



16 **Abstract**

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

32 **Introduction**

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

42 Of the many bacterial factors that contribute to the pathogenesis of *P. aeruginosa*, the  
43 type III secretion system is important for bacterial persistence in the presence of elaborate host  
44 defense mechanisms and has been associated with poor clinical outcomes in individuals with  
45 VAP (13, 32). While the genes encoding the type III secretion apparatus are present in all *P.*  
46 *aeruginosa* isolates, the repertoire of effector-encoding genes is variable. Approximately 28% of  
47 strains from acute infections encode a potent cytotoxin, ExoU (8), which is a marker of highly  
48 virulent *P. aeruginosa* strains isolated from patients with VAP (35). In animal models, ExoU  
49 secretion greatly augments the virulence of *P. aeruginosa* (1, 20, 21, 37). The contribution of  
50 ExoU to virulence is attributable to its phospholipase A<sub>2</sub> activity (27) (34). Upon injection into  
51 host cells, ExoU is activated and targeted to the plasma membrane where it cleaves membrane  
52 phospholipids resulting in rapid and complete cell lysis (10, 14, 27-29, 34). In addition to ExoU,  
53 *P. aeruginosa* isolates can encode other effectors, including ExoS, ExoT, and ExoY in various  
54 combinations (8). ExoS and ExoT are bi-functional enzymes with 75% amino acid identity and

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

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

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

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

83

## 84 **Materials and Methods**

85

### 86 *Bacterial strains and growth conditions*

87 The bacterial strains, mutants, and plasmids used in this study are listed in Table 1. PA99  
88 is a *P. aeruginosa* clinical isolate that naturally encodes the *exoU*, *exoS*, and *exoT* genes but  
89 lacks the gene for *exoY* (8). PA99null and PA99sec<sup>r</sup> (PA99Δ*pscJ*) were generated from PA99 by  
90 in-frame deletions as previously described (37). Complementation restored the mutants to an  
91 expected level of virulence, and neither mutant displayed a growth defect on laboratory medium  
92 (37). TOP10 chemically competent *Escherichia coli* cells (Invitrogen) were used for cloning.

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

97

### 98 *Generation of effector-Bla fusions*

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

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101 miniCTX $exoU\Delta stopAgeI$ . The mature fragment of TEM-1  $\beta$ -lactamase was amplified from  
102 pBR322 with engineered AgeI sites using primers BlacAgeUp (5'-  
103 AAAACCGGTACACCCAGAAACGCTGGTGA-3') and BlacAgeDwn (3'-  
104 AAAACCGGTATTACCAATGCTTAATCAGTGA-5') and ligated into  
105 miniCTX $exoU\Delta stopAgeI$ . A Bla fusion of a non-cytotoxic ExoU variant (ExoU(S142A)) was  
106 created by removing the stop codon of *exoU(S142A)* by site-directed mutagenesis using  
107 miniCTX $exoU(S142A)$  (6) as a template followed by amplification of the resulting  
108 *exoU(S142A)\Delta stop* construct with existing NsiI and engineered AgeI sites at its 5' and 3' ends  
109 using primers *exoU5'* (5'-AAAATGCATATCCAATCGTTGG-3') and *exoUAgeI* (3'-  
110 AAAACCGGTATCGGCCATGTGAACTCCTTATTCC-5'). The amplified fragment was  
111 ligated into miniCTX $exoU\Delta stopAgeI$  digested with NsiI and AgeI to generate  
112 miniCTX $exoU(S142A)\Delta stopAgeI$ . In a second reaction, the *bla* fragment was ligated into  
113 miniCTX $exoU(S142A)\Delta stopAgeI$  digested with AgeI to create miniCTX $exoU(S142A)-bla$ .  
114 A GST-Bla fusion that is expressed under the control of the native *exoU* promoter was  
115 generated by amplifying the open reading frame of glutathione S-transferase from pGEX-5X1  
116 using primers GSTNsiI (5'-AAAATGCATATGTCCCCTATACTAGGTTATTGG-3') and  
117 GSTAgeI (3'-AAAACCGGTTTCAGTCAGTCACGATGAATTCCCG-5') and ligated into  
118 miniCTX $exoU\Delta stopAgeI$  digested with NsiI and AgeI to generate miniCTX $gstAgeI$ . The stop  
119 codon of *gst* was altered by site-directed mutagenesis. In a subsequent reaction, the *bla* fragment  
120 was ligated into AgeI-digested miniCTX $gstAgeI$  to create miniCTX $gst-bla$ .  
121 All plasmids were confirmed by sequence analysis. In each case, the resulting construct  
122 (miniCTX $exoU-bla$ , miniCTX $exoU(S142A)-bla$ , or miniCTX $gst-bla$ ) was transformed into *E.*  
123 *coli* strain S17.1 (38) and, following conjugation, was introduced into a neutral site in the

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124 PA99null chromosome via integrase-mediated recombination at the *attB* site using previously  
125 described approaches (16) to generate PA99null+ExoU-Bla, PA99null+ExoU(S142A)-Bla, and  
126 PA99null+GST-Bla, respectively. miniCTX $exoU$ -bla and miniCTX $exoU$ (S142A)-bla were also  
127 introduced into the chromosome of PA99sec<sup>r</sup> to create strains PA99sec<sup>r</sup>+ExoU-Bla and  
128 PA99sec<sup>r</sup>+ExoU(S142A)-Bla. Integration was confirmed by PCR amplification of each  
129 construct from intact bacterial colonies.

130

### 131 *Immunoblot analysis*

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

134

### 135 *Fluorescence microscopy*

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

146

147 *Lactate dehydrogenase-release cytotoxicity assays*

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

154

155 *Mouse model of acute pneumonia*

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

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

166

167 *Analysis of ExoU-injected cells by flow cytometry*

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



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170 right side of the heart to remove circulating blood cells. Lungs were excised and pressed through  
171 40  $\mu\text{m}$  filters (Falcon). The filters were rinsed repeatedly with PBS. For BAL experiments,  
172 mouse lungs were lavaged by instilling and withdrawing 1 ml of PBS three times. Recovered  
173 cells were gently pelleted by centrifugation at  $500 \times g$  for 5 min. The supernatant was removed  
174 and red blood cells were lysed by addition of 3 ml cold sterile  $\text{H}_2\text{O}$  for 30 sec. Three ml of  $2\times$   
175 normal saline (1.8% NaCl) was quickly added to prevent additional cell lysis. Remaining cells  
176 were pelleted and resuspended in PBS, and trypan blue-excluding cells were quantified using a  
177 hemacytometer.

178         A total of  $1-2 \times 10^5$  cells were aliquoted per well of a V-bottom 96-well plate (Nunc),  
179 resuspended in 100  $\mu\text{L}$  PBS and CCF2-AM (Invitrogen) at  $1\times$  final concentration, and incubated  
180 at room temperature protected from light for 1 h. Cells were pelleted, and surface Fc receptors  
181 and non-specific binding sites were blocked by incubation in 10% rat serum (Sigma) and  $\alpha$ -  
182 CD16/32 in Fluorescence Activated Cell Sorting (FACS) buffer (1% bovine serum albumin,  
183 0.1%  $\text{NaN}_3$  in PBS) for 5-15 min on ice. Cell discriminatory antibodies were added at the  
184 appropriate dilutions in FACS buffer, and the final volume in each well was adjusted to 125  $\mu\text{L}$ .  
185 Antibodies and cells were incubated for 15-30 min on ice. Cells were pelleted by centrifugation  
186 at  $500 \times g$  for 5 min and resuspended in 2% paraformaldehyde in PBS for 2 min. An equal  
187 volume of FACS buffer was added, and cells were pelleted again. Cells were resuspended in  
188 FACS buffer and transferred into Falcon 2052 tubes after passage through 70  $\mu\text{m}$  Nitex filters.  
189 Antibodies were used at the following final dilutions:  $\alpha$ -CD16/32 1:50,  $\alpha$ -CD45 1:1,500,  $\alpha$ -Gr1  
190 1:1,500,  $\alpha$ -F4/80 1:50,  $\alpha$ -CD4 1:500,  $\alpha$ -CD8 1:500,  $\alpha$ -CD19 1:500,  $\alpha$ -CD49 1:500,  
191  $\alpha$ -CD11b 1:2,500,  $\alpha$ -CD11c 1:500, isotype controls 1:100 each. All antibodies were purchased  
192 from eBioscience.

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

209

### 210 *Isolation of type II pneumocytes from mouse lungs*

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

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216 10 min. Contaminating leukocytes were removed by an indirect magnetic separation technique.  
217 Cells were incubated at 37°C for 30 min with biotinylated antibodies at the following  
218 concentrations:  $\alpha$ -CD16/32 1:1,500,  $\alpha$ -TER119 1:500,  $\alpha$ -CD45 1:700,  $\alpha$ -CD90 1:700. Cells were  
219 pelleted, resuspended in DMEM + 10% FBS, and incubated with streptavidin magnetic beads for  
220 30 min at room temperature with rotation, then taped to a magnet for 15 min to remove all  
221 biotinylated cells. The supernatant containing the alveolar epithelial cells was removed and  
222 centrifuged at  $500 \times g$  for 10 min, and trypan blue-excluding cells were quantified using a  
223 hemacytometer. Cells were incubated with CCF2-AM and  $\alpha$ -CD45 as described above and  
224 analyzed by flow cytometry. CD45<sup>+</sup> cells were excluded from FACS analysis of type II  
225 pneumocyte preparations.

226

### 227 *Statistical methods*

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

236

### 237 **Results**

238

239 *ExoU-Bla fusions are secreted in vitro.*

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

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

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262 Bla were associated with blue fluorescence when expressed from PA99sec<sup>r</sup>, a PA99 mutant with  
263 a deletion in the *pscJ* gene, which encodes an essential component of the type III secretion  
264 apparatus (Fig. 1H and data not shown). Likewise *P. aeruginosa* expressing a glutathione-S-  
265 transferase-Bla fusion protein (GST-Bla) did not cause blue fluorescence (Fig. 1I), confirming  
266 that cleavage of the CCF2-AM substrate requires type III translocation of ExoU and is not due to  
267 engulfment of bacteria by J774 cells or non-specific movement of the  $\beta$ -lactamase moiety into  
268 these cells. These observations confirm that ExoU-Bla fusion proteins are translocated into host  
269 cells in vitro and that ExoU-injected cells are detectable in vitro using this reporter assay.

270

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

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

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

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

303  
304 *A substantial portion of phagocytic cells are injected with ExoU(S142A) in the lung.*

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

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308 pneumonia. For these experiments, cells were recovered from whole lung tissue of infected mice,  
309 incubated with CCF2-AM, stained with cell discriminatory antibodies, and analyzed by flow  
310 cytometry. We initially investigated the cell types injected with ExoU(S142A) at 18 h post-  
311 infection, when large numbers of inflammatory cells are present in the lung (6). In this  
312 experiment, approximately one-half million cells recovered from the lung of an infected mouse  
313 were injected with ExoU(S142A) (Fig. 3A). Of this population of injected cells, 99%  
314 (452,000/458,000) were inflammatory cells and over 80% (383,000/458,000) were phagocytic  
315 cells (Fig. 3A). Within the population of injected phagocytic cells, neutrophils were the  
316 predominant cell type (60%) followed by monocytes (27%) and macrophages (5%), while  
317 dendritic cells comprised less than 1% of the injected cell population (Fig. 3A). In addition to  
318 these phagocytic cell types, we also examined CD4<sup>+</sup> and CD8<sup>+</sup> T cells, B cells, and NK cells.  
319 Together lymphocytes comprised < 2% of injected cells recovered from the lung (Fig. 3A). The  
320 majority of cells (> 85%) incorporated CCF2-AM efficiently, and no obvious difference in  
321 CCF2-AM uptake by cell type was observed (data not shown). Therefore a single cell type was  
322 not specifically targeted for injection with ExoU, but phagocytic cells, particularly neutrophils,  
323 comprised the majority of cells injected with ExoU(S142A) in vivo.

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

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331 1% of lymphocytes ( $p < 0.01$ , neutrophils compared to each cell type; Fig. 3C). Therefore a  
332 higher proportion of neutrophils were injected with ExoU(S142A) than other cell types.

333 Most *P. aeruginosa* isolates secrete multiple effector molecules. To assess the effect of  
334 co-secretion of ExoS and/or ExoT on translocation of ExoU in vivo, we examined the cell types  
335 injected with ExoU(S142A)-Bla during infection with *P. aeruginosa* secreting ExoU(S142A)-  
336 Bla in combination with ExoS alone (PA99S+ExoU(S142A)-Bla) or both ExoT and ExoS  
337 (PA99ST+ExoU(S142A)-Bla) (Fig. 4). For these experiments, we used a lower bacterial  
338 inoculum ( $6 \times 10^6$  CFU/mouse) since secretion of ExoS results in more severe illness (37).  
339 Similar to infection with PA99null+ExoU(S142A)-Bla, phagocytic cell types comprised over  
340 95% of injected cells in the lung (Figure 4A). We assessed the proportion of neutrophils,  
341 monocytes, and macrophages among injected cells at 18 h post-infection. Interestingly, the  
342 repertoire of recruited inflammatory cells was somewhat different when ExoS alone or ExoS and  
343 ExoT were secreted with ExoU. We observed slightly higher numbers of injected monocytes  
344 relative to neutrophils when ExoS was secreted compared to infection with  
345 PA99null+ExoU(S142A)-Bla, a result which was also reflected in the total cells recovered from  
346 the lungs of these animals (Figure 4B). This difference may be due to previously undescribed  
347 effects of ExoS. Still, the relative size of the various cell populations dictated the number of that  
348 cell type injected with ExoU(S142A) even in the presence of other effectors (compare Figs. 4A  
349 and 4B). Thus, secretion of additional effectors does not dramatically alter the specificity of  
350 injection in vivo.

351

352 *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

376 types other than leukocytes, we isolated type II pneumocytes from the lungs of mice infected  
377 with PA99null+ExoU(S142A)-Bla by digestion with elastase followed by removal of leukocytes  
378 by magnetic separation. Cells were incubated with CCF2-AM, and injection was measured by  
379 flow cytometry (Fig. 6). Any contaminating leukocytes were removed from the analysis of type  
380 II pneumocytes by exclusion of CD45<sup>+</sup> cells. BAL samples, which contained mostly leukocytes,  
381 were analyzed in parallel. Interestingly, while 15% of cells in the BAL fluid were intoxicated  
382 with ExoU(S142A), minimal injection of ExoU(S142A)-Bla into type II pneumocytes (1%) was  
383 observed (Fig. 6B). Both cell populations incorporated CCF2-AM with similar efficiency (>  
384 85%). This result indicates that not all cell types present in the lungs are appreciably injected  
385 with ExoU during acute pneumonia.

386

387 *ExoU injection specificity changes over time.*

388 Finally we examined whether the cell types injected with ExoU(S142A) changed over  
389 time (Fig. 7). For these experiments, we infected mice with PA99null+ExoU(S142A)-Bla or  
390 appropriate control strains and sacrificed animals after 3, 6, 12 or 18 h. Cells were recovered  
391 from the whole lungs, incubated with CCF2-AM, stained with cell discriminatory antibodies, and  
392 analyzed by flow cytometry. The number of cells recovered from the infected mouse lungs  
393 steadily increased over the first 18 h following inoculation (Fig. 7A), reflecting the recruitment  
394 of inflammatory cells to the site of infection. At all time points examined, the majority of cells  
395 recovered from the lungs were leukocytes (data not shown). At 3 h post-infection, a substantial  
396 number of recovered cells were resident (alveolar) macrophages (Fig. 7B). A robust neutrophilic  
397 infiltrate was apparent at 6 h and reached a maximum at 12 h after infection. Monocyte  
398 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

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

425

## 426 **Discussion**

427

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

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

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445 compared to other cell types. This may simply reflect increased numbers of *P. aeruginosa*  
446 bacteria binding to neutrophils because of the large surface area and phagocytic nature of these  
447 cells, or because a larger proportion of neutrophils migrate into the airways and come into  
448 contact with bacteria. In any case, our results indicate that phagocytic cells are a primary target  
449 of ExoU in vivo. Since most ExoU<sup>+</sup> clinical isolates also secrete ExoT and many secrete ExoY, it  
450 may be that these effector proteins are similarly targeted. ExoS, however, is rarely secreted by  
451 ExoU<sup>+</sup> isolates, and ExoS-secreting strains constitute a distinct clonal group from ExoU<sup>+</sup> strains  
452 (8, 40). For these reasons, it remains possible that ExoS-secreting strains differ in their  
453 pathogenesis and that this effector protein is injected into distinct populations of host cells.  
454 Examination of the cell types injected with other *P. aeruginosa* effectors during acute pneumonia  
455 is currently underway.

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

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468 may prevent effective clearance from the lung and contribute to the enhanced disease observed  
469 with ExoU<sup>+</sup> 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<sup>+</sup> *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<sup>+</sup> *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

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

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

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



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655



656 **Figure Legends**

657 **Figure 1. ExoU-Bla fusion proteins are injected into host cells in vitro.** (A) Immunoblot  
 658 analysis of culture supernatants from *P. aeruginosa* strains secreting ExoU-Bla or  
 659 ExoU(S142A)-Bla grown in type III secretion-inducing conditions using polyclonal ExoU  
 660 antiserum. The same volume of supernatant was used for each sample. (B-I) J774 cells were  
 661 incubated with *P. aeruginosa* bacteria at an MOI of 10 followed by treatment with CCF2-AM for  
 662 1 h. Cells were examined using a filter set that allows visualization of both green and blue  
 663 fluorescence simultaneously. Green cells contain intact CCF2-AM, and blue cells contain  
 664 cleaved CCF2-AM, indicating translocation of the ExoU-Bla fusion. Representative fields are  
 665 shown from the following conditions: uninfected J774 cells (B) without or (C) with CCF2-AM  
 666 substrate; cells incubated with CCF2-AM after infection with (D) PA99null+ExoU, (E)  
 667 PA99null+ExoU(S142A), (F) PA99null+ExoU-Bla, (G) PA99null+ExoU(S142A)-Bla, (H)  
 668 PA99secr<sup>-</sup>+ExoU(S142A)-Bla, or (I) PA99null+GST-Bla. Scale bars, 100  $\mu$ m.

669  
 670 **Figure 2. Detection of cells injected with ExoU-Bla fusion proteins in vivo.** FACS analysis  
 671 was performed on cells recovered by BAL at 18 h post-infection. Green fluorescence (*y*-axis)  
 672 indicates the presence of CCF2-AM in the cell, and blue fluorescence (*x*-axis) indicates  
 673 translocation of the ExoU(S142A)-Bla fusion protein. The percentage of blue (injected) cells is  
 674 indicated in the upper right corner of each dot plot. (A) PA99null+ExoU(S142A), (B) PA99secr<sup>-</sup>  
 675 +ExoU(S142A)-Bla, and (C) PA99null+GST-Bla resulted in only green cells. (D) Infection with  
 676 PA99null+ExoU-Bla did not yield a significant proportion of blue cells relative to control  
 677 strains. (E) ExoU(S142A)-Bla translocation was detectable in vivo; approximately 40% of cells  
 678 recovered from BAL fluid were intoxicated with ExoU(S142A)-Bla. Data shown are from a

679 representative experiment; similar results were obtained in at least three independent  
680 experiments. Results varied from day-to-day, with mean percent injected cells ranging from  
681 15%-40%.

682

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

694

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

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

704

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

713

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

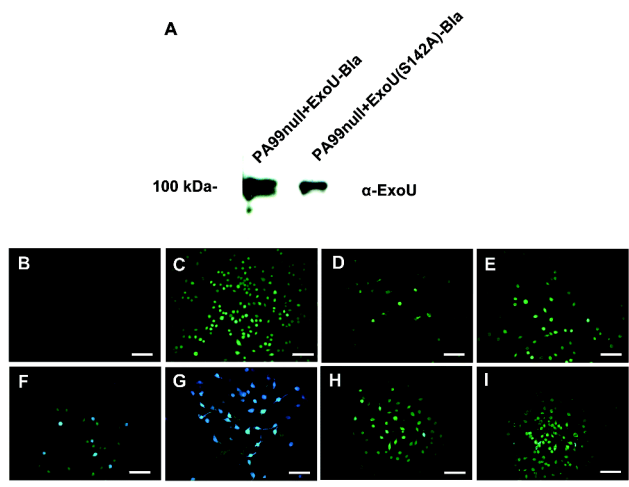
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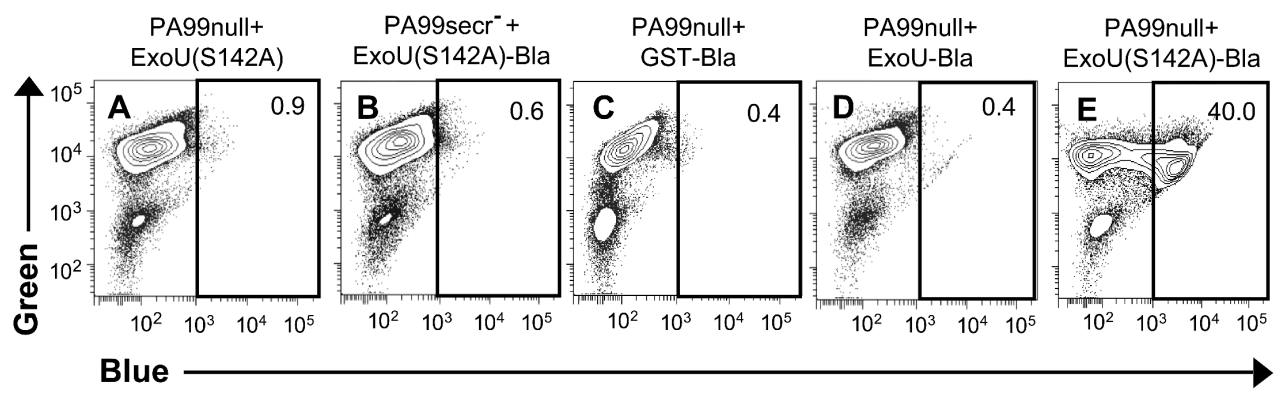
725 **Figure 7. Cells injected with ExoU(S142A) in the lung over time.** FACS analysis was  
726 performed on cells recovered from whole lungs of mice infected with PA99null+ExoU(S142A)-  
727 Bla at 3, 6, 12 and 18 h post-infection. (A) Total leukocytes recovered from the lungs of mice  
728 infected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

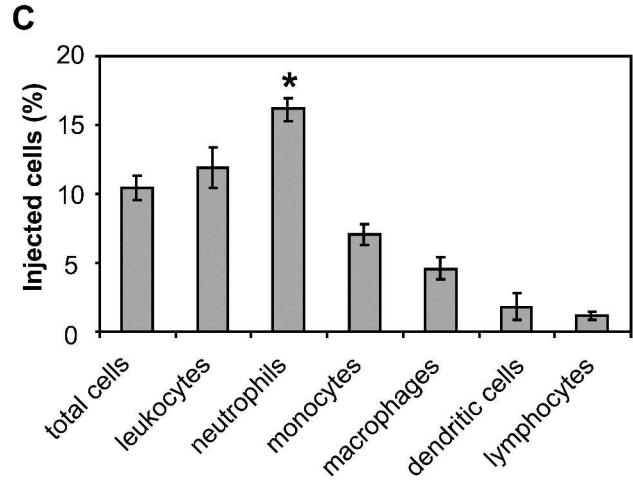
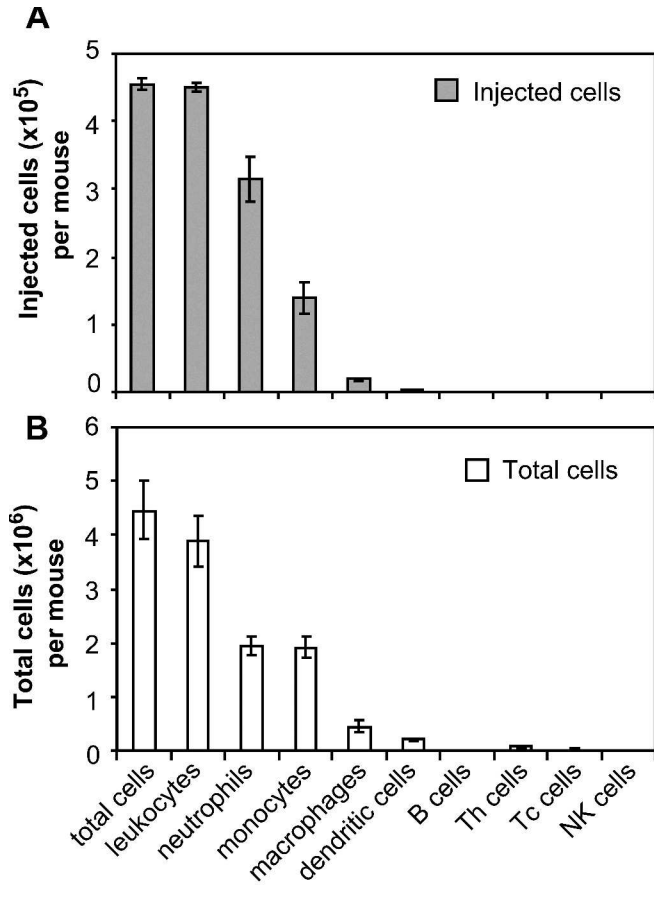
734 **Table 1. *P. aeruginosa* strains and plasmids used in this study.**

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

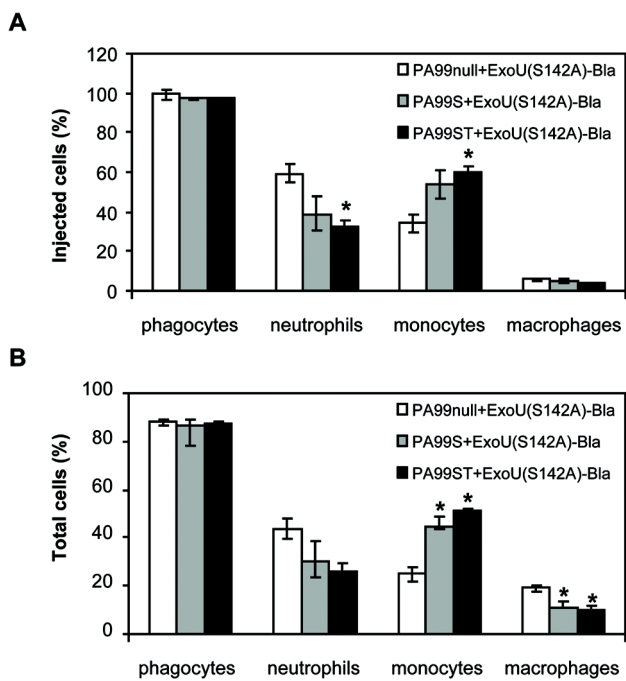
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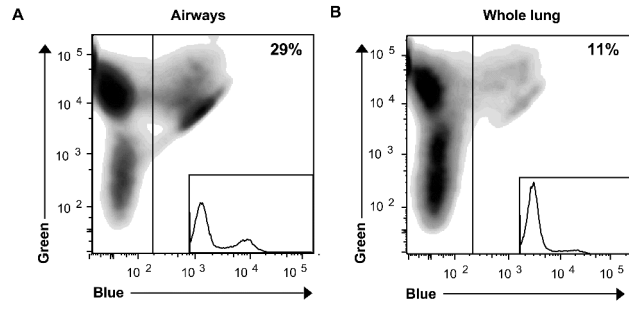


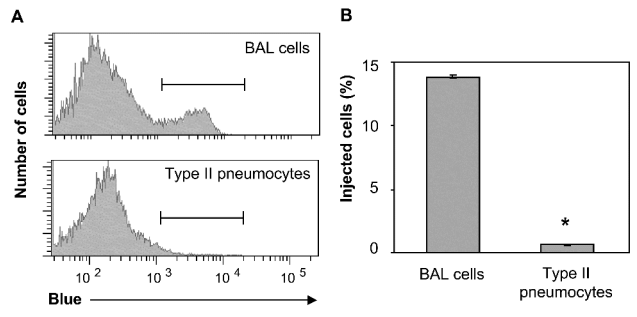




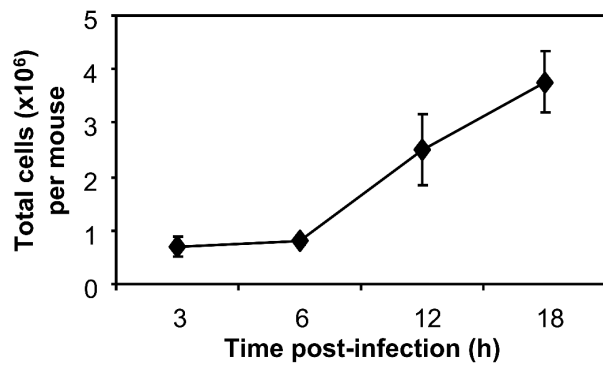




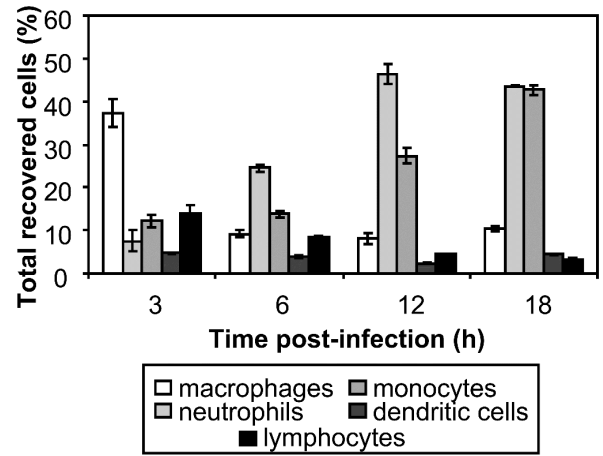




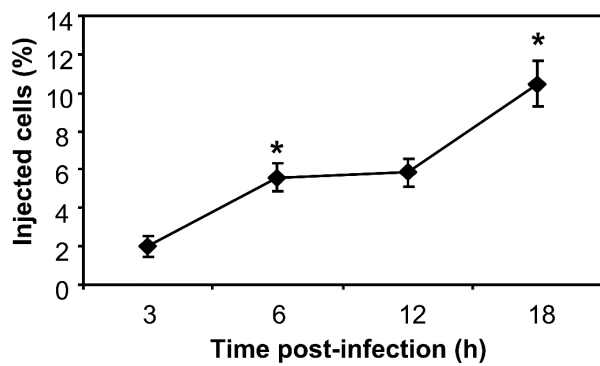
**A**



**B**



**C**



**D**

