PSEUDOMONAS AERUGINOSA TYPE IV PILUS AND RESISTANCE TO THE ANTIMICROBIAL PROPERTIES OF SURFACTANT PROTEIN-A

BY

ROMMEL MAX TAN SIM LAN TAN

DISSERTATION

Submitted in partial fulfillment of the requirements

for the degree of Doctor of Philosophy in VMS-Veterinary Pathobiology

in the Graduate College of the

University of Illinois at Urbana-Champaign, 2013

Doctoral Committee:

Associate Professor Gee W. Lau, Chair and Director of Research

Professor Matthew A. Wallig

Professor Carol W. Maddox

Assistant Professor Sidonie N. Lavergne

ABSTRACT

The lung contains numerous innate immune cells and effector proteins. One important component of this immune system is the surfactant protein-A (SP-A), which facilitates microbial clearance by opsonization and membrane permeabilization [1]. *Pseudomonas aeruginosa* (PA) is a major Gram-negative bacterial pathogen commonly associated with chronic lung infection in cystic fibrosis. In order to resist the antibacterial effects of SP-A, PA expresses various structural and secreted virulence factors. Previously, Wu et al (2003) have shown that *Escherichia coli* lipopolysaccharides (LPS) are important in resisting SP-A-mediated membrane permeabilization. Zhang et al (2005) performed a comparative signature-tagged mutagenesis screen to identify PA virulence factors needed to resist SP-A-mediated lung clearance, and identified PchA (isochorismate synthase) and PtsP (phosphoenolpyruvate protein phosphotransferase) as important for resisting SP-A-mediated membrane permeability. Zhang et al (2007) further showed that PA flagellum is important for resisting SP-A-mediated membrane permeabilization. Finally, Kuang et al (2011) have shown PA elastase degrades SP-A, allowing an avenue for PA to escape SP-A-mediated opsonization and membrane permeabilization.

In this study, we demonstrated that type IV pilus (Tfp) is important in the resistance of lung clearance both in the presence and absence of SP-A. The Tfp-deficient mutant, *ΔpilA,* is severely attenuated in an acute pneumonia model of infection in the lungs of wild-type mice that it allows similar bacterial load as PAO1 in the lungs of SP-A$^{-/-}$ mice. The *ΔpilA* bacteria are more susceptible to SP-A-mediated aggregation and opsonization. In addition, the integrity of the outer membranes of *ΔpilA* bacteria is compromised, rendering them more susceptible to SP-A-mediated membrane permeabilization. By using Tfp extension and retraction mutants, we demonstrate that the increased susceptibility of *ΔpilA* to SP-A-mediated opsonization is caused

by the total absence of Tfp from *PA* cells. Finally, we provide evidence that increased expression of an 18 kDa nonpilus adhesin OprH in *ΔpilA*, may explain why there is an increased susceptibility to SP-A-mediated phagocytosis.

In addition, we also have shown that Tfp glycosylation with O-antigen subunits allows for increased resistance to SP-A. We have also shown the glycosyltransferase mutant, 1244G7, which is deficient in O-antigen, is more susceptible to SP-A-mediated lung clearance and phagocytosis, but not membrane permeability. Finally, we have shown that the increase susceptibility of 1244G7 is associated with exposure of putative mannose residues.

ACKNOWLEDGEMENT

I would like to thank my advisor, Dr. Gee W. Lau, for his support and guidance for my thesis projects. I would also like to thank my committee members, Drs. Matthew Wallig, Carol Maddox and Sidonie Lavergne, for their advice and comments for these projects. I would like to thank Eli Lilly and Company for funding the fellowship of the first three years of my combined Residency/PhD program. I would like to thank my laboratory colleagues, Drs. Zhizhou Kuang, Yonghua Hao and Luchang Zhu, for invaluable advice, training, technical expertise and assistance in completing these projects. I would like to thank my fellow PhD student Mr. Mingyan Han for his assistance and training in using the fluorescence microscope. I would like to thank Dr. Francis X. McCormack for providing the bronchoalveolar lavages of alveolar proteinosis patients, from which we purified SP-A. I would like to thank Dr. Joanne Engel of University of California-San Francisco for providing various Tfp mutant strains of PA, as well as Dr. Castric of Duquesne University for providing the PA strains 1244 and 1244G7.

This project was supported by the University of Illinois Research Board Arnold O. Beckman Award, the American Lung Association DeSouza Research Award (DS-192835-N) and the NIH (HL090699) to GW Lau.

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LIST OF ABBREVIATIONS

Akt	Protein kinase B
AlgC	Alginate C
AlgR	Alginate R
asialoGM1	Asialo ganglioside monosialic acid 1
asialoGM2	Asialo ganglioside monosialic acid 2
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BALF	Bronchoalveolar lavage fluid
cAMP	Cyclic adenosine monophosphate
CD14	Cell differentiation 14
CD91	Cell differentiation 91
CD209	Cell differentiation 209
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming unit

CheY	Chemotaxis Y
ChpA	Chemosensory receptor A
cif	CFTR inhibitory factor
comp	Complement
Crc	Carbon metabolism regulator
CRD	Carbohydrate recognition domain
C-type	Calcium dependent-type
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule nonintegrin
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELF-97	Enzyme labeled fluorescence-97
eNAC	Epithelial sodium channel
FBS	Fetal bovine serum
FimS	Fimbriae U
FimU	Fimbriae U
FleQR	Flagella QR (Flagella gene are nomenclarized as either Fli, Fle or Flg)

FliD	Flagella D (Flagella gene are nomenclarized as either Fli, Fle or Flg)
Flp	Fibrillar
FucNAc	N-acetylfucosamine
Gal	Galactose
GalNAc	N-acetylgalactosamine
GFP	Green fluorescent protein
Glu5	Glutamic acid at amino acid residue 5
HIV	Human immunodeficiency virus
HRP	Horse radish peroxidase
H&E	Hematoxylin and eosin
hSP-A	Human SP-A
IACUC	Institution of animal care and use committee
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
KO	Knockout
LasB	Elastase B
LB	Luria Bertani

LC-MS	Liquid chromatography-mass spectrophotometry
LD_{50}	Lethal dose 50
LPS	Lipopolysaccharide
LBP	LPS binding protein
MAPK	Mitogen-activated protein kinase
MBL	Mannose binding lectin
MD-2	Myeloid differentiation-2
MexXY	MDR (multi-drug resistance) efflux pump XY
MexZ	MDR (multi-drug resistance) efflux pump Z
MIP-2	Macrophage inflammatory protein-2
mRNA	Messenger ribonucleic acid
MucA	Mucoid A
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OD	Optical density
OprM	Outer membrane protein M
OprH	Outer membrane protein H

OprH1	Outer membrane protein H1
PA	*Pseudomonas aeruginosa*
PAPI-1	PA Pathogenecity Island-1
PAPI-2	PA Pathogenecity Island-2
PBS	Phosphate buffered saline
PchA	Pyochelin A (isochorismate synthase gene)
PilA	Pilin A
PilB	Pilin B
PilC	Pilin C
PilD	Pilin D
PilE	Pilin E
PilQ	Pilin Q
PilR	Pilin R
PilRS	Pilin RS
PilS	Pilin S
PilT/U	Pilin T/U
PilV/W/X/Y1/Y2	Pilin V/W/X/Y1/Y2

PI3K	Phosphatidylinositol-3-kinase
PtsP	Phosphotransferase P (Phosphoenolpyruvate protein phosphotransferase gene)
PVDF	Polyvinylidene fluoride
p65	Peptide 65
RFU	Relative fluorescent unit
ROS	Reactive oxygen species
RpoN	RNA (ribonucleic acid) polymerase N
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SE	Standard error
SIRPα	Signal inhibitor regulator protein-α
SP-A	Surfactant Protein-A
SP-A1	Surfactant Protein-A1
SP-A2	Surfactant Protein-A2
SP-B	Surfactant Protein-B
SP-C	Surfactant Protein-C
SP-D	Surfactant Protein-D

TEM	Transmission electron microscope
Tfp	Type IV pilus
Tfpa	Type IVa pilus
Tfpb	Type IVb pilus
TfpO/PilO	Type IV pilus O/Pilin O
TfpW	Type IV pilus W
TfpX	Type IV pilus X
TfpY	Type IV pilus Y
TfpZ	Type IV pilus Z
TLR2	Toll-like receptor-2
TLR4	Toll-like receptor-4
TNF-α	Tumor necrosis factor-α
USP10	Ubiquitin specific peptidase 10
Vfr	Virulence factor regulator
WT	Wild-type
α1	Alpha 1 (at amino acid residue 1)
σ^{22}	Sigma factor 22

5NβOHC$_4$7NFmPse	Pseudaminic acid

CHAPTER 1: GENERAL INTRODUCTION

I. *Pseudomonas aeruginosa* (PA)

A. Cystic Fibrosis (CF)

CF is the most common genetic disease in Caucasians, affecting approximately 30,000 people in the United States or approximately 1 in 2,500 Caucasian newborns [2, 3]. Despite advances in medical therapy and infection prevention, the average life expectancy of CF patients is about 37-40 years of age [2, 4]. CF is caused by an autosomal recessive mutation of the ATP-dependent and cAMP-regulated epithelial chloride channel, CF transmembrane conductance regulator (CFTR) [3, 5, 6]. This mutation causes epithelial dysfunction that leads to mucus obstruction, bronchopneumonia and chronic bacterial lung infection [7]. In addition to bronchopneumonia, CF could also cause pancreatic insufficiency, diabetes mellitus, biliary duct cirrhosis and dilation, and vas deferens and seminal vesicle aplasia or ectasia (Fig. 1a) [5, 8]. However, bronchopneumonia remains the major cause of morbidity and mortality in CF patients [8].

1. CFTR

Over 1800 CFTR mutations have been described [6, 9]. There are six classes of mutation: 1) premature termination signal, 2) defective processing, 3) defective phosphorylation, 4) defective conductance, 5) decrease production, and 6) defective regulation of other channels [3]. The first three types of mutation are associated also with pancreatic insufficiency [5]. Defective processing constitutes the most common class of CFTR mutation; of which, a three base pair deletion, F508del, is the most common [3, 5]. Absent or defective CFTR causes intracellular retention of chloride and hyperabsorption of sodium, as CFTR is an inhibitor of the epithelial

sodium channel eNaC [9]. There are several hypotheses about the mechanism by which the dysfunction in epithelial fluid regulation leads to chronic bacterial bronchopneumonia. Firstly, it is thought that the more acidic nature of trans-Golgi network in CF epithelial cells, caused by altered sodium conductance, leads to increased adherence of PA [10]. Secondly, epithelial cells with mutated CFTR have defective uptake of PA, allowing the bacteria to multiply in the alveolar lumen [11]. Thirdly, due to the defective chloride channel, airway surface fluid sodium and chloride concentrations are thought to increase to twice normal [12].The increase in NaCl concentration is thought to disrupt bactericidal activity of airway fluid antimicrobial factors, including the cationic peptide, β-defensin-1 [13, 14]. This hypothesis of hypertonic airway fluid concentration is disputed by others [15]. Instead, they observed that airway fluid in CF epithelial cells is isotonic; but there is a higher rate of both ions and water absorptions, leading to loss of periciliary fluid and hyperviscosity of the mucus [15]. This sticky mucus leads to impaired mucociliary clearance of microbial pathogen. Finally, studies have shown that CF lungs have impaired ability to regulate immune function. For example, the anti-inflammatory cytokine, IL-10, is depleted in CF airway epithelial fluid, which may lead to enhanced inflammation [16]. CF lung epithelia have been shown to have an exaggerated proinflammatory response [17]. All the mentioned mechanisms increase the retention of bacteria in the airway epithelium, allowing chronic infection. This chronic infection eventually leads to a succession of ineffective chronic inflammation, destruction of pulmonary tissue by inflammatory cells and mediators and finally, loss of pulmonary function (Fig. 1 b & c) [6].

2. Role of *P. aeruginosa* in the pathogenesis of CF

Chronic lung infection is the major cause of morbidity and mortality of CF patients [8, 18]. Common bacterial pathogens associated with chronic pulmonary infection in CF are PA,

Staphylococcus aureus, *Haemophilus influenzae*, *Burkholderia cephacia* complex, *Achromobacter xylosoxidans* and *Stenotrophomonas maltophila* [5, 19]. Among these, PA is the most common pathogen, noted in 52.5% of patients in the United States Cystic Fibrosis Foundation Patient Registry [2].

PA is an opportunistic, Gram-negative bacterial pathogen, commonly associated with acute or chronic infection of mechanically damaged (ventilator associated pneumonia), mechanically obstructed (cystic fibrosis, chronic obstructive pulmonary disease) or immunocompromised (HIV, malignancies, immunosuppressive drugs) lungs [20, 21]. In addition to lung infections, PA is also prevalent in contact lens associated keratitis, and a major cause of burn infections [22, 23]. The ability of PA to cause disease is due to its large genome affording wide range of cell-associated and excreted virulence factors and its genetic flexibility that affords it to adopt to different environmental conditions [20, 24]. Notable among these virulence factors are: lipopolysaccharide (LPS), flagellum, pyocyanin and type IV pilus.

The first step of the chronic lung infection pathogenesis involves the adherence of PA to airway epithelial cells and respiratory mucins by expression of various virulence factors including, type IV pilus (Tfp), non-pilus adhesins and flagella [6, 25-28]. The epithelial receptors of Tfp are the glycosphingolipids, asialoGM1 and asialoGM2, specifically to the disaccharide GalNAcβ1-4Gal [27, 29, 30]. It is noted that the percentage of epithelial cells expressing asialoGM1 is increased in CF patients [27]. Other studies have shown that asialoGM1 also binds to LPS and flagella of PA [31, 32]. On the other hand, flagella cap protein FliD and flagellin are noted to bind with the glycoconjugate bearing Lewis X, present in respiratory mucins [28]. It is also noted that mucins of CF patients have high sialyl Lewis X expression [33, 34].

Because of faulty mucociliary clearance, PA is able to establish long term colonization and infection in the CF airway epithelium [5, 15]. Another mechanism by which PA avoids phagocytosis by recruited neutrophils and resident macrophages is by penetrating deep within thick hypoxic mucus plugs and forming macrocolonies [35]. The combination of increased epithelial oxygen utilization as conferred by increase absorption of ions, very thick mucus, and oxygen consumption by both the bacterial macrocolonies and infiltrating neutrophils produce a hypoxic environment in these mucus plugs [35]. This hypoxic condition stimulates PA to produce the exopolysaccharide alginate, a major component of PA biofilms [35]. Biofilm further helps PA to avoid contact with phagocytes as well as decreases the penetration of antibiotics [35]. In addition to alginate, PA biofilms also contain rhamnolipids that can inactivate epithelial cilia, disrupt ion transport and enhance mucin release [36]. The expression of rhamnolipids, along with other virulence factors of PA, are regulated by quorum sensing molecules called homoserine lactones and quinolones [37]. PA biofilms also contain extracellular bacterial DNA that are important for activing neutrophils, contributing to the chronic inflammation characteristic of CF [38]. After establishing chronic colonization, PA initiates some phenotypic adaptations that prevent detection by the host lung immune system [39]. One such adaptation is the repression of flagellin production, by the presence of neutrophil elastase [40]. This repression is associated with resistance to phagocytosis as well as reduced proinflammatory response because flagellin is recognized by the host Toll like receptor-5 (TLR5) [41, 42]. CF isolates also lack Tfp; and pilus-deficient mutants have been shown to have lower proinflammatory cytokine production [17, 26]. In contrast, other phenotypic modifications increase inflammatory response, which is in line with CF pathogenesis towards lung tissue destruction. The LPS of PA isolates from CF patients contains more acylated lipid A, which has a more robust inflammatory

response than non-CF strains [43]. In addition, lipid A acylation is associated with increased resistance to host antimicrobial peptides [44, 45]. PA in CF lung also undergoes genetic mutations that facilitate selection of strains having traits beneficial for chronic infection. One such example is MexZ, the negative regulator of the multidrug efflux pump, MexXY-OprM, and the negative regulator of alginate biosynthetic genes, MucA [24, 46]. MucA also regulates the RNA polymerase sigma factor σ^{22}, which negatively regulates Tfp and flagella [4]. PA also secretes some elastases that degrade antibodies, complement proteins, inflammatory cytokines and chemokines and surfactant protein A [47]. Another class of secreted virulence factors are the redox-active phenazines. Among the phenazines, pyocyanin increases the proinflammatory chemokine, IL-8 [48]. Finally, the PA toxin, CFTR inhibitory factor (cif), decreases the number of CFTR by inhibiting the deubiquitinating enzyme, USP10 [49].

B. Type IV pilus (Tfp)

Tfp are bacterial unipolar appendages, composed of homopolymer of thousands of pilin (PilA) subunits, found in many Gram-negative and one Gram-positive bacteria (*Clostridium spp.*) (Fig. 2) [50-53]. They are approximately 6-8 nm in diameter, and up to 4 μm in length [54]. In addition to PilA, there also a number of less abundant minor pilins: FimU, PilV/W/X/Y1/Y2 and PilE) [53, 54]. Each pilin subunit is composed of a hydrophobic, N-terminal α-helix, β-sheets, an αβ loop and a D-region marked by two cysteine residues that form disulfide bond [52]. The α-helix, which forms a hydrophobic core, contains an *N*-methylated terminal phenylalanine (α1) and a conserved glutamic acid (Glu5) [52]. The D-region of the final pilin subunit at the tip of a Tfp serves as ligand for receptor binding [52]. Unlike other pili, assembly of Tfp requires more than a dozen components, notable of which are: the subunit pilin (PilA), the outer membrane secretin (PilQ), the inner membrane platform protein that recruits ATPases (PilC), the

assembly polymerase (PilB), the disassembly polymerase (PilT/U) and the prepilin peptidase (PilD) (Fig. 3) [50, 53]. The expression of PilA is under the control of the alternative sigma factor, RpoN [55]. In addition, PilA expression is under the control of the two-component systems, PilR-PilS and AlgR-FimS, the complex chemosensory system ChpA, the global carbon metabolism regulator Crc, and the virulence factor regulator Vfr [56-61].

There are two major subfamilies of Tfp: Tfpa found in PA and *Neisseria gonorrhoeae*, and Tfpb found in enteric pathogens such as *Vibrio cholerae* and enteropathogenic *Escherichia coli* [53, 62, 63]. A subclass of Tfpb, Flp pili, is also encoded in PA [62]. These pili are not involved in motility, but are important for bacterial aggregation and biofilm formation [62]. Also, a PA strain, PA14, contains the pathogenicity island PAPI-1, which contains genes that encode for a Tfpb [63]. This Tfpb is involved in the conjugative transfer of PAPI-1 [63].

PA can be grouped into 5 alleles based on the accessory gene flanking pilA; two of which are glycosyltransferases that decorate pilin with sugar (Group I and Group IV) [64]. Group I alleles (i.e. 1244 strain), which contain a glycosyltransferase that attaches one O-antigen repeating subunit per pilin subunit, are highly associated with CF and environmental isolates [64]. The O-antigen repeating subunit was noted to be the trisaccharide, pseudaminic acid (5NβOHC$_4$7NFmPse)-(2→4)-Xylose-(1→3)-N-acetylfucosamine (FucNAc), bound to the serine residue 148 at the carboxyl terminal of the pilin subunit (Fig. 4) [65, 66]. The O-antigen glycan decorated in the Tfp is the product of the same O-antigen biosynthetic pathway for the LPS O-antigen of the same strain [67].

Among the many functions associated with Tfp are adhesion, twitching and swarming motility, DNA uptake, microcolony formation in biofilm, secretion of protease and colonization factors and eliciting immune response [50, 53]. Tfp is important for the initial attachment of PA

to airway epithelium. The residues 128-144 of the D-region of the tip pilin serves as the ligand for binding to airway epithelial receptor [52]. Several studies have indicated that the airway epithelial receptors of Tfp are the glycosphingolipids, asialoGM1 and asialoGM2 [27, 29, 30]. However, these remain controversial, as other studies show that clinical strains and some laboratory strains fail to bind to such receptors [68, 69]. Recent studies have shown that Tfp binds to the N-glycans in the apical surface of airway epithelial cells, facilitating internalization and activation of phosphatidylinositol-3-kinase (PI3K)/Akt activation [51, 70]. Adhesion to epithelial cells allows for injection of type III secretion system effector proteins [71]. Alternatively, internalization into airway epithelial cells can either be a pathway for PA to bypass the epithelial barrier and proceed to cause systemic infection, or be a defense mechanism of the host by shedding infected epithelia [51, 72]. Internalization of PA also has been shown to cause cytotoxicity to airway epithelium [70].

Another important function of Tfp is twitching motility [53]. Unlike swimming motility which occurs in fluid medium, twitching motility occurs in moist, smooth, solid or semi-solid surfaces such as the airway mucosal epithelia [73]. PA moves in a highly organized fashion, aggregated in rafts, and aligned along their long axis [73]. This is facilitated by sequences of attachment to receptor or inert surface, retraction, and disengagement [52, 74]. This, in turn, is facilitated by the reversible disassembly (retraction) and assembly (extension) of pilin subunits [52]. Extension is facilitated by the ATPase motor PilB; while retraction is facilitated by the ATPase PilT/PilU [57, 75]. These motor ATPases, in turn, are regulated by the Chp chemosensory system, composed of the histidine kinase ChpA and the CheY-like response regulators, PilG (for extension) and PilH (for retraction) (Fig. 5) [57].

Tfp also serves as the receptor for bacteriophage such as PO4 and F116 [76, 77].

Tfp has been shown to be important in surface attachment, forming microcolonies and remodeling during biofilm formation [75, 78]. More specifically, the twitching motility appears to be important for expansive migration and preventing further expansion of existing microcolonies [79]. PA biofilms are highly structured bacterial communities adhered to a surface, embedded within an alginate polysaccharide matrix as opposed to the free-swimming planktonic bacteria [78]. PA within biofilms have phenotypic and genotypic changes compared to planktonic bacteria due to adaptive mutations. PA within biofilms have been shown to upregulate *algC*, the alginate biosynthetic gene [80]. In addition, PA in biofilms are resistant to antibiotic treatment because of diffusion barrier created by the alginate matrix as well as selection of resistant bacteria [81]. In line with the mechanism of diffusion barrier, one of the genetic determinants that allows for antibiotic resistance is *ndvB*, which is required for production of periplasmic glucans [82]. Another mechanism by which PA become resistant to antibiotics is by lowering the metabolic activity as most antibiotics target metabolically active cells [83]. Another mechanism of resistance is the hyperproduction of beta-lactamase and overproduction of efflux pumps [83].

II. Surfactant protein A (SP-A)

The lung's surfactant contains phospholipids and four major surfactant proteins: SP-A, B, C and D [84, 85]. SP-A and SP-D are collagen-containing C-type (calcium-dependent) lectins (collectins); of which, SP-A is the most abundant [84, 85]. Another member of the collectin family is the Mannose Binding Lectin (MBL) of the alternative complement pathway [86]. SP-A is produced by type II pneumocytes, bronchiolar Clara cells and the epithelium and glands of large airways (trachea, bronchi) [86-88]. Native SP-A is a bouquet-shaped, octadecamer (18 subunits), composed of six trimers [84]. Each monomer contains an N-terminal, triple helical

collagen region that binds to eukaryotic receptor (SP-A receptor 210), and a C-terminal, globular carbohydrate recognition domain (CRD) that binds to microbial carbohydrates [84, 85, 89]. Two functional genes in chromosome 10 encode for human SP-A: *SP-A1* and *SP-A2* [86, 90]. It is thought that a trimer of native SP-A consists of two SP-A1 molecules and one SP-A2 molecule [91]. Of the more than 30 alleles characterized, ten occur at high frequency of more than 1% in the general population: SP-A1 alleles (6A, $6A^2$, $6A^3$, $6A^4$), SP-A2 alleles (1A, $1A^0$, $1A^1$, $1A^2$, $1A^3$, $1A^5$) [90, 91]. There are only 10 amino acid differences between these more than 30 alleles [84]. SP-A1 contains an extra cysteine residue that is thought to allow greater oligomerization than SP-A2 [91]. Although SP-A1 has been shown to produce higher order oligomers, SP-A2 has been shown to induce more phagocytosis, greater aggregation and higher proinflammatory cytokine production [90-99].

Through the CRD, SP-A binds and aggregates microbes, enhancing phagocytosis and facilitating lung clearance [1, 47, 84, 100-104]. In addition to opsonic phagocytosis, SP-A enhances phagocytosis directly [85, 105]. SP-A also increases production of reactive oxygen species (ROS) and nitric oxide by alveolar macrophages [106, 107]. SP-$A^{-/-}$ mice are more susceptible to lung infection, with decreased bacterial clearance and macrophage phagocytosis [85, 108-110]. SP-A exerts antibacterial effects either as an opsonin that enhances phagocytosis by effector cells, or by directly permeabilizing microbial membranes (Fig. 6) [1, 47, 101, 102]. SP-A also stimulates chemotaxis of alveolar macrophages and neutrophils [111, 112].

SP-A has also been shown to have anti-inflammatory/immune modulatory effects. Contrary to previous studies, SP-A has been shown to decrease ROS production by decreasing NADPH oxidase activity and has been shown to decrease nitric oxide production [110, 113, 114]. It was observed that both endogenous and exogenous SP-As inhibit LPS-induced production of TNF-α

[114]. Similarly, TNF-α and MIP-2 levels are higher in SP-A$^{-/-}$ mice [110]. In contrast, SP-A has also been shown to increase TNF-α mRNA and protein levels [115, 116]. It is speculated that SP-A and LPS share TLR4 as a receptor, explaining their similar and competitively inhibitory effects [115]. Inhibition by SP-A of LPS-induced immune response was shown to be via direct interaction of SP-A with LPS, preventing interaction of LPS and LPS binding protein (LBP) with TLR4 [117]. SP-A has also been shown to bind to components of the LPS receptor complex, including TLR4, CD14 and MD-2, regulating inflammatory response [118-120]. However, contrary to this, a recent study showed that SP-A does not co-localize with TLR4 [121]. Another study has shown that SP-A inhibition of cytokine production is independent of the CD14 pathway [122]. In addition to TLR4, SP-A has also been shown to bind with TLR2, also downregulating TNF-α secretion [123]. More recently, SP-A regulation of immune response has been shown to be due to decreased phosphorylation of IκBα, a regulator of NF-κB, decreased nuclear translocation of p65, decreased phosphorylation of MAPK family, and decreased phosphorylation of Akt [124]. A recent article showed that SP-A modulates LPS induced inflammatory effects by promoting trafficking of TLR4 from early endosome to the post-Golgi compartment through β-arrestin 2 [121]. The contradictory roles of SP-A during inflammatory response is best illustrated mechanistically by Gardai et al [125]. These authors show that SP-A has a dual function (both as a pro-inflammatory and anti-inflammatory mediator) depending on its orientation and binding [125]. When there are no foreign organisms or cellular debris, the globular CRD of SP-A binds to the signal inhibitor regulator protein α (SIRPα) of resident cells and blocks inflammatory mediator production [125]. However, when there are foreign organisms or cellular debris, the globular CRD binds to them, while the collagen domain of SP-A binds to calreticulin/CD91 of cells to induce production of pro-inflammatory mediators [125].

Several studies have shown that SP-A levels are decreased in patients with CF [126-128]. One study showed that SP-A levels in the bronchoalveolar lavage of CF patients decreased to 2.65 μg/ml from 12.35 μg/ml of control individuals [127]. It has been shown that one of the causes of this decrease is due to proteolytic degradation of SP-A into smaller molecular weight (< 20 kD) residues [128]. And, this degradation has been shown to be caused by PA elastase LasB and protease IV, providing an escape from SP-A-mediated phagocytosis and aggregation [47, 129-131]. In addition to proteolytic degradation by PA enzymes, SP-A is thought to be decreased in CF due to epithelial cell damage, caused by neutrophilic enzymes [132]. In addition to epithelial cell damage, peroxynitrite from infiltrating neutrophils causes nitration and decrease function of SP-A [133]. However, one study actually showed that SP-A concentration is higher in CF [134]. Thus, the concentrations of SP-A within CF airways may depend on the patient population that was sampled within individual studies.

In addition to elastase and protease IV, PA uses several other virulence factors to resist the antibacterial effects of SP-A. SP-A has been shown to bind and enhance phagocytosis only to rough but not smooth strains of bacteria, indicating that it binds to the lipid A moiety of LPS [118, 135, 136]. It is hypothesized that the steric constraint of the bulky O-antigen and core polysaccharide prevents binding of SP-A to lipid A [118]. By extension, this means that O-antigen and core polysaccharide are important to resistance to SP-A-mediated opsonic phagocytosis. Rough strains of bacteria have also been shown to be more susceptible to SP-A-mediated inhibition of TNF-α production and secretion as well as membrane permeability [1, 118].

More recently, a comparative signature-tagged mutagenesis screen was performed using a library of mutants to identify virulence factors of PA that allows for resistance to SP-A [100].

They found that *ΔpchA* (isochorismate synthase) and *ΔptsP* (phosphoenolpyruvate protein phosphotransferase) to be more suscpetible to SP-A-mediated membrane permeability, but not opsonization [100]. *ΔflgE* (flagella hook protein), *ΔfliC* (flagellin) and mucoid, nonmotile CF PA isolates (lacking flagella) are also more susceptible to SP-A-mediated membrane permeability [101]. This increased susceptibility of flagella mutants is attributed to the inadequate amount of LPS, producing membrane instability [101]. Another study showed that *ΔfliC* is unable to produce adequate amounts of exoprotease needed to degrade SP-A [102].

III. Rationale of the project

Morbidity and mortality of CF patients are most commonly associated with the chronic lung infections caused by PA [8, 18]. During chronic PA infection in CF cases, PA undergoes phenotypic changes that allow for increased survival and decreased detection by the innate and adapative immune systems [39]. More specifically, PA changes into a mucoid, nonmotile phenotype [35, 40]. The nonmotile phenotype is due to the absence of both flagella and Tfp [17, 26, 40, 41, 53]. Previous investigators have shown that the flagellum is important for resistance of PA to SP-A-mediated antibacterial effects [101, 102]. Since SP-A levels are severely depleted in the CF airways, PA can safely turn off the flagella expression [126-128]. Thus, a novel intervention approach in treating CF patients might involve infusion of exogenous SP-A to help clear susceptible PA lacking flagellum. The first aim of this dissertation is to explore whether Tfp is important for resistance of PA to SP-A-mediated antibacterial effects, in the hopes of deciphering whether CF isolates that lack Tfp would be ideal candidate for treatment with exogenous recombinant SP-A. As discussed above, because the steric constraint of the bulky O-antigen and core polysaccharide prevents binding of SP-A to lipid A [118], I hypothesized that O-antigen glycosylation of Tfp is a mechanism of resistance to SP-A. Thus, the second aim of

my dissertation was to decipher the functional interactions between Tfp glycosylation with O-antigen and resistance to SP-A.

IV. FIGURES

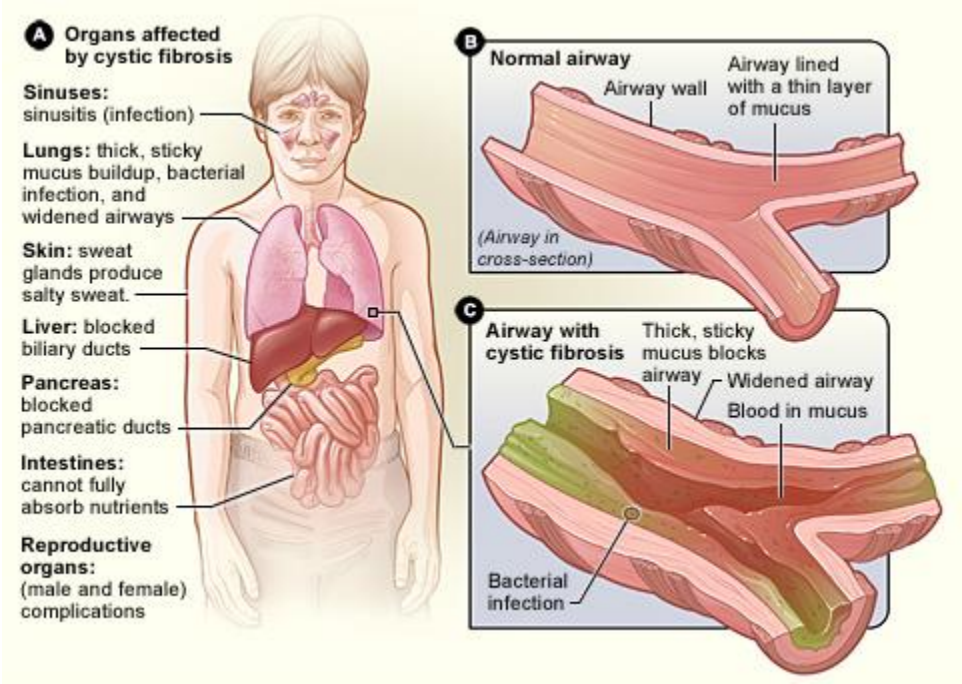

Fig. 1. (a) Organs affected by CF. **(b)** normal airway **(c)** cystic fibrosis airway. This drawing is taken from the NIH NHLBI website.

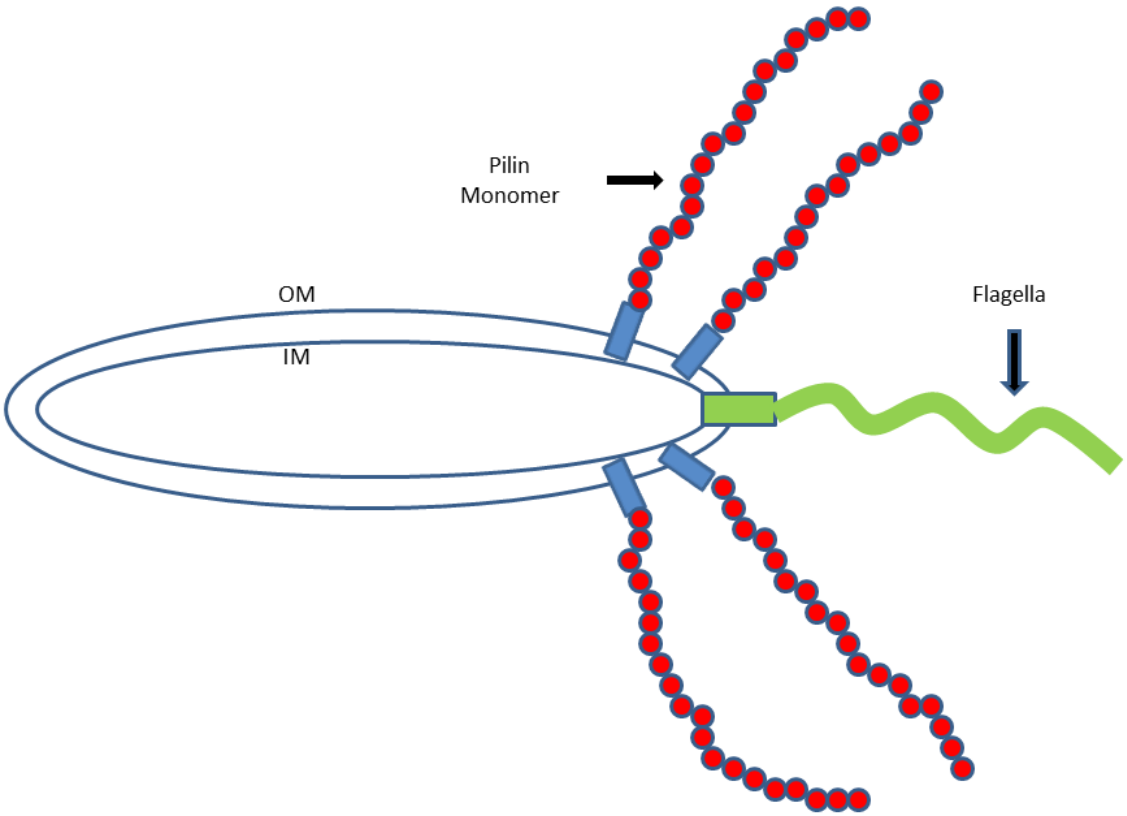

Fig. 2. A schematic drawing of a PA with unipolar appendages, Tfp and flagella. This drawing is adapted from [137]. IM=inner membrane; OM=outer membrane.

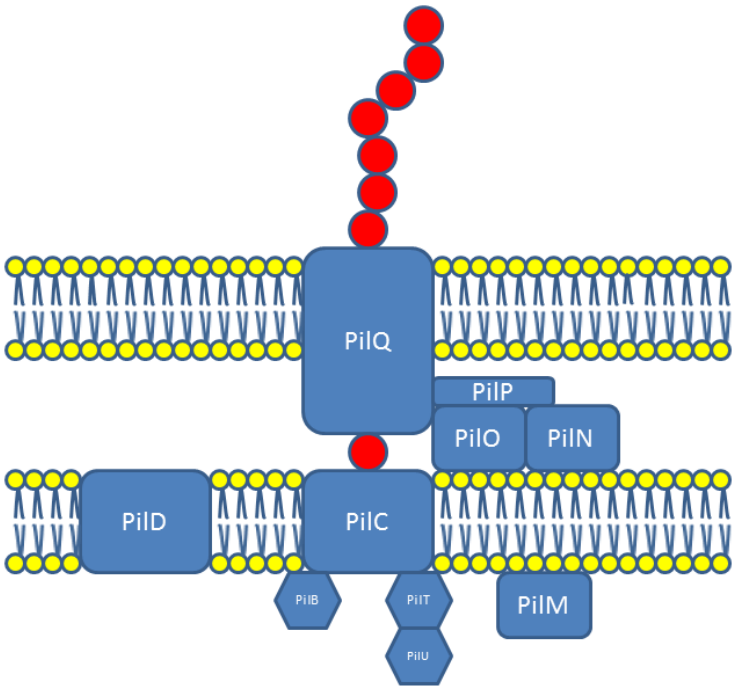

Fig. 3. A schematic drawing of Tfp assembly complex. This drawing is adapted from [53].

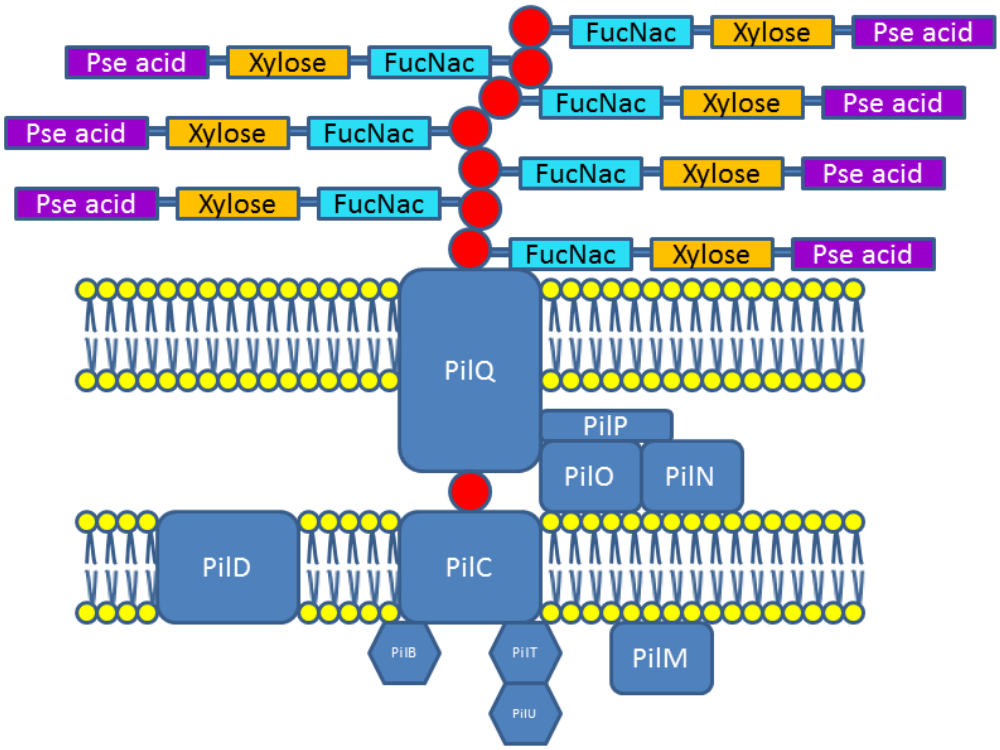

Fig. 4. A schematic drawing of the O-antigen glycosylation of the PA strain 1244 Tfp. FucNac= N-acetylfucosamine; Pse acid=Pseudaminic acid.

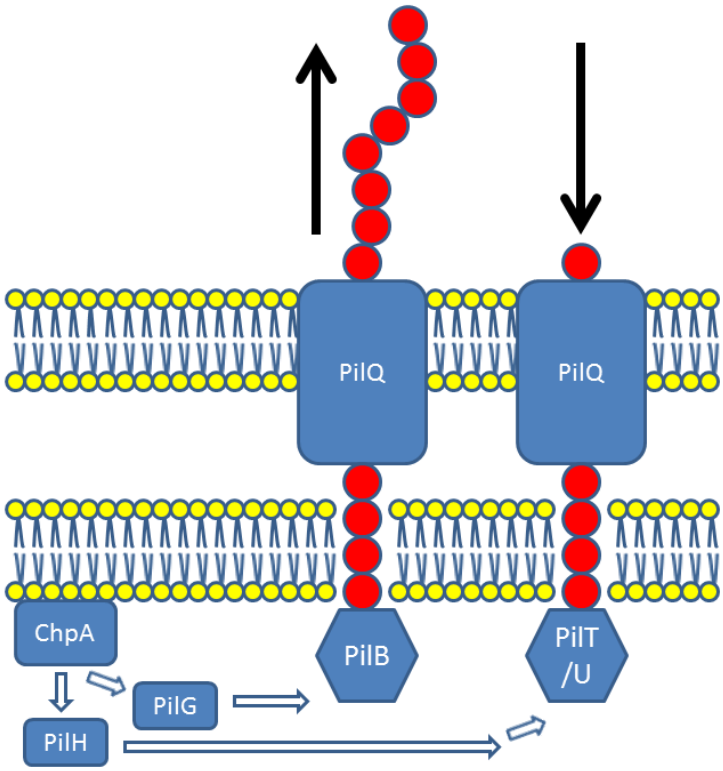

Fig. 5. A schematic drawing of Chp chemosensory regulation system of Tfp. This drawing is adapted from [57]. Up-direction bold black arrow=extension; down-direction bold black arrow=retraction.

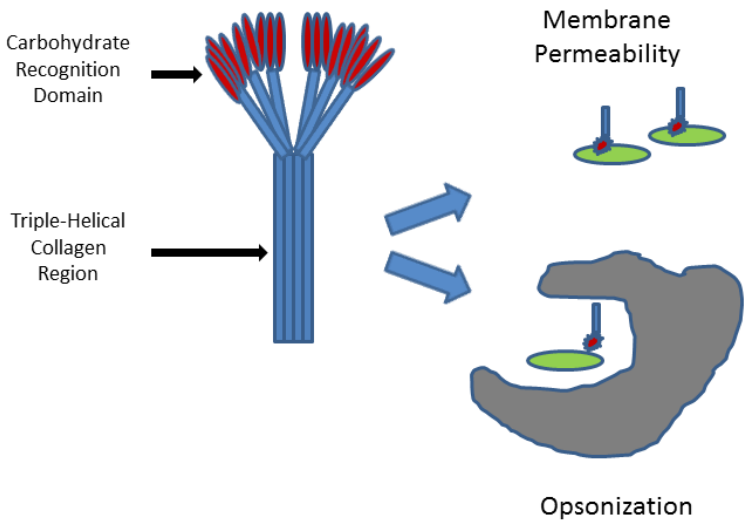

Fig. 6. A schematic drawing of an SP-A and its two main antibacterial functions. This drawing is adapted from [138].

CHAPTER 2: THE IMPORTANCE OF TYPE IV PILUS OF *PSEUDOMONAS AERUGINOSA* FOR RESISTANCE TO SURFACTANT PROTEIN-A-MEDIATED ANTIBACTERIAL EFFECTS[1]

ABSTRACT

Previously, our laboratory has shown that other PA virulence factors, namely LPS, flagella, elastase, PchA and Ptsp, are important for resistance to SP-A-mediated antibacterial effects. In this study, we investigated whether type IV pilus (Tfp) of PA was also important in the resistance of PA to antibacterial effects of SP-A. First, we used C3H wild-type and SP-knockout (SP-KO) mice to investigate the importance of Tfp in resisting SP-A-mediated lung clearance. Then, to further explore the mechanism by which Tfp allows for this resistance, we used gentamicin exclusion assay and two commercially available membrane permeability kits to investigate Tfp's importance for resistance to SP-A-mediated phagocytosis and membrane permeability, respectively. We also determined whether Tfp is important for SP-A-mediated aggregation as well as direct killing.

To explain the mechanism by resistance is afforded, we tested previously known mechanism by other virulence factors. Having discovered that such mechanism does not apply to Tfp, we explored the possibility of increased nonpilus adhesins in Tfp mutants using gentamicin exclusion assay, ligand blot and mass spectrophotometry. Finally, we explored whether the absence of surface Tfp is the cause of increased susceptibility of Tfp mutant to SP-A-mediated phagocytosis.

[1] Reprinted, with permission, from Tan, R. et al. Type IV pilus of *Pseudomonas aeruginosa* confers resistance to antimicrobial activities of the pulmonary surfactant protein-A, Journal of Innate Immunity, Manuscript# JIN354304.indd DOI# 10.1159/000354304. © S. Karger AG, Basel.

INTRODUCTION

PA is an opportunistic Gram-negative bacterial pathogen commonly associated with acute or chronic infection of mechanically damaged (ventilator associated pneumonia), immunocompromised (HIV, malignancies, immunosuppressive drugs) and mechanically obstructed (cystic fibrosis, chronic obstructive pulmonary disease) lungs [20]. In addition to lung infections, PA is also prevalent in contact lens associated keratitis, and a major cause of burn infections [22, 23]. The reason for PA prevalence in many infections lies in its ability to elaborate a wide array of virulence factors, to form biofilms [25], as well as its intrinsic high levels of resistance to many antibiotics [139].

Among the virulence factors expressed by PA is the unipolarly-localized surface appendage, Tfp [25]. Tfp-deficiency attenuates the ability of PA to induce epithelial cytotoxicity, pneumonia, septicemia and mortality in mice [77, 140]. Tfp is important for twitching motility, epithelial adhesion, substratum attachment and biofilm formation, and phage attachment and uptake [25, 52]. Tfp is made up of pilin monomers, encoded by the *pilA* gene [52, 141]. The assembly and disassembly of Tfp are powered by the ATPases PilB and PilT/U, respectively, producing twitching motility [52, 57]. These extension and retraction proteins are regulated by the Chp chemosensory system [57]. The asialoGM1 [25, 52, 141, 142] and N-glycan chains [51, 70] located on the apical surface of the host epithelium are the main receptors for Tfp.

The lung's surfactant contains phospholipids and four major surfactant proteins: SP-A, B, C and D [84, 85]. The naturally occurring octadecameric SP-A is the most abundant [84, 85]. Each monomer contains a 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 [84, 85, 89]. Binding of CRD domain to microbial carbohydrates aggregates microbes, enhancing their phagocytosis [1, 47, 84, 100-102]. In addition to opsonic phagocytosis, SP-A enhances phagocytosis directly [85, 105]. SP-$A^{-/-}$ mice are more susceptible to lung infection, with decreased bacterial clearance and reduced macrophage phagocytosis [85, 108, 109]. Most recently, we and others have shown that SP-A directly permeabilizing microbial membranes [1, 47, 101, 102].

Previously, our laboratory has shown that several virulence factors of PA, including LPS, phosphoenolpyruvate transferase, isochorismate synthase, flagellum, and elastase B, confer resistance to SP-A-mediated antimicrobial effects [1, 47, 100-102]. In this project, we show that Tfp also plays a major role in PA resistance to SP-A-mediated phagocytosis and membrane permeabilization.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The wild-type PA strain PAO1, *ΔpilA* and the genetically-complemented strain *ΔpilA* (comp), *ΔchpA, ΔpilG, ΔpilB, ΔpilH, ΔpilT* and *ΔpilU* were generously provided by Professor Joanne Engel (University of California-San Francisco) [57]. The *ΔrpoN* was purchased from the University of Washington Genome Sciences [143] (Table 1). PA strains were grown in Luria-Bertani (LB) broth (Fisher Scientific, Fair Lawn, NJ) and stored at -80^0C in 30% glycerol (Sigma-Aldrich Co., St. Louis, MO). Before each experiment, bacteria were streaked from frozen stock onto LB agar for 18 hr at 37^0C. One colony from this streak was then cultured in 5 ml LB

broth to stationary phase (OD 600 nm ~ 3.0). OD is measured using a spectrophotometer, Genesys 10 UV (Thermo Scientific, Waltham, MA).

Mouse Clearance Assay

Wild-type C3H/HeN (SP-$A^{+/+}$) mice were purchased from Harlan Laboratory (South Easton, MA). Isogenic SP-$A^{-/-}$ mice were gifts from Dr. Francis McCormack (University of Cincinnati College of Medicine). Mouse experiments complied with the guidelines of the University of Illinois Institution of Animal Care and Use Committee (IACUC). SP-$A^{+/+}$ and SP-$A^{-/-}$ mice (n = 5) were given a single intranasal inoculation of 1×10^7 PAO1 or *ΔpilA* strain ~ 18 hr after infection, mice were euthanized using CO_2; and mouse lungs were harvested for histology or bacterial enumeration as we have previously described [1, 100].

In Vivo Phagocytosis Assay

The phagocytosis rates between different PA strains were compared using the gentamicin exclusion assay as previously described [47]. Briefly, C3H/HeN and C3H SP-$A^{-/-}$ mice (n = 3) were given a single intranasal inoculation of 1×10^7 PAO1 or *ΔpilA* strain. The mouse lungs were lavaged two hr later to collect the alveolar macrophages. The white blood cells were then incubated in PBS with 100 μg/ml gentamicin (Life Technologies, Grand Island, NY) to kill the remaining extracellular bacteria. The macrophages were lysed with 1% Triton X-100 solution (Fisher Scientific, Fair Lawn, NJ) for 5 minutes and serially diluted for PA enumeration (colony counting). The ratio of CFU counts between C3H/HeN and C3H SP-$A^{-/-}$ mice was computed for the fold increase of phagocytosis mediated by SP-A.

Purification of Human hSP-A

Discarded lung washings from anonymous alveolar proteinosis patients were generously provided by Professor Francis McCormack (University of Cincinnati College of Medicine). hSP-A was purified as previously described in [144]. Briefly, raw lung washings, equilibrated with 1 mM $CaCl_2$ (Sigma-Aldrich Co., St. Louis, MO), was passed through Sepharose 6B® (GE Healthcare, Uppsala, Sweden) column laden with mannose (Sigma-Aldrich Co., St. Louis, MO). The captured SP-A is then eluted using 2 mM EDTA 5 mM Tris-HCl (pH 7.4) elution buffer. The eluted fractions were dialyzed using 150 mM NaCl 5 mM Tris-HCl (pH 7.4) dialysis buffer to remove EDTA. The purity of hSP-A preparations was confirmed by Coomasie blue analysis.

Murine Macrophage Cell Line and *In Vitro* Phagocytosis Assay

Murine RAW 264.7 (ATCC# TIB-71) macrophages were maintained in DMEM supplemented with 10% FBS (Phenix Research Products, Candler, NC) at 5% CO2 and 37^0C [47]. The phagocytosis rates between different PA strains were compared using the gentamicin exclusion assay. Briefly, 1×10^6 RAW 264.7 macrophages/ml were plated in 6-well cell culture plates overnight at 37^0C, 5% CO2. PA strains were pre-incubated with 12.5, 25 or 50 µg/ml hSP-A in the presence of 2 mM $CaCl_2$ for 1, 6 or 12 hr in a shaker at 37^0C. The resulting mixture was then incubated with the RAW 264.7 cells at a ratio of 10 bacteria:1 macrophage for 1.5 hr at 37^0C. The macrophages were then washed, and incubated with DMEM (Corning Inc, Manassas, VA) with 100 µg/ml gentamicin (Life Technologies, Grand Island, NY) to kill the remaining extracellular bacteria. The macrophages were lysed with 1% Triton X-100 solution (Fisher Scientific, Fair Lawn, NJ) for 5 minutes, and serially diluted for enumeration (colony counting).

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 GFP gene by electroporation as previously described [47]. Bacterial aggregation were performed using stationary phase PA incubated with 25 µg/ml hSP-A and 2 mM $CaCl_2$ for one hr in a shaker at 37^0C. Afterwards, 6 µl of mixture were placed in a glass slide. A cover slip was added, and sealed. Bacterial clusters were enumerated from 10 independent fields under a fluorescence microscope.

Membrane Permeabilization Assay

Membrane permeability effects of hSP-A were observed using both the thiol-specific fluorophore ThioGlo® (Calbiochem, San Diego, CA) and phosphatase substrate Enzyme Labeled Fluorescence-97 (ELF-97®) (Molecular Probes, Carlsbad, CA), as previously described [1, 47, 102]. ThioGlo® measures thiol containing proteins that leaked out of bacteria; while ELF-97® is a phosphatase substrate that goes inside the leaky bacteria. Stationary phase PA bacteria (n=3) were washed, and incubated with either 50 µg/ml hSP-A for 15 minutes at 37^0C or 50 µg/ml total protein of bronchoalveolar lavage from either C3H WT or SP-A KO mice. For the ThioGlo assay, the bacterial-SP-A mixture was sedimented, and the supernatant was incubated with 10 µM ThioGlo reagent. Fluorescence was measured at excitation wavelength 405 nm and emission wavelength 535 nm. For the ELF-97 assay, the bacterial-SP-A mixture was directly incubated with 100 µM ELF-97 reagent. Fluorescence was measured at excitation 355 nm and emission

535 nm every 3 minutes for the duration of 90 - 120 min. Fluorescence measurement was read using a spectrophotometer SpectraMax Gemini EM® (Molecular Devices, Sunnyvale, CA).

Bacterial Viability Assay

Stationary phase PA bacteria were washed, and incubated with either 50 µg/ml hSP-A for 15 minutes at 37^0C or 50 µg/ml total protein of bronchoalveolar lavage from either C3H WT or SP-A KO mice. The bacterial were then stained with a combination of SYTO 9® and propidium iodide from the commercial bacterial viability kit, LIVE/DEAD BacLight® (Molecular Probes, Eugene, OR). The counts of dead and live bacteria were then counted in ten independent high power fields (400X) using a fluorescent microscope (Q Imaging, Surrey, BC).

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, CA). Stationary phase PA bacteria (n=3) were centrifuge at 8000 rpm for 2 minutes. The supernatant was collected and measured for elastase level. Fluorescence was measured at excitation 546 nm and emission 575 nm. Fluorescence measurement was read using a spectrophotometer SpectraMax Gemini EM® (Molecular Devices, Sunnyvale, CA).

Pyocyanin Assay

Pyocyanin in the bacterial-free culture supernatant of stationary phase PA (n=3) from 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, 30

mM Fe(NO3)3) were measured at OD 690 nm [102]. OD is measured using a spectrophotometer, Genesys 10 UV (Thermo Scientific, Waltham, MA).

SDS Lysis Assay

To assess membrane stability of PA strains, bacterial cell lysis was performed with 0.25% SDS as previously described [101]. Stationary phase PA bacteria (OD 600 nm ~ 3.0) (n=3) were washed, and resuspended in PBS with 0.25% SDS (Sigma-Aldrich Co., St. Louis, MO). The OD 600 nm was measured every 10 min for 1 hr. OD is measured using a spectrophotometer, Genesys 10 UV (Thermo Scientific, Waltham, MA).

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, 1 M Tris-Hcl pH6.8) and boiled for 10 min. 3 µl of 20 µg/µl Proteinase K (Qiagen Sciences, Germantown, MD) was added, and mixture was incubated for 1 hr at 60^0C. The resulting mixture was separated in a SDS-PAGE gel. The resolved gel was incubated with a fixing solution (25% v/v isopropyl alcohol, 7% v/v acetic acid) overnight at 4^0C, oxidized (0.7% periodic acid, 2.7% ethanol, 0.3% v/v acetic acid) for 5 min with gentle agitation, and washed with water for 30 minutes 3 times. The gel was then placed in staining solution (4% v/v 1 M NaOH, 5% v/v NH4OH, 2% AgNO3) and shaken for 10 minutes, followed by four 10-minute washes in water. Finally, the gel was developed (0.02% citric acid, 0.05% v/v formaldehyde) for 20 minutes. The development was then stopped using a stop solution (0.8% v/v acetic acid). Photograph of the SDS-PAGE gel is taken.

Transmission Electron Microscopy (TEM)

PA strains were grown to OD 600 nm ~3.0 in LB broth at 37^0C. Bacteria were washed with PBS, and subsequently fixed using Karnovsky fixative in phosphate buffered 2% Glutaraldeyde and 2.5 % Paraformaldehyde. TEM was performed at the University of Illinois Material Research Laboratory. Briefly, microwave fixation was used with this fixative and the tissue is then washed in cacodylate buffer with no further additives. The tissue was dehydrated in a series of increasing concentrations of ethanol. Acetonitrile was used as the transition fluid between Ethanol and the Epoxy. Infiltration series was done with an epoxy mixture using the epon substitute Lx112. The resulting blocks were polymerizred at 9^0C overnight, trimmed and ultrathin sectioned with diamond knives. Sections were stained with Uranyl Acetate and Lead Citrate, and examined or photographed at 25,000X with an H600 Transmission Electron Microscope (Hitachi High Technologies America Inc, Schaumburg, IL).

Ligand Blot

Biotinylated SP-A was used to identify potential receptors in *ΔpilA* as previously described [89]. Briefly, SP-A was biotinylated using Biotin-N-hydroxysuccinimide ester (Pierce Chemical Co, Rockford, IL). Stationary phase bacteria were ruptured under 14,000 psi in a French Press (Spectronic Instruments, Rochester, NY). Membranes were isolated using 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, and boiled for 5 minutes, and loaded onto 15% SDS-PAGE gels. Gels were either stained using Coomassie blue or transferred to PVDF membranes (Biorad, Hercules, CA). Membranes were blocked for 2 hr, and subsequently

incubated with 3 µg/ml biotinylated SP-A. After three washings, streptavidin-conjugated HRP (EMD Millipore, Darmstadt, Germany) was added. Signal was detected using commercially available Western blot stain and substrate (Pierce Chemical Co, Rockford, IL). Ligand blot signal were analyzed with Image J software (rsbweb.nih.gov). Corresponding band in gels stained with Coomassie to the signal detected in PVDF membrane, were sent to the Chemistry Department for detection of suspected ligand receptor using LC-MS/MS.

Statistical Analysis

All comparisons of means are done using one-way student t-test. Significant difference was considered at $p<0.05$.

RESULTS

The role of Type IV Pilus in 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 Δ*pilA* 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 Δ*pilA* bacteria in SP-$A^{-/-}$ were 2.74 log ($p=0.0001$) and 1.97 log ($p=0.0007$) higher than in SP-$A^{+/+}$ mice, respectively (Fig. 7a). The number of Δ*pilA* bacteria in SP-$A^{-/-}$ mice was statistically indistinguishable ($p=0.15$) when compared to the number of PAO1 the SP-$A^{+/+}$mice, suggesting that Δ*pilA* was more virulence during lung infection in the absence of SP-

A. However, the viable counts of Δ*pilA* were 1.4 log lower (p=0.002) than PAO1 in SP-$A^{+/+}$ mice, and was also 2.16 log lower (p=0.001) 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 is only 0.77 log, this suggests that Δ*pilA* is in general less virulent. In addition, other lung innate immune factors within alveolar space may compensate for the absence of SP-A. Thus, a straightforward link between Tfp and SP-A cannot be easily established (Fig. 7a). 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. 7b).

The aforementioned quantitative observations were supported by histopathology (Fig. 8). 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. 8a) whereas the Δ*pilA* mutant only caused mild bronchopneumonia in the lungs of SP-$A^{+/+}$ mice (Fig. 8b). In contrast, PAO1 caused severe bronchopneumonia in SP-$A^{-/-}$ mice, with large amounts of pulmonary infiltrates and consolidation (Fig. 8c). Importantly, Δ*pilA* caused more severe pulmonary infiltrate in SP-$A^{-/-}$ mice (Fig. 8d) than the SP-$A^{+/+}$ mice (Fig. 8b). The severity of Δ*pilA*-mediated bronchopneumonia in SP-$A^{-/-}$ lungs (Fig. 8d) were similar to that caused by PAO1 in SP-$A^{+/+}$ lungs (Fig. 8a), suggesting that the virulence levels of ΔpilA 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 an important protective role against anti-PA activity mediated by SP-A.

Type IV Pilus 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 *ΔpilA* bacteria were more susceptible to SP-A-mediated opsonization. In the presence of 25 μg/ml SP-A, *ΔpilA* bacteria were phagocytized 5 times more efficiently (p=0.0001) than the wild-type PAO1 (Fig. 9a). The genetically-complemented *ΔpilA*(comp) bacteria were as resistant (p=0.04) to SP-A-mediated opsonization as PAO1 (Fig. 9a). These results were confirmed when using different concentrations of SP-A, which consistently showed that, in the presence of SP-A, *ΔpilA* was 2-3 times (p=0.0003 for 12.5 μg/ml; p=0.004 for 25 μg/ml; p=0.02 for 50 μg/ml) more susceptible to phagocytosis by macrophages than PAO1 (Fig. 9b).

We also examined the phagocytosis of *ΔpilA* in a time-dependent manner. Again, *ΔpilA* was more susceptible to SP-A-mediated opsonization at 1 (p=0.004) and 6 (p=0.002) hr after exposure to RAW 264.7 macrophages (Fig. 9c). However, prolonged exposure (12 hr) abolished phagocytosis, most probably because both PAO1 and *ΔpilA* bacteria degraded SP-A by secreting exoproteases, as we have previously described [47, 102]. These observations were confirmed by an *in vivo* phagocytosis assay, which showed that *ΔpilA* was 4.5 times (p=0.02) more susceptible to SP-A-mediated phagocytosis than PAO1 (Fig. 9d). The *in vivo* phagocytosis assay measures the total phagocytosis activity involving both alveolar macrophages and neutrophils (Fig 9d). Collectively, these results indicate that Tfp is important for resistance of SP-A-mediated opsonization.

Type IV Pilus is Important for Resistance of SP-A-Mediated Aggregation

One of the mechanisms by which SP-A enhances microbial phagocytosis is by increasing their aggregation, allowing more efficient phagocytosis [105]. We compared the aggregation of

wild-type PAO1 versus *ΔpilA* by SP-A *in vitro*. The *ΔpilA* mutant bacteria were aggregated by SP-A 4-fold (p=0.00002) higher than PAO1 per high power field (Fig. 10a-e). Overall, these results show that Tfp is important for resistance of SP-A-mediated aggregation.

Type IV Pilus is Important for Resistance of SP-A-Mediated Membrane Permeabilization

Apart from its ability to opsonize and facilitate the phagocytosis of microbes by macrophages, SP-A is also capable of directly killing bacteria by membrane permeabilization [1, 100, 101]. We examined the susceptibility of *ΔpilA* to SP-A-mediated membrane permeabilization, by measuring the diffusion of an impermeable phosphatase substrate ELF97 into PA cells, and by measuring the leakage of thiol-containing proteins. After 120 min of exposure, the ELF97 assay indicates that *ΔpilA* bacteria were permeabilized 2.1 fold higher than the PAO1 (Fig. 11a). In addition, the leakage of thiol-containing proteins increased by 1.81 fold (p=0.002) relative to PAO1 (Fig. 11b).

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 *ΔpilA* (Fig 11c). The *ΔpilA* bacteria were more susceptible (p=0.00001) to BALF from SP-$A^{+/+}$ mice than PAO1. In contrast, BALF from SP-$A^{-/-}$ only possessed low levels (~ 900-1000 RFU) 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 cause low levels of *PA* killing, the *ΔpilA* was consistently shown to be significantly more susceptible to direct killing by hSP-A (p=0.0002 for 1 hr; p=0.003 for 6 hr) (Fig 11d) and BALF of SP-$A^{+/+}$ mice (p=0.002) (Fig 11e).

These results suggest that SP-A-mediated membrane permeabilization contribute minimally to *PA* clearance.

Type IV Pilus-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 studied previously [47, 100-102], Δ*pilA* is uniquely more susceptible to both SP-A-mediated opsonization and membrane permeabilization. We examined both the pilin extension (*ΔchpA, ΔpilG* and *ΔpilB*) and the retraction (*ΔpilH, ΔpilT* and *ΔpilU*) mutants to determine if the presence of Tfp on the cell surface of PA is required for resistance to SP-A-mediated opsonization. As shown in Fig. 12a, mere absence of Tfp on the bacterial surface in the extension mutants (*ΔchpA, ΔpilG, ΔpilB*) does not increase (p=0.4 for *ΔchpA*; p=0.06 for *ΔpilT*; p=0.4 for *ΔpilU*) susceptibility to SP-A-mediated phagocytosis. Interestingly, the retraction mutants, *ΔpilT* and *ΔpilU*, which are hyperpiliated, are more resistant by 2.5 fold (p=0.0007 for *ΔpilT*; p=0.002 for *ΔpilU*) to SP-A-mediated opsonization (Fig. 12b). Both extension and retraction mutants were as resistant to SP-A-mediated membrane permeabilization as PAO1 (Fig. 12c-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, expression of additional Tfp (e.g., *ΔpilT* and *ΔpilU* mutants) offers in increased resistance to SP-A-mediated phagocytosis.

Increased Susceptibility of *ΔpilA* to SP-A-Mediated Phagocytosis is Probably Due to a Compensatory Increase in Nonpilus Adhesins

Previous work in our laboratory has shown PA confers resistance to SP-A-mediated phagocytosis by increasing the degradation of SP-A through elastase B [47, 102], through mechanisms regulated by both flagellum and quorum-sensing. In addition, this process is regulated through 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, *ΔpilA* produced wild-type levels of both quorum sensing regulated exoprotease activities (Fig. 13a) and the redox-active secondary metabolite pyocyanin (Fig. 13b). Furthermore, the *ΔpilA* lost its susceptibility to SP-A-mediated phagocytosis after 12 hr (Fig. 13c), suggesting that SP-A was degraded. Thus, the lack of SP-A degradation is not the cause of enhanced opsonization of *ΔpilA* by SP-A.

Previous studies have shown that *ΔpilA* has higher binding affinity to host epithelial cells than the hyperpiliated *ΔpilT* and *ΔpilU* mutants [26, 55, 145, 146]. This is speculated to be caused by over-expression of alternative "nonpilus adhesins" in *ΔpilA*. The alternative sigma factor RpoN has been shown to regulate the expression of these adhesins [26, 55, 145, 146]. We hypothesized that one or more nonpilus adhesins may serve as ligands for binding to SP-A. We compared the phagocytosis of *ΔpilA* versus *ΔrpoN::ISphoA/hah* in the presence of SP-A. Fig. 14a shows *ΔrpoN::ISphoA/hah*, which lacks the adhesins, is more resistant by 3-fold (p=0.02) and 8.5-fold (p=0.008) to SP-A-mediated phagocytosis than both with-type PAO1 and *ΔpilA*. Importantly, ligand blot and subsequent Image J analyses shows that the *ΔpilA* overexpresses by 2-fold a protein of ~ 18 kDa compared to both PAO1 and *rpoN::ISphoA/hah* (Fig. 14b, c). LCMS/MS and micro-sequencing analyses indicated that this putative adhesin is the outer membrane protein H1 precursor OprH (PA1178) (Fig. 14d). 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. 14e).

ΔpilA Has Reduced Membrane Stability, Rendering it Susceptible to SP-A-Mediated Membrane Permeabilization

Next, we examined the potential mechanisms of increased susceptibility of *ΔpilA* to SP-A-mediated membrane permeabilization. Previously, we have shown that the loss of LPS [1, 100, 101] and flagellum [47, 101, 102] destabilizes membrane integrity, rendering them more susceptible to SP-A-mediated membrane permeabilization. In addition, Abeyrathne *et al* have shown that the O-antigen ligase mutant, *ΔwaaL*, which is unable to attach O-antigen to the core polysaccharide of LPS, has decreased or absent Tfp and flagella [147]. 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 showed no discernible differences between *ΔpilA* and PAO1 (Fig. 15a-c). However, *ΔpilA* bacteria were more susceptible to lysis by 0.25% SDS, suggesting that the loss of pilin compromises the integrity of the outer membranes in the mutant bacteria (Fig. 15d).

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, phosphoenolpyruvate phosphotransferase that confer resistance to SP-A-mediated membrane permeabilization [1, 47, 100-102]. Flagellum also regulates quorum sensing-mediated expression of elastase B that degrades and disables SP-A-mediated opsonization during phagocytosis [1, 47, 100-102]. 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 conclusion: (i) *ΔpilA* is preferentially cleared in the lungs of SP-$A^{+/+}$ mice compared to SP-$A^{-/-}$ mice; (ii) *ΔpilA* is more susceptible to SP-A-mediated aggregation and opsonization; (iii) *ΔpilA* is more susceptible to SP-A-mediated membrane permeabilization; and (iv) the genetically complemented *ΔpilA* (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 current report seems to contradict previous findings, which show that Tfp-deficient PA strains, including *ΔpilA*, are more resistant to phagocytosis by macrophages [41, 148, 149]. However, this discrepancy can be explained because of different experimental contexts. 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 [41, 148, 149], rather than the quantitative method we used with the gentamicin exclusion assay.

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 (*ΔchpA, ΔpilG* and *ΔpilH*) 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 interacts with SP-A. This is supported by previous studies showing that PA mutants with no Tfp (*ΔpilA*) have higher adhesion to epithelial cells than hyperpiliated Tfp extension mutants strains *ΔpilT* and *ΔpilU*, suggesting that Tfp-independent adhesins are responsible for increased binding to epithelial cells [26, 140]. Importantly, both hyperpiliated Tfp mutants strains *ΔpilT* and *ΔpilU* are significantly more resistant than the wild-type PAO1 to SP-A-mediated phagocytosis. These observations suggest that additional Tfp expressed by both *ΔpilT* and *ΔpilU* mutants may have masked the putative ligands, decreasing SP-A-mediated binding, aggregation and subsequent opsonization and enhanced phagocytosis by macrophages.

With ligand blot, we have identified the putative nonpilus adhesion which is overexpressed in *ΔpilA* that may serve as ligands for SP-A binding. This nonpilus adhesion is probably regulated by the alternative sigma factor RpoN. Previous work has shown that RpoN positively regulates both pilin and flagellin production through their respective two-component systems PilRS and FleQR [146, 150]. Thus, *ΔrpoN* is deficient in the expression of Tfp and flagellum in PA. Interestingly, *ΔpilA* has been used as a model to study the expression of alternative nonpilus adhesins. In contrast, *ΔrpoN* has been used as a model for the lack of nonpilus adhesins [55, 145, 146]. 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 *ΔrpoN* is even more resistant to SP-A-mediated phagocytosis than PAO1. Several studies have tried to identify of these nonpilus adhesins, with most of them focusing on those that bind to mucins [151-154]. Other studies focus on the role of nonpilus adhesins in nonopsonic phagocytosis [55, 145, 154]. 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-sequenicng 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 [155, 156]. Interestingly, OprH has direct interactions with lipopolysaccharide [155]. Another outer membrane protein, P2 of *Hemophilus influenzae*, has been noted to be a receptor of SP-A [157]. We are currently constructing nonpolar deletion mutant and study the complex interaction of OprH with Tfp, RpoN, and SP-A. Furthermore, we are examining the sequence and functional homology between OprH and P2.

Tfp interacts with glycoconjugates (e.g., asialoGM1) on the host epithelium [25]. Similarly, SP-A also interacts with glycoconjugates through its CRD domain. Thus, another possible mechanism of preferential clearance of ΔpilA 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 SP-A, the integrity of the outer membranes is compromised, rendering the *ΔpilA* bacteria more permeable to the phosphotase substrate ELF97 into the cells and increased leakage of thiol containing intracellular proteins out of the cells. Previously, we have shown the the wild-type strain PAO1 is highly resistant to direct

killing by SP-A [102]. Although *ΔpilA* is more susceptible to SP-A-mediated membrane permeabilization, this only results in modest 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 *ΔpilA*. However, *ΔpilA* 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 of PA is important for resistance to SP-A-mediated opsonization and membrane permeabilization. We provide evidence that Tfp functions to camouflage nonpilus adhesins, and prevents the binding of SP-A to these ligands and reduces 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 improve the clearance of PA by augmenting the efficiency of SP-A in killing this important respiratory pathogen.

FIGURES AND TABLES

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
P. aeruginosa strains		
PAO1	Wild-type	[57]
PAO1 *ΔpilA*	In frame deletion of the *pilA* gene	[57]
ΔpilA (comp)	Genetically-complemented *ΔpilA* mutant	[57]
PAO1 – GFP	PAO1 harboring pUCP19-*gfp*	This study
ΔpilA – GFP	*ΔpilA* harboring pUCP19-*gfp*	This study
ΔchpA	In frame deletion of the *chpA* gene	[57]
ΔpilG	In frame deletion of the *pilG* gene	[57]
ΔpilB	In frame deletion of the *pilB* gene	[57]
ΔpilH	In frame deletion of the *pilH* gene	[57]
$\Delta pilT^{\text{CTX-}pilU}$	In frame deletion of the *pilT*; *pilU* and 1 kb upstream sequence at *attB* site	[57]
ΔpilU	In frame deletion of the *pilU* gene	[57]

rpoN::ISphoA/hah	Mutant harboring a *ISphoA/hah* transposon insertion into the *rpoN* gene	University of Washington Genome Sciences
Plasmids		
pUCP19-*gfp*	pUCP19 plasmic expressing a green fluorescence protein	[47]

This table is adapted from Tan et al. Type IV pilus of *Pseudomonas aeruginosa* confers resistance to antimicrobial activities of the pulmonary surfactant protein-A. Journal of Innate Immunity. Manuscript# JIN354304.indd DOI# 10.1159/000354304. © S. Karger AG, Basel.

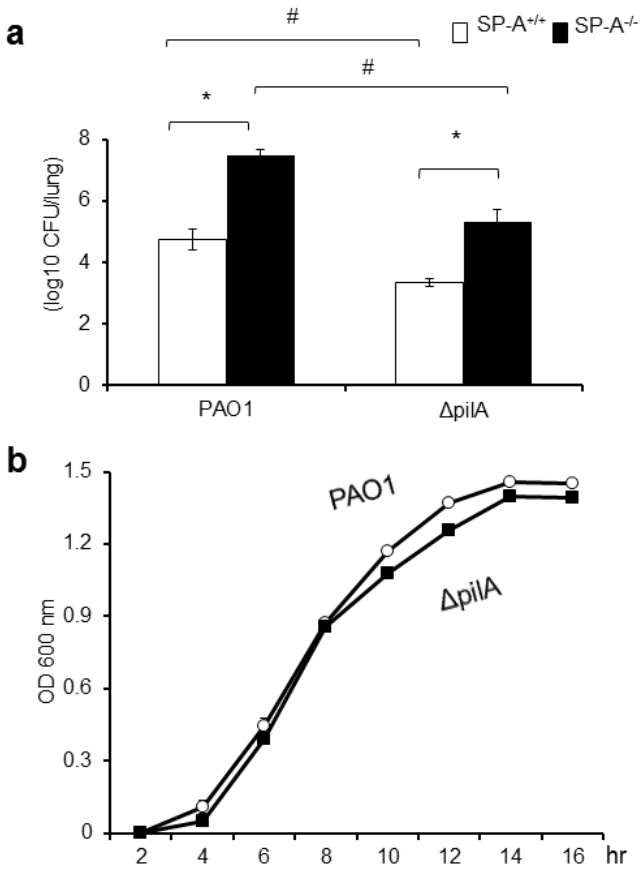

Fig. 7. *ΔpilA* bacteria are more susceptible to clearance by SP-A. **(a)** Respiratory tract infections with wild-type PAO1 versus Δ*pilA* were performed by intranasal inoculation of anesthetized SP-$A^{+/+}$ or SP-$A^{-/-}$ mice. Mouse lungs were harvested 18 hours post infection for bacterial enumeration. Data are the mean CFU ± SE (n = 5 per group). $^{*}p < 0.05$ when comparing the bacterial loads between SP-$A^{+/+}$ versus SP-$A^{-/-}$ infected by PAO1 or Δ*pilA*. $^{\#}p < 0.05$ when comparing the bacterial loads between PAO1 versus Δ*pilA* infecting the SP-$A^{+/+}$ or SP-$A^{-/-}$ mice. **(b)** The growth kinetics of PAO1 and *ΔpilA* bacteria were determined by measuring OD 600nm. The experiments were performed three times independently in triplicates. The representative growth curve from one out of three independent experiments is shown. This figure is adapted from Tan et al. Type IV pilus of *Pseudomonas aeruginosa* confers resistance to antimicrobial

activities of the pulmonary surfactant protein-A. Journal of Innate Immunity. Manuscript# JIN354304.indd DOI# 10.1159/000354304.

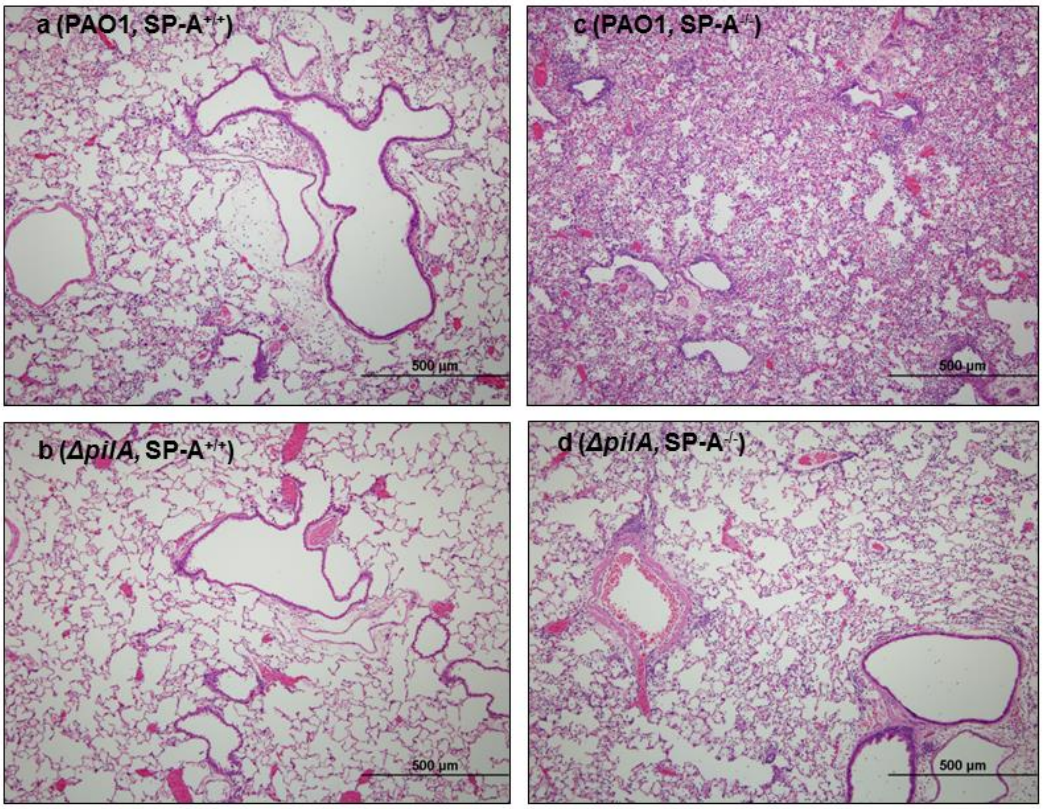

Fig. 8. Histopathology of *PA*-infected lungs. SP-$A^{+/+}$ and SP-$A^{-/-}$ mice were infected with PAO1 or *ΔpilA* as described in Fig. 1. Representative H&E-stained lung sections from SP-$A^{+/+}$ and SP-$A^{-/-}$ mice (n = 5) 18-hour post intranasal instillation of PAO1 **(a, c)** and Δ*pilA* **(b, d)** bacteria. This figure is adapted from Tan et al. Type IV pilus of *Pseudomonas aeruginosa* confers resistance to antimicrobial activities of the pulmonary surfactant protein-A. Journal of Innate Immunity. Manuscript# JIN354304.indd DOI# 10.1159/000354304. © S. Karger AG, Basel.

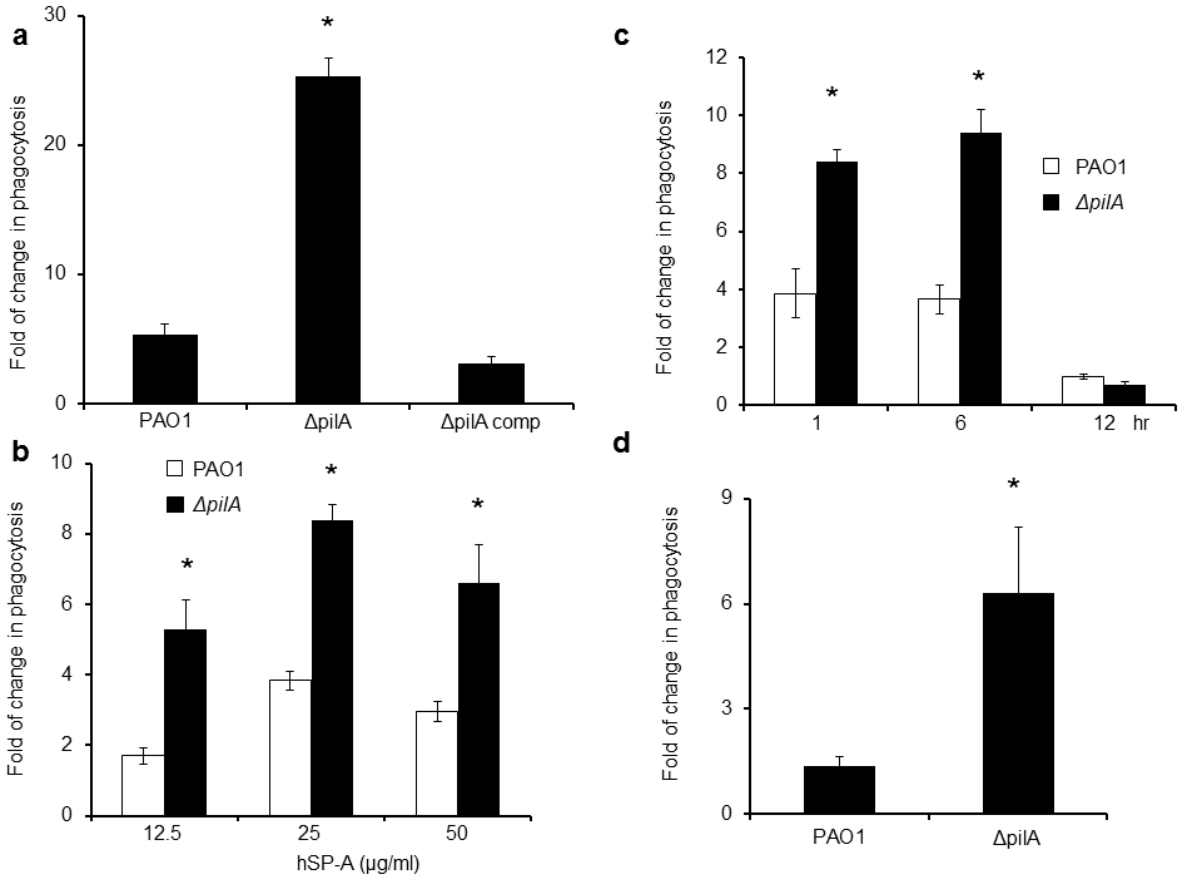

Fig. 9. The *ΔpilA* mutant is more susceptible to SP-A-mediated opsonization. **(a-c)** RAW 264.7 macrophages were infected with either PAO1 or *ΔpilA* in the presence or absence of hSP-A. The ratio of ingested bacteria was expressed as fold increase in phagocytosed bacteria due to the effect of hSP-A. **(a)** Phagocytosis of PAO1, *ΔpilA* and genetically complemented ΔpilA(comp) in the presence of 25 μg/ml hSP-A. **(b)** Phagocytosis of PAO1 and *ΔpilA* in the presence of different concentrations of hSP-A. **(c)** Time dependent phagocytosis of PAO1 versus *ΔpilA* in the presence of 25 μg/ml hSP-A. **(d)** *In vivo* phagocytosis of PAO1 versus *ΔpilA* in SP-$A^{+/+}$ and SP-$A^{-/-}$ mice (n = 3). All phagocytosis experiments were independently performed three times in triplicates. The mean ± standard deviation from one representative experiment is shown. *p < 0.05 when comparing the number of phagocytosed Δ*pilA* against PAO1. This figure is adapted from Tan et al. Type IV pilus of *Pseudomonas aeruginosa* confers resistance to antimicrobial

activities of the pulmonary surfactant protein-A. Journal of Innate Immunity. Manuscript# JIN354304.indd DOI# 10.1159/000354304.

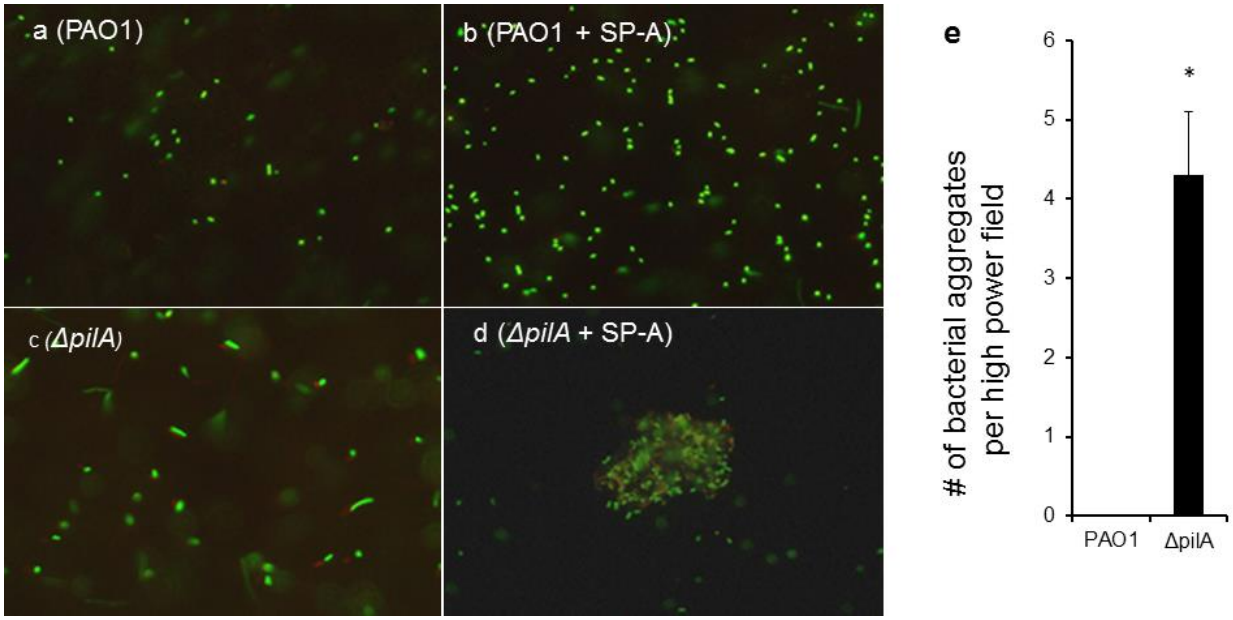

Fig. 10. *ΔpilA* bacteria are more susceptible to SP-A-mediated aggregation. GFP-expressing PAO1 and *ΔpilA* bacteria were incubated with 25 µg/ml hSP-A for 1 hour, and examined under a confocal fluorescence microscope. **(a)** PAO1 without hSP-A. **(b)** PAO1 with hSP-A. **(c)** *ΔpilA* without hSP-A. **(d)** *ΔpilA* with hSP-A. **(e)** The number of aggregates was averaged from 10 independent high power field. *$p < 0.05$ when comparing the number aggregates in Δ*pilA* against PAO1. This figure is adapted from Tan et al. Type IV pilus of *Pseudomonas aeruginosa* confers resistance to antimicrobial activities of the pulmonary surfactant protein-A. Journal of Innate Immunity. Manuscript# JIN354304.indd DOI# 10.1159/000354304. © S. Karger AG, Basel.

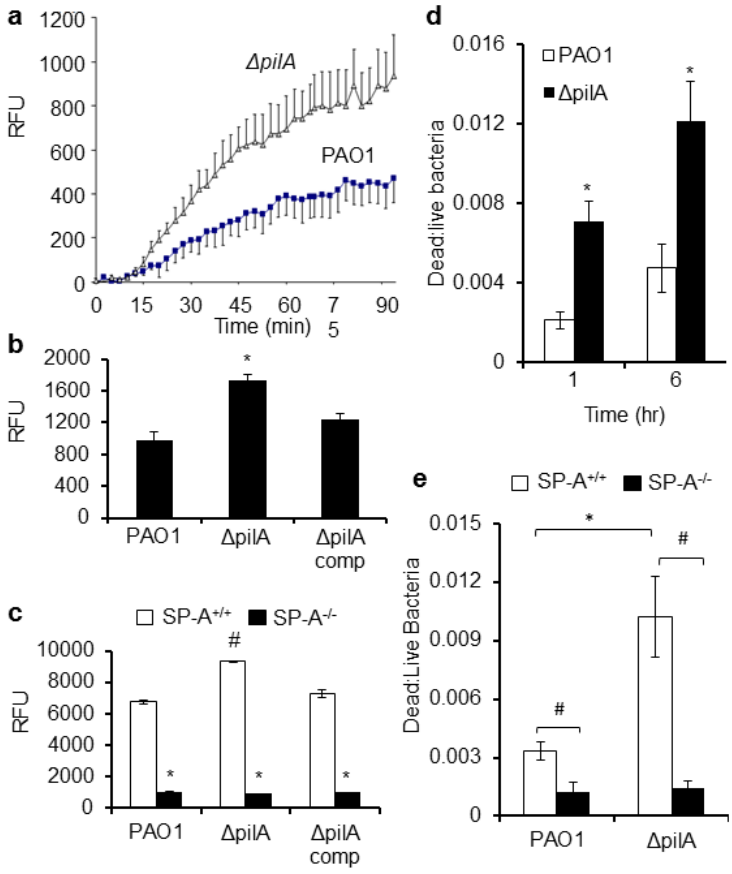

Fig. 11. *ΔpilA* are more susceptible to SP-A-mediated membrane permeabilization. **(a)** ELF-97® assay. PAO1 and *ΔpilA* were preincubated with 50 µg/ml hSP-A for 15 minutes before the addition of ELF-97®. Absorbance was measured every 3 minutes at excitation wavelength 355 nm and emission wavelength 535 nm, for a total of 90 minutes. **(b)** *In vitro* ThioGlo® assay. PAO1, *ΔpilA* and *ΔpilA* comp were preincubated with 50 µg/ml hSP-A for 15 minutes. The bacterial-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 *ΔpilA* against PAO1 and ΔpilA comp. **(c)** *Ex vivo* ThioGlo® assay. PAO1, *ΔpilA* and *ΔpilA* comp were preincubated with 50 µg/ml total protein of BALF from either SP-$A^{+/+}$ mice or SP-$A^{-/-}$ mice for one hour. The bacterial 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 *ΔpilA* against PAO1 and *ΔpilA* in BALF from SP-$A^{+/+}$ mice. *$p < 0.05$ when comparing the RFU of each *PA* strain in BALF from SP-$A^{+/+}$ versus SP-$A^{-/-}$ mice. **(d)** *In vitro PA* killing by hSP-A. PAO1 and *ΔpilA* were preincubated with 50 μg/ml hSP-A for one or six hour(s). The bacteria were then stained with a mixture of SYTO 9® and propidium iodide for 15 minutes. 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 *ΔpilA* versus PAO1 bacteria in the presence of hSP-A. **(e)** *Ex vivo PA* killing by mouse SP-A within BALF. PAO1 and *ΔpilA* were preincubated with 50 μg/ml total protein of BALF from either SP-$A^{+/+}$ mice or SP-$A^{-/-}$ mice for one hour. 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-$A^{+/+}$ versus SP-$A^{-/-}$ mice. *$p < 0.05$ when comparing the ratio of dead to live PAO1 versus *ΔpilA* bacteria treated with BALF from SP-$A^{+/+}$ mice. Experiments were performed independently three times in triplicates (for ELF® and ThioGlo® assays) or decuplicate (for Live Dead® assay). Data from one typical experiment are shown. This figure is adapted from Tan et al. Type IV pilus of *Pseudomonas aeruginosa* confers resistance to antimicrobial activities of the pulmonary surfactant protein-A. Journal of Innate Immunity. Manuscript# JIN354304.indd DOI# 10.1159/000354304. © S. Karger AG, Basel.

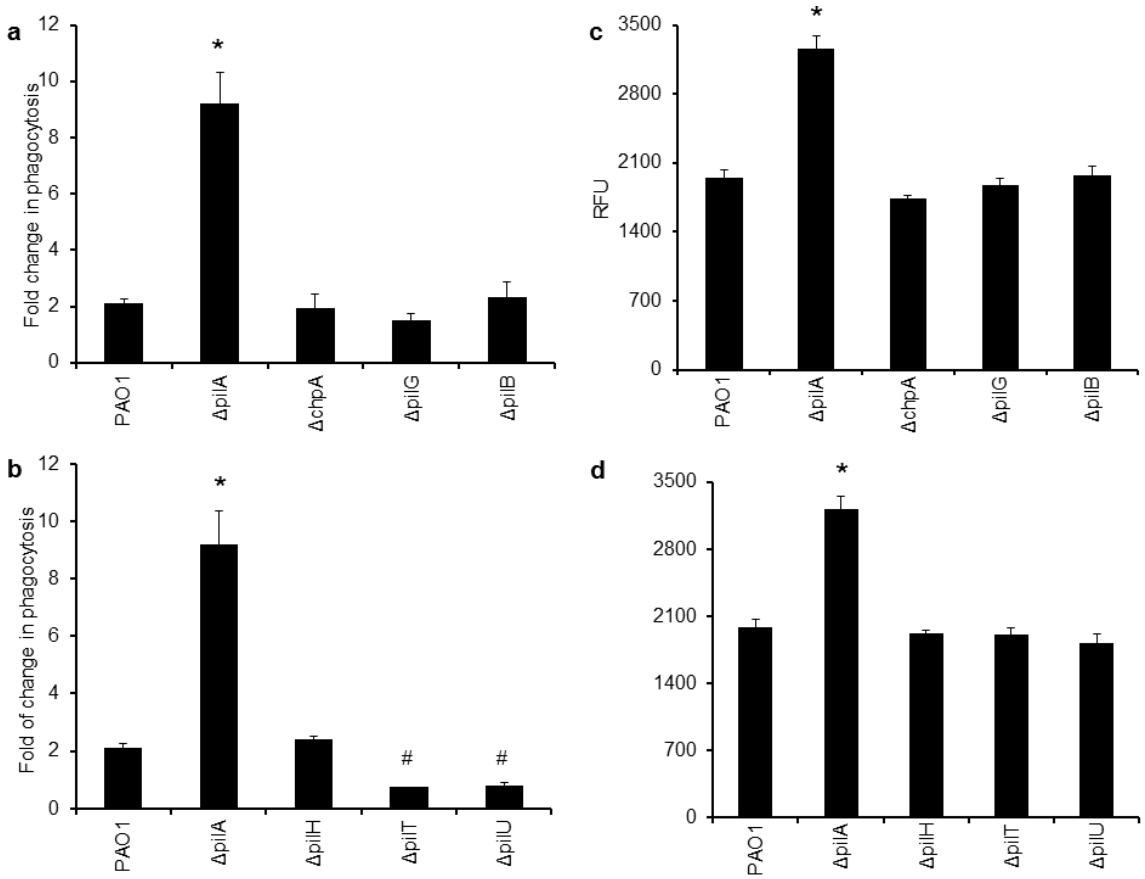

Fig. 12. The susceptibility of Tfp extension and retraction mutants to SP-A-mediated opsonization. **(a-b)** RAW 264.7 macrophages were infected with PAO1, *ΔpilA*, the extension or retraction mutants in the presence or absence of hSP-A. The ratio of ingested bacteria between those exposed to hSP-A versus those unexposed was expressed as fold increase in phagocytosis. **(a)** Phagocytosis of PAO1, *ΔpilA* and extension mutants *ΔchpA*, *ΔpilG*, *ΔpilB*. **(b)** Phagocytosis of PAO1, *ΔpilA* and retraction mutants *ΔpilH*, *ΔpilT*, *ΔpilU*. $^{*}p < 0.05$ when comparing phagocytosis of PAO1 versus *ΔpilA* mutant. $^{\#}p < 0.05$ when comparing phagocytosis of PAO1 versus *ΔpilT* and *ΔpilU* mutants. All experiments were independently performed three times in triplicates. The mean ± standard deviation from one representative experiment is shown. **(c-d)** Membrane permeabilization of *PA* strains by hSP-A. **(c)** Membrane permeabilization of PAO1, *ΔpilA* and extension mutants *ΔchpA*, *ΔpilG*, *ΔpilB*. **(d)** Membrane permeability of PAO1, *ΔpilA*

and retraction mutants *ΔpilH*, *ΔpilT*, *ΔpilU*. All experiments were independently performed three times in triplicates. The mean ± standard deviation from one representative experiment is shown. This figure is adapted from Tan et al. Type IV pilus of *Pseudomonas aeruginosa* confers resistance to antimicrobial activities of the pulmonary surfactant protein-A. Journal of Innate Immunity. Manuscript# JIN354304.indd DOI# 10.1159/000354304.

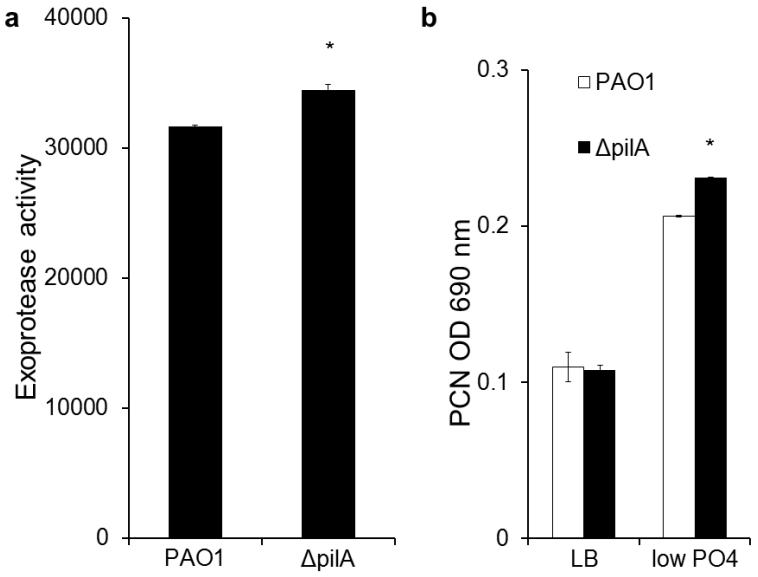

Fig. 13. Resistance to SP-A-mediated phagocytosis is independent of quorum sensing. **(a)** Exoprotease activity of PAO1 versus *ΔpilA*. **(b)** Pyocyanin production of PAO1 versus *ΔpilA*. *$p < 0.05$ when comparing the exoprotease activity and pyocyanin OD 690nm in Δ*pilA* against PAO1. PO4=phosphate. This figure is adapted from Tan et al. Type IV pilus of *Pseudomonas aeruginosa* confers resistance to antimicrobial activities of the pulmonary surfactant protein-A. Journal of Innate Immunity. Manuscript# JIN354304.indd DOI# 10.1159/000354304. © S. Karger AG, Basel.

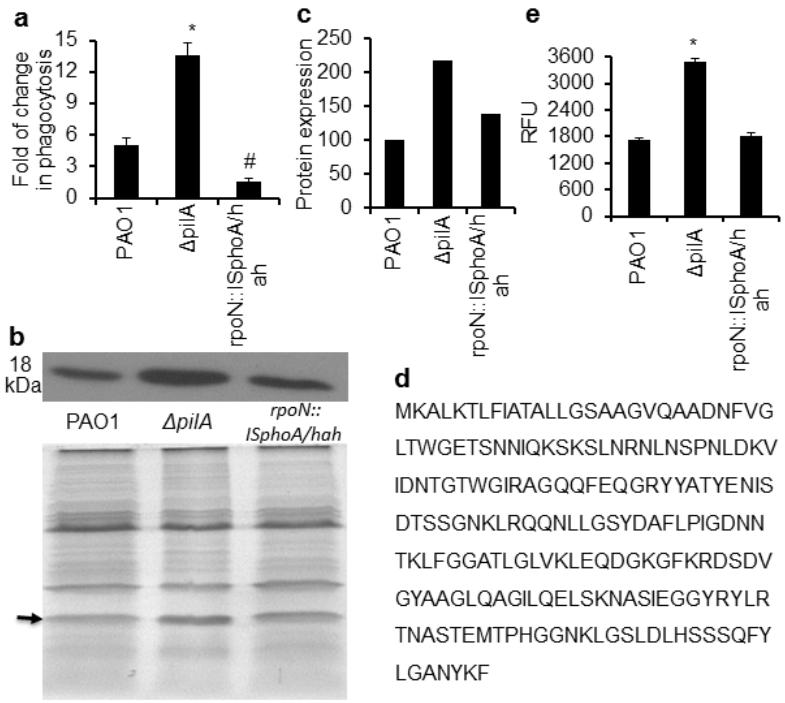

Fig. 14. Resistance to SP-A-mediated phagocytosis is regulated by RpoN. **(a)** Comparison of bacterial phagocytosis by RAW 264.7 macrophages between PAO1, *ΔpilA* and *rpoN::ISphoA/hah* in the presence or absence of 25 μg/ml hSP-A. All experiments were independently performed three times in triplicates. The mean ± standard deviation from one representative experiment is shown. $^{*}p < 0.05$ when comparing PAO1 versus *ΔpilA*. $^{\#}p < 0.05$ when comparing PAO1 versus *rpoN::ISphoA/hah*. **(b)** Ligand blot analysis of nonpilus adhesins in PAO1, *ΔpilA* 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, *ΔpilA* and *rpoN::ISphoA/hah*. The experiments were independently performed three times in triplicates. The mean ± standard deviation from one representative experiment is shown. $^{*}p < 0.05$ when comparing PAO1 versus *ΔpilA* and *rpoN::ISphoA/hah*. This figure is adapted from Tan et al. Type IV pilus of *Pseudomonas aeruginosa* confers resistance to antimicrobial activities of the pulmonary surfactant protein-A.

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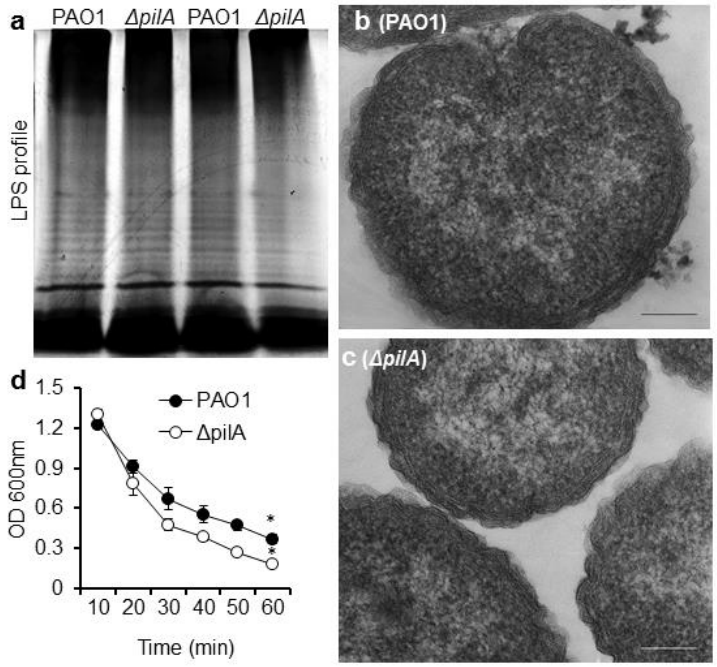


Fig. 15. *ΔpilA* is more susceptible to SDS-mediated cell lysis. **(a)** LPS analysis of PAO1 and *ΔpilA* as visualized using silver stain. **(b & c)** TEM of PAO1 and *ΔpilA*, respectively. Bar = 100 nm. **(d)** SDS lysis assay. PAO1 and *ΔpilA* bacteria were incubated in 0.25% SDS solution. OD 600nm was measured every 10 minutes. Bacterial lysis experiments were independently performed three times in triplicates. The mean from one representative experiment is shown. **p* < 0.05 when comparing the OD 600 nm in Δ*pilA* against PAO1.This figure is adapted from Tan et al. Type IV pilus of *Pseudomonas aeruginosa* confers resistance to antimicrobial activities of the pulmonary surfactant protein-A. Journal of Innate Immunity. Manuscript# JIN354304.indd DOI# 10.1159/000354304. © S. Karger AG, Basel.

CHAPTER 3: O-ANTIGEN GLYCOSYLATION OF TYPE IV PILIN CONFERS RESISTANCE OF PSEUDOMONAS AERUGINOSA STRAIN 1244 TO SURFACTANT PROTEIN-A-MEDIATED OPSONIZATION

ABSTRACT

Previously, another investigator has shown that the addition of O-antigen subunit to Tfp pilin monomer allows for increased fitness to the strain 1244 over its cognate glycosyltransferase mutant. Here, we show that one of the possible mechanism of this increase fitness is the increased resistance of strain 1244 to SP-A-mediated phagocytosis. We have shown that while 12447 does not cause mortality, 1244 causes mortality in the absence of SP-A. Using C3H WT and SP-A KO mice, we are able to show that while 1244G7 is statistically susceptible to SP-A-mediated lung clearance; while 1244 is not. Using piperacillin exclusion assay, we have shown that 1244G7 is more susceptible to SP-A-mediated phagocytosis. Both 1244 and 1244G7 are equally susceptible to SP-A-mediated membrane permeability. Finally, we have shown that the increased susceptibility of 1244G7 to SP-A-mediated phagocytosis is associated with increased exposure of putative mannose receptors.

INTRODUCTION

Pseudomonas aeruginosa (PA) can be separated into five groups based on the presence or absence of varying downstream accessory gene flanking the Type IV pilin gene *pilA* [64]. The PilA of these five PA groups have varying amino acid sequence, length and presence of post-

translational modification [64, 158]. The downstream accessory genes function either for pilin post-translational glycosylation/modification (Group I and Group IV) or in modulation of pilus assembly (Group III and Group V) [54, 64, 159].

Group I alleles (i.e. 1244 strain), which contain a glycosyltransferase (PilO/TfpO) that attaches one O-antigen repeating subunit per pilin subunit, is highly associated with CF and environmental isolates [64]. The O-antigen repeating subunit was noted to be the trisaccharide, pseudaminic acid (5NβOHC$_4$7NFmPse)-(2→4)-Xylose-(1→3)-N-acetylfucosamine (FucNAc), bound to the serine residue 148 at the carboxyl terminal of the pilin subunit [65, 66]. The O-antigen glycan decorating PilA is the product of the same O-antigen biosynthetic pathway for the LPS O-antigen of the same strain [67]. Group II alleles do not contain an accessory flanking gene, and includes common laboratory strains, PAO1 and PAK [64]. Group III alleles contain the accessory gene, TfpY, and includes the human clinical isolate, PA14 [64]. PA14 was shown to produce lower LD_{50} and higher mortality rate than PAO1 [160]. This difference of virulence was partially attributed to *ybtQ*, a gene present in PA14 but not PAO1 [160]. Also, PA14, contains the pathogenicity island PAPI-1 and PAPI-2, which have been shown to contribute to virulence [161]. Group IV alleles contain two accessory genes, TfpW and TfpX [64]. The PilA of Group IV alleles are glycosylated with homo-oligomer of α-1,5-linked $_D$-arabinofuranose, which are similar to the lipoarabinomannan polymer found in the cell wall of *Mycobacteria spp.* [158]. Group V alleles contain the accessory gene, TfpZ [64]. TfpY of Group III alleles and TfpZ of Group V alleles have been shown to be important for the surface expression of PilA [159].

There are few studies exploring the virulence of the clinical strain 1244. It has been shown that strain 1244 is more virulent than its isogenic nonpilated mutants [77]. TfpO/PilO of Group I

alleles have been shown to allow for increased fitness of strain 1244 isolates when competed against its cognate glycosyltransferase mutant, 1244G7 [162].

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The wild-type PA strain 1244, the Δ*tfpO* mutant 1244G7, and the genetically-complemented strain 1244G7 (comp) were generously provided by Professor Peter Castric (Duquesne University) [162]. Strain 1244 were grown in plain Luria-Bertani (LB) broth (Fisher Scientific, Fair Lawn, NJ); 1244G7 strains were grown in LB containing 75 µg/ml gentamicin (Life Technologies, Grand Island), NY; and 1244G7 (comp) strains were grown in LB containing 200 µg/ml carbenicillin (Lab Scientific Inc, Highlands, NJ) at 37^0C overnight. They were then stored at -80^0C in 30% glycerol (Sigma-Aldrich, St Louis, MO). Before each experiment, bacteria were streaked from frozen stock onto LB agar with or without antibiotic for 18 hr at 37^0C. One colony from this streak was then cultured in 5 ml LB broth to stationary phase (OD 600 nm ~ 3.0). OD is measured using a spectrophotometer, Genesys 10 UV (Thermo Scientific, Waltham, MA).

Mouse Clearance Assay

Wild-type C3H/HeN (SP-$A^{+/+}$) mice were purchased from Harlan Laboratory (South Easton, MA). Isogenic SP-$A^{-/-}$ mice were gifts from Dr. Francis McCormack (University of Cincinnati College of Medicine). Mouse experiments complied with the guidelines of the University of Illinois Institution of Animal Care and Use Committee (IACUC). SP-$A^{+/+}$ and SP-

$A^{-/-}$ mice (n = 10) were given a single intranasal inoculation of 1×10^7 PA strains 1244 or 1244G7 strain ~ 18 hr after infection, mouse lungs were harvested for histology or bacterial enumeration as we have previously described [1, 100].

In Vivo Phagocytosis Assay

The phagocytosis rates between different PA strains were compared using a modified gentamicin exclusion assay as previously described. Briefly, C3H/HeN and C3H SP-$A^{-/-}$ mice (n = 3) were given a single intranasal inoculation of 1×10^7 1244 or 1244G7. After two hr, the mouse lungs were lavaged to collect the alveolar macrophages and neutrophils. The white blood cells were then incubated in PBS with 100 µg/ml piperacillin (Sigma-Aldrich, St Louis, MO) to kill the remaining extracellular bacteria [163]. Piperacillin was used instead of gentamicin as 1244G7 contain a gentamicin resistance cassette [162]. The macrophages were lysed with 1% Triton X-100 solution (Fisher Scientific, Fair Lawn, NJ) and serially diluted for PA enumeration. The ratio of CFU counts between C3H/HeN and C3H SP-$A^{-/-}$ mice was computed for the fold increase of phagocytosis mediated by SP-A.

Purification of Human hSP-A

Discarded lung washings from anonymous alveolar proteinosis patients were generously provided by Professor Francis McCormack (University of Cincinnati College of Medicine). hSP-A was purified as previously described in [144]. Briefly, raw lung washings, equilibrated with 1 mM $CaCl_2$ (Sigma-Aldrich, St Louis, MO), was passed through Sepharose 6B® column (GE Healthcare, Uppsala, Sweden) ladened with mannose (Sigma-Aldrich, St Louis, MO). The captured SP-A is then eluted using 2 mM EDTA 5 mM Tris-HCl (pH 7.4) elution buffer. The

eluted fractions were dialyzed using the dialysis buffer (150 mM NaCl 5 mM Tris-HCl (pH 7.4)) to remove EDTA (Sigma-Aldrich, St Louis, MO). The purity of hSP-A preparations was confirmed by Coomasie blue analysis.

Murine Macrophage Cell Line and *In Vitro* Phagocytosis Assay

Murine RAW 264.7 (ATCC# TIB-71) macrophages were maintained in DMEM (Corning Inc, Mannassas, VA) supplemented with 10% FBS (Phenix Research Products, Candler, NC) at 5% CO2 and 37^0C [47]. The phagocytosis rates between different PA strains were compared using the modified gentamicin exclusion assay. Briefly, 1×10^6 RAW 264.7 macrophages/ml were plated in 6-well cell culture plates overnight at 37^0C, 5% CO2. RAW 264.7 cells were pre-incubated with or without 1 mM D-mannose (Sigma-Aldrich, St Louis, MO) for 2 hours. PA strains were pre-incubated with 12.5, 25 or 50 µg/ml hSP-A in the presence of 2 mM $CaCl_2$ for 1, 6 or 12 hr in a shaker at 37^0C. The resulting mixture was then incubated with the RAW 264.7 cells at a ratio of 10 bacteria:1 macrophage for 1.5 hr. The macrophages were then washed, and incubated with DMEM with 100 µg/ml piperacillin (Sigma-Aldrich, St Louis, MO) to kill the remaining extracellular bacteria. The macrophages were lysed with 1% Triton X-100 solution (Fisher Scientific, Fair Lawn, NJ), 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.

Membrane Permeabilization Assay

Membrane permeability effects of hSP-A were observed using both the thiol-specific fluorophore ThioGlo® (Calbiochem, San Diego, CA), as previously described [1]. Stationary

phase PA bacteria were washed, and incubated with either 50 µg/ml hSP-A for 15 minutes at 37^0C or 50 µg/ml total protein of bronchoalveolar lavage from either C3H WT or SP-A KO mice. For the ThioGlo assay, the bacterial-SP-A mixture was sedimented, and the supernatant was incubated with 10 µM ThioGlo reagent. Fluorescence was measured at excitation wavelength 405 nm and emission wavelength 535 nm. Fluorescence measurement was read using a spectrophotometer SpectraMax Gemini EM® (Molecular Devices, Sunnyvale, CA).

Statistical Analysis

All comparisons of means are done using one-way student t-test. Significant difference was considered at $p<0.05$.

RESULTS

The O-antigen Subunit on Tfp is Important for Resistance to SP-A-Mediated Lung Clearance

To determine the contribution of O-antigen on Tfp to resistance SP-A-mediated clearance, we compared the lung infection by the wild-type *PA* 1244 and the isogenic glycosyltransferase-deficient Δ*tfpO* mutant 1244G7 in a mouse model of acute pneumonia. Eighteen hr after intranasal inoculation with 1244, SP-$A^{+/+}$ mice showed no mortality, while SP-$A^{-/-}$ showed 3/5 mortality. In contrast, SP-$A^{-/-}$ mice infected with 1244G7 showed no mortality (Fig. 16c). The number of viable 1244G7 bacteria in SP-$A^{-/-}$ mice is significantly higher (2.39 log) (0.0003) than in SP-$A^{+/+}$ mice. In contrast, the burden of 1244 bacteria was only 1 log higher ($p=0.06$) in the SP-$A^{-/-}$ mice than the SP-$A^{+/+}$ mice (Fig. 16a). These data suggest that the

presence of O-antigen on pilins of Tfp allows for increased resistance to SP-A-mediated lung clearance.

The aforementioned virulence observations were further supported by histopathological analysis of infected lungs (Fig. 17). 1244 caused severe bronchopneumonia (Fig. 17c) whereas the Δ*tfpO* mutant 1244G7 only caused mild to moderate bronchopneumonia in the lungs of SP-$A^{-/-}$ mice (Fig. 17d). In contrast, both 1244 and 1244G7 caused only mild to moderate bronchopneumonia in SP-$A^{+/+}$ mice (Fig. 17 a-b).

The Presence of O-antigen Subunit on Pilin of Type IV Pilus Allows for Increased Resistance to SP-A-Mediated Phagocytosis

To decipher the mechanism of increased resistance to SP-A-mediated lung clearance afforded by the presence of O-antigen on pilins, we examined whether 1244G7 bacteria were more susceptible to SP-A-mediated opsonization. In the presence of 12.5, 25 and 50 µg/ml SP-A, 1244G7 bacteria were phagocytized 2-2.5 times more ($p=0.02$ for 12.5 µg/ml; $p=0.0001$ for 25 µg/ml; $p=0.02$ for 50 µg/ml) efficiently than the wild-type 1244 (Fig. 18a). We also examined the phagocytosis of 1244G7 in a time-dependent manner. We found that 1244G7 was more susceptible to SP-A-mediated opsonization than 1244 only at 1 hr ($p=0.0001$) after exposure to RAW 264.7 macrophages (Fig. 18b). Prolonged exposure abolished the difference of phagocytic efficiency between 1244 and 1244G7, most probably due to production of exoproteases that degrade SP-A [47, 102]. These observations were confirmed by an 2 hr *in vivo* phagocytosis assay, which showed that 1244G7 was 4.5 times ($p=0.003$) more susceptible to SP-A-mediated phagocytosis than 1244 (Fig. 18c). The *in vivo* phagocytosis assay measures the total phagocytosis activity involving both alveolar macrophages and neutrophils.

The Increase Susceptibility of 1244G7 is due to Increased Susceptibility to Uptake by Mannose Receptor

Previously, it was determined that SP-A increases the surface expression of mannose receptors in macrophages, resulting in increased phagocytosis [164, 165]. In order to determine whether the increase susceptibility of 1244G7 is due to increase susceptibility to uptake via mannose receptor, we pre-incubated RAW 264.7 cells with 1 mM D-mannose. We have shown that the increase susceptibility of 1244G7 to SP-A-mediated phagocytosis is abrogated ($p=0.04$) by the addition of mannose; while the addition of mannose did not affect the susceptibility of 1244 (Fig. 19).

The O-antigen on the Pilin of Type IV Pilus Does Not Confer Increased Resistance to SP-A-Mediated Membrane Permeabilization

As mentioned previously, SP-A enhances microbial clearance by opsonization and membrane permeabilization. To further examine whether the presence of O-antigen on the pilin of Tfp allows also for increased resistance to SP-A-mediated membrane permeability, we used a commercially available membrane permeability kit measuring leaked thiol containing proteins. All three strains 1244, 1244G7 and 1244G7 (comp) were equally susceptible to SP-A-mediated permeability (Fig. 20), suggesting that susceptibility to membrane permeabilization is independent of pilin modification with the O- antigen.

DISCUSSION

The pilin subunits of the Group 1 PA strains, including 1244, are glycosylated with O-antigen containing pseudaminic acid by the TfpO glycosyltransferase. TfpO of Group I PA has been shown to be important for the overall fitness of 1244 in the host. In a mixed infection within mouse lungs, the Δ*tfpO* mutant 1244G7 is less competitive against the wild-type strain 1244, [162]. However, these authors did not decipher the possible mechanism associated to this. One of their suggested mechanisms is that the glycosylated 1244 has enhanced resistance towards innate host defenses [162]. We propose that one of the mechanisms of higher fitness in the wild-type 1244 is due to the increase resistance to SP-A-mediated effects, afforded by the addition of O-antigen subunits to each pilin monomer on Tfp. Using mouse model of acute pneumonia infection, we have shown that 1244 caused mortality (3/5) when inoculated into SP-$A^{-/-}$ mice, but no mortality when inoculated in SP-$A^{+/+}$ mice. This shows the importance of SP-A in innate immune resistance to the clinical strain 1244. Importantly, while 1244 caused mortality in the SP-$A^{-/-}$ mice, the cognate glycosyltransferase Δ*tfpO* strain, 1244G7, did not. This implicates the O-antigen subunit as the cause of higher virulence in 1244 than 1244G7 in SP-$A^{-/-}$ mice. Using the bacterial burden within infected lungs as an indicator of lung clearance, we have shown that the log CFU is not statistically significantly different ($p=0.06$) between SP-$A^{+/+}$ and SP-$A^{-/-}$ mice after infection by 1244 infection. In contrast, the log CFU of 1244G7 strain is significantly higher in ths SP-$A^{-/-}$ mice than in the SP-$A^{+/+}$ mice ($p=0.0003$). These results suggest that the presence of O-antigen on the pilin subunits of Tfp confer resistance to antimicrobial properties of SP-A. This observations was reflected in the histopathology as 1244

in SP-A$^{-/-}$ mice showed severe bronchopneumonia; while 1244 in SP-A$^{+/+}$ and 1244G7 in both SP-A$^{+/+}$ and SP-A$^{-/-}$ only showed mild to moderate bronchopneumonia.

To further decipher the mechanism of SP-A resistance by 1244, we explored whether the O-antigen on the pilin subunits of Tfp confer resistance to SP-A-mediated opsonization or membrane permeability. The presence of O-antigen on pilin subunits of Tfp does not confer increased resistance to SP-A-mediated membrane permeability.

We have shown that the wild-type clinical strain 1244 is consistently more resistant to SP-A-mediated phagocytosis than its O-antigen-deficient mutant 1244G7 during *in vitro* phagocytosis assays. These results were confirmed by the *in vivo* phagocytosis assay, using bronchoalveolar lavage macrophage and neutrophils from SP-A$^{+/+}$ and SP-A$^{-/-}$ mice, showing similar results.

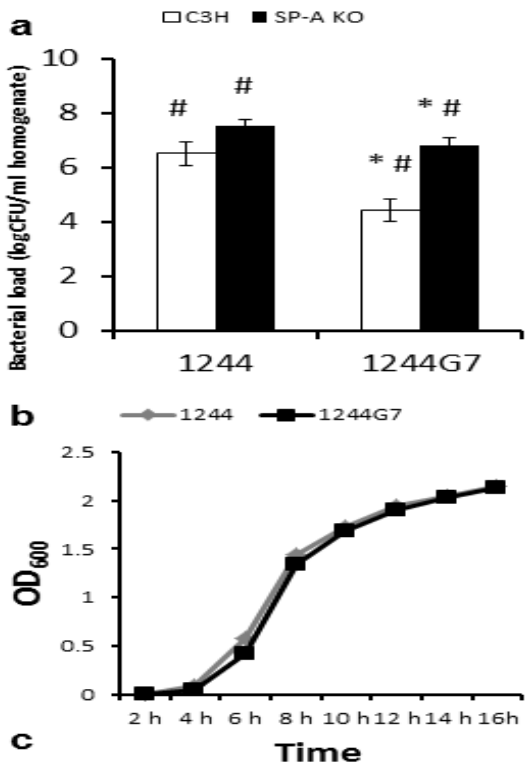
Previous studies in other microbes have implicate the importance of O-antigen for resistance to phagocytosis, including *Vibrio anguillarum, Burkholderia cenocepacia Salmonella enterica, Escherichia coli, Haemophilus ducreyi,* and *Neisseria gonorrhoeae* [166-171]. In *E. coli*, *S. enterica, H. ducreyi* and *N. gonorrhoeae,* it was noted that O-antigen allows for resistance to phagocytosis by masking the N-acetylglucosamine residues of LPS core polysaccharide, which is the ligand for the dendritic cell-specific intercellular adhesion molecule nonintegrin (DC-SIGN)/CD209 [169, 171]. In *V. anguillarum,* it is speculated that O-antigen mask putative mannose residues that interact with the mannose receptors of skin epithelial cells [166]. We show that provision of mannose as a competitive inhibitor for mannose receptor, abrogated the increase susceptibility of 1244G7 to SP-A. These results suggest that the additional O-antigen mask putative mannose residues, preventing its interaction with mannose receptors that have been upregulated in the presence of SP-A.

In conclusion, we have shown that the addition of O-antigen subunit to Tfp confers increased virulence; and resistance to this virulence is associated with the presence of SP-A. We have also shown the O-antigen subunit in Tfp allows for resistance to SP-A-mediated lung clearance and phagocytosis, but not membrane permeability. Finally, we have shown that the increased susceptibility of the non-glycosylated mutant to SP-A-mediated phagocytosis is associated with the exposure of mannose residues.

FIGURES AND TABLES

TABLE 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference
P. aeruginosa strains		
1244	Wild-type	[162]
1244G7	In frame deletion of the *tfpO* gene	[162]
1244G7 (comp)	*ΔtfpO* mutant complemented with a copy of wild-type gene *in trans*	[162]



	SP-A$^{+/+}$	SP-A$^{-/-}$
1244	0/5	3/5
1244G7	0/5	0/6

Fig. 16. The addition of O-antigen subunit to Tfp allows for increased virulence and resistance to SP-A-mediated phagocytosis. **(a)** Respiratory tract infections with wild-type 1244 versus 1244G7 were performed by intranasal inoculation of anesthetized SP-A$^{+/+}$ or SP-A$^{-/-}$ mice. Mouse lungs were harvested 18 hours post infection for bacterial enumeration. Data are the mean CFU ± SE (n = 5-6 per group). $^{*}p < 0.05$ when comparing the bacterial loads between SP-A$^{+/+}$ versus SP-A$^{-/-}$ infected by 1244 or 1244G7. $^{\#}p < 0.05$ when comparing the bacterial loads between 1244 versus 1244G7 infecting the SP-A$^{+/+}$ or SP-A$^{-/-}$ mice. **(b)** The growth kinetics of 1244 and 1244G7 bacteria were determined by measuring OD 600nm. The experiments were performed three times independently in triplicates. The representative growth curve from one out

of three independent experiments is shown. **(c)** The mortality rate of SP-A$^{+/+}$ and SP-A$^{-/-}$ mice infected with either 1244 or 1244G7.

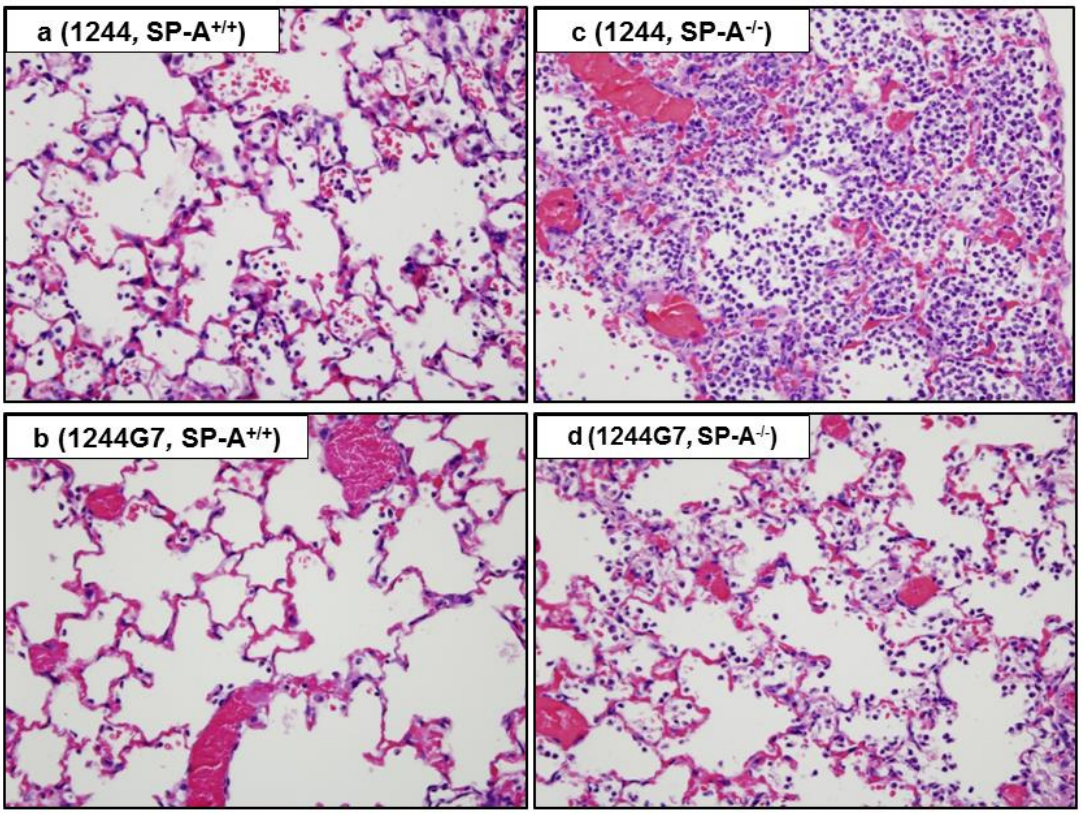


Fig. 17. Histopathology of *PA*-infected lungs. SP-$A^{+/+}$ and SP-$A^{-/-}$ mice were infected with 1244 or 1244G7 as described in Fig. 10. Representative H&E-stained lung sections from SP-$A^{+/+}$ and SP-$A^{-/-}$ mice (n = 5) 18-hour post intranasal instillation of 1244 **(a, c)** and 1244G7 **(b, d)** bacteria.

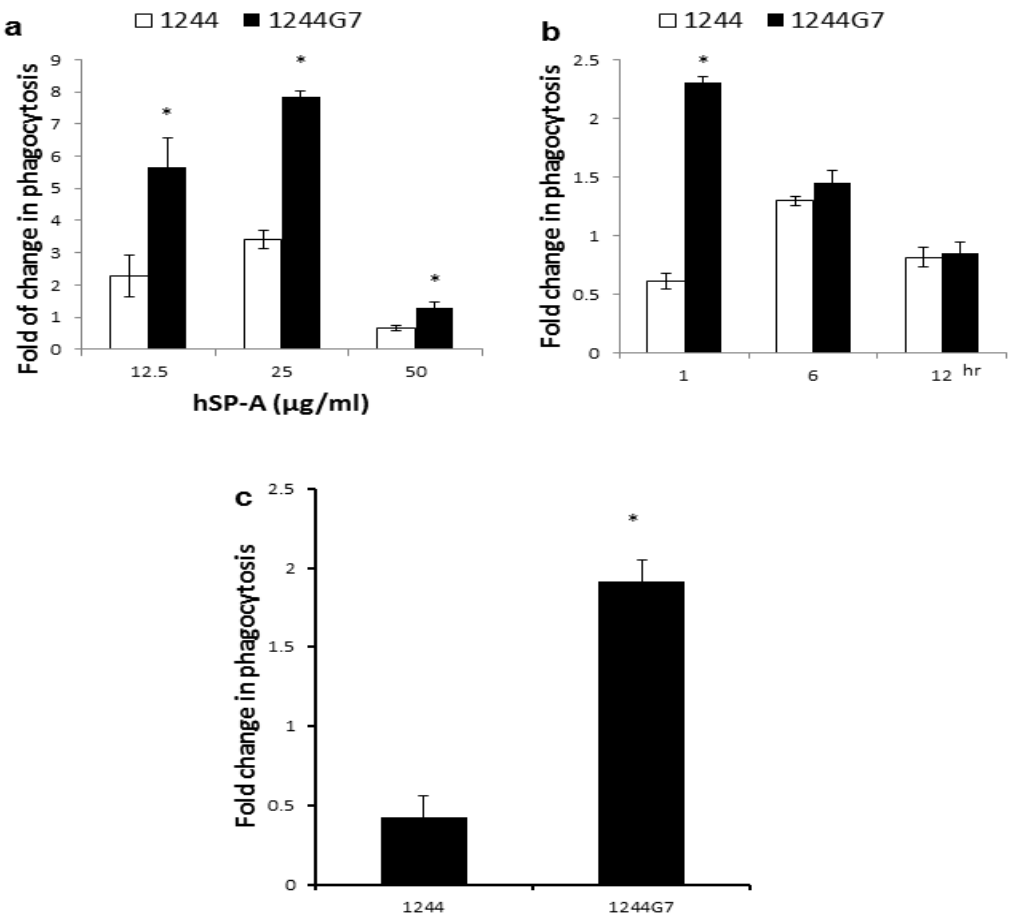


Fig. 18. The 1244G7 mutant is more susceptible to SP-A-mediated opsonization. **(a-c)** RAW 264.7 macrophages were infected with either 1244 or 1244G7 in the presence or absence of hSP-A. The ratio of ingested bacteria was expressed as fold increase in phagocytosed bacteria due to the effect of hSP-A. **(a)** Phagocytosis of 1244 and 1244G7 in the presence of different concentrations of hSP-A. **(b)** Time dependent phagocytosis of 1244 versus 1244G7 in the presence of 25 µg/ml hSP-A. **(c)** *In vivo* phagocytosis of 1244 versus 1244G7 in SP-$A^{+/+}$ and SP-$A^{-/-}$ mice (n = 3). All phagocytosis experiments were independently performed three times in triplicates. The mean ± standard deviation from one representative experiment is shown. *p < 0.05 when comparing the number of phagocytosed 1244G7 against 1244.

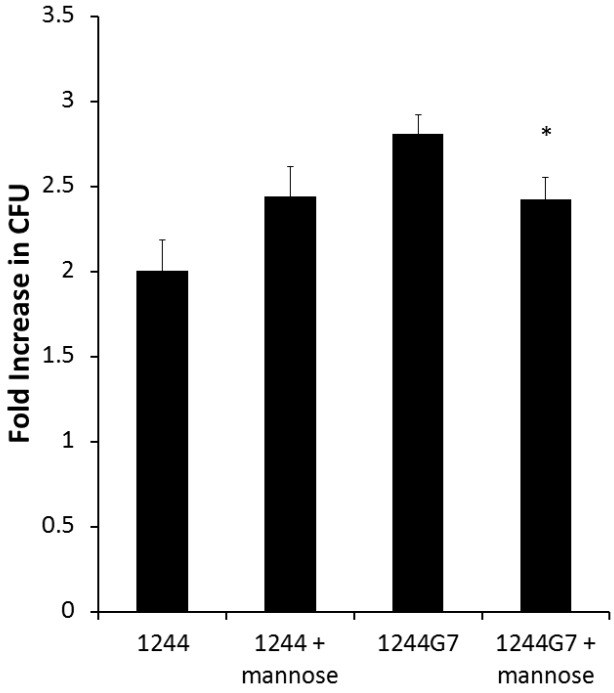


Fig 19. The increase susceptibility of 1244G7 mutant is associated with increase susceptibility to mannose receptor uptake. RAW 264.7 macrophages were pre-incubated with or without 1 mM D-mannose. It was then infected with either 1244 or 1244G7 in the presence or absence of hSP-A. The ratio of ingested bacteria was expressed as fold increase in phagocytosed bacteria due to the effect of hSP-A. *$p < 0.05$ when comparing the number of phagocytosed 1244G7 with the presence of 1 mM mannose against untreated 1244G7.

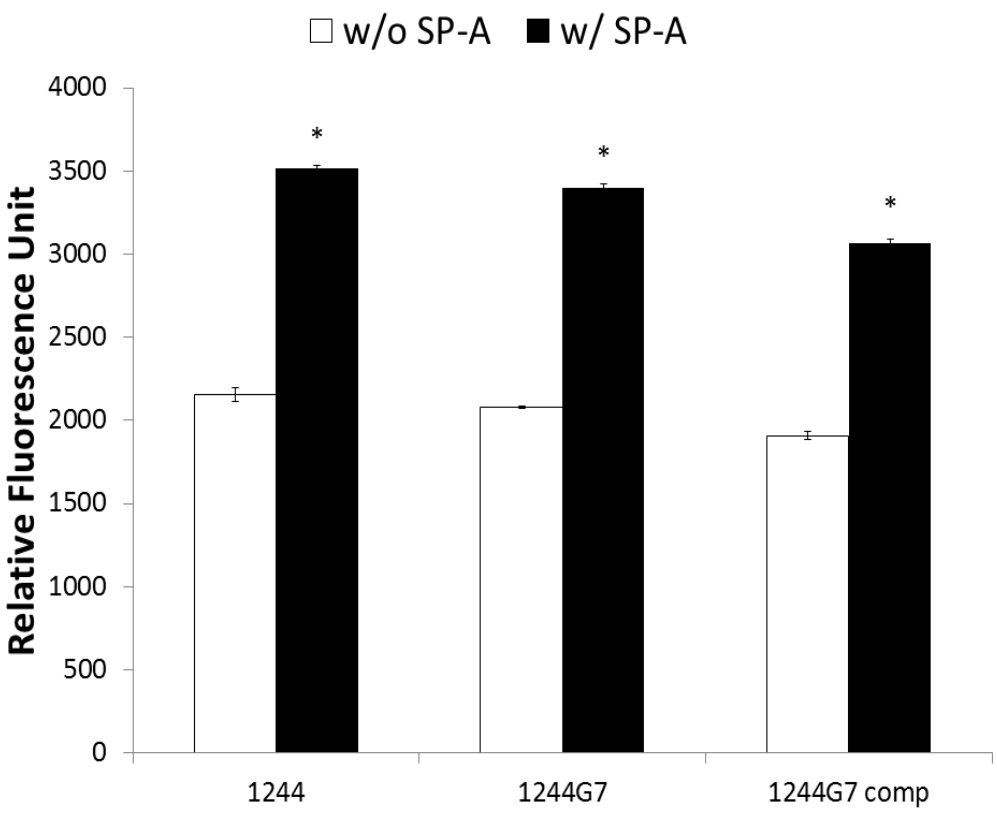


Fig. 20. The addition of O-antigen subunit does not allow for increased resistance to SP-A-mediated membrane permeabilization. *In vitro* ThioGlo® assay. 1244, 1244G7 and 1244G7 comp were preincubated with 50 µg/ml hSP-A for 15 minutes. The bacterial-free supernatants were then mixed with ThioGlo®. Absorbance was measured at excitation wavelength 405 nm and emission wavelength 535 nm. w=with; w/o=without. *$p < 0.05$ when comparing the relative fluorescence unit (RFU) of SP-A treated versus non-treated bacteria.

CHAPTER 4: SUMMARY

During my preliminary examination, I proposed two hypotheses: 1. Tfp is important for PA resistance to SP-A-mediated antibacterial effects; 2. The addition of O-antigen moiety to Tfp allows for increase resistance to SP-A-mediated effects. Both of these hypotheses have been verified (see Chapter 2 and 3).

For my first aim, I first attempted to show the importance of Tfp to SP-A-mediated lung clearance. Because the relative decrease of bacterial load between from SP-$A^{-/-}$ mice and SP-$A^{+/+}$ mice for both PAO1 and Δ*pilA* is similar, a straightforward link between Tfp and SP-A cannot be easily established. What is clear is that Tfp is important for resistance against lung clearance, both in the presence and absence of SP-A. One possible explanation to this is that other lung innate immune factors (i.e. phagocytic cells, cytokines, chemokines) within the airway spaces may compensate for the absence of SP-A. Next, I explored which specific aspect of SP-A-mediated antibacterial effects to which Tfp affords resistance to. Using the gentamicin exclusion assay, I have shown that Δ*pilA* is consistently more susceptible to SP-A-mediated phagocytosis. To further show that this is consistent *in vivo*, I used C3H mice and their SP-A KO counterparts to evaluate *in vivo* phagocytosis. Again, I have shown that Δ*pilA* is more susceptible than wild-type to SP-A. More importantly, I have shown that Δ*pilA* complemented with a copy of wild-type *pilA* gene regains its resistance to SP-A. SP-A mediated-phagocytosis is facilitated by enhanced aggregation of bacteria. By using GFP expressing PA strains, I have shown that Δ*pilA-GFP* is susceptible to SP-A-mediated aggregation; while wild-type PAO1-GFP strain is not. Another antibacterial mechanism of SP-A is by directly killing cells via membrane permeabilization. Using two commercially available membrane permeability assay kits, ELF-97

(which measures a fluorophore-linked phosphatase substrate that enters leaky bacteria) and ThioGlo (which measures thiol containing proteins that leak out of the bacteria), I have shown consistently that Δ*pilA* is more susceptible than wild-type PAO1 to SP-A-mediated membrane permeabilization. This was further confirmed using bronchoalveolar lavage (BAL) from C3H wild-type and SP-A KO mice, where BAL the SP-A-containing wild-type mice permeabilized the Δ*pilA* mutant more readily than the BAL from the SP-$A^{-/-}$ mice. Finally, purified hSP-A or BAL from mice consistently kills more Δ*pilA* bacteria than its isogenic wild-type PAO1.

Further exploring the underlying mechanisms by which Tfp resist SP-A-mediated phagocytosis, I used both the extension and retraction gene mutants of Tfp to determine whether this increase susceptibility by Δ*pilA* is associated with the mere absence of surface Tfp or the total absence of Tfp both on the bacterial surface and intracellularly. Extension mutants, which don't expresses surface Tfp but have Tfp protein intracellularly, did not show increased susceptibility to SP-A-mediated phagocytosis. This suggests that it is the total absence of Tfp (both intracellular and in the surface) allows for increased susceptibility to SP-A-mediated phagocytosis. Interestingly, retraction mutants, Δ*pilT* and Δ*pilU*, showed even higher levels of resistance to SP-A-mediated phagocytosis than the wild-type PAO1. These results suggest that hyperpilation (the phenotype of Δ*pilT* and Δ*pilU*) is covering/masking an unknown ligand for SP-A, allowing increased resistance to SP-A-mediated phagocytosis. I hypothesized that the ligands was one of the myriad nonpilus adhesins whose expression were compensatorily increased in the absence of PilA, causing an increase susceptibility to SP-A-mediated phagocytosis. To test this, I compared the SP-A-mediated phagocytosis on PA strains PAO1, Δ*pilA* and Δ*rpoN*. RpoN has been shown to positively regulate the expression of Tfp and non-Tfp adhesins. My results show that Δ*rpoN* bacteria are more resistance to SP-A-mediated

phagocytosis than both Δ*pilA* and wild-type, supporting my hypothesis. To further explore this hypothesis, I use ligand blot to determine whether there is an SP-A binding ligand (nonpilus adhesin) that is more expressed in Δ*pilA* but not wild-type and Δ*rpoN*. I have shown that an 18 kDa membrane protein is more strongly expressed in Δ*pilA* than both wild-type and Δ*rpoN*. By mass spectrophotometry, I have determined that this ligand is OprH.

For my second aim, I proposed that the presence of sugar moiety, specifically O-antigen subunits, on pilin monomers allows the clinical strain 1244 to have increased resistance to SP-A-mediated antibacterial effects. Using the mouse clearance assay, I was able to show that the Δ*tfpO* glycosyltransferase mutant strain 1244G7 has a significantly increased clearance in wild-type C3H compare SP-A KO mice, while 1244 did not. This shows that the absence of O-antigen decorating the pilin subunits on Tfp increases 1244G7 susceptibility to SP-A-mediated lung clearance. More importantly, I was able to show that while 1244G7 did not produce mortality in SP-A KO mice, 1244 did, showing that the additional O-antigen allows for increased virulence. Using piperacillin exclusion assay, I was able to show that 1244G7 is more susceptible than 1244 to SP-A-mediated phagocytosis, but not SP-A-mediated membrane permeability. Finally, using mannose as a competitive inhibitor, I was able to show that the increased susceptibility of 1244G7 to SP-A-mediated phagocytosis is abrogated, suggesting that 1244's increased resistance is due to masking of putative mannose moiety by the O-antigen subunits.

Future direction for our project would include confirming the importance of OprH as an SP-A ligand. To undertake this, a nonpolar deletion mutant (*ΔoprH*) and as well as a *ΔoprH* strain genetically-complemented by a copy of the wild-type gene *in trans* would need to be constructed. These would then be tested against the existing SP-A functional assay in our laboratory, including gentamicin exclusion assay and membrane permeability kits. We also plan

to study the complex interaction of OprH with RpoN. Using real time-reverse transcriptase-polymerase chain reaction (RT-PCR), we plan to quantify whether *oprH* mRNA is decreased in the absence of *rpoN*. Furthermore, we plan to examine the sequence and functional homology between OprH with other existing protein SP-A ligand such as *Haemophilus influenzae* P2 and *Staphylococcus aureus* EAP [89, 157]. Interestingly both OprH and P2 are outer membrane porin proteins, possessing β-barrel structures [155-157, 172]. Further examination of porins of other Gram-negative bacteria such as LKT of *Mannheimia hemolytica*, Apx of *Actinobacillus pleuropneumonia* and their possible function as SP-A receptors is warranted. Adhesins of Gram-positive bacteria such as EAP of *Staphylococcus aureus* could also be explored as receptors of SP-A.

Also, further investigations describing the mechanism for the increased resistance allowed by the addition of O-antigen to Tfp against SP-A-mediated antibacterial effects are needed. Currently, we have two working hypotheses. Firstly, it has been shown that bacterial hydrophobicity is associated with increased phagocytosis [173]. Also, it has been shown that the glycosyltransferase mutant, 1244G7, is more hydrophobic than the WT, 1244 [162]. They speculate that this hydrophobicity is due to the loss of negatively charged trisaccharides (O-antigen repeating subunits) [162]. Hence, we speculate that 1244G7 is more susceptible than SP-A-mediated phagocytosis due to increased hydrophobicity. This is very plausible as SP-A have been shown to bind to hydrophobic ligand, namely lipid A [135]. To test this, first, we plan to purify the Tfp of both 1244 and 1244G7. Then, we would like to test whether the addition of Tfp of glycosylated 1244 would abrogate the increased susceptibility of 1244G7 to SP-A-mediated phagocytosis. The theory is that the purified charged Tfp of 1244 would repel or hinder the interaction of SP-A with the hydrophobic Tfp of 1244G7. An alternative experiment would be by

adding either the purified trisaccharide O-antigen subunit or the terminal sugar, pseudaminic acid. We would also use SP-A ligand blot to determine whether SP-A binds to these Tfps. Also, we would test whether it binds preferentially to 1244 or 1244G7 Tfp. Alternatively, a co-immunoprecipitation of Tfp and SP-A could be performed. We predict that 1244 Tfp would bind more avidly to SP-A than 1244G7 Tfp.

Secondly, it has been shown that SP-A binds to and enhance phagocytosis only in rough, but not smooth strains of *E. coli* [118]. This is explained by the fact that SP-A binds to lipid A [135]. It is hypothesized that the steric constraint of the bulky O-antigen and core polysaccharide prevents binding of SP-A to lipid A [118]. LPS O-antigen has also been shown to be important to resistance of non-SP-A-mediated phagocytosis by many authors [166-171]. Again, this is explained due to the steric block caused by the O-antigen to the the N-acetylglucosamine residue in the core polysaccharides, which is the ligand for DC-SIGN, the phagocytic receptors in dendritic cells [171]. In one paper, it has been shown that the resistance caused by O-antigen is associated with putative mannose receptors [166]. Currently, we have shown evidence that a similar mechanism exists with the 1244 PA strain. We plan to further refine our experiments with mannose inhibition by using lower concentration of mannose. Alternatively, we want to test other known sugars that are known to bind to SP-A, such as fucose and N-acetylglucosamine or combinations there of.

Taken together, I have shown that Tfp and its accessory sugar structures are important for resistance to SP-A-mediated antibacterial effects. Hence, a novel approach to CF treatment using exogenous SP-A administration, may be useful, as Tfp is missing in CF isolates.

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