IAI Accepts, published online ahead of print on 25 January 2010
Infect. Immun. doi:10.1128/IAI.01134-09

The *Pseudomonas aeruginosa* cytotoxin ExoU is injected into phagocytic cells during acute pneumonia

Running title: *P. aeruginosa* toxin ExoU is injected into phagocytes

Maureen H. Diaz[1,†] and Alan R. Hauser[1,2,*]

[1]Department of Microbiology-Immunology and [2]Department of Medicine, Northwestern University, Chicago, IL, USA

* Corresponding author. Mailing address: 303 E. Chicago Ave. Searle 6-495, Chicago, IL 60611. Phone: (312) 503-1044. Fax: (312) 503-1339. Email: ahauser@northwestern.edu.

[†] Present address: Centers for Disease Control and Prevention, Division of Bacterial Diseases, Respiratory Disease Branch, 1600 Clifton Rd. N.E., Mail Stop G-03, Atlanta, GA 30333

Abstract

ExoU, a cytotoxin translocated into host cells via the type III secretion system of *Pseudomonas aeruginosa*, is associated with increased mortality and disease severity. We previously showed that impairment of recruited phagocytic cells allowed survival of ExoU-secreting *P. aeruginosa* in the lung. Here we analyzed cell types injected with ExoU in vivo using translational fusions of ExoU with a β-lactamase reporter (ExoU-Bla). Cells injected with ExoU-Bla were detectable in vitro but not in vivo, presumably due to the rapid cytotoxicity induced by this toxin. Therefore, we used a non-cytotoxic ExoU variant, designated ExoU(S142A)-Bla, to analyze injection in vivo. We determined that phagocytic cells in the lung are frequently injected with ExoU(S142A). Early during infection, resident macrophages constituted the majority of ExoU-injected cells, but neutrophils and monocytes became the predominant injected cell types upon recruitment into the lung. We observed a modest preference for injection of neutrophils relative to other cell types, but in general the repertoire of injected immune cells reflected the relative abundance of these cells in the lung. Our results indicate that phagocytic cells in the lung are injected with ExoU and further support a role of ExoU-mediated impairment of phagocytes in the pathogenesis of pneumonia caused by *P. aeruginosa*.

Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium found ubiquitously in both natural and manmade environments including hospitals. In the healthcare setting, it is a frequent cause of acute infections such as urinary tract infections, burn and wound infections, sepsis, and severe pneumonia (23). The risk of *P. aeruginosa* pneumonia is increased in hospitalized patients who are immunosuppressed or require mechanical ventilation. In fact, *P. aeruginosa* accounts for almost 20% of all pneumonia cases in the intensive care unit (ICU) and is the leading cause of ventilator-associated pneumonia (VAP) (30). Additionally, as a causative agent of VAP, *P. aeruginosa* has a higher attributable mortality rate compared to most other bacterial etiologies, making it a particularly dangerous pathogen (4).

Of the many bacterial factors that contribute to the pathogenesis of *P. aeruginosa*, the type III secretion system is important for bacterial persistence in the presence of elaborate host defense mechanisms and has been associated with poor clinical outcomes in individuals with VAP (13, 32). While the genes encoding the type III secretion apparatus are present in all *P. aeruginosa* isolates, the repertoire of effector-encoding genes is variable. Approximately 28% of strains from acute infections encode a potent cytotoxin, ExoU (8), which is a marker of highly virulent *P. aeruginosa* strains isolated from patients with VAP (35). In animal models, ExoU secretion greatly augments the virulence of *P. aeruginosa* (1, 20, 21, 37). The contribution of ExoU to virulence is attributable to its phospholipase A_2 activity (27) (34). Upon injection into host cells, ExoU is activated and targeted to the plasma membrane where it cleaves membrane phospholipids resulting in rapid and complete cell lysis (10, 14, 27-29, 34). In addition to ExoU, *P. aeruginosa* isolates can encode other effectors, including ExoS, ExoT, and ExoY in various combinations (8). ExoS and ExoT are bi-functional enzymes with 75% amino acid identity and

similar functional domains, each encoding GTPase activating protein (GAP) and adenosine diphosphate-ribosyltransferase (ADPRT) activities (2). ExoY is an adenylate cyclase (41). Interestingly, most strains contain either *exoS* or *exoU*, but strains containing both genes are uncommon (8).

Previously, we showed that ExoU-secreting *P. aeruginosa* strains are capable of persisting in the lungs of infected animals despite eliciting an exaggerated immune response consisting of infiltrating inflammatory cells. Recruited phagocytic cells were impaired by ExoU during acute lung infection, resulting in a localized paucity of functional phagocytes within the airways, allowing bacteria to persist and cause severe disease (6). However, whether phagocytes were directly injected with ExoU or compromised indirectly following intoxication of other cell types was unclear.

Recently a fluorogenic β-lactam substrate, CCF2-AM, has been used to detect translocation of bacterial proteins into host cells in vitro and in vivo (3, 11, 17, 24, 25). Upon diffusion into host cells, intact CCF2-AM exhibits fluorescence resonance energy transfer (FRET) resulting in green fluorescence. Cleavage of the substrate by a β-lactamase molecule disrupts FRET, resulting in a shift to blue fluorescence (3, 42). Fusion of a bacterial protein, in this case ExoU, with a β-lactamase tag allows detection of protein translocation into cells by virtue of the change in fluorescence emission. Here we have used CCF2-AM to identify cell types intoxicated with ExoU in a mouse model of acute pneumonia. We found that phagocytic cells were injected with ExoU in the lung. Resident alveolar macrophages were injected with ExoU in the earliest stages of infection, but as neutrophils and monocytes were rapidly recruited to the lung, they became the predominant cell types injected with ExoU. In comparison, only a small number of lymphocytes and type II alveolar epithelial cells were injected in vivo. Cells

injected with ExoU were detected as early as 3 h after infection and increased steadily to a maximum of approximately 10% of all recovered cells at 18 h post-infection. These findings are consistent with a model whereby extensive injection of phagocytic cells with ExoU incapacitates these cells, which facilitates persistence of *P. aeruginosa* in the lung and progression to severe disease.

Materials and Methods

Bacterial strains and growth conditions

The bacterial strains, mutants, and plasmids used in this study are listed in Table 1. PA99 is a *P. aeruginosa* clinical isolate that naturally encodes the *exoU*, *exoS*, and *exoT* genes but lacks the gene for *exoY* (8). PA99null and PA99secr$^-$ (PA99Δ*pscJ*) were generated from PA99 by in-frame deletions as previously described (37). Complementation restored the mutants to an expected level of virulence, and neither mutant displayed a growth defect on laboratory medium (37). TOP10 chemically competent *Escherichia coli* cells (Invitrogen) were used for cloning.

Bacterial strains were streaked from frozen cultures onto Luria-Bertani (LB) agar or Vogel Bonner minimal (VBM) agar (39). For infections, overnight cultures of *P. aeruginosa* grown in 5 ml MINS medium (26) at 37ºC were diluted into fresh medium and regrown to exponential phase.

Generation of effector-Bla fusions

The stop codon of *exoU* was altered, and an AgeI site was engineered at the 3' end of *exoU* by site-directed mutagenesis using the plasmid miniCTX*exoU* (29) as template to generate

miniCTX*exoU*ΔstopAgeI. The mature fragment of TEM-1 β-lactamase was amplified from pBR322 with engineered AgeI sites using primers BlacAgeUp (5'-AAAACCGGTACACCCAGAAACGCTGGTGA-3') and BlacAgeDwn (3'-AAAACCGGTATTACCAATGCTTAATCAGTGA-5') and ligated into miniCTX*exoU*ΔstopAgeI. A Bla fusion of a non-cytotoxic ExoU variant (ExoU(S142A)) was created by removing the stop codon of *exoU(S142A)* by site-directed mutagenesis using miniCTX*exoU(S142A)* (6) as a template followed by amplification of the resulting *exoU(S142A)*Δstop construct with existing NsiI and engineered AgeI sites at its 5' and 3' ends using primers exoU5' (5'-AAAATGCATATCCAATCGTTGG-3') and exoUAgeI (3'-AAAACCGGTATCGGCCATGTGAACTCCTTATTCC-5'). The amplified fragment was ligated into miniCTX*exoU*ΔstopAgeI digested with NsiI and AgeI to generate miniCTX*exoU(S142A)*ΔstopAgeI. In a second reaction, the *bla* fragment was ligated into miniCTX*exoU(S142A)*ΔstopAgeI digested with AgeI to create miniCTX*exoU(S142A)-bla*.

A GST-Bla fusion that is expressed under the control of the native *exoU* promoter was generated by amplifying the open reading frame of glutathione S-transferase from pGEX-5X1 using primers GSTNsiI (5'-AAAATGCATATGTCCCCTATACTAGGTTATTGG-3') and GSTAgeI (3'-AAAACCGGTTCAGTCAGTCACGATGAATTCCCG-5') and ligated into miniCTX*exoU*ΔstopAgeI digested with NsiI and AgeI to generate miniCTX*gst*AgeI. The stop codon of *gst* was altered by site-directed mutagenesis. In a subsequent reaction, the *bla* fragment was ligated into AgeI-digested miniCTX*gst*AgeI to create miniCTX*gst-bla*.

All plasmids were confirmed by sequence analysis. In each case, the resulting construct (miniCTX*exoU-bla*, miniCTX*exoU(S142A)-bla*, or miniCTX*gst-bla*) was transformed into *E. coli* strain S17.1 (38) and, following conjugation, was introduced into a neutral site in the

PA99null chromosome via integrase-mediated recombination at the *attB* site using previously described approaches (16) to generate PA99null+ExoU-Bla, PA99null+ExoU(S142A)-Bla, and PA99null+GST-Bla, respectively. miniCTX*exoU-bla* and miniCTX*exoU(S142A)-bla* were also introduced into the chromosome of PA99secr$^-$ to create strains PA99secr$^-$+ExoU-Bla and PA99secr$^-$+ExoU(S142A)-Bla. Integration was confirmed by PCR amplification of each construct from intact bacterial colonies.

Immunoblot analysis

Immunoblot analysis for secretion of ExoU-Bla fusion proteins were performed using polyclonal ExoU antiserum as previously described (35).

Fluorescence microscopy

J774 macrophage-like cells were cultured in high-glucose Dulbecco's MEM with 10% fetal bovine serum (FBS) and grown on glass coverslips in a 24-well plate overnight. Cells were incubated with *P. aeruginosa* at an MOI of 10 for 3 h. Cells were washed once with Hank's Balanced Salt Solution (HBSS) and incubated with CCF2-AM at 1× final concentration for 1 h at room temperature protected from light. Cells were washed with HBSS to eliminate excess CCF2-AM and fixed with 3.7% formaldehyde. Coverslips were placed on slides with 1 drop of Fluoromount-G (Southern Biotechnology Associates, Inc.) and sealed with nail polish. Cells were viewed with a Leica DMR microscope powered by a 100-W mercury lamp and equipped with an Axiocam MR3 color camera. Pictures were taken using AxioVision release 4.6.3 software.

Lactate dehydrogenase-release cytotoxicity assays

J774 macrophage-like cells were incubated with bacteria at an MOI of 10. At the appropriate time-points, a 50 µl aliquot of supernatant was removed and assayed for release of lactate degydrogenase (LDH, CytoTox 96 Non-radioactive Cytotoxicity Assay, Promega). Cell lysis was quantified by measuring absorbance at 490 nm and normalizing to total cell lysis achieved by adding 0.05% Triton X-100. Background lysis in the absence of bacteria was subtracted from all values.

Mouse model of acute pneumonia

Studies of acute pneumonia were conducted using the aspiration mouse model described by Comolli et al. (5). Briefly, bacteria were collected by centrifugation and resuspended to the appropriate concentration in phosphate-buffered saline (PBS). Six- to eight-week-old female BALB/c mice were anesthetized by intraperitoneal injection of a mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml). Mice were intranasally inoculated with 1.2×10^7 CFU of bacteria in 50 µL PBS, as determined by optical density. Inocula were confirmed by plating of serial dilutions.

Animals were purchased from Harlan and housed in the containment ward of the Center for Comparative Medicine at Northwestern University. All experiments were performed in accordance with the guidelines of the Northwestern University Animal Care and Use Committee.

Analysis of ExoU-injected cells by flow cytometry

At the appropriate time-points, mice were anesthetized and sacrificed by cervical dislocation and thoracotomy. Lungs were perfused and flushed by injection of 2 ml PBS into the

right side of the heart to remove circulating blood cells. Lungs were excised and pressed through 40 μm filters (Falcon). The filters were rinsed repeatedly with PBS. For BAL experiments, mouse lungs were lavaged by instilling and withdrawing 1 ml of PBS three times. Recovered cells were gently pelleted by centrifugation at 500 × *g* for 5 min. The supernatant was removed and red blood cells were lysed by addition of 3 ml cold sterile H_2O for 30 sec. Three ml of 2× normal saline (1.8% NaCl) was quickly added to prevent additional cell lysis. Remaining cells were pelleted and resuspended in PBS, and trypan blue-excluding cells were quantified using a hemacytometer.

A total of 1-2 × 10^5 cells were aliquoted per well of a V-bottom 96-well plate (Nunc), resuspended in 100 μL PBS and CCF2-AM (Invitrogen) at 1× final concentration, and incubated at room temperature protected from light for 1 h. Cells were pelleted, and surface Fc receptors and non-specific binding sites were blocked by incubation in 10% rat serum (Sigma) and α-CD16/32 in Fluorescence Activated Cell Sorting (FACS) buffer (1% bovine serum albumin, 0.1% NaN_3 in PBS) for 5-15 min on ice. Cell discriminatory antibodies were added at the appropriate dilutions in FACS buffer, and the final volume in each well was adjusted to 125 μl. Antibodies and cells were incubated for 15-30 min on ice. Cells were pelleted by centrifugation at 500 × *g* for 5 min and resuspended in 2% paraformaldehyde in PBS for 2 min. An equal volume of FACS buffer was added, and cells were pelleted again. Cells were resuspended in FACS buffer and transferred into Falcon 2052 tubes after passage through 70 μm Nitex filters. Antibodies were used at the following final dilutions: α-CD16/32 1:50, α–CD45 1:1,500, α–Gr1 1:1,500, α–F4/80 1:50, α–CD4 1:500, α–CD8 1:500, α–CD19 1:500, α–CD49 1:500, α–CD11b 1:2,500, α–CD11c 1:500, isotype controls 1:100 each. All antibodies were purchased from eBioscience.

Cells were analyzed by flow cytometry on a Becton Dickinson FACSCantoII. Instrument settings were determined each day using the appropriate positive and negative control samples. After data collection, cell debris was removed from analysis by gating for forward- and side-scatter. The remaining cells were considered to represent the total number of cells extracted from the lung. Innate immune cells were defined as follows: $CD11b^{+}Gr1^{hi}$ as neutrophils; $CD11b^{+}Gr1^{int}$ as recruited monocytes, $Gr1^{-}F4/80^{+}$ as resident macrophages, $CD11c^{int}Gr1^{-}$ as dendritic cells. (In separate samples, $CD11c^{int}$ cells were shown to be $F480^{-}$, indicating that these were dendritic cells rather than $CD11c^{+}$ alveolar macrophages.) Recruited mononuclear cells that had migrated into tissue were considered monocytes and were distinguished from resident macrophages by expression of Ly6C. Recruited monocytes express intermediate levels of Ly6C (recognized by α-Gr1 antibody) on their surface while mature macrophages do not (12). Lymphocyte subtypes were identified as follows: $CD4^{+}$ helper T cells, $CD8^{+}$ cytotoxic T cells, $CD19^{+}$ B cells, and $CD49^{+}$ NK cells. The total number of viable inflammatory cell types per mouse lung was determined by equating the number of total cells with normal scatter characteristics measured by flow cytometry to the number of trypan blue-negative cells counted in the hemacytometer.

Isolation of type II pneumocytes from mouse lungs

Murine type II pneumocytes were isolated from lungs using a modification of the method described by Ridge et al. (31). Briefly, lungs were perfused via the pulmonary artery, lavaged six times, and digested with elastase for 20 min (30 U/mL; Worthington Biochemical). Cells were isolated from collected lavage fluid for parallel analysis. Digested lung tissue was minced in the presence of FBS and DNAse, filtered through 70 µm cell strainers and centrifuged at 500 × g for

10 min. Contaminating leukocytes were removed by an indirect magnetic separation technique. Cells were incubated at 37ºC for 30 min with biotinylated antibodies at the following concentrations: α-CD16/32 1:1,500, α-TER119 1:500, α-CD45 1:700, α-CD90 1:700. Cells were pelleted, resuspended in DMEM + 10% FBS, and incubated with streptavidin magnetic beads for 30 min at room temperature with rotation, then taped to a magnet for 15 min to remove all biotinylated cells. The supernatant containing the alveolar epithelial cells was removed and centrifuged at 500 × g for 10 min, and trypan blue-excluding cells were quantified using a hemacytometer. Cells were incubated with CCF2-AM and α-CD45 as described above and analyzed by flow cytometry. $CD45^+$ cells were excluded from FACS analysis of type II pneumocyte preparations.

Statistical methods

Analyses of bacterial load differences were performed by Student's t test. Prior to analysis, all colonization data were natural log-transformed so as to fit a normal distribution. The use of parametric tests on transformed colonization data was justified by analysis of a large set of control data that confirmed that colonization data were log-normally distributed. Proportions of each cell type injected were compared by analysis of variance (ANOVA). For ANOVA comparisons with $P < 0.05$, adjustment for multiple unplanned comparisons was performed using the Tukey-Kramer Honestly Significant Difference (HSD) test with $\alpha=0.05$. Analyses of differences in LDH release were performed by Student's t test.

Results

ExoU-Bla fusions are secreted in vitro.

To analyze translocation of ExoU, we generated C-terminal translational fusions of the full-length catalytically active ExoU or a catalytically inactive non-cytotoxic ExoU variant, designated ExoU(S142A), with the mature fragment of the TEM-1 β-lactamase (Bla). The constructs encoding these fusion proteins were introduced into a neutral site in the chromosome of PA99null, a strain with an intact type III secretion system but with disruptions in each of its effector-encoding genes (37). We assessed the ability of these strains, named PA99null+ExoU-Bla and PA99null+ExoU(S142A)-Bla, to secrete the respective fusion proteins by immunoblot analysis of supernatants from cultures grown in type III secretion-inducing conditions. Both fusion proteins were detected in culture supernatants (Fig. 1A), indicating that addition of Bla does not prevent movement of ExoU through the type III secretion needle.

We next examined translocation of the ExoU-Bla fusion proteins into J774 macrophage-like cells in vitro using fluorescence microscopy (Fig. 1 B-I). Following co-incubation with PA99null+ExoU-Bla, PA99null+ExoU(S142A)-Bla, or control strains, J774 cells were treated with the substrate CCF2-AM, which fluoresces upon diffusion into host cells. Intact CCF2-AM exhibits FRET resulting in green fluorescence, and cleavage of this substrate by a β-lactamase molecule disrupts FRET, resulting in a shift to blue fluorescence emission. Cells containing translocated ExoU-Bla (Fig. 1F) or ExoU(S142A)-Bla (Fig. 1G) were identified by blue fluorescence compared to uninfected cells (Fig. 1C) and cells infected with *P. aeruginosa* secreting ExoU or ExoU(S142A) without the Bla tag (Fig. 1D and Fig 1E, respectively). Notably, few cells remained after 3 h of incubation with either PA99null+ExoU (Fig. 1D) or PA99null+ExoU-Bla (Fig. 1F) as a result of rapid cell lysis induced by this effector, and remaining cells appeared severely damaged. Importantly, neither ExoU-Bla nor ExoU(S142A)-

Bla were associated with blue fluorescence when expressed from PA99secr$^-$, a PA99 mutant with a deletion in the *pscJ* gene, which encodes an essential component of the type III secretion apparatus (Fig. 1H and data not shown). Likewise *P. aeruginosa* expressing a glutathione-S-transferase-Bla fusion protein (GST-Bla) did not cause blue fluorescence (Fig. 1I), confirming that cleavage of the CCF2-AM substrate requires type III translocation of ExoU and is not due to engulfment of bacteria by J774 cells or non-specific movement of the β-lactamase moiety into these cells. These observations confirm that ExoU-Bla fusion proteins are translocated into host cells in vitro and that ExoU-injected cells are detectable in vitro using this reporter assay.

Secretion of ExoU(S142A)-Bla but not ExoU-Bla is detectable in vivo.

ExoU is injected into many cell types in vitro; however the relevant cellular targets in vivo during infection have not been determined. We intranasally infected mice with *P. aeruginosa* secreting either wildtype or catalytically inactive ExoU-Bla fusions or appropriate control strains. After 18 h, cells were recovered by bronchoalveolar lavage (BAL), incubated with CCF2-AM, and analyzed by flow cytometry. Infection with PA99null+ExoU(S142A) without the Bla moiety resulted in only green fluorescence (Fig. 2A). Similar to what we observed in vitro, blue cells were not detected in lavage fluid of mice infected with a non-secreting *P. aeruginosa* strain expressing either ExoU(S142A)-Bla or ExoU-Bla (Fig. 2B and data not shown) or with *P. aeruginosa* expressing GST-Bla (Fig. 2C). During infection with PA99null+ExoU-Bla, we were unable to detect cells containing translocated ExoU-Bla in BAL fluid (Fig. 2D) or whole lung tissue (data not shown). In contrast, a significant proportion (40%) of cells recovered from the lungs of mice infected with PA99null+ExoU(S142A)-Bla were injected with fusion protein (Fig. 2E). The inability to detect cells containing catalytically active

ExoU-Bla is likely due to the rapid cell death induced by this molecule since in vitro observations have shown that ExoU induces fast and complete cell lysis (6). Consistent with this explanation is that our in vitro CCF2-AM experiments using PA99null+ExoU-Bla also resulted in relatively few blue-fluorescent cells (Fig. 1F). Because ExoU-induced cell lysis precluded in vivo detection of injected cells, we utilized the non-cytotoxic ExoU(S142A)-Bla fusion for quantification and identification of injected cells in vivo.

Since secretion of catalytically active ExoU facilitates bacterial persistence in the lung, higher numbers of bacteria are present over the course of infection with an ExoU$^+$ strain relative to an ExoU$^-$ strain or *P. aeruginosa* expressing the non-cytotoxic ExoU variant (ExoU(S142A)) (6). To more accurately model infection with *P. aeruginosa* secreting catalytically active ExoU, we infected mice with a higher dose of PA99null+ExoU(S142A)-Bla (1.2×10^7 CFU/mouse) that resulted in similar numbers of bacteria present in the lungs after 18 h of infection relative to a standard inoculum (1.2×10^6 CFU) of PA99null+ExoU (data not shown). Infection with this dose resulted in the recruitment of high numbers of inflammatory cells to the lung over time (data not shown). These conditions approximate the bacterial burden and bacterium-to-host cell ratio observed during infection with *P. aeruginosa* secreting catalytically active ExoU (6), indicating that secretion of ExoU(S142A) under these conditions is a suitable model to study ExoU secretion. Therefore, we used the higher dose for all experiments in this study.

A substantial portion of phagocytic cells are injected with ExoU(S142A) in the lung.

Previous studies indicated that ExoU contributes to virulence by compromising phagocytic cells intended to eradicate bacteria from the lung (6). Based on these observations, we aimed to directly identify the cell types that were injected with ExoU during acute

pneumonia. For these experiments, cells were recovered from whole lung tissue of infected mice, incubated with CCF2-AM, stained with cell discriminatory antibodies, and analyzed by flow cytometry. We initially investigated the cell types injected with ExoU(S142A) at 18 h post-infection, when large numbers of inflammatory cells are present in the lung (6). In this experiment, approximately one-half million cells recovered from the lung of an infected mouse were injected with ExoU(S142A) (Fig. 3A). Of this population of injected cells, 99% (452,000/458,000) were inflammatory cells and over 80% (383,000/458,000) were phagocytic cells (Fig. 3A). Within the population of injected phagocytic cells, neutrophils were the predominant cell type (60%) followed by monocytes (27%) and macrophages (5%), while dendritic cells comprised less than 1% of the injected cell population (Fig. 3A). In addition to these phagocytic cell types, we also examined $CD4^+$ and $CD8^+$ T cells, B cells, and NK cells. Together lymphocytes comprised < 2% of injected cells recovered from the lung (Fig. 3A). The majority of cells (> 85%) incorporated CCF2-AM efficiently, and no obvious difference in CCF2-AM uptake by cell type was observed (data not shown). Therefore a single cell type was not specifically targeted for injection with ExoU, but phagocytic cells, particularly neutrophils, comprised the majority of cells injected with ExoU(S142A) in vivo.

To examine whether the size of the various cell populations within the lung dictated the number of that cell type injected with ExoU(S142A), we determined the total numbers of each cell type recovered from the lungs (Fig. 3B) and calculated the proportion of each cell type that was injected (Fig. 3C). Although the composition of injected cells largely mirrored the overall population of cell types in the lung (compare Fig. 3A to Fig. 3B), there was a modest preference for neutrophils. Of all the neutrophils recovered from the lung, 16% were injected with ExoU(S142A) compared to 7% of monocytes, 5% of macrophages, 2% of dendritic cells, and

1% of lymphocytes ($p < 0.01$, neutrophils compared to each cell type; Fig. 3C). Therefore a higher proportion of neutrophils were injected with ExoU(S142A) than other cell types.

Most *P. aeruginosa* isolates secrete multiple effector molecules. To assess the effect of co-secretion of ExoS and/or ExoT on translocation of ExoU in vivo, we examined the cell types injected with ExoU(S142A)-Bla during infection with *P. aeruginosa* secreting ExoU(S142A)-Bla in combination with ExoS alone (PA99S+ExoU(S142A)-Bla) or both ExoT and ExoS (PA99ST+ExoU(S142A)-Bla) (Fig. 4). For these experiments, we used a lower bacterial inoculum (6×10^6 CFU/mouse) since secretion of ExoS results in more severe illness (37). Similar to infection with PA99null+ExoU(S142A)-Bla, phagocytic cell types comprised over 95% of injected cells in the lung (Figure 4A). We assessed the proportion of neutrophils, monocytes, and macrophages among injected cells at 18 h post-infection. Interestingly, the repertoire of recruited inflammatory cells was somewhat different when ExoS alone or ExoS and ExoT were secreted with ExoU. We observed slightly higher numbers of injected monocytes relative to neutrophils when ExoS was secreted compared to infection with PA99null+ExoU(S142A)-Bla, a result which was also reflected in the total cells recovered from the lungs of these animals (Figure 4B). This difference may be due to previously undescribed effects of ExoS. Still, the relative size of the various cell populations dictated the number of that cell type injected with ExoU(S142A) even in the presence of other effectors (compare Figs. 4A and 4B). Thus, secretion of additional effectors does not dramatically alter the specificity of injection in vivo.

ExoU intoxication preferentially occurs in the airways of infected animals.

353 We next addressed the question of where in the lungs injection with ExoU(S142A) was
354 occurring. Since *P. aeruginosa* causes a bronchopneumonia in which the majority of bacteria
355 reside in the airways and alveoli (7, 22), and since type III secretion requires direct contact
356 between the bacterium and a host cell, we anticipated that injection would primarily occur in
357 these lung compartments. Proportions of injected cells in the airways (recovered by BAL) were
358 compared to those in the entire lung (recovered from whole lung tissue) (Fig. 5). A significantly
359 higher proportion of ExoU(S142)-intoxicated cells were recovered from the airways and alveoli
360 (29%, Fig. 5A) relative to cells recovered from whole lung tissue (11%, Fig. 5B) ($p < 0.05$). The
361 relative abundance of ExoU-injected cells in the airway indicates that type III secretion
362 predominantly occurs in the airspace of infected lungs.
363
364 *Type II alveolar epithelial cells are not appreciably injected with ExoU(S142A).*
365 The protocols used in the preceding experiments are not conducive for the purification of
366 cell types other than immune cells. Thus approximately 99% of cells recovered from the lungs
367 of infected mice were resident or recruited leukocytes (Fig. 3B and data not shown). Since ExoU
368 injects and lyses numerous epithelial cell types in vitro and secretion appears to occur primarily
369 in the airways and alveoli of infected mice, we wished to determine whether alveolar epithelial
370 cells (pneumocytes), were injected with ExoU during infection. Although type I pneumocytes
371 comprise the majority of the alveolar surface area, these cells are not amenable to isolation from
372 mouse lungs. Type II pneumocytes comprise much less of the epithelial surface but nonetheless
373 are extremely important in lung physiology and response to infection. Type II pneumocytes
374 differentiate into type I cells to maintain the epithelium and also provide protection from
375 pathogens by secreting surfactant into the alveolar space. To assess secretion of ExoU into cell

types other than leukocytes, we isolated type II pneumocytes from the lungs of mice infected with PA99null+ExoU(S142A)-Bla by digestion with elastase followed by removal of leukocytes by magnetic separation. Cells were incubated with CCF2-AM, and injection was measured by flow cytometry (Fig. 6). Any contaminating leukocytes were removed from the analysis of type II pneumocytes by exclusion of $CD45^+$ cells. BAL samples, which contained mostly leukocytes, were analyzed in parallel. Interestingly, while 15% of cells in the BAL fluid were intoxicated with ExoU(S142A), minimal injection of ExoU(S142A)-Bla into type II pneumocytes (1%) was observed (Fig. 6B). Both cell populations incorporated CCF2-AM with similar efficiency (> 85%). This result indicates that not all cell types present in the lungs are appreciably injected with ExoU during acute pneumonia.

ExoU injection specificity changes over time.

Finally we examined whether the cell types injected with ExoU(S142A) changed over time (Fig. 7). For these experiments, we infected mice with PA99null+ExoU(S142A)-Bla or appropriate control strains and sacrificed animals after 3, 6, 12 or 18 h. Cells were recovered from the whole lungs, incubated with CCF2-AM, stained with cell discriminatory antibodies, and analyzed by flow cytometry. The number of cells recovered from the infected mouse lungs steadily increased over the first 18 h following inoculation (Fig. 7A), reflecting the recruitment of inflammatory cells to the site of infection. At all time points examined, the majority of cells recovered from the lungs were leukocytes (data not shown). At 3 h post-infection, a substantial number of recovered cells were resident (alveolar) macrophages (Fig. 7B). A robust neutrophilic infiltrate was apparent at 6 h and reached a maximum at 12 h after infection. Monocyte recruitment was somewhat more delayed and continued to increase through 18 h post-infection.

399 The increase in monocytes may reflect the recruitment of macrophage precursors intended to
400 replenish the resident macrophage population. The timing of mononuclear cell recruitment
401 observed in our model is similar to that described by other investigators (15). Relatively few
402 dendritic cells and lymphocytes were recovered at all time points.

403 The total number of cells injected with ExoU(S142A) increased over the first 18 h of the
404 infections. After only 3 h, approximately 2% of total recovered cells were already injected, but
405 this proportion rose to approximately 10% by 18 h (Fig. 7C). This increase was statistically
406 significant ($p < 0.05$). Thus even as more inflammatory cells entered the lungs, a larger
407 proportion of these cells were injected with ExoU(S142A).

408 At all time points tested, neutrophils, monocytes, and macrophages comprised the
409 majority of injected cells recovered from the lungs whereas dendritic cells and lymphocytes each
410 comprised less than 5% of injected cells (Fig. 7D). Therefore, the proportion of each cell type in
411 the injected cell population reflected to a large extent the composition of the total leukocytes
412 recovered from the lung at each time point (compare Figs. 7B and 7D). At 3 h post-infection,
413 alveolar macrophages were the predominant cell type among injected cells (58%) while
414 neutrophils and monocytes accounted for only 17% and 18%, respectively (Fig. 7D). Likewise,
415 alveolar macrophages represented approximately 40% of the total cells recovered from the lungs
416 at this early time point, before neutrophils and monocytes had yet been appreciably recruited to
417 lungs. At 6 h post-infection, 70% of injected cells recovered from the lung were neutrophils and
418 18% were monocytes while only 5% were macrophages. Again, this proportion closely reflected
419 the relative abundance of each cell type in the total cell population at this time point (Fig. 7B). At
420 12 and 18 h, neutrophils remained the predominant cell type injected with ExoU(S142A) even
421 though the proportion of monocytes present in the lung increased substantially (Fig. 7B). In

summary, alveolar macrophages, resident cells of the lung, were injected early during infection prior to the recruitment of neutrophils and monocytes but as neutrophils and mononuclear phagocytes were recruited to the lungs, they became the most commonly injected cell types.

Discussion

Using a FRET-based reporter assay, we have directly shown that phagocytic cells are extensively injected with ExoU during acute pneumonia caused by *P. aeruginosa*. Resident alveolar macrophages were injected early during infection while recruited neutrophils and monocytes were injected slightly later as these cells entered the lungs in response to the invading microbes. To our knowledge, this is the first identification of cell types targeted by a *P. aeruginosa* type III effector in vivo and the first examination of cell injection during pneumonia for any type III secretion system. As such, these results provide important insights into the mechanisms by which bacteria cause pneumonia.

Using a non-cytotoxic ExoU variant, we found that the cell types injected with ExoU reflect the overall repertoire of immune cells in the lungs over the first 18 h of infection. Injection was observed as early as 3 h post-infection, at a time when few inflammatory cells were present in the lungs. Thus resident alveolar macrophages comprised the majority of the initially injected cells in the lung. The subsequent influx of neutrophils and monocytes into the lung correlated with an increase in injection of these cell types, suggesting that cell type targeting of ExoU is to a large extent determined by the availability of cells rather than a specific preference of the type III secretion apparatus or *P. aeruginosa* adhesins for particular cell types. However, a somewhat higher proportion of neutrophils in the lungs were injected with ExoU

compared to other cell types. This may simply reflect increased numbers of *P. aeruginosa* bacteria binding to neutrophils because of the large surface area and phagocytic nature of these cells, or because a larger proportion of neutrophils migrate into the airways and come into contact with bacteria. In any case, our results indicate that phagocytic cells are a primary target of ExoU in vivo. Since most ExoU$^+$ clinical isolates also secrete ExoT and many secrete ExoY, it may be that these effector proteins are similarly targeted. ExoS, however, is rarely secreted by ExoU$^+$ isolates, and ExoS-secreting strains constitute a distinct clonal group from ExoU$^+$ strains (8, 40). For these reasons, it remains possible that ExoS-secreting strains differ in their pathogenesis and that this effector protein is injected into distinct populations of host cells. Examination of the cell types injected with other *P. aeruginosa* effectors during acute pneumonia is currently underway.

Targeting of phagocytic cells, including neutrophils, has previously been implicated in the pathogenesis of other Gram-negative bacterial pathogens. Geddes and colleagues showed that *Salmonella enterica* targets splenic neutrophils for injection with type III effectors to promote intracellular survival in vivo (11). Similarly, Marketon et al. and Koberle et al. observed injection of neutrophils, macrophages, and dendritic cells in the spleen during infection with *Yersinia* spp. (18, 24). In these cases, targeting of splenic innate immune cells was shown to be a mechanism of evading or disrupting host immune responses. Our results demonstrate that neutrophils are also targets for type III secretion in the lung. Previous experimental observations suggested that impairment of recruited phagocytic cells by ExoU was important for *P. aeruginosa* survival in the lung and progression to severe disease. In agreement with this functional evidence, we found that *P. aeruginosa* injected ExoU into neutrophils and macrophages during pneumonia. Impairment or killing of these cells by direct injection of ExoU

468 may prevent effective clearance from the lung and contribute to the enhanced disease observed
469 with ExoU$^+$ strains.

470 Interestingly, we did not observe significant injection of type II pneumocytes during
471 infection. There are several possible explanations for this finding. Infiltrating inflammatory
472 cells may bind *P. aeruginosa* with increased affinity, thereby reducing the number of bacteria
473 available to bind to epithelial cells. Alternatively, type II pneumocytes may be resistant to
474 injection with ExoU. If this is the case, damage to the lung epithelium, previously observed
475 during infection with ExoU$^+$ *P. aeruginosa* (20), may be an indirect effect caused by release of
476 damaging inflammatory mediators from ExoU-lysed phagocytic cells rather than direct killing by
477 injection of ExoU into epithelial cells. Additional studies will be necessary to understand the
478 interaction between pulmonary epithelial cells and ExoU.

479 Injection with ExoU disproportionately occurred in the airspace compared to other
480 compartments of the lung during early pneumonia. This finding is consistent with several
481 previous observations. First, the majority of *P. aeruginosa* bacteria reside in the airways and
482 alveoli of the lungs during pneumonia (7, 22). Since type III secretion requires direct contact
483 between the bacterium and the host cell, this implies that secretion would primarily occur in the
484 airspace. Second, we previously observed a relative reduction of viable neutrophils in the
485 airways and alveoli relative to whole lung compartments of mice infected with ExoU$^+$ *P.*
486 *aeruginosa* (6), suggesting that neutrophils are injected with ExoU upon transmigration across
487 the respiratory epithelium. Thus, in early pneumonia, type III secretion may be most actively
488 employed against immune cells as they enter the airways and alveoli.

489 We were unable to detect secretion of catalytically active ExoU-Bla into host cells in
490 vivo. This is likely due to the rapid cell lysis induced by this molecule since cells injected with

ExoU(S142A)-Bla, a non-cytotoxic ExoU variant, were detectable at all time points examined. Previously it has been shown that ExoU induces rapid and complete cell lysis of neutrophils in vitro, which would preclude detection of ExoU-injected cells in vivo (6). Similarly, other investigators have also been unable to detect cells containing catalytically active ExoU (9, 27, 33). Our inability to detect cells injected with catalytically active ExoU further supports the idea that ExoU induces rapid lysis of phagocytic cells in vivo. The use of a non-catalytic variant of ExoU rather than wild-type ExoU is one limitation of this study because the lack of cell killing by ExoU alters bacterial survival, disease progression and the host immune response. We attempted to more accurately mimic infection with a strain producing wild-type ExoU by increasing the infecting dose to increase the overall number of bacteria in the lung and enhance recruitment of inflammatory cells to the lung. Nonetheless, it remains possible that the dynamics of translocation may be somewhat different in the context of wild-type ExoU. Another potential limitation is that throughout this study we utilized a strain which secreted ExoU(S142A)-Bla alone, in the absence of other effector proteins. Clinical isolates of *P. aeruginosa* usually secrete combinations of type III effector proteins. However, this does not appear to be a major limitation since the presence of ExoS and ExoT did not dramatically alter the types of cells injected with ExoU and since the injected cell populations continued to closely mirror the total phagocytic cell populations present in the lung (Fig. 4). This is consistent with published reports indicating that co-secretion of ExoS or ExoT along with ExoU did not substantially alter the severity of pneumonia (36). Nonetheless, co-secretion of other effector proteins could have subtle effects on the total numbers and proportions of host cells injected with ExoU. Interestingly, the repertoire of inflammatory cells recruited to the lung did change when ExoS or ExoS plus ExoT

were secreted along with ExoU(S142A), indicating that the effector proteins did dictate the nature of the inflammatory response.

By directly analyzing injection of cells in the lung during acute infection, this study suggests a model for the mechanism of ExoU during early pneumonia. During the initial stage of infection, alveolar macrophages are injected, which may incapacitate these sentinel cells that are crucial in host defense during early *P. aeruginosa* pneumonia (19). Later ExoU injection into phagocytes may allow *P. aeruginosa* to evade phagocytosis by these immune cells, allowing bacteria to persist in large numbers in the lung. Thus ExoU injection creates an environment in which the bacteria can survive and cause the pathology observed during severe pneumonia.

Acknowledgements

This work was supported by the National Institute of Health (grants AI053674, AI065615, AI053674, and AI075191 (A. R. H.) and T32 GM008061 (M. H. D.)).

We thank Ciara Shaver, Cheryl Olson, Kerry Sheppard, and Karen Ridge for their advice and technical assistance with the experiments.

References

1. Allewelt, M., F. T. Coleman, M. Grout, G. P. Priebe, and G. B. Pier. 2000. Acquisition of expression of the *Pseudomonas aeruginosa* ExoU cytotoxin leads to increased bacterial virulence in a murine model of acute pneumonia and systemic spread. Infect. Immun. 68:3998-4004.

2. Barbieri, J. T., and J. Sun. 2004. *Pseudomonas aeruginosa* ExoS and ExoT. Rev. Physiol. Biochem. Pharmacol. 152:79-92.

3. Charpentier, X., and E. Oswald. 2004. Identification of the secretion and translocation domain of the enteropathogenic and enterohemorrhagic *Escherichia coli* effector Cif, using TEM-1 beta-lactamase as a new fluorescence-based reporter. J. Bacteriol. 186:5486-5495.

4. Chastre, J., and J. Y. Fagon. 2002. Ventilator-associated pneumonia. Am J Respir Crit Care Med 165:867-903.

5. Comolli, J. C., A. R. Hauser, L. Waite, C. B. Whitchurch, J. S. Mattick, and J. N. Engel. 1999. *Pseudomonas aeruginosa* gene products PilT and PilU are required for cytotoxicity in vitro and virulence in a mouse model of acute pneumonia. Infect. Immun. 67:3625-3630.

6. Diaz, M. H., C. M. Shaver, J. D. King, S. Musunuri, J. A. Kazzaz, and A. R. Hauser. 2008. *Pseudomonas aeruginosa* induces localized immunosuppression during pneumonia in mammals. Infect. Immun. 76:4414-4421.

547 7. DiMango, E., H. Zar, R. Bryan, and A. Prince. 1995. Diverse *Pseudomonas aeruginosa*
548 gene products stimulate respiratory epithelial cells to produce interleukin-8. J. Clin.
549 Invest. 96:2204-2210.

550 8. Feltman, H., G. Schulert, S. Khan, M. Jain, L. Peterson, and A. R. Hauser. 2001.
551 Prevalence of type III secretion genes in clinical and environmental isolates of
552 *Pseudomonas aeruginosa*. Microbiology (Reading, England) 147:2659-2669.

553 9. Finck-Barbançon, V., and D. W. Frank. 2001. Multiple domains are required for the toxic
554 activity of *Pseudomonas aeruginosa* ExoU. J. Bacteriol. 183:4330-4344.

555 10. Finck-Barbançon, V., J. Goranson, L. Zhu, T. Sawa, J. P. Wiener-Kronish, S. M. J.
556 Fleiszig, C. Wu, L. Mende-Mueller, and D. Frank. 1997. ExoU expression by
557 *Pseudomonas aeruginosa* correlates with acute cytotoxicity and epithelial injury. Mol.
558 Microbiol. 25:547-557.

559 11. Geddes, K., F. Cruz, and F. Heffron. 2007. Analysis of cells targeted by Salmonella type
560 III secretion in vivo. PLoS Pathog 3:e196.

561 12. Gordon, S., and P. R. Taylor. 2005. Monocyte and macrophage heterogeneity. Nat. Rev.
562 Immunol. 5:953-964.

563 13. Hauser, A. R., E. Cobb, M. Bodí, D. Mariscal, J. Vallés, J. N. Engel, and J. Rello. 2002.
564 Type III protein secretion is associated with poor clinical outcomes in patients with
565 ventilator-associated pneumonia caused by *Pseudomonas aeruginosa*. Crit. Care Med.
566 30:521-528.

14. Hauser, A. R., P. J. Kang, and J. Engel. 1998. PepA, a novel secreted protein of *Pseudomonas aeruginosa,* is necessary for cytotoxicity and virulence. Mol. Microbiol. 27:807-818.

15. Henderson, R. B., J. A. Hobbs, M. Mathies, and N. Hogg. 2003. Rapid recruitment of inflammatory monocytes is independent of neutrophil migration. Blood 102:328-335.

16. Hoang, T. T., A. J. Kutchma, A. Becher, and H. P. Schweizer. 2000. Integration-proficient plasmids for *Pseudomonas aeruginosa*: site- specific integration and use for engineering of reporter and expression strains. Plasmid 43:59-72.

17. Koberle, M., A. Klein-Gunther, M. Schutz, M. Fritz, S. Berchtold, E. Tolosa, I. B. Autenrieth, and E. Bohn. 2009. Yersinia enterocolitica targets cells of the innate and adaptive immune system by injection of Yops in a mouse infection model. PLoS Pathog 5:e1000551.

18. Koberle, M., A. Klein-Gunther, M. Schutz, M. Fritz, S. Berchtold, E. Tolosa, I. B. Autenrieth, and E. Bohn. 2009. *Yersinia enterocolitica* targets cells of the innate and adaptive immune system by injection of Yops in a mouse infection model. PLoS Pathog. 5:e1000551.

19. Kooguchi, K., S. Hashimoto, A. Kobayashi, Y. Kitamura, I. Kudoh, J. Wiener-Kronish, and T. Sawa. 1998. Role of alveolar macrophages in initiation and regulation of inflammation in *Pseudomonas aeruginosa* pneumonia. Infect. Immun. 66:3164-3169.

586 20. Kurahashi, K., O. Kajikawa, T. Sawa, M. Ohara, M. A. Gropper, D. W. Frank, T. R.
587 Martin, and J. P. Wiener-Kronish. 1999. Pathogenesis of septic shock in *Pseudomonas*
588 *aeruginosa* pneumonia. J. Clin. Invest. 104:743-750.

589 21. Lee, V. T., R. S. Smith, B. Tummler, and S. Lory. 2005. Activities of *Pseudomonas*
590 *aeruginosa* effectors secreted by the type III secretion system in vitro and during
591 infection. Infect. Immun. 73:1695-1705.

592 22. Lyczak, J. B., C. L. Cannon, and G. B. Pier. 2002. Lung infections associated with cystic
593 fibrosis. Clin. Microbiol. Rev. 15:194-222.

594 23. Mandell, G. L., J. E. Bennett, and R. Dolin. 2005. Mandell, Douglas, and Bennett's
595 Principles and Practice of Infectious Diseases, 6th ed. Elsevier, Philadelphia.

596 24. Marketon, M. M., R. W. DePaolo, K. L. DeBord, B. Jabri, and O. Schneewind. 2005.
597 Plague bacteria target immune cells during infection. Science (New York, N.Y 309:1739-
598 1741.

599 25. Mills, E., K. Baruch, X. Charpentier, S. Kobi, and I. Rosenshine. 2008. Real-time
600 analysis of effector translocation by the type III secretion system of enteropathogenic
601 Escherichia coli. Cell Host Microbe 3:104-113.

602 26. Nicas, T. I., and B. H. Iglewski. 1984. Isolation and characterization of transposon-
603 induced mutants of *Pseudomonas aeruginosa* deficient in production of exoenzyme S.
604 Infect. Immun. 45:470-474.

605 27. Phillips, R. M., D. A. Six, E. A. Dennis, and P. Ghosh. 2003. In vivo phospholipase
606 activity of the *Pseudomonas aeruginosa* cytotoxin ExoU and protection of mammalian
607 cells with phospholipase A2 inhibitors. J. Biol. Chem. 278:41326-41332.

608 28. Rabin, S. D., J. L. Veesenmeyer, K. T. Bieging, and A. R. Hauser. 2006. A C-terminal
609 domain targets the *Pseudomonas aeruginosa* cytotoxin ExoU to the plasma membrane of
610 host cells. Infect. Immun. 74:2552-2561.

611 29. Rabin, S. D. P., and A. R. Hauser. 2005. Functional regions of the *Pseudomonas*
612 *aeruginosa* cytotoxin ExoU. Infect. Immun. 73:573-582.

613 30. Rello, J., E. Diaz, and A. Rodriguez. 2005. Etiology of ventilator-associated pneumonia.
614 Clin. Chest Med. 26:87-95.

615 31. Ridge, K. M., D. H. Rutschman, P. Factor, A. I. Katz, A. M. Bertorello, and J. L.
616 Sznajder. 1997. Differential expression of Na-K-ATPase isoforms in rat alveolar
617 epithelial cells. Am J Physiol 273:L246-255.

618 32. Roy-Burman, A., R. H. Savel, S. Racine, B. L. Swanson, N. S. Revadigar, J. Fujimoto, T.
619 Sawa, D. W. Frank, and J. P. Wiener-Kronish. 2001. Type III protein secretion is
620 associated with death in lower respiratory and systemic *Pseudomonas aeruginosa*
621 infections. J. Infect. Dis. 183:1767-1774.

622 33. Sato, H., and D. W. Frank. 2004. ExoU is a potent intracellular phospholipase. Mol.
623 Microbiol. 53:1279-1290.

624 34. Sato, H., D. W. Frank, C. J. Hillard, J. B. Feix, R. R. Pankhaniya, K. Moriyama, V.
625 Finck-Barbancon, A. Buchaklian, M. Lei, R. M. Long, J. Wiener-Kronish, and T. Sawa.
626 2003. The mechanism of action of the *Pseudomonas aeruginosa*-encoded type III
627 cytotoxin, ExoU. EMBO J. 22:2959-2969.

628 35. Schulert, G. S., H. Feltman, S. D. P. Rabin, C. G. Martin, S. E. Battle, J. Rello, and A. R.
629 Hauser. 2003. Secretion of the toxin ExoU is a marker for highly virulent *Pseudomonas*
630 *aeruginosa* isolates obtained from patients with hospital-acquired pneumonia. J. Infect.
631 Dis. 188:1695-1706.

632 36. Shaver, C. M., and A. R. Hauser. 2006. Interactions between effector proteins of the
633 Pseudomonas aeruginosa type III secretion system do not significantly affect several
634 measures of disease severity in mammals. Microbiology (Reading, England) 152:143-
635 152.

636 37. Shaver, C. M., and A. R. Hauser. 2004. Relative contributions of *Pseudomonas*
637 *aeruginosa* ExoU, ExoS, and ExoT to virulence in the lung. Infect. Immun. 72:6969-
638 6977.

639 38. Simon, R., U. Priefer, and A. Puhler. 1983. A broad host range mobilization system for in
640 vivo genetic engineering: transposon mutagenesis in gram negative bacteria.
641 Biotechnology 1:784-791.

642 39. Vogel, H. J., and D. M. Bonner. 1956. Acetylornithinase of *Escherichia coli* partial
643 purification and some properties. J. Biol. Chem. 218:97-106.

644 40. Wolfgang, M. C., B. R. Kulasekara, X. Liang, D. Boyd, K. Wu, Q. Yang, C. G. Miyada,
645 and S. Lory. 2003. Conservation of genome content and virulence determinants among
646 clinical and environmental isolates of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. U
647 S A 100:8484-8489.

648 41. Yahr, T. L., A. J. Vallis, M. K. Hancock, J. T. Barbieri, and D. W. Frank. 1998. ExoY, an
649 adenylate cyclase secreted by the *Pseudomonas aeruginosa* type III system. Proc. Natl.
650 Acad. Sci. USA 95:13899-13904.

651 42. Zlokarnik, G., P. A. Negulescu, T. E. Knapp, L. Mere, N. Burres, L. Feng, M. Whitney,
652 K. Roemer, and R. Y. Tsien. 1998. Quantitation of transcription and clonal selection of
653 single living cells with beta-lactamase as reporter. Science (New York, N.Y 279:84-88.
654
655

Figure Legends

Figure 1. ExoU-Bla fusion proteins are injected into host cells in vitro. (A) Immunoblot analysis of culture supernatants from *P. aeruginosa* strains secreting ExoU-Bla or ExoU(S142A)-Bla grown in type III secretion-inducing conditions using polyclonal ExoU antiserum. The same volume of supernatant was used for each sample. (B-I) J774 cells were incubated with *P. aeruginosa* bacteria at an MOI of 10 followed by treatment with CCF2-AM for 1 h. Cells were examined using a filter set that allows visualization of both green and blue fluorescence simultaneously. Green cells contain intact CCF2-AM, and blue cells contain cleaved CCF2-AM, indicating translocation of the ExoU-Bla fusion. Representative fields are shown from the following conditions: uninfected J774 cells (B) without or (C) with CCF2-AM substrate; cells incubated with CCF2-AM after infection with (D) PA99null+ExoU, (E) PA99null+ExoU(S142A), (F) PA99null+ExoU-Bla, (G) PA99null+ExoU(S142A)-Bla, (H) PA99secr$^-$+ExoU(S142A)-Bla, or (I) PA99null+GST-Bla. Scale bars, 100 µm.

Figure 2. Detection of cells injected with ExoU-Bla fusion proteins in vivo. FACS analysis was performed on cells recovered by BAL at 18 h post-infection. Green fluorescence (*y*-axis) indicates the presence of CCF2-AM in the cell, and blue fluorescence (*x*-axis) indicates translocation of the ExoU(S142A)-Bla fusion protein. The percentage of blue (injected) cells is indicated in the upper right corner of each dot plot. (A) PA99null+ExoU(S142A), (B) PA99secr$^-$ +ExoU(S142A)-Bla, and (C) PA99null+GST-Bla resulted in only green cells. (D) Infection with PA99null+ExoU-Bla did not yield a significant proportion of blue cells relative to control strains. (E) ExoU(S142A)-Bla translocation was detectable in vivo; approximately 40% of cells recovered from BAL fluid were intoxicated with ExoU(S142A)-Bla. Data shown are from a

representative experiment; similar results were obtained in at least three independent experiments. Results varied from day-to-day, with mean percent injected cells ranging from 15%-40%.

Figure 3. Analysis of injected and total cells recovered from the lungs of mice infected with *P. aeruginosa*. FACS analysis was performed on cells recovered from the whole lungs of mice infected with PA99null+ExoU(S142A)-Bla at 18 h post-infection. Injected cells (A) and total cells (B) per mouse of each immune cell type recovered from whole lungs. Cells per mouse were determined by equating the number of total cells with normal scatter characteristics measured by flow cytometry to the number of trypan-blue-negative cells counted in the hemacytometer. (C) Proportion of each of the cell subpopulations purified from the lungs that were injected with ExoU(S142A)-Bla. A significantly higher proportion of neutrophils were injected relative to monocytes, macrophages, dendritic cells, or lymphocytes. Data are means ± SEM; n ≥ 3 per group; similar results were obtained in at least three independent experiments. Results varied from day-to-day, with mean percent injected cells ranging from 7%-15%. * $p < 0.01$.

Figure 4. Secretion of ExoU(S142A)-Bla in the presence of other effectors in vivo. FACS analysis was performed on cells recovered from the lungs of mice infected with PA99null strain engineered to secrete ExoU(S142A)-Bla alone (PA99null+ExoU(S142A)-Bla); ExoU(S142A)-Bla along with ExoS (PA99S+ExoU(S142A)-Bla); or ExoU(S142A)-Bla along with ExoS and ExoT (PA99ST+ExoU(S142A)-Bla) at 18 h post-infection. **(A)** Percentage of phagocytic cell types in the injected cell population recovered from infected lungs. **(B)** Proportion of phagocytic cell types among total cells recovered from infected lungs. Data are means ± SEM from a

representative experiment; n = 3 per group. Similar results were obtained in two independent experiments. * $p < 0.05$.

Figure 5. ExoU injection occurs in the airspace of infected lungs. FACS analysis was performed on cells recovered from BAL fluid (A) or whole lungs (B). Green fluorescence (*y*-axis) indicates presence of CCF2-AM in the cells, and blue fluorescence (*x*-axis) indicates translocation of the ExoU(S142)-Bla fusion protein. The percentage of blue (injected) cells is indicated in the upper right corner of each density plot. Inset shows histogram of blue fluorescence. The proportion of injected cells in the BAL fluid (29%) was approximately three times that in the whole lung sample (11%). Representative plots are shown; similar results were obtained in at least three experiments with a total of 8 mice; $p < 0.05$.

Figure 6. Type II alveolar epithelial cells (pneumocytes) are not appreciably injected with ExoU(S142A). FACS analysis was performed on type II pneumocytes isolated from lungs of mice infected with PA99null+ExoU(S142A)-Bla at 18 h post-infection. Cells recovered by BAL were analyzed in parallel. (A) Histogram of blue fluorescence of cells from BAL fluid (top panel) or type II pneumocyte preparations (bottom panel). Blue cells indicate injection with ExoU(S142A)-Bla. Brackets indicate cells exhibiting blue fluorescence. Representative plots are shown from a total of 3 experiments. (B) Percentage of cells in BAL fluid or type II pneumocyte preparations that were injected with ExoU(S142A)-Bla. Data are means ± SEM of triplicate samples of cells pooled from three mice; similar results were obtained in three independent experiments. * $p < 0.00001$.

Figure 7. Cells injected with ExoU(S142A) in the lung over time. FACS analysis was performed on cells recovered from whole lungs of mice infected with PA99null+ExoU(S142A)-Bla at 3, 6, 12 and 18 h post-infection. (A) Total leukocytes recovered from the lungs of mice infected with ExoU(S142A)-Bla. Total lung leukocytes increased over the course of infection. (B) Proportion of cell types among total cells recovered from the lung. (C) Percentage of total cells that were injected with ExoU(S142A). (* $p < 0.05$ relative to preceding time point) (D) Percentage of immune cell types in the injected cell population. Lymphocyte subpopulations (T cells, B cells, and NK cells) are combined. Data are means ± SEM; $n \geq 3$ per group; similar results were obtained in at least two independent experiments.

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

Bacterial strain or plasmid	Relevant characteristics	Reference or source
P. aeruginosa strains		
PA99	Clinical isolate, naturally secretes ExoS, ExoT, and ExoU	(8)
PA99null	Secretes no effectors; PA99Δ*exoSexoTexoU*; gentamicin resistant	(37)
PA99secr-	Secretes no effectors, does not make secretion apparatus; PA99Δ*pscJ*	(37)
PA99T	Secretes only ExoT; PA99null complemented with *exoT* in the *att* locus	(37)
PA99null+ExoU	PA99null complemented with *exoU* in the *att* locus	This work
PA99null+ExoU(S142A)	PA99null complemented with *exoU(S142A)* in the *att* locus	(6)
PA99null+ExoU-Bla	PA99null complemented with *exoU-bla* in the *att* locus	This work
PA99null+ExoU(S142A)-Bla	PA99null complemented with *exoU(S142A)-bla* in the *att* locus	This work
PA99null+GST-Bla	PA99null complemented with *gst-bla* under control of native *exoU* promoter in the *att* locus	This work
PA99secr$^-$ + ExoU-Bla	PA99secr$^-$ complemented with *exoU-bla* in the *att* locus	This work
PA99secr$^-$ + ExoU(S142A)-Bla	PA99secr$^-$ complemented with *exoU(S142A)-bla* in the *att* locus	This work
Plasmids		
mini-CTX-1	plasmid for chromosomal gene integration into *attB* locus; Tet^r	H. Schweizer, (16)
mini-CTX*exoU*	*exoU* gene ligated into mini-CTX-1; Tet^r	(29)
mini-CTX*exoU-bla*	miniCTX*exoU* with C-terminal bla tag; Tet^r	This work
mini-CTX*exoU(S142A)-bla*	miniCTX*exoU(S142A)* with C-terminal bla tag; Tet^r	This work
mini-CTX*gst-bla*	*gst-bla* replaces *exoU* coding sequence in miniCTX*exoU-bla*; Tet^r	This work

735