Type II Secretion System of *Pseudomonas aeruginosa*: In Vivo Evidence of a Significant Role in Death Due to Lung Infection

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Background. The role of toxins secreted by the type II secretion system (T2SS) of *Pseudomonas aeruginosa* during lung infection has been uncertain despite decades of research.

Methods. Using a model of pneumonia in Toll-like receptor (TLR) $2,4^{-/-}$ mice, we reexamined the role of the T2SS system. Flagellin-deficient mutants of *P. aeruginosa*, with mutations in the T2SS and/or T3SS, were used to infect mice. Mice were followed up for survival, with some killed at different intervals to study bacterial clearance, inflammatory responses, and lung pathology.

Results. Strains carrying either secretion system were lethal for mice. Double mutants were avirulent. The $T3SS^+$ strains killed mice within a day, and the $T2SS^+$ strains killed them later. Mice infected with a strain that had only the T2SS were unable to eradicate the organism from the lungs, whereas those infected with a T2SS-T3SS double deletion were able to clear this mutant. Death caused by the $T2SS^+$ strain was accompanied by a >50-fold increase in bacterial counts and higher numbers of viable intracellular bacteria.

Conclusions. The T2SS of *P. aeruginosa* may play a role in death from pneumonia, but its action is delayed. These data suggest that antitoxin strategies against this organism will require measures against the toxins secreted by both T2SS and T3SS.

Pneumonia due to *Pseudomonas aeruginosa* carries a high mortality rate [1] attributed to its secreted exotoxins [2] and possibly the inflammatory response to bacterial products [3]. This bacterium is known to possess 5 protein secretion systems of which the type II secretion system (T2SS) and type III secretion system (T3SS) secrete the majority of known toxins. The T2SS secretes exotoxin A, LasA and LasB proteases, type IV protease, and phospholipase H, as well as lipolytic enzymes [4]. The T3SS secretes exotoxins U, S, T, and Y [5]. The latter system uses a membrane-spanning structure and needle to inject toxins into mammalian cells [6] whereas the T2SS is composed of multiprotein secretons encoded by the *xcp* and *hxc* operons [7] as well as an additional secretin, XqhA, that functions in T2 secretion when *xcpQ*, the major secretin, is mutated [8].

The biologic roles of the exoproducts of these systems have been under study for decades with clarity only about the role of the T3SS. Its major toxins, ExoU and ExoS cause death in animal models of pulmonary infection [9] and possible humans [10]. However, most studies with few exceptions have failed to demonstrate that the T2SS toxins are important virulence factors during pulmonary infections [11–13]. There are several possible reasons for this: (1) no studies have addressed the role of the T2SS as a whole in an acute pneumonia model; (2) multiple toxins may be involved in death confounding mutational analysis when the roles of single toxins were addressed; (3) the full repertoire of T2 secreted toxins is not known, and other unidentified toxins may be effectors in mortality; (4) studies were

Received 29 June 2010; accepted 29 December 2010.

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Potential conflicts of interest: none reported.

Presented in part: American Society of Microbiology General Meeting, San Diego, 26 May 2010. Poster B - 2575.

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The Journal of Infectious Diseases 2011;203:1369–77

0022-1899 (print)/1537-6613 (online)/2011/20310-0001$14.00
DOI: 10.1093/infdis/jir045

done in the presence of a fully functional T3SS; and (5) any contribution of inflammation as a cause of death was not circumvented.

In prior studies, we demonstrated that Toll-like receptor (TLR) $2,4^{-/-}$ mice are hypersusceptible to low inocula of a flagellin-negative mutant of *P. aeruginosa*, owing to the lack of recognition of lipopolysaccharide and flagellin [14]. In those studies, bronchoalveolar lavage (BAL) samples of infected mice demonstrated a grossly defective early inflammatory response [14], Thus, we hypothesized that death was due to bacterial toxins and not inflammation. Using this model, we conclusively demonstrate that both the T2SS and the T3SS play independent roles in death due to *Pseudomonas* lung infection and that the XcpQ secretin is the major outer membrane pathway used by the T2SS effectors.

MATERIALS AND METHODS

Animals

$TLR2^{-/-}$ and $TLR4^{-/-}$ mice obtained from S. Akira were backcrossed 8 times with C57BL/6 to ensure similar genetic backgrounds. $TLR2,4^{-/-}$ mice were generated by breeding $TLR2^{-/-}$ and $TLR4^{-/-}$ mice. Male mice were used for the experiments. Mice were fed and housed under standard conditions with air filtration and cared for in accordance with Pasteur Institute guidelines and in compliance with European Animal Welfare regulations.

Animal Infection

Mice were anesthetized by intraperitoneal injection of a mixture of ketamine-xylazine, and infected via the intratracheal route, as described elsewhere [15]. A 50-μL bacterial suspension containing $0.5–1.0 \times 10^7$ colony-forming units (CFU) was administered. In some mice survival was observed for 1 week after infection, and in others BAL was performed on groups of three mice after pentobarbital euthanasia at 6, 24, or 44 h after infection. The BAL fluids were diluted and plated on LB agar plates to obtain total viable bacterial counts. In some animals viable intracellular bacteria were enumerated by incubating washed BAL cells in tobramycin for 30 min, to kill extracellular bacteria, and lysing the cells with .1% triton X - 100 before plating on L-agar plates. Cell counts were measured in the BAL fluids and cell differential counts were determined after cytospin centrifugation and staining with Diff-Quik products. Murine cytokine concentrations in BAL fluid were determined using DuoSet enzyme-linked immunosorbent assay kits (R&D Systems).

Bacterial Strains

All bacterial strains and plasmid vectors used in this study are described in Table1. For mutant construction, *P. aeruginosa* was grown in Luria broth at 37°C with shaking at 250 rpm or on 1.5% L-agar plates with or without antibiotics. For sucrose selection, plates containing 10% sucrose were used. To detect secretion of proteases, wild-type or mutant bacteria were spotted on 1.5% Casein-Milk agar plates. For the animal challenges, bacteria were grown overnight in Luria broth, transferred to fresh medium and grown for 4–5 h to mid–log phase. The cultures were centrifuged at 4000 *g* for 15 min, and the cell pellets were washed twice with phosphate-buffered saline and suspended in its original volume. The optical density was adjusted to give the approximate desired inoculum and verified by plate counts.

CONSTRUCTION OF MUTANTS.

fliC Mutants

P. aeruginosa mutant strains PAKΔ*pscC* ($T3SS^-$), PAKΔ*xcp* ($T2SS^-$), and PAKΔ*pscC*Δ*xcp* ($T2SS^-/T3SS^-$) were obtained from Stephen Lory [16, 17]. These well-characterized mutants were used in studies of the role of the T3SS in toxicity to cells [17]. The *fliC* mutants of these strains were engineered as described elsewhere, using a disrupted allele of the *fliC* gene of strain PAK [18]. This provided 1 set of *fliC* mutant strains for the first series of experiments.

pscF and *xcpQ* Mutants

An in-frame partial gene deletion of 138 bp was made in *pscF*, the gene encoding the major needle protein of the T3SS, by cloning 1-kb DNA fragments up and downstream of the region to be deleted into the vector $pEX_{18}Gm$ [19]. Plasmid $pEX_pΔpscF$ was used to introduce a partial deletion of the *pscF* gene in strain PAKΔ*fliC*, using sucrose selection, as described elsewhere [19]. An in-frame partial deletion in *xcpQ* was made by first cloning a 3.2-kb DNA fragment containing the entire *xcpQ* gene into $pEX_{18}Gm$ and then excising a 1109-bp Sal I fragment from *xcpQ*. This plasmid, $pEX_pΔxcpQ$, was used to generate PAK mutants in PAKΔ*fliC* and PAKΔ*fliC*Δ*pscF*, providing a second independent mutation in the T2SS (PAKΔ*fliC*Δ*xcpQ*) as well as a T2SS-T3SS mutant (PAKΔ*fliC*Δ*pscF*Δ*xcpQ*). Next, we engineered these latter mutations in strain PA14 to examine whether our observations held with another *P. aeruginosa* strain, and lastly, the same T2SS and T3SS mutations were engineered in the wild-type flagellated strain PAK.

xcpQ Complementation of PAK Δ*fliC*Δ*pscF*Δ*xcpQ*

Chromosomal complementation of the PAKΔ*fliC*Δ*pscF*Δ*xcpQ* mutant was achieved at the *att* site by cloning a 5.2-kb *Bam*HI fragment from cosmid PAX24 that contains the *xcp* operon of *P. aeruginosa* strain PAO1 [20] into the mini-CTX1 vector [21]. This construct was transformed into *Escherichia coli* S17 and mated into PAKΔ*fliC*Δ*pscF*Δ*xcpQ* mutant. The tetracycline-resistant colonies were resolved through *flp* excision [21]. The mutant with an insertion of the mini-CTX1 vector

Table 1. Bacterial Strains and Plasmids Used in Study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
Pseudomonas aeruginosa		
PAK	Wild-type clinical isolate	D. Bradley
PAK Δ*pscC*	Deletion of bp 30–1701 in *pscC* in strain PAK	Wolfgang et al [16]
PAK Δ*fliC*-Δ*pscC*	*fliC* mutation in PAK Δ*pscC*	Current study
PAK Δ*xcp*	Deletion in *xcp* operon in strain PAK	Lee et al [17]
PAK Δ*fliC*-Δ*xcp*	*fliC* mutation in PAK Δ*xcp*	Current study
PAK Δ*pscC*-Δ*xcp*	Double mutant of strain PAK carrying both *pscC* and *xcp* operon deletions	Lee et al [17]
PAK Δ*fliC*	In-frame deletion of *fliC* gene in strain PAK	Dasgupta et al [18]
PAK Δ*fliC*-Δ*pscC*-Δ*xcp*	*fliC* mutation in PAK Δ*pscC*-Δ*xcp*	Current study
PAK Δ*xcpQ*	Partial deletion in *xcpQ* gene in strain PAK	Current study
PAK Δ*fliC*-Δ*xcpQ*	*fliC* mutation in PAK Δ*xcpQ*	Current study
PAK Δ*pscF*	In-frame partial deletion of *pscF* from bp 78–213 in strain PAK	Current study
PAK Δ*fliC*-Δ*pscF*	*pscF* mutation in PAK Δ*fliC*	Current study
PAK Δ*xcpQ*-Δ*pscF*	*pscF* mutation in PAK Δ*xcpQ*	Current study
PAK Δ*fliC*-Δ*pscF*-Δ*xcpQ*	*fliC* mutation in PAK Δ*xcpQ*-Δ*pscF*	Current study
PA14	Wild-type strain	F. Ausubel
PA14Δ*fliC*	*fliC* mutation in PA14	Current study
PA14Δ*xcpQ*	Partial deletion in *xcpQ* gene in PA14	Current study
PA14Δ*fliC*-Δ*xcpQ*	*fliC* mutation in PA14Δ*xcpQ*	Current study
PA14Δ*pscF*	*pscF* mutation in PA14	Current study
PA14Δ*fliC*-Δ*pscF*	*fliC* mutation in PA14Δ*pscF*	Current study
PA14-Δ*xcpQ*-Δ*pscF*	*pscF* mutation in PA14Δ*xcpQ*	Current study
PA14Δ*fliC*-Δ*pscF*-Δ*xcpQ*	*fliC* mutation in PA14Δ*xcpQ*-Δ*pscF*	Current study
Plasmid		
$pEX_{18}Gm$	Gm^R; *oriT*$^+$ *sacB*$^+$, gene replacement vector with MCS from pUC18	Hoang et al [19]
PAX24	*P. aeruginosa xcpP* to *–Z* cluster in pLAFR3 (IncP Tc^r)	Filloux et al [20]
Mini-CTX1	Tc^r; self-proficient integration vector with *tet*, Ω-*FRT-attP* MCS, *ori*, *int*, and *oriT*	Hoang et al [21]
Mini-CTX-5.2*xcp*	*P. aeruginosa xcpPQ* on a 5.2-kb *Bam*HI fragment from PAX24 cloned in mini-CTX1	Current study
$pEX_pΔpscF$	$pEX_{18}Gm$ containing an in-frame deletion of *pscF* gene from bp 78–213 of strain PAK	Current study
$pEX_pΔxcpQ$	$pEX_{18}Gm$ containing a 1109-bp *Sal* I fragment deletion in *xcpQ* of strain PAK	Current study

into the *att* site was also constructed and used as a control. Complementation was verified by polymerase chain reaction and examining for restoration of protease secretion on casein-milk agar plates.

Histologic Studies

Mice were infected with 0.5–1 $\times$ 10^7 CFU of the mutants to be studied and euthanized with pentobarbital at different intervals after infection, and their lungs were fixed in formalin, sectioned, and stained with hematoxylin-eosin.

Statistical Calculations

Cytokine levels, polymorphonuclear neutrophil (PMN) counts, and pathogen counts were expressed as means ± standard errors of the mean. Differences between groups were assessed for statistical significance, using analysis of variance followed by Fisher's test or *t* test, as appropriate. Differences were considered statistically significant at $P < .05$.

RESULTS

Role of T2SS and T3SS in Death Due to Lung Infection

$TLR2,4^{-/-}$ mice were infected with the T2SS− and/or T3SS− defective strains and observed for mortality. A PAKΔ*fliC* mutant that caused 100% mortality [14] served as the control. In most experiments, PAK mutants were flagellin defective; therefore, the notation "PAKΔ*fliC*" will not be repeated unless required for clarification. *Δxcp* is a T2SS mutant, in which there is a deletion of the *xcp* operon, including *xcpQ* encoding the secretin XcpQ. *ΔpscC* is a T3SS mutant, in which part of the gene encoding PscC, an outer membrane protein, is deleted. *ΔpscCΔxcp* is a double mutant of the 2 secretion systems. The PAKΔ*fliC* mutant rapidly killed all mice within 24 h (Figure 1A). This dose of bacteria, 0.5–1.0 $\times$ 10^7 CFU, does not cause death in wild-type mice (data not shown), because the innate host defense eradicates the infection [15]. Unequivocally, the *Δxcp* mutant ($T3SS^+$) killed all the mice within 24 h. The *ΔpscC* mutant

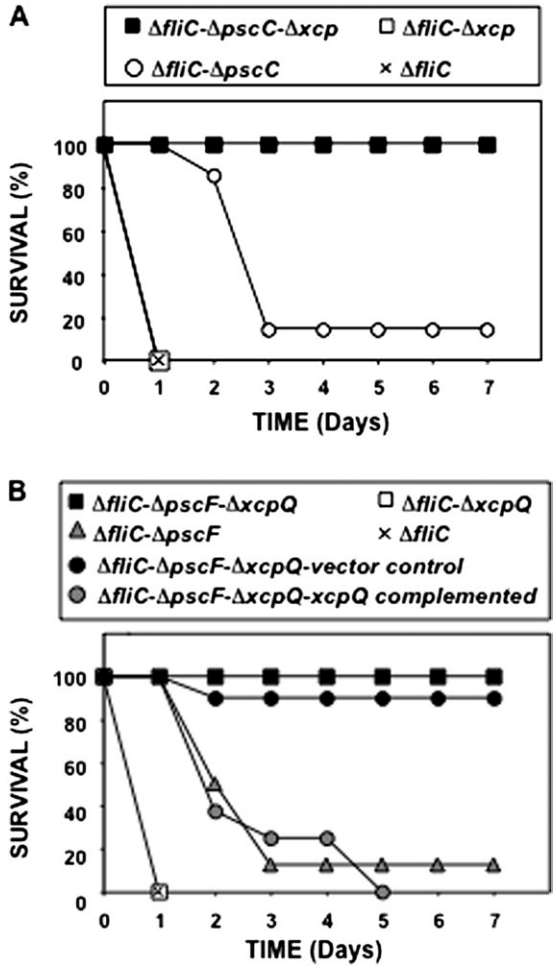

Figure 1. *A,* Survival of Toll-like receptor (TLR) 2,4 $^{-/-}$ mice infected with type II secretion system (T2SS) and type III secretion system (T3SS) mutants of *Pseudomonas aeruginosa* strain PAK. Mice were infected with the *P. aeruginosa* mutants, and their survival was monitored for ≥1 week. PAKΔ*fliC* is shown as a positive control strain. *B,* Survival of TLR2,4 $^{-/-}$ mice infected with mutants in the T2SS secretin, XcpQ, and the T3SS needle of *P. aeruginosa*. Mice were infected with *P. aeruginosa* PAK mutants, and their survival monitored for ≥1 week. Mutant PAKΔ*fliC*Δ*pscF*Δ*xcpQ* was complemented by insertion of a mini-CTX vector (control) or the same vector with a DNA fragment that carries the *xcpQ* gene and its promoter. PAKΔ*fliC* is shown as a positive control strain.

(T2SS$^+$) killed 8 of 9 mice, but deaths began after 24 h, with most occurring between days 2 and 3. In contrast, *ΔpscCΔxcp* (T3SS$^-$/T2SS$^-$) was avirulent (Figure 1A). Thus, each toxin secretion system played an independent role in causing death.

Role of XcpQ Secretin in T2SS-Mediated Death in Lung Acute Infections

P. aeruginosa possesses 3 secretins, XcpQ, HxcQ and XqhA, that are known to be involved in T2 secretion [22]. We deleted *xcpQ* to provide another mutation in the T2SS as well as to ascertain whether this was the secretin involved in secretion of the factors

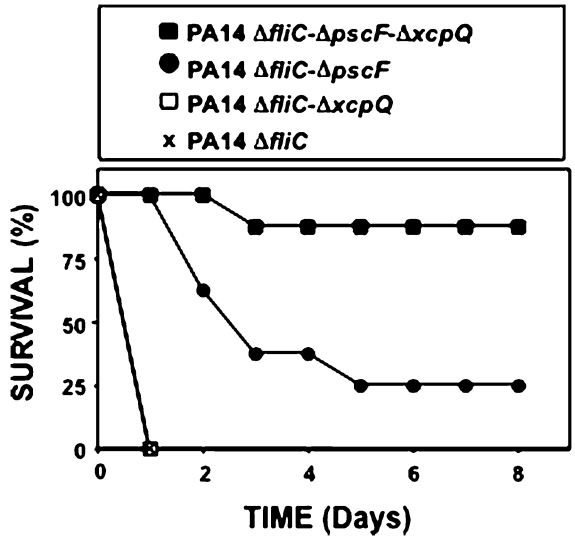

Figure 2. Survival of Toll-like receptor 2,4$^{-/-}$ mice infected with secretion mutants of *Pseudomonas aeruginosa* strain PA14. Mice were infected with *P. aeruginosa* PA14 mutants similar to those used for strain PAK, and their survival was monitored for ≥1 week. The type II secretion system of this strain also killed mice, and its deletion reduced mortality due to this strain.

responsible for death. To test whether another independent mutation in the T3SS demonstrated a similar phenotype as the *pscC* mutant, we engineered a mutation in *pscF*, encoding the major protein of the T3SS needle and a double-secretion mutant, *ΔpscFΔxcpQ*. TLR2,4$^{-/-}$ mice were infected as described earlier. This second set of mutants had the same virulence phenotypes as the first, demonstrating that each secretion system had an independent effect (Figure 1B), and that XcpQ was the critical secretin, with no measurable contribution from the alternate secretion pathway through HxcQ or XqhA. The *ΔxcpQ* mutant (T3SS$^+$) rapidly killed all mice, the *ΔpscF* mutant (T2SS$^+$) killed 9 of 10 mice, again with the delay seen with the previous T2SS$^+$ strain, and *ΔpscFΔxcpQ* (T3SS$^-$/T2SS$^-$) was avirulent (Figure 1B). To confirm that this specific secretin deletion led to the loss of virulence rather than a secondary mutation, we complemented *xcpQ* in *ΔpscFΔxcpQ* and challenged mice with this strain. As a control for these experiments, the mini-CTX vector was inserted into the *att* site of *ΔpscFΔxcpQ*. The complemented secretin mutation restored virulence, confirming the important role of the T2SS and XcpQ in virulence (Figure 1B).

Effect of T2SS and T3SS Mutations in *P. aeruginosa* Strain PA14

Strain PA14 is believed to be one of the most virulent *P. aeruginosa* strain studied. We therefore engineered flagellin and secretion system mutants in this strain and examined the virulence of these mutants at 2×10^7 CFU per animal. Again, we observed the same independent effects of the T2SS and T3S, with the double mutant being less avirulent (Figure 2).

Gross and Microscopic Pathologic Lung Findings in Mice Infected with T2SS and T3SS Mutants

To characterize the role of the toxin secretion systems at the pathologic level, histopathologic analysis of the lungs was performed in mice 6, 24, or 44 h after infection with strains having mutations in the secretion systems, as indicated in Figure 3. Gross examination of the lungs at 6 h after infection (Figure 3A top panel) showed an intense inflammatory response among mice infected with strains that had a functioning T3SS (both $T2SS^+/T3SS^+$ and $T2SS^-/T3SS^+$). No data were obtained beyond this time with these 2 strains, because mice were dead within 24 h. In contrast, infection with a strain having only a functioning T2SS demonstrated gross hemorrhagic lesions only at 24 h after infection, which progressed with time (Figure 3B top panel). Mice infected with the $T2SS^-/T3SS^-$ mutant demonstrated some gross inflammation on the surface of the lungs at 24 h, but this did not progress. Microscopic examination of the lungs (Figure 3 bottom panels) demonstrated changes that correlated with the survival data and the gross pathologic findings. At 6 h after infection only mice infected with strains having an intact T3SS ($T2SS^+/T3SS^+$ and $T2SS^-/T3SS^+$) showed pathologic changes, characterized by randomly distributed infiltrates of neutrophils, located in bronchiolar and alveolar spaces, often associated with focal necrosis of the bronchiolar overlying epithelium and alveolar walls. At this time, mice that were infected with the strain having only the T2SS did not demonstrate histopathologic changes, nor did mice infected with the $T2SS^-/T3SS^-$ strain. At 24 and 44 h after infection, mice infected with $T2SS^+/T3SS^-$ and $T2SS^-/T3SS^-$ strains displayed similar inflammatory lesions—infiltrates of neutrophils located in the bronchiolar and alveolar spaces—but the extent and severity of the lesions differed between the 2 mutants (Figure 3B bottom panel). At 44 h after infection, the changes were more extensive and severe for the $T2SS^+/T3SS^-$ mutant, whereas the mild inflammatory response seen in the double mutant was receding. Thus, the pathologic effects of the T2SS on the lungs are delayed, and they correlated with the survival studies, whereas the effects of the T3SS were seen 6 h after the challenge, and the animals died within 24 h.

Effect of the T2SS on Bacterial Clearance and Host Innate Responses

Infected mice were euthanized, and BAL performed. Mice infected with $T3SS^+$ mutants could be studied only at 6 h after infection. Mice infected with mutants that were $T2SS^+/T3SS^-$ or $T2SS^-/T3SS^-$ were studied at 6, 24, and 44 h after infection. Regardless of the mutant used, the number of bacteria in the BAL increased 5–10-fold at 6 h after challenge, compared with

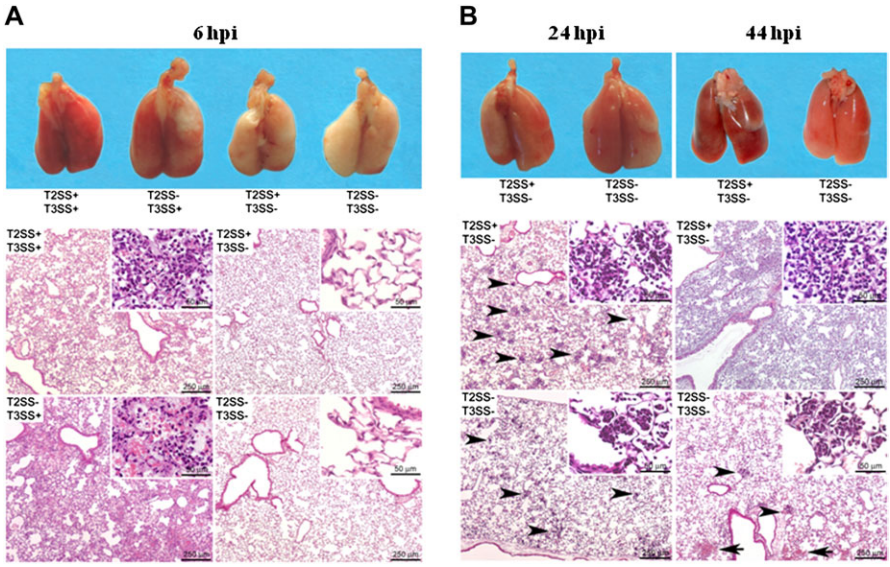

Figure 3. Macroscopic and microscopic pathologic findings in the lungs of Toll-like receptor (TLR) $2,4^{-/-}$ mice infected with secretion mutants of *Pseudomonas aeruginosa*. Groups of 3 mice were infected with type II secretion system (T2SS) and type III secretion system (T3SS) mutants, as indicated, and were euthanized at different intervals after infection. Mice that were infected with mutants PAKΔ*fliC* and PAKΔ*fliC*Δ*xcpQ*, both having a competent T3SS, could be studied only at 6 h after infection (hpi), because they died before 24 h. *A*, At 6 h, lesions were already visible grossly (Fig. 3A top) and microscopically (Fig. 3A bottom) in the lungs of mice infected with mutants possessing a competent T3SS (description in text). By contrast, at this time point, the lungs of the mice infected with the $T2SS^+$ mutant or the double-secretion mutant $T2SS^-/T3SS^-$ did not show significant macroscopic (Fig. 3A top) or microscopic lesions (Fig. 3A bottom). *B*, At 24 h, both gross (Fig. 3B top) and histologic (Fig. 3B bottom) changes were apparent in animals infected with both the $T2SS^+$ and the double-secretion mutant but were more severe with the $T2SS^+$ strain, with findings characterized by randomly distributed intra-alveolar neutrophils infiltrates (*arrowheads, insets*). At 44 h, mice infected with the double-secretion mutant showed minimal gross pathologic changes (Fig. 3B top) and a decrease in cell infiltrates (Fig. 3B bottom) with rare hemorrhages (*arrowheads*). By contrast, lesions were more severe and extensive, and progressed to hemorrhage and consolidation with the $T2SS^+$ strain. This coincided with deaths that began on day 2 after infection.

the administered inoculum (Figure 4A). Interesting differences were observed at later time points between mice infected with *ΔpscFΔxcpQ* ($T2SS^-/T3SS^-$) and those infected with *ΔpscF* ($T2SS^+/T3SS^-$). In mice infected with *ΔpscFΔxcpQ*, BAL bacterial counts fell rapidly in the next 20 h, but in those infected with *ΔpscF*, counts rose to >50-fold more than the initial inoculum (Figure 4A), implying a defect in the host defenses in the presence of the T2SS. The number of PMNs recovered from the BAL fluids did not differ significantly at 24 h (Figure 4B) from that at other time points (data not shown). Thus, the difference in colony counts cannot be explained by differences in the number of PMNs recruited. We therefore examined whether there was a defect in phagocytic killing when the T2SS was present by measuring viable intracellular bacterial counts in total BAL PMNs (Figure 4B). There were 4-fold more viable intracellular bacteria in the cells of the $T2SS^+$ infected group than in the $T2SS^-$ group ($P = .0186$).

We also analyzed the synthesis of cytokines under the same experimental conditions (Figure 5 A–F). Globally, there was a tendency for a higher production in the presence of the T2SS, except for the tumor necrosis factor α response at 24 h, ruling out the subversion of these responses by the T2SS. However, there was one finding of possible significance in the host response to the T2SS competent mutant, elevated levels of the anti-inflammatory cytokine interleukin-10 (Figure 5F), which has been shown to inhibit *P. aeruginosa* clearance from the lungs and to worsen survival rates [23, 24], as well as affecting survival in other types of infections [25].

Role of the T2SS and T3SS in Immunocompetent Mice

Given the clear-cut distinction between the action of the secretion systems in $TLR2,4^{-/-}$ mice, we examined whether this difference held for mice that had functioning TLRs 2, 4, and 5 by infecting wild-type mice with flagellated secretion system mutants (Figure 6). Even in these mice, we note that both secretion systems function independently, but 5–10-fold more bacteria were required to kill 100% of wild-type mice with the wild-type strain or the $T3SS^+$ strain of *P. aeruginosa*. Deaths were even more delayed under these circumstances.

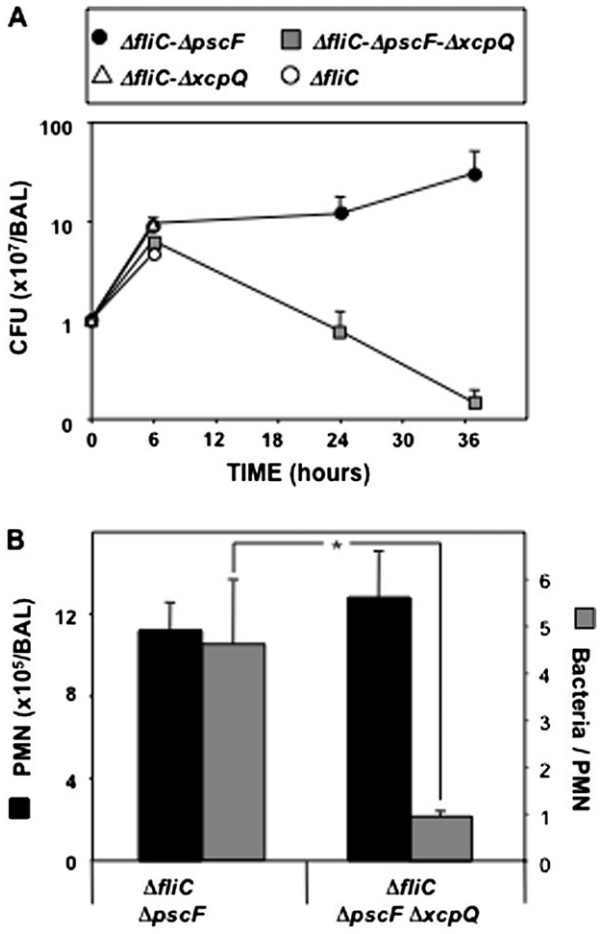

Figure 4. *A,* Bacterial proliferation in the bronchoalveolar lavage (BAL) fluid of Toll-like receptor (TLR) $2,4^{-/-}$ mice infected with type II secretion system (T2SS) mutant strains of *Pseudomonas aeruginosa* strain PAK ($T2SS^+$ and $T2SS^-$). Groups of 5–6 mice were infected with $T2SS^+$ and $T2SS^-$ strains and were euthanized at 6, 24 and 36 h after infection. BAL was performed, and samples were processed to obtain viable bacterial counts. Data are presented as means ± standard errors of the mean for 5–6 mice per time point. Differences between groups were assessed 36 h after infection for statistical significance, using analysis of variance followed by Fisher's test; $*P < .05$. CFU, colony-forming units. *B,* Polymorphonuclear neutrophil (PMN) and intracellular bacterial counts in the BAL fluid of $TLR2,4^{-/-}$ mice infected with $T2SS^+$ and $T2SS^-$ strains of *P. aeruginosa* strain PAK. Groups of 5–6 mice were infected with T2SS and T3SS mutants and were euthanized at 24 h after infection. BAL was performed, and samples were processed to obtain PMN counts. Left ordinate shows the PMN counts; right ordinate, number of viable bacteria per PMN. Data are presented as means ± standard errors of the mean for 5–6 mice per time point. Differences between groups were assessed for statistical significance, using the *t* test; $*P = .0186$.

DISCUSSION

These studies demonstrate unequivocally that the T2SS of *P. aeruginosa* is capable of causing death in lung infections, an issue that has been unsettled. They also demonstrate that the onset of action differs between the T2SS and the T3SS , with the T3SS acting rapidly within 24 h, and the T2SS causing death at a slower rate, differences that were not hitherto appreciated and no doubt contributed to the lack of an appreciation of the role of the T2SS. It is not known whether this difference in the timing of lung injury is due to the fact that activation of the T2SS requires that bacteria reach high concentrations to achieve quorum sensing [26] or due to the fact that pathologic lesions are late in appearing; however, because the bacterial count in the lungs had increased significantly by 24 h, the observations are consistent with a role of quorum sensing in mediating lung injury and death.

These studies also narrow down the possible pathways used for T2 toxin secretion. It has long been suspected that the Xcp

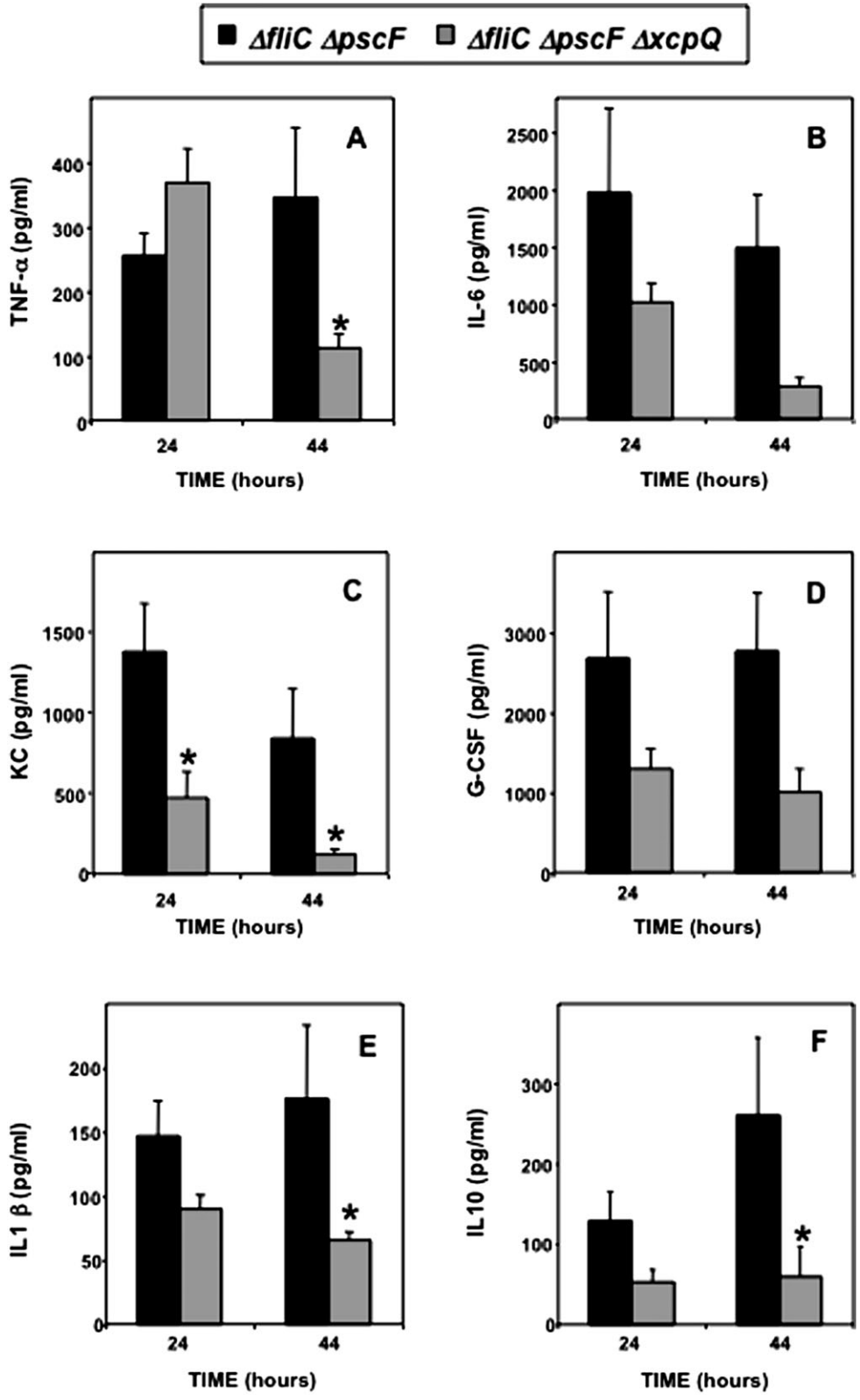

Figure 5. Cytokine contents of the bronchoalveolar lavage (BAL) fluid of TLR2,4$^{-/-}$ mice resulting from infections with secretion mutants of *Pseudomonas aeruginosa* strain PAK. Groups of 5–6 mice were infected with type II secretion system (T2SS) and type III secretion system (T3SS) mutants, as indicated, and were euthanized at 24 or 44 h after infection. BAL was performed, and samples were processed to measure different cytokines, including granulocyte colony-stimulating factor (G-CSF), interleukin (IL), KC cytokine, and tumor necrosis factor (TNF). Data are presented as means ± standard errors of the mean for 5–6 mice per time point. Differences between groups were assessed for statistical significance at both 24 and 44 h after infection, using analysis of variance followed by Fisher's test; $^{*}P < .05$.

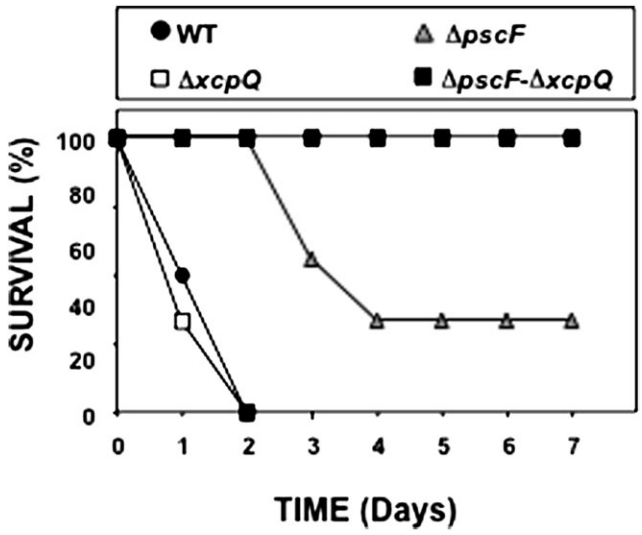

Figure 6. Survival of wild-type (WT) mice infected with flagellated strain PAK carrying mutations in the type II secretion system (T2SS) and type III secretion system (T3SS). Mice were infected with 5×10^7 colony-forming units of each indicated strain, the dose of the wild-type strain required to kill 100% of wild-type mice [15]. The independent effects of the secretion systems are seen, but deaths caused by both secretion systems are delayed compared with outcomes in TLR $2,4^{-/-}$ mice infected with the *fliC* mutant, in which the T3SS system kills all mice in <24 h at a lower dose of bacteria and deaths due to the T2SS begin after 24 h.

secreton was responsible for secretion of the better-known T2 secreted toxins of *P. aeruginosa* [27], but the discovery of the Hxc secreton [7] has raised the possibility that this pathway may also be used for novel toxin secretion. Additionally, there is XqhA [8], an alternative secretin that enables low-level secretion of XcpQ substrates. Neither of these, however, appear to play significant roles in virulence in this model, because deletion of *xcpQ* alone renders the organism avirulent in the absence of T3 secretion.

The ability of *P. aeruginosa* to avoid phagocytic clearance by PMNs is a major virulence determinant. Successful evasion of phagocytosis by *P. aeruginosa* is believed to be primarily dependent on the presence of a functional T3SS, because this system has been demonstrated to kill neutrophils [28]. However, it also appears that a functional T2SS causes a clearance defect, as demonstrated in the present study by rising bacterial counts when the T2SS is present and more viable bacteria within the neutrophils. The sole possible insight into this defect involved the elevated concentrations of the anti-inflammatory cytokine interleukin-10 in BAL fluid. This cytokine has been demonstrated to impair both neutrophil and alveolar macrophage bactericidal activity [29, 30], and antibody against it improves survival in *P. aeruginosa* lung infection in a cecal ligation puncture model of immunosuppression [25]. Thus, there may be 2 independent actions of the T2SS as a whole that are not fully explained by our knowledge of the secreted products—a toxic effect on the mouse lung and an independent one on host defenses. In vitro, none of the major T2 secreted enzymes (ExoA, LasB, and phospholipase H) kill neutrophils [31], even though subtle effects on function have been reported [32]. In vivo, a mutant in a regulatory gene for ExoA, has been shown to impair host defenses in the lungs of wild-type mice by allowing bacterial counts to reach higher levels, but this mutation did not affect survival of mice and it had no effect on neutrophil migration into the airways [33]. Elucidating which toxins are having these effects(s) will require a detailed characterization of the secreted products that use the XcpQ secretin and testing of mutants in the secreted products.

It may be argued that the T3SS is the first system to act, and because it kills more rapidly, there may not be a role for the T2SS. However, not all *P. aeruginosa* strains are competent for T3 secretion. Sokol et al [34] examined 124 clinical isolates from burns and bacteremia and found that only 38% of the isolates secreted ExoS, whereas 80% secreted ExoA. Assuming that strains having ExoU were missed (approximately one-third of those that are ExoS positive), this suggests that only half of these strains were T3 secretion competent. Roy-Burman et al [10] examined 71 non CF lung isolates and found that only 66% secreted ExoS or ExoU. Hauser et al [35] examined 35 selected strains from patients with pneumonia and found that 74% were T3SS competent, but they further pointed out that among these same isolates, secretion of ExoS was not consistently associated with virulence in a mouse model of pneumonia [36], suggesting that other virulence factors played a role in death. We have also examined 100 blood isolates for T2 secretion, using elastolytic activity as a proxy for T2 secretion, and have found that 99% of these isolates are T2 secretion competent (data not shown). Thus, these 2 systems may be viewed together as comprising a fail-safe system for defense against whatever host the organism encounters and are both integral to pathogenesis, neither more important than the other.

The findings in this study are also of practical significance in the development of active or passive vaccines or treatments for *P. aeruginosa* if one wishes to target the toxin-producing systems. Targeting either the T3SS or the T2SS, or any of their products alone, will not be optimal. Although it has been demonstrated that targeting PcrV of strain PA103 is protective [37], it should be pointed out that this strain demonstrates defects in T2 secretion; it does not secrete the major protease LasB and lacks a flagellum [38] and is therefore not representative of a large number of *P. aeruginosa* strains. Thus, some surface-exposed component of the T2SS or a specific toxic secretion product needs to be included in a vaccine designed to target secretions.

Besides demonstrating a role for the T2SS, this study also demonstrates the potential of using TLR-knockout mice to elucidate pathogenesis under circumstances where there is host compromise. A reasonable assumption is that for a significant infection caused by this opportunistic bacterium [39] to occur there must be failure of innate immunity which we have replicated by using these mice. Although challenge with larger numbers of

bacteria will overcome the innate immune response of normal mice, this is also likely to cause an inflammatory response that may confound an analysis of the effects on individual toxins, especially if multiple toxins act together to cause death.

Funding

This work was supported in part by grants from Vaincre la Mucoviscidose and Legs Poix to M.C. and National Institutes of Health grant 1R01AI078770-01A1 to R.R.

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