quantified by normalizing the amount of ExoT at each time to the corresponding amount of GAPDH. The ratio is plotted as a percentage of the ratio at time 0 after addition of antibiotics. The blots and plots are representative of at least two experiments.


A
Time post inoculation into MinS w/o calcium (hrs)
ExoT-HA
ExoT (K49R, K74R)-HA
ExoT (K49R)-HA
ExoT (K74R)-HA
2 3
Pellet
ExoT
PilA
Supernatant
ExoT

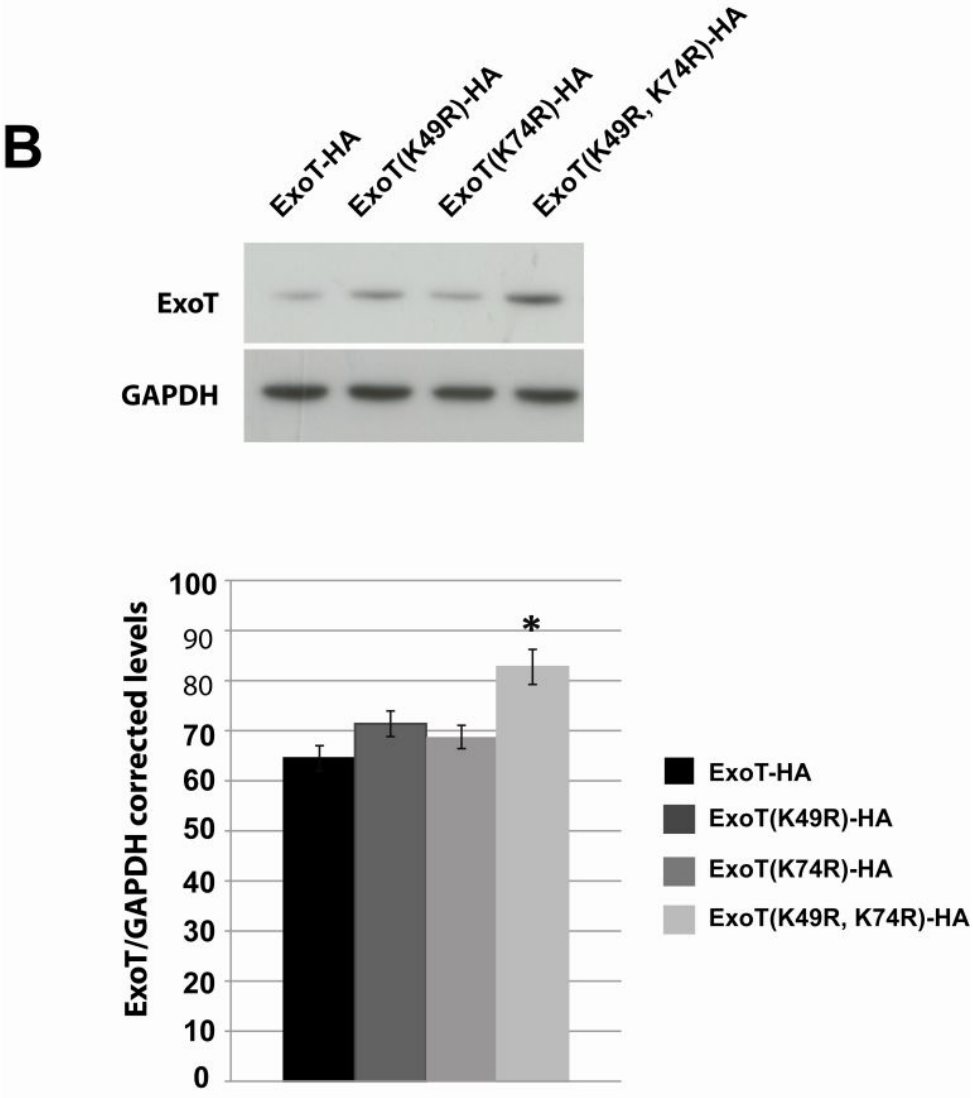
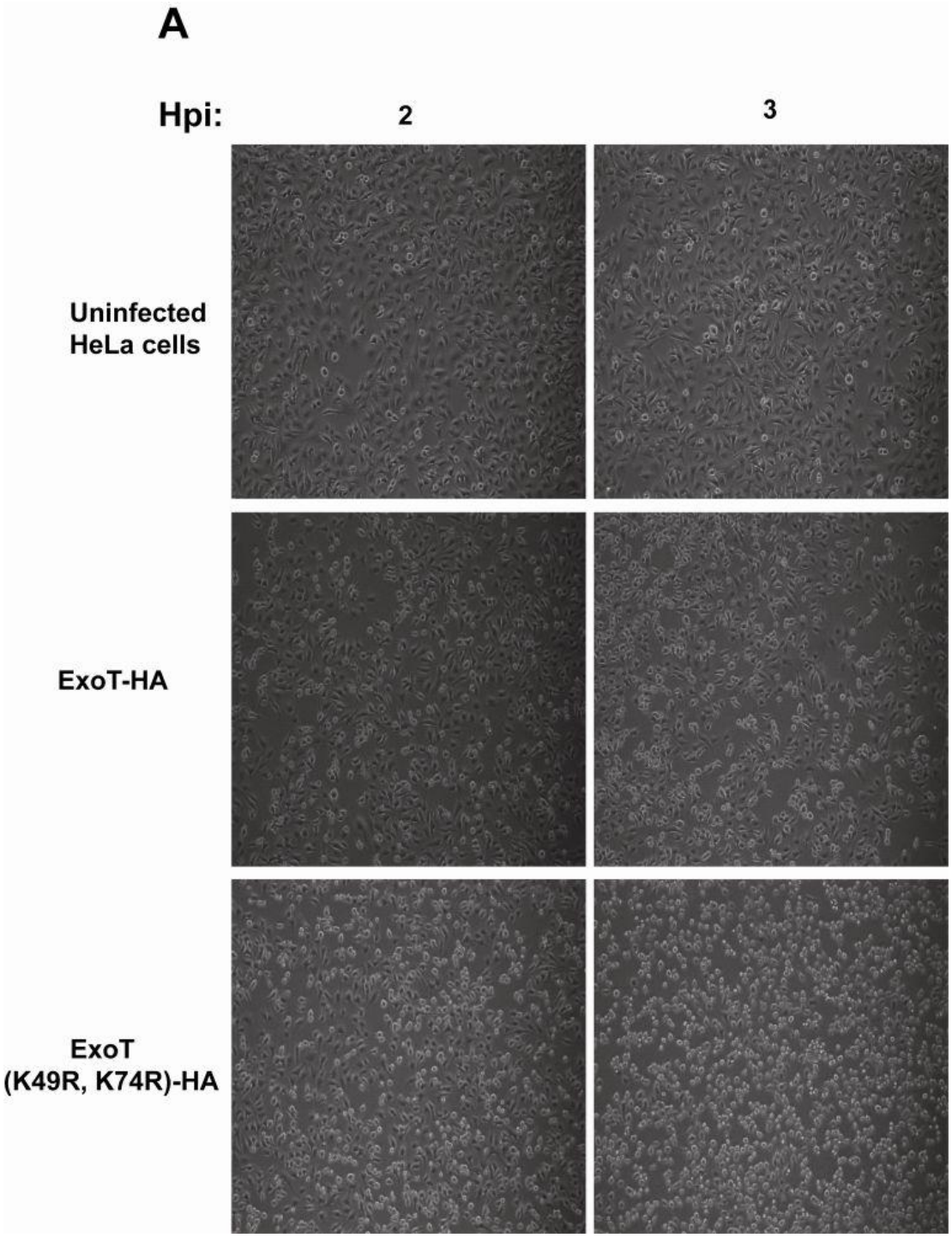

B
ExoT-HA
ExoT(K49R)-HA
ExoT(K74R)-HA
ExoT(K49R, K74R)-HA
ExoT
GAPDH
ExoT/GAPDH corrected levels
100
90
80
70
60
50
40
30
20
10
0
*
ExoT-HA
ExoT(K49R)-HA
ExoT(K74R)-HA
ExoT(K49R, K74R)-HA

Figure 4. K49R mutation affects secretion but not production or translocation of ExoT. (A) Production and secretion of ExoT. After dilution into MinS medium without calcium, the bacterial pellets (cell associated) and supernatant (secreted) were collected at the indicated times and immunoblotted with antibodies to HA (to detect ExoT) or to PilA (loading control). (B) ExoT translocation assays were performed as described in Figure 3B. Host cells were lysed after bacterial removal at 1.5 hpi. The blot is representative of 4 independent experiments (D). The ratio of ExoT to GAPDH is plotted for each strain. Statistical analysis was performed using one-way-ANOVA with Tukey post-test (n=4).* $P< 0.05$.


A
Hpi:
2
3
Uninfected HeLa cells
ExoT-HA
ExoT (K49R, K74R)-HA

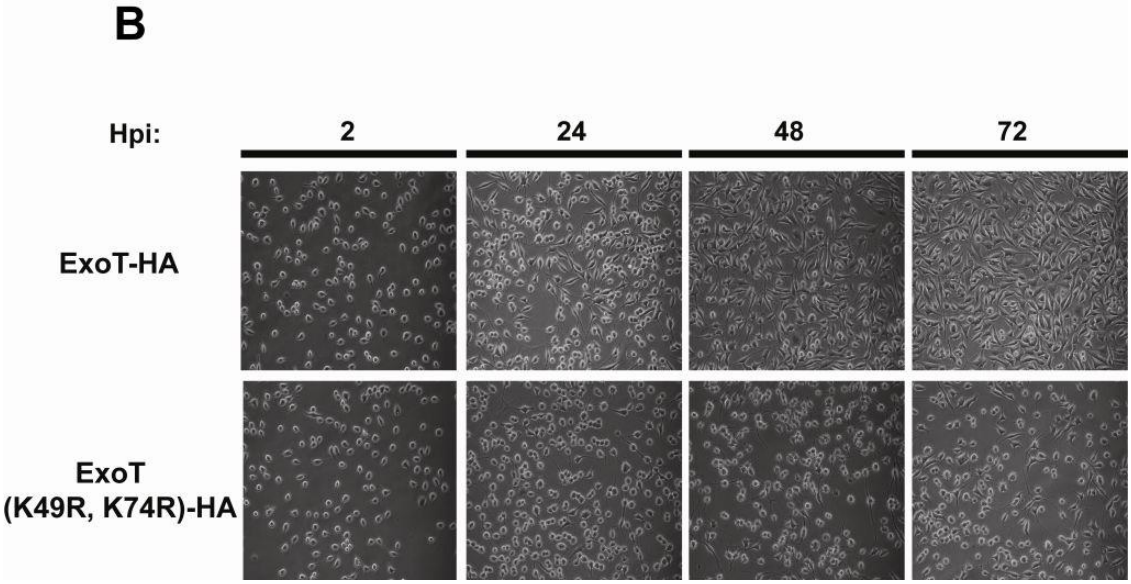


Figure 5. *P. aeruginosa* expressing ExoT (K49R, K74R) causes increased host cell morphology changes. (A) HeLa cells were infected with PA103ΔUΔT::CTX-*exoT-HA* or PA103ΔUΔT::CTX-*exoT* (K49R, K74R)-*HA* (MOI = 200) for 2 hrs or 3 hrs and phase images were obtained by light microscopy at 200X. The images shown are representative from 3 independent experiments **(B)** L929 cells were inoculated with PA103ΔUΔT::CTX-*exoT-HA* or PA103ΔUΔT::CTX-*exoT* (K49R,K74R)-*HA* (MOI=200) for 2 hrs. Bacteria were removed and fresh medium with antibiotics was added to the cells. Phase images were obtained at the indicated times by light microscopy at 200X. The images shown are representative from 3 independent experiments. Note that the cells infected with *P. aeruginosa* expressing ExoT (K49R, K74R) did not recover normal morphology within 72 hrs after removal of the bacteria.

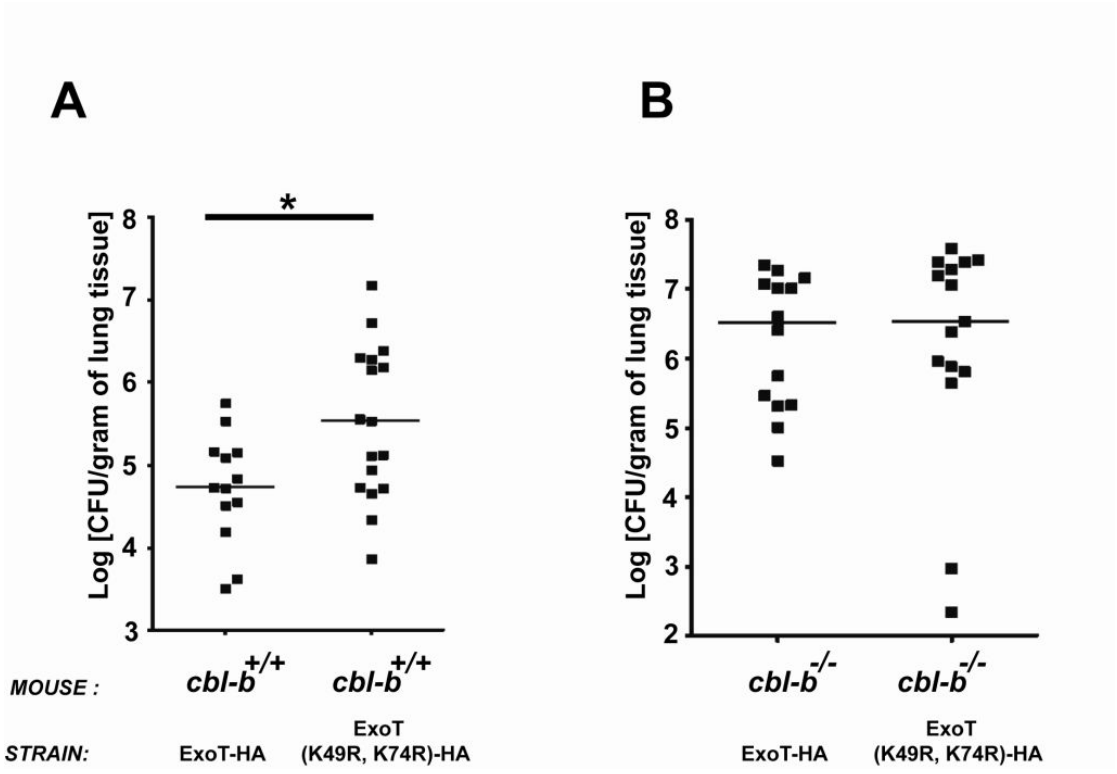


Figure 6. Infection with *P. aeruginosa* expressing ExoT(K49R, K74R) results in higher bacterial burden in the lungs of wild type mice, but not in *cbl-b*$^{-/-}$ mice. C57BL/6 (*cbl-b*$^{+/+}$) **(A)** or C57BL/6 (*cbl-b*$^{-/-}$) **(B)** were infected intranasally with 5 x 10^6 of PA103ΔUΔT::CTX-*exoT*-HA or PA103ΔUΔT::CTX-*exoT*(K49R,K74R)-HA. At 18 hpi, the mice were sacrificed and bacterial CFU were enumerated from the lung. Statistical analyses were performed by the 2-tailed Mann-Whitney *U* test. *P<0.05

MATERIALS AND METHODS:

Bacterial strains and plasmids:

Bacterial strains and plasmids used in this study are described on **Table 1**. To avoid ExoU-mediated cytotoxicity, all experiments were performed in PA103ΔU strain background, a mutant that encodes and secretes only ExoT. *P. aeruginosa* and *E. coli* strains were grown in Luria-Bertani (LB) medium, unless otherwise noted. Antibiotics were used at the following concentrations: 15μg/ml and 80 μg/ml of tetracycline for *E. coli* and *P. aeruginosa*, respectively; 70 μg/ml of kanamycin for *E. coli*; 100 μg/ml of ampicillin for *E. coli*; 450μg/ml amikacin and 150μg/ml chloramphenicol for *P. aeruginosa*.

Creation of point mutations in lysine residues of ExoT:

The ExoT gene with its native promoter was digested with HindIII and EcoRI from the parent plasmid mini-CTX-ExoT-HA-lacZ (Endoh and Engel 2009) and ligated into HindIII-EcoRI-cleaved-pOK12. Since the gene obtained from the parent plasmid contained threonine at position 59, and all the ExoT sequences known to date contain an

alanine residue at this site, this residue was mutated back to alanine using the primer T59TOALA59 (see Table 3). Briefly, 30 cycles of PCR, with 68°C annealing temperature, were performed with Herculase Enhanced Polymerase (STRATAGENE-600260) using this single primer. The PCR fragments were purified with QIAquick PCR Purification Kit following the manufacturer's directions, and then treated with DpnI (NEB) for 1hr at 37°C to remove any remaining parent vector. DH5alpha, Library Efficiency competent cells (INVITROGEN) were then transformed with the single stranded DNA PCR fragment following manufacturer directions. Plasmids from single colonies were purified by using QIAprep spin miniprep columns (Qiagen), verified by sequencing (see Table 2 for sequencing primers; sequencing was carried out at ELIM biopharmaceuticals, inc.), restricted with HindIII-EcoRI, and ligated back into HindIII-EcoRI-restricted- mini-CTX-lacZ (Hoang, Kutchma et al. 2000; Endoh and Engel 2009). Point mutations on lysine residues were created following the protocol described above for threonine to alanine mutation. Briefly, ExoT-HA-pOK12 (with alanine at position 59), was mutated one lysine at a time with the primers described on Table 3. Once the constructs were ligated back to the HindIII-EcoRI-cleaved-mini-CTX-LacZ, the vector was introduced into *P. aeruginosa* by electroporation. Tetracycline resistant *P. aeruginosa* colonies were selected following overnight incubation at 37°C in LB (+80 μg/ml tetracycline) plates. Single colonies were tested for expression and translocation of the toxin into host cells by western blot against ExoT-HA using anti-HA mAbs (SIGMA).

Translocation and degradation of toxins:

Translocation and degradation of ExoT was performed as previously described with minor modifications (Balachandran, Dragone et al. 2007). Briefly, toxins were translocated into host cells by inoculating $1x10^6$ L929 cells in 6-well dishes with the appropriate *P. aeruginosa* strain at MOI=200, followed by a co-incubation for 1.5hrs at 37°C, 5% CO_2. Bacteria were removed by washing with phosphate buffered saline (PBS) (without calcium or magnesium salts), followed by addition of new medium with amikacin (450ug/ml) and chloramphenicol (150ug/ml) to kill remaining bacteria and prevent further bacterial protein expression, respectively. Cells were lysed with 100ul of 1% Triton X-100 containing complete protease cocktail inhibitors (ROCHE-11697498001) at the specified time points and toxin levels were determined by western blot by using mouse anti-HA mAbs (SIGMA) at 1:4000 dilution in blocking buffer. To control for loading efficiency, the blots were probed with anti-GAPDH mAbs (MILLIPORE) at 1:10,000 dilution in blocking buffer. The ratio of the ExoT-HA band and the GAPDH band was plotted as a percentage for each time point after addition of antibiotics (1.5hpi). To determine the role of proteasome in ExoT degradation in L929 cells, DMSO or MG-132 proteasome inhibitor (SIGMA) was added to the medium at the specified concentrations 30 min before infection.

Phagocytosis assays of P. aeruginosa in bone marrow derived macrophages (BMDMs)

For bone marrow collection, BMDMs were differentiated from the bone marrow from femurs of wild type or *cbl-b*$^{-/-}$ mice for 6 days in bone marrow-derived macrophage medium (BMM) containing RPMI, 10% fetal calf serum, 2mM L-glutamine, 110ug/ml sodium pyruvate, penicillin/streptomycin, and a total of 30% (vol/vol) L929 supernatant medium, which was a source of colony stimulating factors. BMDMs were cultured at 37°C, 5%CO_2. To test the difference in phagocytosis rates between wild type and *cbl-b*$^{-/-}$ innate immune cells, 5x10^5 BMDMs from wild type and *cbl-b*$^{-/-}$ mice were infected with PA103ΔU or PA103ΔUΔT (MOI=100). At 2 hpi, the bacteria were removed by washing with PBS, and new medium containing amikacin (450ug/ml) was added to kill the remaining extracellular bacteria for 1hr at 37°C, 5% CO_2. Host cells were lysed with Triton X-100 (final concentration 1%), and released bacteria were enumerated by plating onto LB agar for 18hrs at 37°C.

Quantitation of ExoT production and secretion by immunoblot analysis

P. aeruginosa strains were grown overnight shaking (250 RPM, at 37°C) in 5 ml of LB, and washed once in PBS (without calcium or magnesium salts). This was followed by inoculation of 2x10^9 CFUs into 25ml of MinS-calcium. The production and secretion

of ExoT was determined every hour by collecting 4ml of culture and separating bacterial pellet samples from supernatant samples by centrifugation (7.5Krpm, 5min, 4°C). Bugbuster (200ul) was added to bacterial pellets, followed by shaking at room temperature for 15 min. Following centrifugation at 14k rpm for 5min at room temperature, an equal volume of 2X Laemmli sample buffer was added to the supernatant containing secreted ExoT, boiled for 5 min, and electrophoresed on a 12% NuPAGE (INVITROGEN) gel. For secreted proteins, supernatants from MinS-calcium culture were obtained, and filtered with 0.2μm acrodisc syringe filters (PALL life sciences). Filtrate (4ml) was then centrifuged through 10Kda centrifuge filters (AMIKON) to concentrate ExoT, and an equal volume of 2X sample buffer was added. The mixtures were boiled and electrophoresed on a 12% NuPAGE (INVITROGEN) gel and immunoblotted. Immunoblot analysis was performed as previously described (Garrity-Ryan, Shafikhani et al. 2004) using anti-HA mAbs (SIGMA), at 1:4000 dilution overnight. Goat anti-mouse immunoglobulin G horseradish peroxidase conjugate diluted at 1:3,333 was used as a secondary antibody to detect ExoT-HA. As a loading control, anti-PilA antibodies against PA103 PilA were utilized at a dilution of 1:100,000. Goat anti-rabbit immunoglobulin G horseradish peroxidase conjugate diluted at 1:3,333 was used as a secondary antibody to detect PilA.

Cell rounding assays

HeLa cells (ATCC) were plated at a density of $1x10^6$ cells/well (6 well plate) in MEM-Eagle medium with 10% Fetal Calf Serum (FCS). Prior to addition of bacteria, the cells were washed once with D-PBS, and 2ml of MEM-Lite was added to each well. Cells were then inoculated with $2x10^8$ CFU of appropriate *P. aeruginosa* strain (PA103ΔUΔT::CTX-*exoT*-HA or PA103ΔUΔT::CTX-*exoT*(K49R, K74R)-HA), and incubated for the specified times at 37°C, 5%CO_2 (2hpi and 3hpi). Phase microscopy images were obtained at time 2hpi and 3hpi. Phase microscopy images were obtained using a Nikon Eclipse TE2000-E fluorescence microscope, using a CCD camera, and processed by Simple PCI imaging software (Compix, Inc.).

For L929 cell rounding and recovery assay, L929 cells were plated at a density of $1x10^6$ cells/well (6 well plate) in RPMI medium with 10% FCS. Prior to adding bacteria, cells were washed with D-PBS and new RPMI medium without FCS was added. *P. aeruginosa* strain (MOI=200) was added, and incubated for 2hrs at 37°C, 5%CO_2. Bacteria were removed by washing with PBS (without calcium or magnesium salts), and fresh RPMI containing 10% FCS, amikacin (450μg/ml), and chloramphenicol (150ug/ml) was added to cells. Phase microscopy images were obtained as described at various times post removal of antibiotics.

Animal experiments:

Eight-to-twelve-week-old C57BL/6 (*cbl-b*$^{+/+}$) and Cbl-b knockout (*cbl-b*$^{-/-}$ backcrossed at least 7 generations into C57BL/6) mice were infected intranasally with $5x10^6$ *P. aeruginosa* expressing wild type ExoT-HA or ExoT(K49R, K74R)-HA. Animals were sacrificed 18 hours post infection and lungs were harvested and homogenized in PBS. Bacterial counts were determined by serial dilutions in LB plates. Statistical analysis was performed using 2-tailed Mann-Whitney test (*$P<0.05$ was considered significant*). For myeloperoxidase (MPO) assays, mice were sacrificed at the specified type points. One lung was utilized for bacterial counts, as described above, and the other was used for MPO assay as previously described (Su, Johansen et al. 2008). All experiments used in this study were approved by the University of California, San Francisco Committee on Animal Research.

Table 1. *Pseudomonas aeruginosa* strains and plasmids used in this study

STRAINS AND PLASMIDS	CHARACTERISTICS	REFERENCES
PA103 ΔU	**PA103 with an in-frame deletion of *exoU***	(Garrity-Ryan, Kazmierczak et al. 2000)
PA103 ΔUΔT	**PA103ΔU with an *xylE aacC1* cassette replacing aa 36–348 of *exoT***	(Garrity-Ryan, Kazmierczak et al. 2000)
PA103ΔUΔT::CTX *exoT* -HA	**PA103ΔUΔT Gm^r; mini-CTX- *exoT* -HA-LacZ integrated into *attB* site, Tet^r**	This study
PA103ΔUΔT::CTX *exoT* (K49R)-HA	**PA103ΔUΔT Gm^r; mini-CTX- *exoT* (K49R)-HA-LacZ integrated into *attB* site, Tet^r**	This study
PA103ΔUΔT::CTX *exoT* (K74R)-HA	**PA103ΔUΔT Gm^r; mini-CTX- *exoT* (K74R)-HA-LacZ integrated into *attB* site, Tet^r**	This study
PA103ΔUΔT::CTX *exoT* (K49R, K74R)-HA	**PA103ΔUΔT Gm^r; mini-CTX- *exoT* (K49R, K74R)-HA-LacZ integrated into *attB* site, Tet^r**	This study
PA103ΔUΔT::CTX *exoT* (K74R, K238R, K310R, K319R, K334R, K390R, K401R, K441R)-HA (CTX)	**PA103ΔUΔT Gm^r; mini-CTX- *exoT* (K74R K238R K310R, K319R, K334R, K390R, K401R, K441R)-HA-LacZ integrated into *attB* site, Tet^r**	This study
PA103ΔUΔT::CTX *exoT* (K49R, K74R, K238R,K310R, K319R, K334R, K390R, K401R, K441R)-HA (CTX)	**PA103ΔUΔT Gm^r; mini-CTX- *exoT* (K49R, K74R K238R K310R, K319R, K334R, K390R, K401R, K441R)-HA-LacZ integrated into *attB* site, Tet^r**	This study

Table 1. *Pseudomonas aeruginosa* strains and plasmids used in this study

STRAINS AND PLASMIDS	CHARACTERISTICS	REFERENCES
ExoT (A59T)-HA-LacZ -CTX	**mini-CTX- *exoT* (A59T)-HA-LacZ; Tet^r**	(Endoh and Engel 2009)
ExoT-HA-LacZ-CTX	**mini-CTX- *exoT* -HA-LacZ; Tet^r**	This study
ExoT(K49R)-HA-LacZ-CTX	**mini-CTX- *exoT* (K49R)-HA-LacZ; Tet^r**	This study
ExoT(K74R)-HA-LacZ-CTX	**mini-CTX- *exoT* (K74R)-HA-LacZ; Tet^r**	This study
ExoT(K49R, K74R)-HA-LacZ-CTX	**mini-CTX- *exoT* (K49R, K74R)-HA-LacZ; Tet^r**	This study
ExoT(K74R, K238R, K310R, K319R, K334R, K390R, K401R, K441R)-HA (CTX)	**mini-CTX- *exoT* (K74R K238R K310R, K319R, K334R, K390R, K401R, K441R)-HA-LacZ Tet^r**	This study
ExoT(K49R, K74R, K238R, K310R, K319R, K334R, K390R, K401R, K441R)-HA (CTX)	**mini-CTX- *exoT* (K49R, K74R K238R K310R, K319R, K334R, K390R, K401R, K441R)-HA-LacZ Tet^r**	This study
pOK12	***E. coli* cloning vector; Kan^r**	(Vieira and Messing 1991)
ExoT (A59T)-HA-LacZ pOK12	**pOK12- *exoT* (A59T)-HA; Kan^r**	This study
ExoT-HA-pOK12	**pOK12- *exoT* -HA; Kan^r**	This study
ExoT(K49R)-HA-pOK12	**pOK12- *exoT* (K49R)-HA; Kan^r**	This study
ExoT(K74R)-HA-pOK12	**pOK12- *exoT* (K74R)-HA; Kan^r**	This study
ExoT(K49R, K74R)-HA-pOK12	**pOK12- *exoT* (K49R, K74R)-HA; Kan^r**	This study
ExoT(K74R, K238R, K310R, K319R, K334R, K390R, K401R, K441R)-HA pOK12	**pOK12- *exoT* (K74R K238R K310R, K319R, K334R, K390R, K401R, K441R)-HA Kan^r**	This study
ExoT(K49R, K74R, K238R, K310R, K319R, K334R, K390R, K401R, K441R)-HA pOK12	**pOK12- *exoT* (K49R, K74R K238R K310R, K319R, K334R, K390R, K401R, K441R)-HA Kan^r**	This study

Table 2. Primers used for ExoT sequencing.

Name	sequence
AL1	**(5’ TTCTCCGCCCCGGTGTAGGCG’3)**
AL2	**(5’ CAAGTCCGCCCAAGGTCAGG’3)**
AL3	**(5’ TCAGGACGCGCCGCCTGCCGC’3)**
AL4	**(5’ CAGGTGCTGGTACTCGCCGTTGG’3)**
AL5	**(5’ AGCAGTACCTCGGCGAACACC’3)**
AL6	**(5’ TATGGGTAGGCCAGGTCGAGG’3)**

Table 3. Primers used to create point mutations in ExoT

Name	sequence
T59TOALA59	**(5’CTCTCCCGCCTGGGGGCCGCCCTCGCGCGTC 3’)**
LYS49ARG EXOT	**(5’ CGCCAGGAAGCACCGCGGGGCGAGGGCCTGCTC 3’)**
LYS74TOARG74	**(5’ ATCGAGTGGCTGGGCCGACTGCTGGGGAGCCGT 3’)**
1stadprtKtoR	**(5' GGCGAGCCTGTCGACCGGGCGCTGGCGGATGGC 3')**
2thlysADPRTtoARG	**(5' CCGCGTTCGAAcgGAGCGGACCGGCTGAACAGG 3')**
3thlysADPRTtoARG	**(5' GCTGAACAGGTCGTGcgGACCTTCCGCGGCACC 3')**
4thlysADPRTtoARG	**(5' GCCTTCGAGGCGGTGCGAGAGGGCCAGGTCGGC 3')**
5thadprtKtoR	**(5' GAGATCCTCTACGACCGGGGGACCGACATGCGC 3')**
6thadprtKtoR	**(5' CTTCTCAGTGCCCGGGATGGGCAGGGTGTGACC 3')**
7thlysADPRTtoARG	**(5' ACGGATCGTTCAGGCCGGCCCCAGGAACAGGACC3')**

CHAPTER 3

Role of Type I IFN in *Pseudomonas aeruginosa* intracellular replication, host colonization and organ dissemination

INTRODUCTION:

Pseudomonas aeruginosa is a versatile and well equipped opportunistic pathogen that can cause substantial disease in immunocompromised individuals (Engel and Balachandran 2009; Hauser 2009). The combination of cell associated and secreted virulence factors make this organism a formidable foe for individuals who cannot establish a functional immune response during infection, such as those who are neutropenic, or those that undergo extensive surgery, chemotherapy, or extensive burn wounds (Mandell, Bennett et al. 2010). *P. aeruginosa* hospital associated pneumonia has

an attributable mortality rate close to 40%, and it can significantly increase the length of hospital stay in patients who acquire nosocomial infections (Bou, Lorente et al. 2009; Fujitani, Sun et al.). *P. aeruginosa* is also able to chronically colonize the lungs of Cystic Fibrosis patients, leading to persistent lung damage and even death (Lau, Hassett et al. 2005; Engel and Balachandran 2009). Although epithelial cell injury, immunocompromise, or a genetic defect is generally required for *P. aeruginosa* to establish infection, it is clear that this organism possesses a large arsenal of virulence factors that allow it to manipulate or evade the innate immune system (Lau, Hassett et al. 2005; Engel and Balachandran 2009; Hauser 2009).

The innate immune system employs both extracellular and intracellular sensors that detect the presence of a pathogen. Recognition of microorganisms by the host innate immune system is dependent on pattern recognition receptors (PRRs), which are able to detect conserved microbial structures known as pathogen associated molecular patterns (PAMPs). These PAMPs can include sugars, flagellin, and cell wall components like peptidoglycan and lipopolysaccharide among others (Martinon, Mayor et al. 2009). Recognition of PAMPs initiates an inflammatory response in the host by activating a large number of genes associated with cytokine and chemokine production (Miao, Ernst et al. 2008). To date, the best studied PRRs are Toll-like receptors (TLRs), and their activation is well known for inducing a diverse number of different immunological responses (Beutler 2004). Type I interferons are important immunoregulatory cytokines during infections, which can be activated by TLRs, among other signals (Perry, Chen et al. 2005). They can be subdivided into interferon alpha (IFN-α) and interferon beta (IFN-

β). Binding of these molecules to IFN-α/β-receptors on the surface of cells leads to the activation of STAT1 and STAT2, which is followed by expression of genes that are regulated by IFN-stimulated response elements (Perry, Chen et al. 2005). Recent studies have demonstrated that some bacteria are potent inducers of type I interferons (O'Connell, Saha et al. 2004; Stetson and Medzhitov 2006). During bacterial infection, activation of type I interferon gene expression is dependent on the recognition of various molecules, including LPS, multidrug resistance transporters, bacterial DNA, and flagellin (Perry, Chen et al. 2005; Stetson and Medzhitov 2006; Crimmins, Herskovits et al. 2008). Induction of type I interferon can either benefit the pathogen or protect the host, depending upon the pathogen, the animal model, and/or the magnitude of interferon induction (O'Connell, Saha et al. 2004; Stetson and Medzhitov 2006; Coers, Vance et al. 2007; Crimmins, Herskovits et al. 2008). Our laboratory is interested in understanding whether *Pseudomonas aeruginosa* induces type I interferon expression in macrophages, and if so, what molecules in the bacterium are recognized, and in determining whether type I interferon expression benefits the host or the pathogen *in vitro* and *in vivo*. Here, we show that interferon beta (IFN-β) expression is induced in bone marrow derived macrophages during *P. aeruginosa* infection, and that expression of this gene is independent of type III secreted effector delivery or functional type III secretion system recognition by the host. We also found that IFN-β expression is dependent on Myd88/TRIF signaling in BMDMs, and that exogenous IFN-β increases intracellular survival of *P. aeruginosa* in wild type and Myd88/TRIF deficient BMDMs. Although the mechanism of IFN-β mediated increased intracellular bacterial survival remains elusive,

this study has shown that IFN-β signaling may play an important role in *P. aeruginosa* infections. Understanding whether type I interferon activation during bacterial infection has any beneficial or detrimental effects on the host can prove valuable for the medical community because new treatment strategies could be discovered in the process.

RESULTS:

Type I interferon gene expression is induced in BMDMs during P. aeruginosa infection

To determine whether type I interferon expression is induced during *P. aeruginosa* infection, we performed a limited RTPCR screen in bone marrow derived macrophages (BMDMs) in the presence and absence of PA103ΔU. We found that IFN-β was highly induced in wild type (C57BL/6) BMDMs at 2 hpi compared to uninfected cells (Fig.7A). Since the strain of *P. aeruginosa* utilized in this experiment contains a functional type III secretion system (TTSS) and is able to translocate ExoT, we were interested in determining whether IFN-β expression is dependent on recognition of the TTSS or on the translocated effector. We found that IFN-β expression in wild type BMDMs is independent of the presence of a functional type III secretion (PA103*pscJ::Tn5*), of ExoT translocation (PA103ΔU), or the translocon apparatus (PA103ΔUΔT) (Fig 7A). As

expected, we also observed an increase in expression of many immune related genes during infection of wild type BMDMs, including KC, TNF-α, and mip-2, and likewise, their induction was independent of a functional type III secretion apparatus, translocon, or ExoT (Fig 7B).

Type I interferon gene expression is dependent on Myd88/TRIF signaling pathways during P. aeruginosa infection

Our studies show that *P. aeruginosa* induces type I interferon gene expression. However, it is unclear how this signaling network is induced by this Gram negative opportunistic pathogen. Since expression of IFN-β appears to be independent of type III secretion system recognition, we tested whether host immune signaling networks such as the Myd88 and TRIF pathways were involved in IFN-β induction during *P. aeruginosa* infection. As shown in Fig 8, induction of IFN-β transcription by any of the *P. aeruginosa* strains tested was not observed in BMDMs isolated from Myd88/TRIF deficient mice. This finding suggests that one or both of these immunological signaling pathways is necessary for expression of IFN-β after recognition of *P. aeruginosa* by the host.

Exogenous *Interferon-β* increases intracellular survival of *P. aeruginosa* in wild type and *Myd88/TRIF* deficient BMDMs

Recent studies have demonstrated that absence of type I interferon receptor in macrophages leads to a decrease in intracellular killing of *Legionella pneumophila* (Coers, Vance et al. 2007). This finding suggests that type I interferon activity plays key a role in host protection against some bacterial infections. In order to determine whether IFN-β signaling plays an important role in the killing of *Pseudomonas aeruginosa* by BMDMs, we performed an intracellular bacterial killing assay in the presence or absence of exogenous IFN-β in both wild type and Myd88/TRIF deficient BMDMs. In order to reduce the amount of type III secretion mediated cytotoxicity, we utilized a strain of *P. aeruginosa* that does not produce ExoU or ExoT (PA103ΔUΔT). We found increased intracellular growth of *P. aeruginosa* in the presence of exogenous IFN-β in both WT (C57BL/6) and Myd88/TRIF KO BMDMs at 2 hrs post antibiotic treatment compared to control treated cells (Fig. 9). The greatest effect was seen in Myd88/TRIF deficient cells, consistent with our finding that IFN-β production requires this signaling pathway.

DISCUSSION:

Activation of type I interferons has been extensively studied in viral infections, as well as some parasitic, bacterial, and most recently in fungal infections (Chessler,

Caradonna et al. ; Inglis, Berkes et al. ; Perry, Chen et al. 2005; Crimmins, Herskovits et al. 2008). Recognition of various bacterial PAMPs, such as bacterial DNA, Gram-negative flagellin, multidrug resistance transporters and LPS, is well known for induction of this signaling network. The wide variety of bacterial molecules that lead to activation of this host surveillance system suggests that both extracellular and cytosolic foreign molecules can be recognized by the host. Although a previous study has demonstrated that a mucoid strain of *P. aeruginosa* induces IFN-β production in the mouse lung at 4hrs post infection, it is unclear what tissue produces this cytokine in response to *P. aeruginosa* infection (Carrigan, Junkins et al. 2010). In this study, we have discovered that *P. aeruginosa* induces type I interferon signaling in macrophages, and that recognition of this bacterium is independent of its type III secretion system components. Furthermore, we show that *P. aeruginosa* dependent type I interferon induction requires Myd88/TRIF signaling pathways, and that IFN-β signaling benefits pathogen intracellular survival. The exact trigger mechanism of *P. aeruginosa*-induced type I interferon remains elusive, but our results suggest that activation of this cytokines may be dependent on TLR-signaling since both Myd88 and TRIF are adaptor molecules for many TLRs. Taken together, our study suggests that type I interferon signaling may play a role in host-*P. aeruginosa* interactions. In depth understanding of IFN-β signaling on host physiological effects during *P. aeruginosa* infection may lead to new treatment strategies against this highly virulent opportunistic pathogen.

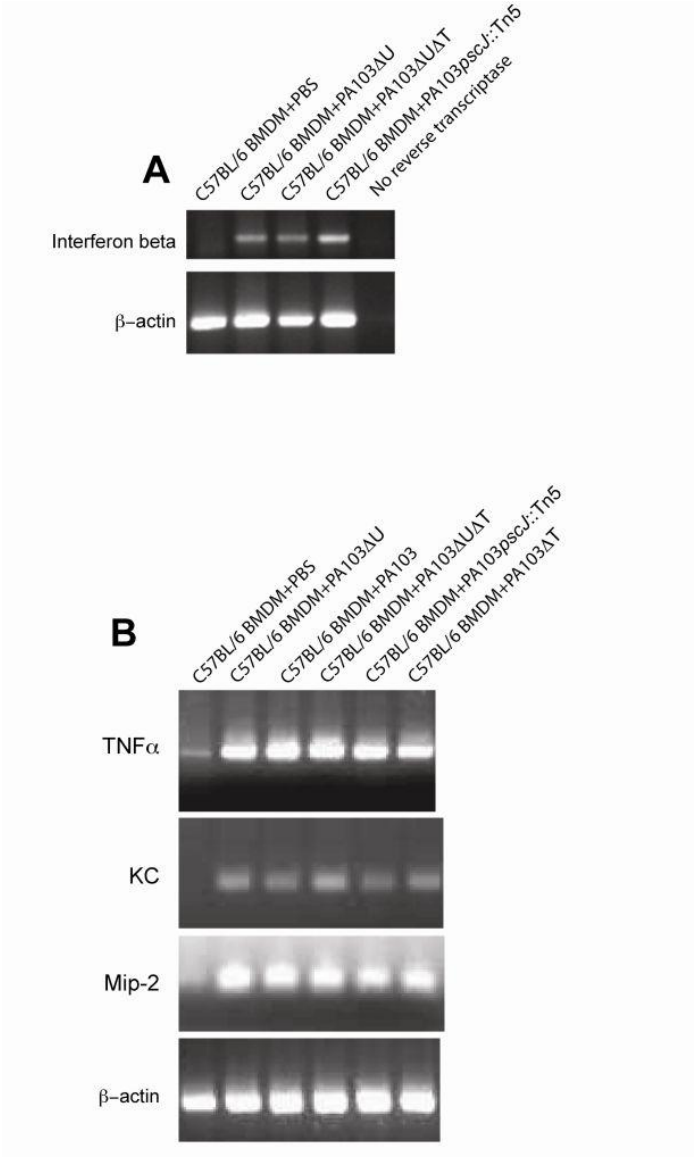


Figure 7. *P. aeruginosa* induces IFN-β and other cytokines in a type III secretion independent manner in BMDMs. BMDMs were infected with the indicated strains (MOI=100). At 2 hpi, RNA was isolated and levels of actin, IFN-β (A), TNF-alpha, KC, and MIP-2 (B) gene expression were assessed by semiquantitative RT-PCR.

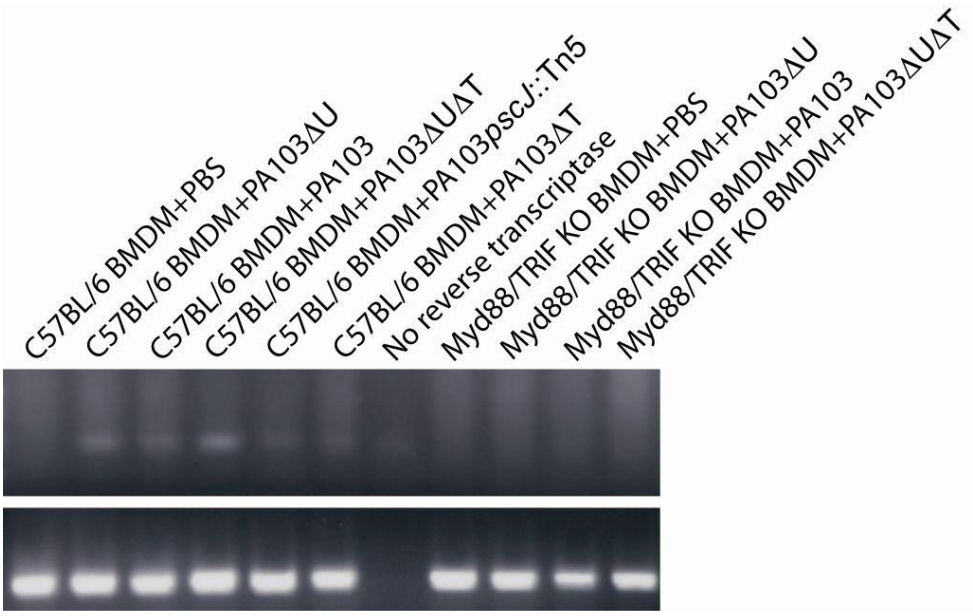


Figure 8. *P. aeruginosa* induction of IFN-β gene expression is Myd88/TRIF-dependent. BMDMs were infected with the indicated *P. aeruginosa* strain (MOI=200). At 2 hpi, BMDM RNA was isolated and semiquantitative RT-PCR for IFN-β and actin expression was performed.

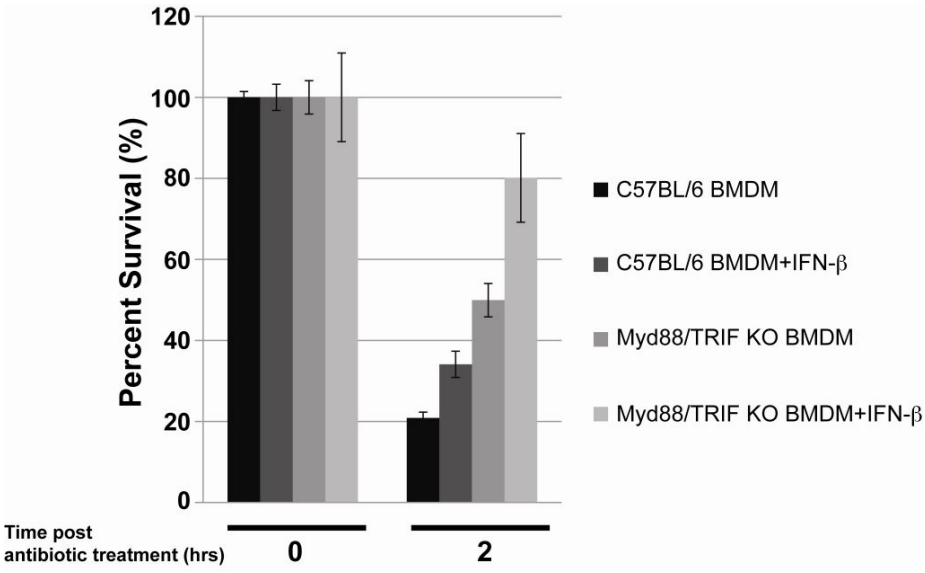


Figure 9. Exogenous interferon beta leads to greater bacterial survival in wild type and Myd88/TRIF deficient BMDMs. BMDMs from wild type (C57BL/6) and Myd88/TRIF KO mice were treated with IFN-β 30 min before adding PA103ΔUΔT (MOI=50). One hour after infection, bacteria were removed by washing and fresh BMDM medium with antibiotics was added. BMDMs were lysed at times 0 and 2 hrs post antibiotic treatment, and bacteria were enumerated by plating lysates in LB agar plates.

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MATERIALS AND METHODS:

Bacterial strains and plasmids:

Bacterial strains used in this study are described on Table 5. *P. aeruginosa* was grown in Luria-Bertani (LB) medium, unless otherwise noted. Antibiotics were used at the following concentrations: 450μg/ml amikacin for *P. aeruginosa*.

RT-PCR in bone marrow derived macrophages

BMDMs from wild type (C57BL/6) mice and Myd88/TRIF deficient mice were collected as described previously (see Materials and methods for Chapter 2). These cells were infected with *P. aeruginosa* (PA103, PA103ΔU, PA103ΔUΔT, PA103ΔT, PA103*pscJ*::*Tn5*) for 2hrs at an MOI=100. Cells were lysed, using RNeasy mini kit (QIAGEN) and RNA was isolated according to the manufacturer's directions. To detect gene expression, RTPCR for IFN-β and other immune related genes was performed. Primers used in this study are listed in Table 4. For RTPCR, M-MLV Reverse Transcriptase (INVITROGEN) was utilized following manufacturer's directions. For semiquantitative PCR to detect cDNA, 31 cycles were performed with the following

parameters: (58°C annealing temperature for 1 minute, 72°C extension 1 minute, and 72°C final extension for 5 min). The PCR products were detected by gel electrophoresis.

Table 4. Primer sequences for RTPCR experiments

Primer name	Sequence
Tnfa forward	5-GGT GCC TAT GTC TCA GCC TCT-3
Tnfa reverse	5-CAT CGG CTG GCA CCA CTA GTT-3
Mip-2 forward	5-GCTGTCCCTCAACGGAA-3
Mip-2 reverse	5-ACATCTGGGCAATGGAAT-3
KC forward	5-CCT TGACCCTGAAGCTCCCTTGGTTC-3
KC reverse	5-CGTGCGTGTTGACCATACAATATG-3
actin F	5-TGGCATTGTTACCAACTGGGACG
actin R	5-GCTTCTCTTTGATGTCACGCACG
murine *ifnb* F	5-GCACTGGGTGGAATGAGACTATTG
murine *ifnb* R	5-TTCTGAGGCATCAACTGACAGGTC

Intracellular bacterial survival in the presence of exogenous IFN-β

BMDMs ($2x10^5$/well) were infected at an MOI=50 for 1hr in 24-well dishes. One set of samples was treated with 0.7ul of exogenous mouse IFN-β (SIGMA) 30 min before addition of bacteria. This was followed by removal of extracellular bacteria at 1hr post infection, and fresh medium with amikacin was added to kill remaining extracellular bacteria. BMDMs were lysed with 1% Triton X-100 at 0 hr, and 2 hrs post-antibiotic

treatment. Lysates were plated in LB agar dishes, and CFUs were counted the following day after an overnight incubation at 37°C.

Table 5. *Pseudomonas aeruginosa* strains and plasmids used in this study

STRAINS AND PLASMIDS	CHARACTERISTICS	REFERENCES
PA103	**Wild type clinical isolate; secretes ExoU and ExoT**	(Liu 1966)
PA103ΔU	**PA103 with an in-frame deletion of *exoU***	(Garrity-Ryan, Kazmierczak et al. 2000)
PA103ΔUΔT	**PA103ΔU with an *xylE aacC1* cassette replacing aa 36–348 of *exoT***	(Garrity-Ryan, Kazmierczak et al. 2000)
PA103ΔT	**PA103 with an *xylE aacC1* cassette replacing aa 36–348 of *exoT***	(Garrity-Ryan, Kazmierczak et al. 2000)
PA103*pscJ*::*Tn5*	**Tn*5* Gmr cassette inserted into *pscJ*; defective in type III secretion**	(Kang, Hauser et al. 1997)

CONCLUDING REMARKS:

The highly virulent opportunistic Gram-negative pathogen *Pseudomonas aeruginosa* has the potential to become a serious health threat for those individuals who are immunocompromised or have some type epithelial barrier injury. Its recent decline in susceptibility to first line antibiotic treatments has also been alarming. It is clear that new approaches to combat human disease caused by this organism will require a broad understanding of its biology, specifically those biochemical mechanisms involved in its pathogenesis. This dissertation work has attempted to understand how the host and this pathogen interact at the molecular level, and how the host is able to defend itself against virulence factors that *P. aeruginosa* injects directly into the cytoplasm of host cells. In this study, we have been able to show explicitly that two amino acid residues in the type III secreted toxin ExoT are the crucial determinants of proteasomal dependent degradation of this toxin. We have also been able show the host E3 ubiquitin ligase Cbl-b targets these lysine residues in the toxin to protect the host in an *in vivo* model. However, it is still unclear whether this Cbl-b protection of the host is mediated through ubiquitination of the toxin. Our current focus is to determine whether Cbl-b can still ubiquitinate a mutant toxin that lacks these lysine residues.

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