Type IV Pilus of *Pseudomonas aeruginosa* Confers Resistance to Antimicrobial Activities of the Pulmonary Surfactant Protein-A

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Key Words

Surfactant protein-A · *Pseudomonas aeruginosa* · Type IV pilus · Opsonization · Membrane permeabilization · Adhesins

Abstract

Pseudomonas aeruginosa (PA) is a Gram-negative bacterial pathogen commonly associated with chronic lung infections. Previously, we have identified several PA virulence factors that are important for resistance to the surfactant protein-A (SP-A), a pulmonary innate immunity protein that mediates bacterial opsonization and membrane permeabilization. In this study, we demonstrate that the type IV pilus (Tfp) is important in the resistance of PA to the antibacterial effects of SP-A. The Tfp-deficient mutant $\Delta pilA$ is severely attenuated in an acute pneumonia model of infection in the lungs of wild-type mice, but is virulent in the lungs of SP-A^{-/-} mice. The $\Delta pilA$ bacteria are more susceptible to SP-A-mediated aggregation and opsonization. In addition, the integrity of the outer membranes of $\Delta pilA$ bacteria is compromised, rendering them more susceptible to SP-A-mediated membrane permeabilization. By comparing Tfp extension and retraction mutants, we demonstrate that the increased susceptibility of $\Delta pilA$ to SP-A-mediated opsonization requires the total ab-

sence of Tfp from PA cells. Finally, we provide evidence of increased expression of nonpilus adhesin OprH that may serve as an SP-A ligand, resulting in increased phagocytosis and preferential pulmonary clearance of $\Delta pilA$.

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Introduction

Pseudomonas aeruginosa (PA) is an opportunistic Gram-negative bacterial pathogen commonly associated with acute or chronic infection of mechanically damaged (ventilator-associated pneumonia), immunocompromised (HIV, malignancies and immunosuppressive drugs) and mechanically obstructed (cystic fibrosis and chronic obstructive pulmonary disease) lungs [1]. In addition to lung infections, PA is also prevalent in contact-lens-associated keratitis and is a major cause of burn infections [2, 3]. The prevalence of PA in many infections can be attributed to its ability to express a wide array of virulence factors and form biofilms [4] as well as its intrinsically high levels of resistance to many antibiotics [5].

Among the virulence factors expressed by PA is the unipolarly localized surface appendage, type IV pilus (Tfp) [4]. Tfp deficiency attenuates the ability of PA to

induce epithelial cytotoxicity, pneumonia, septicemia and mortality in mice [6, 7]. Tfp is important for twitching motility, epithelial adhesion, substratum attachment and biofilm formation as well as phage attachment and uptake [4, 8]. Tfp is made up of monomers of pilin, encoded by the *pilA* gene [8, 9]. The assembly and disassembly of Tfp are powered by the ATPases PilB and PilT/U, respectively, producing twitching motility [8, 10]. These extension and retraction proteins are regulated by the Chp chemosensory system [10]. The asialoGM1 [4, 8, 9, 11] and N-glycan chains [12, 13] located on the apical surface of the host epithelium are the main receptors for Tfp.

The surfactant of the lung contains phospholipids and four major surfactant proteins (SPs): SP-A, B, C and D [14, 15]. The naturally occurring octadecameric SP-A is the most abundant [14, 15]. Each monomer contains an N-terminal, triple-helical collagen region that binds to eukaryotic receptor (SP-A receptor 210), and a C-terminal carbohydrate-recognition domain (CRD) that binds to microbial carbohydrates [14–16]. Binding of the CRD to microbial carbohydrates aggregates microbes, enhancing their phagocytosis [14, 17–21]. In addition to opsonic phagocytosis, SP-A enhances phagocytosis directly [15, 22]. SP-A^{-/-} mice are more susceptible to lung infection, with decreased bacterial clearance and reduced macrophage phagocytosis [15, 23, 24]. Most recently, we and others have shown that SP-A directly permeabilizes microbial membranes [17–21].

Previously, our laboratory has shown that several virulence factors of *PA*, including lipopolysaccharide (LPS), phosphoenolpyruvate transferase, isochorismate synthase, flagellum and elastase B confer resistance to SP-A-mediated antimicrobial effects [18–21]. In this paper, we show that Tfp of *PA* also plays a major role in resistance to SP-A-mediated phagocytosis and membrane permeabilization.

Materials and Methods

Bacterial Strains and Growth Conditions

The wild-type *PA* strain PAO1 and its isogenic derivatives *ΔpilA*, the genetically-complemented strain *ΔpilA-comp*, *ΔchpA*, *ΔpilG*, *ΔpilB*, *ΔpilH*, *ΔpilT* and *ΔpilU* were generously provided by Prof. Joanne Engel (University of California, San Francisco, Calif., USA) [10]. The *rpoN::ISphoA/hah* mutant was purchased from the University of Washington Genome Sciences [25] (table 1). *PA* strains were grown in Luria-Bertani (LB) broth and stored at –80°C in 30% glycerol. Before each experiment, bacteria were streaked from frozen stock onto LB agar for 18 h at 37°C. One colony from this streak was then cultured in 5 ml LB broth to the stationary phase (OD₆₀₀ approx. 3.0).

Mouse Clearance Assay

Wild-type C3H/HeN (SP-A^{+/+}) mice were purchased from Harlan Laboratory (South Easton, Mass., USA). Isogenic SP-A^{-/-} mice were gifts from Dr. Francis McCormack (University of Cincinnati College of Medicine, Cincinnati, Ohio, USA). Mouse experiments complied with the guidelines and were approved by the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee. SP-A^{+/+} and SP-A^{-/-} mice (n = 5) were given a single intranasal inoculation of 1 × 10⁷ PAO1 or *ΔpilA* strain. After 18 h of infection, mouse lungs were harvested for histology or bacterial enumeration as we previously described [18–21].

In vivo Phagocytosis Assay

The phagocytosis rates between different *PA* strains were compared using the gentamicin exclusion assay as previously described [21]. Briefly, SP-A^{+/+} and SP-A^{-/-} mice (n = 3) were given a single intranasal inoculation of 1 × 10⁷ PAO1 or *ΔpilA* cells. After 2 h, mouse lungs were lavaged to collect the alveolar macrophages and neutrophils. The macrophages were then incubated in PBS supplemented with 100 µg/ml gentamicin to kill the remaining extracellular bacteria. The macrophages were lysed with 1% Triton X-100 solution and serially diluted for the enumeration of internalized *PA*. The ratio of CFU counts between SP-A^{+/+} and SP-A^{-/-} mice was computed for the fold increase of phagocytosis mediated by SP-A.

Purification of Human SP-A

Discarded lung washings from anonymous alveolar proteinosis patients were generously provided by Dr. Francis McCormack. Human (h)SP-A was purified as previously described [26]. Briefly, raw lung washings, equilibrated with 1 mM CaCl₂, were passed through a Sepharose® 6B column laden with mannose. The captured SP-A was eluted using the elution buffer (2 mM EDTA and 5 mM Tris-HCl, pH 7.4). The eluted fractions were dialyzed using the dialysis buffer (150 mM NaCl and 5 mM Tris-HCl, pH 7.4) to remove EDTA. The purity of hSP-A preparations was confirmed by Coomassie blue stain.

Murine Macrophage Cell Line and in vitro Phagocytosis Assay

Murine RAW 264.7 macrophages (ATCC# TIB-71) were maintained in DMEM supplemented with 10% FBS at 5% CO₂ and 37°C [21]. The phagocytosis rates between different *PA* strains were compared using the gentamicin exclusion assay. Briefly, 1 × 10⁶ RAW 264.7 macrophages/ml were plated in 6-well cell culture plates overnight in a 37°C incubator with 5% CO₂. *PA* strains were preincubated with 12.5, 25 or 50 µg/ml hSP-A in the presence of 2 mM CaCl₂ for 1, 6 or 12 h in a shaker (300 rpm) at 37°C. The resulting mixture was then incubated with the RAW 264.7 cells at a ratio of bacteria/macrophages of 10:1 for 1.5 h. The macrophages were then washed and incubated in fresh DMEM supplemented with 100 µg/ml gentamicin to kill the remaining extracellular bacteria. The macrophages were lysed with 1% Triton X-100 solution, and serially diluted for enumeration. The ratio of CFU between treated and untreated bacteria was computed for the fold increase of phagocytosis mediated by hSP-A.

Aggregation Assay

PA strains were transformed with the plasmid pUCP19 harboring a green fluorescent protein (GFP) gene by electroporation as previously described [21]. Bacterial aggregation was performed using the stationary-phase *PA* incubated with 25 µg/ml hSP-A and

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
<i>P. aeruginosa</i> strains		
PAO1	wild-type	[10]
PAO1 $\Delta pilA$	in-frame deletion of the <i>pilA</i> gene	[10]
$\Delta pilA$ -comp	genetically-complemented $\Delta pilA$ mutant	[10]
PAO1-GFP	PAO1 harboring pUCP19- <i>gfp</i>	this study
$\Delta pilA$ -GFP	$\Delta pilA$ harboring pUCP19- <i>gfp</i>	this study
$\Delta chpA$	in-frame deletion of the <i>chpA</i> gene	[10]
$\Delta pilG$	in-frame deletion of the <i>pilG</i> gene	[10]
$\Delta pilB$	in-frame deletion of the <i>pilB</i> gene	[10]
$\Delta pilH$	in-frame deletion of the <i>pilH</i> gene	[10]
$\Delta pilT^{CTX-pilU}$	in-frame deletion of the <i>pilT</i> ; <i>pilU</i> and 1 kb upstream sequence at <i>attB</i> site	[10]
$\Delta pilU$	in-frame deletion of the <i>pilU</i> gene	[10]
<i>rpoN::ISphoA/hah</i>	mutant harboring a <i>ISphoA/hah</i> transposon insertion into the <i>rpoN</i> gene	University of Washington Genome Sciences
Plasmids		
pUCP19- <i>gfp</i>	plasmid pUCP19 expressing a GFP	[21]

2 mM CaCl₂ for 1 h in a shaker (300 rpm) at 37°C. Bacterial clusters were enumerated from 10 independent fields under a fluorescence microscope.

Membrane Permeabilization Assay

The membrane permeability effects of hSP-A were measured using both the thiol-specific fluorophore ThioGlo® (Calbiochem, San Diego, Calif., USA) and enzyme-labeled fluorescence phosphatase substrate ELF-97® (Molecular Probes, Carlsbad, Calif., USA), as previously described [17, 20, 21]. Stationary-phase *PA* bacteria were washed and incubated with either 50 µg/ml hSP-A for 15 min at 37°C or with 50 µg/ml total protein of bronchoalveolar lavage fluids (BALF) from either SP-A^{+/+} or SP-A^{-/-} mice. For the ThioGlo assay, the bacteria/SP-A mixture was sedimented, and the supernatant was incubated with 10 µM ThioGlo. Fluorescence was measured at an excitation wavelength of 405 nm and an emission wavelength of 535 nm. For the ELF-97 assay, the bacteria/SP-A mixture was directly incubated with 100 µM ELF-97. Fluorescence was measured at an excitation wavelength of 355 nm and an emission wavelength of 535 nm every 3 min for a period of 90–120 min.

Bacterial Viability Assay

Stationary-phase *PA* bacteria were washed and incubated with either 50 µg/ml hSP-A or 50 µg/ml total protein of BALF from either SP-A^{+/+} or SP-A^{-/-} mice for 60 min or 6 h at 37°C. The bacteria were then stained with a combination of SYTO® 9 and propidium iodide from the commercial bacterial viability kit, LIVE/DEAD® BacLight (Molecular Probes). The numbers of dead and live bacteria were counted in 10 independent high-power fields (×400) using a fluorescent microscope.

Elastase Assay

Elastase production in the culture supernatant of stationary-phase *PA* was evaluated using the Sensolyte™ Red Protease assay kit (AnaSpec Inc., San Jose, Calif., USA). Fluorescence was measured at excitation 546 nm and emission 575 nm.

Pyocyanin Assay

Pyocyanin in the bacteria-free supernatant of stationary-phase *PA* cultured in LB broth or a low-phosphate medium (20 mM succinic acid, 40 mM NH₄Cl, 2 mM K₂SO₄, 0.014 g NAH₂PO₄, 1 M MOPS, 40 mM MgCl₂, 100 mM CaSO₄, 100 mM ZnCl₂, 30 mM MnCl₂ and 30 mM Fe(NO₃) were measured at OD₆₉₀ [20].

SDS Lysis Assay

To assess the membrane stability of *PA* strains, bacterial cell lysis was performed with 0.25% SDS as previously described [19]. Stationary-phase *PA* bacteria (OD₆₀₀ 3.0) were washed and resuspended in PBS with 0.25% SDS. The OD₆₀₀ was measured every 10 min for 1 h.

LPS Analysis

LPS was purified from stationary-phase *PA* strains as follows: the bacteria were sedimented and resuspended in 200 µl lysis buffer (2 g SDS, 4 ml 2-mercaptoethanol, 0.003 g bromophenol blue and 1 M Tris-HCl, pH 6.8) and boiled for 10 min. Three microliters of 20 µg/µl proteinase K were added, and the mixture was incubated for 1 h at 60°C. The resulting mixture was separated in an SDS-PAGE gel. The resolved gel was incubated with a fixing solution (25% v/v isopropyl alcohol and 7% v/v acetic acid) overnight at 4°C, oxidized (0.7% periodic acid, 2.7% ethanol and 0.3% v/v acetic acid) for 5 min with gentle agitation, and washed with water 3 times for 30 min each. The gel was then placed in staining solution (4% v/v 1 M NaOH, 5% v/v NH₄OH and 2% AgNO₃) and shaken for 10 min, followed by four washes in water for 10 min each. Finally, the gel was developed (0.02% citric acid and 0.05% v/v formaldehyde) for 20 min. The development was stopped using a stop solution (0.8% v/v acetic acid).

Transmission Electron Microscopy

PA strains were grown to OD₆₀₀ approximately 3.0 in LB broth at 37°C. Bacteria were washed with PBS, and subsequently fixed

Fig. 1. *ΔpilA* bacteria are more susceptible to clearance by SP-A. **a** Respiratory tract infections with wild-type PAO1 versus *ΔpilA* were performed by intranasal inoculation of anesthetized SP-A^{+/+} or SP-A^{-/-} mice. Mouse lungs were harvested 18 h after infection for bacterial enumeration. Data are the mean CFU ± SE (n = 5 per group). * p < 0.05 when comparing the bacterial loads between SP-A^{+/+} and SP-A^{-/-} infected by PAO1 or *ΔpilA*. # p < 0.05 when comparing the bacterial loads between PAO1 and *ΔpilA* infecting the SP-A^{+/+} or SP-A^{-/-} mice. **b** The growth kinetics of PAO1 and *ΔpilA* bacteria were determined by measuring OD₆₀₀. The experiments were performed independently in triplicate. The representative growth curve from 1 of 3 independent experiments is shown.

using the Karnovsky fixative. Transmission electron microscopy (TEM) was performed at the University of Illinois Material Research Laboratory.

Ligand Blot

Biotinylated SP-A was used to identify potential receptors in *PA* strains as previously described [16]. Briefly, stationary-phase bacteria were ruptured under 14,000 psi in a French press. Membranes were isolated in 100 mM sodium carbonate and centrifuged at 115,000 g. Isolated membranes were solubilized using 50 mM Tris pH 7.4 mixed with loading buffer, boiled for 5 min and then resolved using 15% SDS-PAGE gels. Gels were either stained using Coomassie blue or transferred to PVDF membranes. Membranes were blocked for 2 h, and subsequently incubated with 3 μg/ml biotinylated SP-A. After 3 washings, streptavidin-conjugated HRP was added. Signal was detected using commercially available Western blot stain and substrate. Ligand blot signal was analyzed with the Image J software. Corresponding bands in gels stained with Coomassie blue to the signal detected in the PVDF membrane were sent for analyses at the University of Illinois LC-MS/MS core facility.

Results

Tfp Is Important in Resistance to SP-A-Mediated Lung Clearance

To determine the contribution of Tfp-mediated resistance to SP-A, we compared the virulence of the wild-type *PA* PAO1 and the isogenic *ΔpilA* mutant in a mouse model of acute pneumonia. Eighteen hours after intranasal inoculation with PAO1 or *ΔpilA*, SP-A^{+/+} mice showed signs of infection and respiratory distress but were not moribund. In contrast, PAO1-infected SP-A^{-/-} mice were moribund and had to be euthanized (data not shown). The number of viable PAO1 or *ΔpilA* bacteria in SP-A^{-/-}

were 2.74 log and 1.97 log higher than in SP-A^{+/+} mice, respectively (fig. 1a). The number of *ΔpilA* bacteria in SP-A^{-/-} mice was statistically indistinguishable when compared to the number of PAO1 in the SP-A^{+/+} mice, suggesting that *ΔpilA* was more virulent during lung infection in the absence of SP-A. However, the viable counts of *ΔpilA* were 1.4 log lower than PAO1 in SP-A^{+/+} mice and 2.16 log lower than PAO1 in SP-A^{-/-} mice. Because the relative decrease of bacterial load between PAO1 and *ΔpilA* from SP-A^{-/-} mice to SP-A^{+/+} mice was only 0.77 log, this suggests that *ΔpilA* is in general less virulent. In addition, other innate immune factors of the lung within the alveolar space may compensate for the absence of SP-A. Thus, a straightforward link between Tfp and SP-A cannot be easily established (fig. 1a). The *ΔpilA* showed similar growth kinetics to that of PAO1, suggesting that the difference in mouse clearance was not due to the growth defects in the former (fig. 1b).

The aforementioned quantitative observations were supported by histopathology (fig. 2). In the absence of bacterial infection, histopathological features of SP-A^{-/-} mouse lungs were indistinguishable when compared to the lungs of SP-A^{+/+} mice [data not shown]. PAO1 caused moderate bronchopneumonia (fig. 2a) whereas the *ΔpilA* mutant only caused mild bronchopneumonia in the lungs of SP-A^{+/+} mice (fig. 2b). In contrast, PAO1 caused severe bronchopneumonia in SP-A^{-/-} mice, with large amounts of pulmonary infiltrates and consolidation (fig. 2c). Importantly, *ΔpilA* caused more severe pulmonary infiltrate in SP-A^{-/-} mice (fig. 2d) than the SP-A^{+/+} mice (fig. 2b). The severity of *ΔpilA*-mediated bronchopneumonia in SP-A^{-/-} lungs (fig. 2d) was similar to that caused by PAO1

Fig. 2. Histopathology of PA-infected lungs. SP-A^{+/+} and SP-A^{-/-} mice were infected with PAO1 or $\Delta pilA$ as described in figure 1. Representative HE-stained lung sections from SP-A^{+/+} and SP-A^{-/-} mice (n = 5) 18 h after intranasal instillation of PAO1 (**a**, **c**) and $\Delta pilA$ (**b**, **d**) bacteria.

Fig. 3. The $\Delta pilA$ mutant is more susceptible to SP-A-mediated opsonization. **a–c** RAW 264.7 macrophages were infected with either PAO1 or $\Delta pilA$ in the presence or absence of hSP-A. The ratio of ingested bacteria was expressed as fold increase in phagocytosed bacteria due to the effect of hSP-A. **a** Phagocytosis of PAO1, $\Delta pilA$ and genetically complemented $\Delta pilA$ -comp in the presence of 25 $\mu\text{g/ml}$ hSP-A. **b** Phagocytosis of PAO1 and $\Delta pilA$ in the presence of different concentrations of hSP-A. **c** Time-dependent phagocytosis of PAO1 versus $\Delta pilA$ in the presence of 25 $\mu\text{g/ml}$ hSP-A. **d** In vivo phagocytosis of PAO1 versus $\Delta pilA$ in SP-A^{+/+} and SP-A^{-/-} mice (n = 3). All phagocytosis experiments were independently performed in triplicate. The mean $\pm$ standard deviation from 1 representative experiment is shown. * p < 0.05 when comparing the number of phagocytosed $\Delta pilA$ to PAO1.

Fig. 4. $\Delta pilA$ are more susceptible to SP-A-mediated aggregation. GFP-expressing PAO1 and $\Delta pilA$ bacteria were incubated with 25 $\mu\text{g/ml}$ hSP-A for 1 h and examined under a confocal fluorescence microscope. **a** PAO1 without hSP-A. **b** PAO1 with hSP-A. **c** $\Delta pilA$

without hSP-A. **d** $\Delta pilA$ with hSP-A. **e** The number of aggregates was averaged from 10 independent high-power fields. * $p < 0.05$ when comparing the number aggregates in $\Delta pilA$ to PAO1.

in SP-A^{+/+} lungs (fig. 2a), suggesting that the virulence levels of $\Delta pilA$ in mouse lungs devoid of SP-A was similar to PAO1 in mouse lungs with sufficient SP-A. Collectively, these results indicate that Tfp plays a protective role against anti-PA activity mediated by SP-A.

Tfp Is Important for Resistance of SP-A-Mediated Phagocytosis

To decipher the mechanism of Tfp resistance in SP-A-mediated lung clearance, we examined whether $\Delta pilA$ bacteria were more susceptible to SP-A-mediated opsonization. In the presence of 25 $\mu\text{g/ml}$ of SP-A, $\Delta pilA$ bacteria were phagocytized 5 times more efficiently than the wild-type PAO1 (fig. 3a). The genetically-complemented $\Delta pilA$ -comp bacteria were as resistant to SP-A-mediated opsonization as PAO1 (fig. 3a). These results were confirmed by studies using different concentrations of SP-A, which consistently showed that, in the presence of SP-A, $\Delta pilA$ was more susceptible to phagocytosis by macrophages than PAO1 (fig. 3b). We also examined the phagocytosis of $\Delta pilA$ in a time-dependent manner. Again, $\Delta pilA$ was more susceptible to SP-A-mediated opsonization at 1 and 6 h after exposure to RAW 264.7 macrophages (fig. 3c). However, prolonged exposure (12 h) abolished phagocytosis, most probably because both PAO1 and $\Delta pilA$ bacteria degraded SP-A by secreting

exoproteases, as we have previously described [20, 21]. These observations were confirmed by an in vivo phagocytosis assay, which showed that $\Delta pilA$ was 4.5 times more susceptible to SP-A-mediated opsonization than PAO1 (fig. 3d). The in vivo phagocytosis assay measures the total phagocytosis activity involving both alveolar macrophages and infiltrating neutrophils responding to PA infection. Collectively, these results indicate that Tfp is important for resistance to SP-A-mediated opsonization.

Tfp Is Important for Resistance to SP-A-Mediated Aggregation

One of the mechanisms by which SP-A enhances microbial phagocytosis is by increasing their aggregation, allowing more efficient phagocytosis [22]. We compared the aggregation of wild-type PAO1 versus $\Delta pilA$ by SP-A in vitro. The $\Delta pilA$ mutant bacteria were aggregated by SP-A 4-fold higher than PAO1 per high-power field (fig. 4a–e). Overall, these results show that Tfp confers resistance to SP-A-mediated aggregation.

Tfp Is Important for Resistance to SP-A-Mediated Membrane Permeabilization

Apart from its ability to opsonize and facilitate the phagocytosis of microbes by macrophages, SP-A is also

Fig. 5. $\Delta pilA$ are more susceptible to SP-A-mediated membrane permeabilization. **a** ELF-97 assay. PAO1 and $\Delta pilA$ were preincubated with 50 μ g/ml hSP-A for 15 min before the addition of ELF-97. Absorbance was measured every 3 min at excitation wavelength 355 nm and emission wavelength 535 nm, for a total of 90 min. **b** In vitro ThioGlo assay. PAO1, $\Delta pilA$ and $\Delta pilA$ -comp were preincubated with 50 μ g/ml hSP-A for 15 min. The bacteria-free supernatants were then mixed with ThioGlo. Absorbance was measured at excitation wavelength 405 nm and emission wavelength 535 nm. * $p < 0.05$ when comparing the relative fluorescence unit (RFU) of $\Delta pilA$ to PAO1 and $\Delta pilA$ -comp. **c** Ex vivo ThioGlo assay. PAO1, $\Delta pilA$ and $\Delta pilA$ -comp were preincubated with 50 μ g/ml total protein of BALF from either SP-A^{+/+} mice or SP-A^{-/-} mice for 1 h. The bacteria-free supernatants were then mixed with ThioGlo. Absorbance was measured at excitation wavelength 405 nm and emission wavelength 535 nm. # $p < 0.05$ when comparing the RFU of $\Delta pilA$ to PAO1 and $\Delta pilA$ in BALF from SP-A^{+/+}

mice. * $p < 0.05$ when comparing the RFU of each PA strain in BALF from SP-A^{+/+} to SP-A^{-/-} mice. **d** In vitro PA killing by hSP-A. PAO1 and $\Delta pilA$ were preincubated with 50 μ g/ml hSP-A for 1 or 6 h. The bacteria were then stained with a mixture of SYTO 9 and propidium iodide for 15 min. The ratio of dead-to-live bacteria is counted in 10 high-power fields. * $p < 0.05$ when comparing the ratio of dead-to-live $\Delta pilA$ to PAO1 bacteria in the presence of hSP-A. **e** Ex vivo PA killing by mouse SP-A within BALF. PAO1 and $\Delta pilA$ were preincubated with 50 μ g/ml total protein of BALF from either SP-A^{+/+} mice or SP-A^{-/-} mice for 1 h. Bacterial viability was determined as in **d**. # $p < 0.05$ when comparing the ratio of dead-to-live bacteria between those treated with BALF from SP-A^{+/+} and SP-A^{-/-} mice. * $p < 0.05$ when comparing the ratio of dead-to-live PAO1 to $\Delta pilA$ -treated with BALF from SP-A^{+/+} mice. Experiments were performed independently in triplicate (for ELF-97 and ThioGlo assays) or duplicate (for the LIVE/DEAD assay). Data from 1 typical experiment are shown.

Fig. 6. The susceptibility of Tfp extension and retraction mutants to SP-A-mediated opsonization. **a, b** RAW 264.7 macrophages were infected with PAO1, $\Delta pilA$ and the extension or retraction mutants in the presence or absence of hSP-A. The ratio of ingested bacteria between those exposed to hSP-A to those unexposed was expressed as fold increase in phagocytosis. **a** Phagocytosis of PAO1, $\Delta pilA$ and extension mutants $\Delta chpA$, $\Delta pilG$ and $\Delta pilB$. **b** Phagocytosis of PAO1, $\Delta pilA$ and retraction mutants $\Delta pilH$, $\Delta pilT$ and $\Delta pilU$. * $p < 0.05$ when comparing phagocytosis of PAO1 to the $\Delta pilA$ mutant. # $p < 0.05$ when comparing phagocytosis of PAO1 to the $\Delta pilT$ and $\Delta pilU$ mutants. All experiments were independently performed in triplicate. The mean $\pm$ standard deviation from 1 representative experiment is shown. **c, d** Membrane permeabilization of PA strains by hSP-A. **c** Membrane permeabilization of PAO1, $\Delta pilA$ and extension mutants $\Delta chpA$, $\Delta pilG$ and $\Delta pilB$. **d** Membrane permeability of PAO1, $\Delta pilA$ and retraction mutants $\Delta pilH$, $\Delta pilT$ and $\Delta pilU$. All experiments were independently performed in triplicate. The mean $\pm$ standard deviation from 1 representative experiment is shown.

Fig. 7. Resistance to SP-A-mediated phagocytosis is independent of quorum sensing. **a** Exoprotease activity of PAO1 versus $\Delta pilA$. **b** Pyocyanin production of PAO1 versus $\Delta pilA$.

capable of directly killing bacteria by membrane permeabilization [17–19]. We examined the susceptibility of $\Delta pilA$ to SP-A-mediated membrane permeabilization, by measuring the diffusion of an impermeable phosphatase substrate ELF-97 into PA cells, and by measuring the

leakage of thiol-containing proteins. After 90 min of exposure, the ELF-97 assay indicated that $\Delta pilA$ bacteria were permeabilized 2.1-fold higher than the PAO1 (fig. 5a). In addition, the leakage of thiol-containing proteins increased by 1.81-fold relative to PAO1 (fig. 5b). To confirm our in vitro observations, we compared the ability of BALF of SP-A^{+/+} and SP-A^{-/-} mice to permeabilize the membrane of PAO1 and $\Delta pilA$ (fig. 5c). The $\Delta pilA$ bacteria were more susceptible to BALF from SP-A^{+/+} mice than PAO1. In contrast, BALF from SP-A^{-/-} only possessed low levels of membrane-permeabilizing capability, suggesting that SP-A is an important membrane-permeabilizing protein within the BALF. To determine whether increased susceptibility to membrane permeability leads to direct bacterial killing, we examined the bacterial viability after exposure to hSP-A and to BALF. Although both the hSP-A and the BALF from SP-A^{+/+} mice only caused low levels of PA killing, the $\Delta pilA$ was consistently shown to be significantly more susceptible to direct killing by hSP-A (fig. 5d) and BALF of SP-A^{+/+} mice (fig. 5e). These results suggest that SP-A-mediated membrane permeabilization contributes minimally to PA clearance.

Fig. 8. Resistance to SP-A-mediated phagocytosis is regulated by RpoN. **a** Comparison of bacterial phagocytosis by RAW 264.7 macrophages between PAO1, $\Delta pilA$ and $rpoN::ISphoA/hah$ in the presence or absence of 25 $\mu\text{g/ml}$ hSP-A. All experiments were independently performed in triplicate. The mean $\pm$ standard deviation from 1 representative experiment is shown. * $p < 0.05$ when comparing PAO1 to $\Delta pilA$. # $p < 0.05$ when comparing PAO1 to $rpoN::ISphoA/hah$. **b** Ligand blot analysis of nonpilus adhesins in PAO1, $\Delta pilA$ and $rpoN::ISphoA/hah$. **c** Image J analysis of the ligand blot. Protein expression was normalized to PAO1 as 100%. **d** Protein sequence of OprH. **e** Comparison of membrane permeabilization between PAO1, $\Delta pilA$ and $rpoN::ISphoA/hah$. The experiments were independently performed in triplicate. The mean $\pm$ standard deviation from 1 representative experiment is shown. * $p < 0.05$ when comparing PAO1 to $\Delta pilA$ and $rpoN::ISphoA/hah$.

Tfp-Mediated Resistance to SP-A-Mediated Phagocytosis Is Independent of the Presence of the Appendages on the Cell Surface

Compared to other PA mutants we have studied previously [15, 16, 27, 28], $\Delta pilA$ is uniquely more susceptible to both SP-A-mediated opsonization and membrane permeabilization. We used both the pilin extension ($\Delta chpA$, $\Delta pilG$ and $\Delta pilB$) and retraction ($\Delta pilH$, $\Delta pilT$ and $\Delta pilU$) mutants to determine if the presence of Tfp on the cell surface of PA is required for resistance to both SP-A-mediated opsonization and membrane permeabilization. As shown in figure 6a, the mere absence of Tfp on the bacterial surface in the extension mutants ($\Delta chpA$, $\Delta pilG$ and $\Delta pilB$) does not increase susceptibility to SP-A-mediated phagocytosis. Interestingly, the retraction mutants, $\Delta pilT$ and $\Delta pilU$, which are hyperpilated, are more resistant to SP-A-mediated opsonization (fig. 6b). Both extension and retraction mutants were as resistant to SP-A-mediated membrane permeabilization as PAO1 (fig. 6c, d). Collectively, these results suggest that the presence of pilin anchoring the membranes is adequate to confer resistance to SP-A-mediated phagocytosis. In addition, ex-

pression of additional Tfp (e.g. $\Delta pilT$ and $\Delta pilU$ mutants) offers increased resistance to SP-A-mediated phagocytosis.

Increased Susceptibility of $\Delta pilA$ to SP-A-Mediated Phagocytosis Is Probably Due to a Compensatory Increase in Nonpilus Adhesins

Previous work in our laboratory has shown that PA confers resistance to SP-A-mediated phagocytosis by increasing the degradation of SP-A through elastase B [20, 21], through mechanisms regulated by both flagellum and quorum-sensing. Flagellar-deficient mutants are deficient in quorum sensing, decreasing their ability to produce adequate elastase B to degrade SP-A. However, $\Delta pilA$ produced wild-type levels of both quorum-sensing-regulated exoprotease activities (fig. 7a) and the redox-active secondary metabolite pyocyanin (fig. 7b). Furthermore, the $\Delta pilA$ lost its susceptibility to SP-A-mediated phagocytosis after 12 h (fig. 3c), suggesting that SP-A was degraded. Thus, the lack of quorum-sensing and SP-A degradation is not the cause of enhanced opsonization of $\Delta pilA$ by SP-A.

Fig. 9. $\Delta pilA$ is more susceptible to SDS-mediated cell lysis. **a** LPS analysis of PAO1 and $\Delta pilA$ as visualized using silver stain. **b**, **c** TEM of PAO1 and $\Delta pilA$, respectively. Bar = 100 nm. **d** SDS lysis assay. PAO1 and $\Delta pilA$ bacteria were incubated in 0.25% SDS solution. OD₆₀₀ was measured every 10 min. Bacterial lysis experiments were independently performed in triplicate. The mean from 1 representative experiment is shown.

Previous studies have shown that $\Delta pilA$ has a higher binding affinity to host epithelial cells than the hyperpilated mutants $\Delta pilT$ and $\Delta pilU$ [29–32]. This is speculated to be caused by overexpression of alternative ‘nonpilus adhesins’ in $\Delta pilA$. The alternative sigma factor RpoN has been shown to regulate the expression of these adhesins [29–32]. We hypothesized that one or more of these nonpilus adhesins may serve as ligands for binding to SP-A. We compared the phagocytosis of $\Delta pilA$ versus $rpoN::ISphoA/hah$ in the presence of SP-A. Figure 8a shows $rpoN::ISphoA/hah$, which lacks the adhesins, is more resistant to SP-A-mediated phagocytosis than both wild-type PAO1 and $\Delta pilA$. Importantly, ligand blot analysis shows that the $\Delta pilA$ overexpresses a protein of approximately 18 kDa compared to both PAO1 and $rpoN::ISphoA/hah$ (fig. 8b, c). LCMS/MS and microsequencing analyses indicate that this putative adhesin is the outer membrane protein H1 precursor OprH (PA1178) (fig. 8d). Finally, $rpoN::ISphoA/hah$ does not exhibit increased susceptibility to SP-A-mediated membrane permeabilization, suggesting that these nonpilus

adhesins are dispensable against the pore-forming function of SP-A (fig. 8e).

$\Delta pilA$ Has Reduced Membrane Stability, Rendering It Susceptible to SP-A-Mediated Membrane Permeabilization

Next, we examined the potential mechanisms of increased susceptibility of $\Delta pilA$ to SP-A-mediated membrane permeabilization. Previously, we have shown that the loss of LPS [18, 19] and flagellum [19–21] destabilizes membrane integrity, rendering them more susceptible to SP-A-mediated membrane permeabilization. In addition, Abeyrathne et al. [33] have shown that the O-antigen ligase mutant, $\Delta waaL$, which is unable to attach O-antigen to the core polysaccharide of LPS, has absent or decreased Tfp and flagella. We examined whether the absence of Tfp destabilizes the membranes, through reduced expression of LPS. Qualitative analyses of both the LPS and the TEM images of bacterial membranes show no discernible differences between $\Delta pilA$ and PAO1 (fig. 9a–c). However, $\Delta pilA$ was more slightly more sus-

ceptible to lysis by 0.25% SDS, suggesting that the loss of pilin modestly compromises the integrity of the outer membranes in the mutant bacteria (fig. 9d).

Discussion

SP-A is a major pulmonary innate immunity protein that mediates microbial clearance through opsonization and membrane permeabilization. However, little is known about the mechanisms elaborated by microbial pathogens to confer resistance or susceptibility to SP-A. Previously, we have demonstrated that several *PA* factors: LPS, flagellum, isochorismate synthase and phosphoenolpyruvate phosphotransferase confer resistance to SP-A-mediated membrane permeabilization [18–21]. Flagellum also regulates the quorum-sensing-mediated expression of elastase B that degrades and disables SP-A-mediated opsonization during phagocytosis [19–21]. In this study, we found that Tfp of *PA* is uniquely important for resistance to SP-A-mediated opsonization and membrane permeabilization. Several lines of evidence support this argument: (1) $\Delta pilA$ is preferentially cleared in the lungs of SP-A^{+/+} mice compared to SP-A^{-/-} mice, (2) $\Delta pilA$ is more susceptible to SP-A-mediated aggregation and opsonization, (3) $\Delta pilA$ is more susceptible to SP-A-mediated membrane permeabilization and (4) the genetically complemented $\Delta pilA$ -(comp strain, which carries a copy of the wild-type *pilA* gene in trans, has restored resistance to SP-A-mediated opsonization and membrane permeabilization.

At face value, our report seems to contradict previous findings, which show that Tfp-deficient *PA* strains, including $\Delta pilA$, are more resistant to phagocytosis by macrophages [34–36]. However, this discrepancy can be explained because of different experimental context. Firstly, these previously published studies were pertaining to nonopsonin-mediated, fibronectin-dependent phagocytosis. Secondly, the number of phagocytized bacteria was quantified using a semi-quantitative fluorescent technique, rather than the quantitative method we used with the gentamicin exclusion assay. However, the gentamicin exclusion assay, which has been used for decades to quantify microbial phagocytosis, has its disadvantages. Because phagocytes ingest both individual and SP-A-aggregated bacteria, we cannot rule out the possibility that the vigorous pipetting and vortexing procedures used during serial dilution plating may not completely disaggregate bacteria. However, even if this were the case, $\Delta pilA$, which is more susceptible than PAO1 to SP-A-mediated aggre-

gation, would be undercounted. This would mean that the actual difference in phagocytic factor between $\Delta pilA$ and PAO1 would be even higher, further supporting our results showing that $\Delta pilA$ is more susceptible to SP-A-mediated opsonization and phagocytosis.

A careful examination of data presented in figure 3 (a, b) indicates that there is variation in the efficiency of phagocytosis in different sets of experiments. This is not unusual, due to the fact that bacteria and macrophage cultures are not 'identical' from one set of experiments to the next; for example, the number of bacterial cells used in each experiment certainly differs slightly. Another contributing factor to the variation in phagocytic efficiency is that different batches of SP-A preparations derived by lung washing from different alveolar proteinosis patients may have different antimicrobial activity and potency. Nevertheless, in each experiment described, $\Delta pilA$ is consistently more susceptible to SP-A-mediated opsonization and membrane permeabilization.

Detailed genetic analyses of the *PA* mutants defective in retraction and extension of Tfp reveal rather unexpected mechanisms by which the pilus mediates resistance to SP-A-mediated phagocytosis. For example, the extension gene mutants ($\Delta chpA$, $\Delta pilG$ and $\Delta pilH$) did not show increased susceptibility to SP-A-mediated phagocytosis. These results suggest that a total absence of *pilA* both intracellularly and extracellularly is required to render *PA* becoming susceptible to SP-A-mediated opsonization. We postulate that the absence of *pilA* causes a compensatory increase of alternative ligands that actually interact with SP-A. This is supported by previous studies showing that Tfp-deficient *PA* mutants (e.g. $\Delta pilA$) have higher adhesion to epithelial cells than hyperpilated Tfp extension mutants strains $\Delta pilT$ and $\Delta pilU$, suggesting that Tfp-independent adhesins are responsible for increased binding to epithelial cells [7, 29]. Importantly, both hyperpilated Tfp mutants $\Delta pilT$ and $\Delta pilU$ are significantly more resistant than the wild-type PAO1 to SP-A-mediated phagocytosis. These observations suggest that additional Tfp expressed by both $\Delta pilT$ and $\Delta pilU$ mutants may have masked the putative ligands, decreasing SP-A-mediated binding, aggregation and subsequent opsonization, and enhanced phagocytosis by phagocytes.

Previous work has shown that the alternative sigma factor, RpoN, positively regulates both pilin and flagellin production through their respective 2-component systems *PilRS* and *FleQR* [32, 37]. Thus, RpoN deficiency results in the loss of both Tfp and flagellum in *PA*. Interestingly, $\Delta pilA$ has been used as a model to study the expression of alternative nonpilus adhesins. In contrast,

rpoN mutation has been used as a model for the lack of nonpilus adhesins [30–32]. We speculate that the absence of Tfp causes RpoN to magnify the production of nonpilus adhesins, which serve as ligands for SP-A. This argument is supported by our analyses showing that $\Delta rpoN$ is even more resistant to SP-A-mediated phagocytosis than PAO1. Several studies have tried to determine the identity of these nonpilus adhesins, with most of them focusing on those that bind mucins [27, 28, 38, 39]. Other studies focus on the role of nonpilus adhesins in nonopsonic phagocytosis [30, 31, 39]. However, the identity of these adhesins remains elusive. Another complication is the uncertainty whether the alternative adhesins associated with binding to mucin are the same as those conferring susceptibility to SP-A-mediated phagocytosis. By using ligand blot, we have identified a putative non-pilus adhesin that binds SP-A. LC-MS/MS and micro-sequencing analyses show that the SP-A ligand is the 18-kDa outer membrane protein H1 precursor OprH. OprH expression is governed by low-magnesium environment through the PhoP-PhoQ two-component regulatory system [40, 41]. Interestingly, OprH is noted to have direct interaction with LPS, which is bound by the CRD of SP-A [40]. Another outer membrane protein, P2 of *Hemophilus influenzae*, has been shown to be a receptor of SP-A [42]. We are currently constructing nonpolar deletion mutant and study the genetic, molecular and cellular interactions of OprH with Tfp, RpoN, and SP-A. Furthermore, we are examining the sequence and functional homology between OprH and *H. influenzae* P2.

Tfp interacts with glycoconjugates (e.g. asialoGM1) on the host epithelium [4]. Similarly, SP-A also interacts with glycoconjugates through its CRD. Thus, another possible mechanism of preferential clearance of $\Delta pilA$ mutant from mouse lungs is that SP-A may have blocked Tfp receptors, and preventing the binding of PA to the airway epithelium of wild-type mice. In contrast, in SP-A^{-/-} mice, these Tfp receptors will be exposed, allowing better binding and colonization of PA to the lungs devoid of SP-A. Future studies will include performing comparative in vivo binding of wild-type versus various Tfp mutants to the lung epithelium of SP-A^{+/+} versus SP-A^{-/-} mice.

Our experimental evidence demonstrates that Tfp also mediates resistance to SP-A-mediated membrane permeabilization. Following exposure to hSP-A and to the BALF from SP-A^{+/+} mice, the integrity of the outer membranes is compromised, rendering the $\Delta pilA$ bacteria more permeable to the phosphatase substrate ELF-97 into the cells and increased leakage of thiol-containing intracellular

proteins out of the cells. Previously, we have shown the wild-type strain PAO1 is highly resistant to direct killing by SP-A. Although $\Delta pilA$ is more susceptible to SP-A-mediated membrane permeabilization, this only results in small increase in direct killing of the bacteria, suggesting that the pore-forming process may be transient. Qualitative LPS analysis and TEM examination of membrane structures did not reveal any gross alterations in $\Delta pilA$. However, $\Delta pilA$ bacteria have reduced membrane stability as demonstrated by a modest increase of cell lysis by 0.25% SDS.

In conclusion, we have shown that Tfp is important for resistance to SP-A-mediated opsonization and membrane permeabilization. We provide evidence that Tfp may camouflage nonpilus adhesin, and prevent the binding of SP-A to these ligands, reducing the opsonization and phagocytosis by macrophages. In addition, Tfp is necessary to stabilize bacterial membranes, rendering PA more resistant to SP-A-mediated membrane permeabilization. Adjunctive treatment regimen aimed at inhibiting Tfp may help to improve the clearance of PA by augmenting the efficiency of SP-A in killing this important respiratory pathogen.

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