Study of host-pathogen interactions using a model of *Pseudomonas aeruginosa* infection of lung epithelial cells

by

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Table of Contents **Page #**

List of abbreviations

Amino Acid abbreviations
- A = alanine
- D = aspartate
- E = glutamic acid
- G = glycine
- P = proline
- R = arginine
- S = serine
- V = valine
- X = unspecified
- Y = unspecified

AP-1 (activator protein 1)
BCS (bovine calf serum)
BSA (bovine serum albumin)
CF (cystic fibrosis)
CFTR (cystic fibrosis transmembrane conductance regulator)
CFU/ml (colony forming units per ml)
dsRNA (double stranded RNA)
EC (epithelial cell)
ECM (extracellular matrix)
FAP (fibronectin attachment protein)
FHA (filamentous hemagglutinin)
Fn (fibronectin)
FnBP (fibronectin binding protein)
FSC (forward scatter)
gfp (green fluorescence protein)
GM-CSF (granulocyte macrophage colony stimulating factor)
Gn (gentamicin)
GSK-3 (glycogen synthase kinase-3)
ICAM-1 (intracellular adhesion molecule 1)
IgG (immunoglobulin G)
IL (interleukin)
ILK (integrin linked kinase)
LB (Luria Bertani)
LBA (Luria Bertani agar)
LEC (lung epithelial cell)
Lmn (laminin)
LPS (lipopolysaccharide)
MAPK (mitogen-activated protein kinase)
MMP (matrix metalloproteinase)
MOI (multiplicity of infection)
mRNA (messenger RNA)
NF-κB (nuclear factor κB)
NP (non-piliated)

OD (optical density)
OMP (outer membrane protein)
PBS (phosphate buffered saline)
PCR (polymerase chain reaction)
PI3-K (phosphatidylinositol 3-kinase)
PKB/Akt (protein-kinase B/Akt)
PMN (polymorphonuclear cell)
PMx (polymyxin-B)
PTGS (post-transcriptional gene silencing)
qRT-PCR (quantitative real-time PCR)
RANTES (regulated upon activation, normal T cell expressed and secreted)
RGD (arginine-glycine-aspartate)
RISC (RNA-induced silencing complex)
RNAi (RNA interference)
rpm (revolutions per minute)
siRNA (small interfering RNA)
Syk (spleen tyrosine kinase)
TE (Tris-EDTA)
TLR (toll-like receptor)
TNF (tumour necrosis factor)
Vn (vitronectin)
WT (wild type)

Abstract

Pseudomonas aeruginosa is an opportunistic pulmonary pathogen that can cause acute, life-threatening pneumonia in immunocompromised individuals, it is a common cause of high-mortality ventilator associated pneumonia, and frequently colonizes the lungs of cystic fibrosis patients. As an opportunist, *P. aeruginosa* uses multiple strategies to exploit host cells during pulmonary infections, and many of these mechanisms are not completely understood. The goal of this study was to develop and optimize a set of experimental methods that would allow us to explore the host-pathogen interactions between *P. aeruginosa* and lung epithelial cells, and to use these methods to specifically investigate the role of lung epithelial integrin receptors in *P. aeruginosa* pathogenesis. We labelled a strain of *P. aeruginosa* PAK with green fluorescent protein (*gfp*) gene and optimized conditions using this strain to measure its interaction with A549 lung epithelial cells using flow cytometry, a plate reader based assay and fluorescence microscopy. We then used these methods to investigate the importance of integrin inhibitory peptides or antibodies and integrin ECM ligands for PAK*gfp* interactions with A549 cells. In addition, we measured the effect of inhibiting the functional involvement of integrins using siRNA against integrin linked kinase (ILK) on A549 cellular responses (cytokine release) to PAK. Finally, isogenic PAK mutants and clinical isolates of *P. aeruginosa* were used to determine the importance of various bacterial virulence factors in activation A549 cells. We found that *P. aeruginosa* PAK preferentially binds the $\alpha5\beta1$ integrin ligand fibronectin which can increase PAK*gfp* association with A549 cells, and that ILK mediated signaling is necessary for the maximal cytokine response of A549 to *P. aeruginosa* infection. In addition, infection with PAK mutants lacking flagella or LPS, and all studied *P. aeruginosa* clinical isolates resulted in decreased cytokine release by A549 cells compared to PAK WT. We conclude that *P. aeruginosa* is able to exploit integrins, particularly $\alpha5\beta1$, to activate cellular signaling events in host airway epithelial cells during infection, and that the ability of the bacterium to elicit such responses can vary depending on the phenotype and virulence of the particular *P. aeruginosa* strain.

Chapter 1: Labelling PAK*gfp* and optimizing methods to study host-pathogen interactions

Sections of this chapter have been previously published:

Barnes RJ, Leung KT, Schraft H, & Ulanova M (2008) Chromosomal *gfp* labeling of *Pseudomonas aeruginosa* using a mini-Tn7 transposon: application for studies of bacteria-host interactions Can J Microbiol 54:48-57.

Abstract

Analysis of bacterial interactions with host cells using multiple techniques is essential for studies on microbial pathogenesis and for the development of new antimicrobial therapies. *Pseudomonas aeruginosa* is an important opportunistic pathogen which can cause severe, often life threatening pulmonary infections in individuals with impaired host defense mechanisms. Using a mini-Tn7 transposon delivery system we have chromosomally labelled the strain *P. aeruginosa* PAK with a green fluorescent protein gene (*gfp*) and tested PAK*gfp* as a research tool for studies of bacteria-host interactions. We were able to reliably and rapidly measure the interactions of PAK*gfp* with A549 human lung epithelial cells using flow cytometry, a fluorometric microplate reader-based assay, and fluorescence microscopy. We have also been able to develop and optimize a fluorescence quenching based internalization assay that is quick and easy to use for preliminary investigations. With these analytical tools we have demonstrated the adhesion of PAK*gfp* to the extracellular matrix protein fibronectin and the involvement of fibronectin in PAK*gfp* - A549 cell interactions. PAK*gfp* can be successfully used to explore the effects of various pharmacological compounds on *P. aeruginosa* – host cell interactions in both *in vitro* and *in vivo* systems, with potentially important medical applications.

Introduction

Pseudomonas aeruginosa is a ubiquitous Gram negative, aerobic rod shaped bacterium that is able to inhabit almost any aqueous environment including soil, surface water, sewage, plants, and various foods including vegetables and fruit (Bonten *et al.*, 1999). In the health care setting, *P. aeruginosa* is often found in hand-washing sinks and humidifiers, and is frequently transferred to patients through direct contact by hospital staff (Nseir *et al.*, 2002). *P. aeruginosa* is the one of the most common causes of nosocomial pneumonia, second only to the opportunistic pathogen *Staphylococcus aureus* (NNIS System, 2003). In addition, *P. aeruginosa* often causes acute life-threatening infections in immunocompromised patients (Chatzinikolaou *et al.*, 2000), burn wound victims (Santucci *et al.*, 2003), ventilator-associated pneumonia with high mortality rates (Chastre & Fagon, 2002), and is the major cause of chronic pulmonary infection in cystic fibrosis (CF) patients (Saiman & Siegel, 2004).

Since this bacterium is an opportunistic pathogen, it possesses many virulence factors which contribute to its ability to infect various tissues. *P. aeruginosa* possesses a single flagellum that is critical for motility and important for initial colonization (Prince, 2006); however, due to its high immunogenicity, flagella expression is often lost during the course of chronic infection (Sadikot *et al.*, 2005). The surface of *P. aeruginosa* is covered with short structures called pili (Keizer *et al.*, 2001) which are important for *P. aeruginosa* adhesion to cells (Ramphal *et al.*, 1984; Doig *et al.*, 1988). In addition, *P. aeruginosa* has a type III secretion system that allows the bacterium to secrete four exotoxins (ExoS, ExoT, ExoU and Exo Y) which help establish infection and contribute to tissue damage (reviewed by (Sadikot *et al.*, 2005)). To aid in long term survival in the host, *P. aeruginosa* can also form biofilms which both confer antibiotic resistance and protection from the immune system (Drenkard & Ausubel,

2002), and they can produce siderophores to scavenge iron under the low iron conditions encountered inside the host organism (Takase *et al.*, 2000).

In vivo animal models are very useful for gaining an understanding of host-pathogen interactions in a physiologically relevant context. In particular, host factors involved in *P. aeruginosa* lung infections can be investigated by using transgenic knock-out mice that lack certain host factors, such as MyD88 knock-out mice for studying the role of toll-like receptors (TLR) (Power *et al.*, 2004; Skerrett *et al.*, 2004), or cystic fibrosis transmembrane conductance regulator (CFTR) knock-out mice which mimic CF conditions (Schroeder *et al.*, 2001). However, it still can be difficult to distinguish specific cause and effect relationships due to the complexity of the system. It is therefore common practice to use *in vitro* models to study bacterial interactions with cells at the molecular level. While primary airway epithelial cells most closely mimic the behaviour of the *in vivo* airway epithelium (Armstrong *et al.*, 2004), immortalized cell lines such as the alveolar type II pneumocyte cell line A549 are readily available, easy to culture and commonly used for *in vitro* studies. In addition, several laboratory strains of *P. aeruginosa* including PAO1 (which has its entire genome sequenced) and PAK (described by Pasloske *et al.*, 1985) have been well characterized and are often used to investigate different aspects of lung infection (Feldman *et al.*, 1998; Rajan *et al.*, 2000; Kube *et al.*, 2005).

The green fluorescent protein gene (*gfp*), originally isolated from the jellyfish *Aequorea victoria,* can be cloned into the bacterial genome to create mutants that produce GFP protein and emit a strong green fluorescent signal (Chalfie *et al.*, 1994). Broad or narrow host range plasmids with constitutively expressed *gfp* are commonly used to transfect bacteria (Valdivia *et al.*, 1996), including some plasmids specifically designed to be stable in *Pseudomonas* spp.

(Bloemberg *et al.*, 1997). GFP mutant proteins, such as GFPmut3 that is used in this study, have excitation and emission frequencies similar to FITC, allowing easy detection by most fluorescence read-out systems (Cormack *et al.*, 1996).

P. aeruginosa containing plasmids with *gfp* have been used to study certain aspects of host-pathogen interactions. *P. aeruginosa* PAK mutants carrying a pUCP18 plasmid coding for GFPmut3 have been used to investigate the role of flagella in macrophage uptake during pulmonary infection by flow cytometry analysis (Feldman *et al.*, 1998). A *P. aeruginosa* PAO1/*gfp* mutant with a pUCP19 based vector plasmid has been used to study apoptosis of stimulated epithelial cells by confocal or epi-fluorescence microscopy (Rajan *et al.*, 2000), and also to quantify adherence of *P. aeruginosa* to cystic fibrosis epithelial cell monolayers by measuring fluorescence of lysed cells with a fluorometer (Kube *et al.*, 2005). Similar studies have been performed to investigate other host-pathogen interactions, such as the interaction of a *gfp*-expressing *S. aureus* with integrins expressed on A549 lung epithelial cells using fluorescence microscopy (Liang & Ji, 2006). Plasmids carrying different types of genetic markers, i.e. bioluminescence, have also been applied to measure bacterial interactions with host cells using microplate reader based assays (Swanson *et al.*, 2003), but a bioluminescence marker is not suitable for microscopy or flow cytometry.

Some major drawbacks to plasmid-based systems include the need to maintain plasmids by antibiotic selection, and the possibility that over expressed gene products will interfere with bacterial behaviour (Koch *et al.*, 2001). A superior system to plasmid labelling is the use of transposon insertion systems, incorporating the marker genes directly into the host chromosome (Koch *et al.*, 2001; Lambertsen *et al.*, 2004; Choi *et al.*, 2005). One of the most efficient delivery systems is the mini-Tn7 transposon system, which allows for insertion of desired genes

into Gram-negative non-enterobactericeae species at a well conserved intergenic *att*Tn7 'hot-spot' just downstream from the coding region of the glucosamine-6-phosphate synthetase (*glmS*) gene.

Lambertsen et al (Lambertsen *et al.*, 2004) have developed a panel of mini-Tn7 transposons expressing a variety of fluorescent proteins under the control of constitutively expressed or growth dependent promoter systems. In order for transposition to occur, the delivery plasmid carrying the transposon with marker genes, and the helper plasmid that produces transposase enzyme in *trans* must be introduced simultaneously into the host bacterium. The accuracy and efficiency of this labelling system has been verified using polymerase chain reaction (PCR) and Southern blot analysis of proper insertion, and growth curves and competition assays show that bacterial behaviour has not changed. Advantages of this system include use of stably labelled bacteria in studies of mixed-species communities, or in any system where antibiotic selection to maintain plasmids is not possible.

The gentamicin (Gn) exclusion assay is a commonly used tool to distinguish between the amount of bacteria adhered to and internalized by cells in culture. It was initially developed to study the invasiveness of *Yersinia enterocolitica*, *Escherichia coli* and *Y. pseudotuberculosis* (Mehlman *et al.*, 1977; Devenish & Schiemann, 1981; Vesikari *et al.*, 1982; Isberg & Falkow, 1985). Gn is used because it is one of the few antibiotics that can effectively kill many types of drug resistant bacteria like *P. aeruginosa*, and it does not cross the eukaryotic cell membrane, meaning that it will not be able to kill any bacteria inside the cell. The assay can be performed multiple ways, but we have based our methods on those used by other groups to study the internalization of *P. aeruginosa* into various cells types (Plotkowski *et al.*, 1999; Gagniere & Di Martino, 2004; Leroy-Dudal *et al.*, 2004; Zaas *et al.*, 2005a), and have further optimized

conditions for our specific cells and strain of bacteria. The assay can also be performed using the antibiotic polymyxin B (PMx), since it is also membrane impermeant (Gophna *et al.*, 2001; Schaeffer & Weiss, 2001a; Gophna *et al.*, 2002a). This is advantageous for our study, since our *gfp* labelled PAK is resistant to Gn, but not to PMx.

Most studies of internalization exclusively utilize the antibiotic exclusion assay approach. We are interested in using the green fluorescent properties of our bacteria to investigate internalization using other fluorescence-based approaches which would be much faster and easier to complete. In order to use our fluorescent bacteria, we had to determine the suitable conditions for study, and needed to ensure that the methods used are comparable to standard methods. Firstly, the concentrations of Gn and PMx which ensured effective killing of bacteria had to be optimized. Secondly, the similarity between Gn and PMx in killing extracellular bacteria had to be verified. If PMx killing of extracellular bacteria is comparable to Gn killing, we should be able to substitute it for Gn, allowing us to use PAK*gfp*. Based on our observations that PMx killing of PAK*gfp* results in elimination of the green fluorescent signal, we investigated the possibility of using an internalization assay modeled after the system developed by Voyich and DeLeo (2002). Their system distinguished between intracellular FITC labeled *S. aureus* and extracellular bacteria by quenching the extracellular FITC signals with trypan blue. In our system, the addition of PMx should act in a similar way to 'quench' the extracellular PAK*gfp* signal, while leaving the intracellular bacteria untouched and still green fluorescent. This PMx quenching system could be used to measure PAK*gfp* invasion into adherent cells using a plate reader based method or flow cytometry.

Although the utility of *gfp*-labelled bacteria for studying host-pathogen interactions has been illustrated, no attempts have been made to combine the mini-Tn7 transposon chromosomal

labelling with the development of a complete system to study multiple aspects of such interactions. In this study we have labelled the *P. aeruginosa* strain PAK with *gfp* using the mini-Tn7 transposon delivery system developed by Lambertsen *et al.* (2004), and used the PAK*gfp* to investigate bacterial interactions with human cells using flow cytometry, fluorescence microscopy and microplate reader technology. This system allows for rapid measurements and visualization of bacterial adherence or internalization in a live cell system, as well as the quantification of the effects of various pharmacological compounds on bacteria-host cell interactions.

Materials and Methods

Cell line and bacterial strains

The A549 human alveolar type II epithelial cell line was used in this study (ATCC # CCL-185). A549 cells were maintained in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% heat inactivated iron fortified bovine calf serum (BCS) (SAFC Biosciences, Lenexa, KS) and 1% L-glutamine (Gibco, Carlsbad, CA) without antibiotics. Cells were grown at 37°C with 5% CO_2 and seeded every 4 days when confluency neared 80% (passage number 75-100).

P. aeruginosa strain PAK (kindly provided by Dr RJ Irvin, University of Alberta, Edmonton), described by Pasloske *et al.* (1985), was maintained on sterile Luria Burtani medium (Fischer Scientific, Fair Lawn, NJ) with 1% agar (LBA). The labelled PAK*gfp* constructed in this study was maintained on LBA with 30 μg/ml filter-sterilized Gn (USBiological, Swampscott, MA). *E. coli* S17-1λ containing the pMiniTn7(Gm)$P_{PA1/04/03}$gfp-a transposon plasmid, and *E. coli* SM10-λpir containing the pUX-BF13 helper plasmid (both plasmids kindly

donated by Dr Søren Molin, Technical University of Denmark, Lyngby) were maintained on sterile LBA with 30 μg/ml Gn and 100 μg/ml ampicillin (Sigma-Aldrich) respectively, to maintain their plasmids. All strains were grown at 37°C.

Genomic labelling of PAK with *gfp*

PAK was chromosomally labelled with *gfp* using a system developed by Lambertsen *et al.* (2004). The plasmids pMiniTn7(Gm)$P_{PA1/04/03}$gfp-a and pUX-BF13 necessary for transformation were isolated from their hosts using a Wizard Mini-prep Plasmid isolation kit (Promega, Madison, WI) following the manufacturer's instructions. The final products were resuspended at a concentration of approximately 300 ng/μl in nuclease free water (Promega) for use in electroporation.

The method for electroporation was adapted from Sambrook and Russel (Sambrook & Russell, 2001). All washes and resuspensions were performed with ice-cold sterile double deionized water. Electroporation was performed using an Electroporator 2510 (Eppendorf, Mississauga, ON, Canada) at 2.5 kV, adding approximately 600 ng of each plasmid DNA to 300 μl of prepared cells in a 4 mm gap, 800 μl volume electroporation cuvette (optical density at 600 nm (OD_{600}) of 1:1000 bacteria dilution = 0.5). Following electroporation and 3 h incubation in sterile Super Optimal Catabolite medium, various dilutions were plated onto 30 μg/ml Gn LBA plates for selection of transformants based on both their ability to grow in antibiotics and their green fluorescence.

Testing PAK*gfp*

Three successfully transposed PAK*gfp* isolates were biochemically tested for similarity to PAK wild type (WT) using API 20E test strips (Biomerieux, St Laurent, QC, Canada). Growth curves were generated by measuring the OD_{600} of each mutant and the WT every 30 min, starting at an OD_{600} of 0.05, until the curve reached a plateau. Insertion of the *gfp* cassette into the chromosome (and therefore loss of plasmid) was verified by using a plasmid extraction kit (Promega) on each selected mutant. Chromosomal stability of the insert was determined by performing a 5-day stability test as described by Choi *et al.* (2005). Briefly, the chosen PAK*gfp* strain was grown in sterile LB broth without antibiotics for 5 consecutive days, with dilution by a factor of 1000 each day into new media. Green fluorescence of the culture was monitored daily using a Nikon Eclipse 80i epi-fluorescent microscope (Nikon Canada, Mississauga, ON). After the fifth day, dilutions were spread plated onto non-selective LBA, and following incubation at 37° C for 18 hours, 20 colonies were selected and tested for green fluorescence and for their ability to grow on LBA with 30 μg/ml Gn.

Correct insertion into the *att*Tn7 intergenic region was confirmed using the PCR system according to Lambertsen *et al.* (2004), which verifies correct insertion when a PCR product of approximately 164 bp is obtained. The Tn7-*glmS* primer (5'-AATCTGGCCAAGTCGGTGAC-3') anneals approximately 30 bp from the 3' end of the *glmS* gene, while the Tn7R109 primer (5'-CAGCATAACTGGACTGATTTCAG-3') anneals to the 109th nucleotide of the conserved right end of the miniTn7. Primers were synthesized by Sigma-Genosys (Oakville, ON, Canada). Approximate size of PCR products was estimated by comparing them to Labaid O'GeneRuler 100bp DNA ladder (Fermentas, Burlington, ON, Canada) following electrophoresis, and products were then purified and sequenced (MOBIX, Hamilton, ON, Canada). Sequencing

results were compared to the Genbank database to verify inclusion of a portion of *glmS*, and the intergenic region.

Preparation of bacteria for experiments

PAK*gfp* cultures were grown overnight in sterile LB medium, and diluted by a factor of 20 into fresh sterile LB broth. Cultures were allowed to grow for approximately 1 h, until mid-log phase (OD_{600} of 0.4-0.45) and were centrifuged at 3500 × g, for 20 min at 4°C. Cells were washed twice in 5 ml sterile phosphate-buffered saline (PBS, pH 7.4). Following the final resuspension, cells were diluted to the appropriate OD_{600} for the intended application in sterile adhesion buffer (PBS with 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4, as per Chi *et al.*, 1991). Actual numbers of bacterial used in each experiment were confirmed by drop plating on LBA.

Bacterial adherence to substrates

Microscopic detection of PAKgfp adherence to substrates

For microscopic detection, round glass coverslips were coated with 250 µl of human plasma fibronectin (20 µg/ml Fn) (Sigma), or 1% bovine serum albumin (BSA) (Sigma) at 4°C, overnight. Coverslips were washed twice with 500 µl sterile adhesion buffer. PAK*gfp* at a concentration of 2×10^8 colony forming units per ml (CFU/ml) were added to the coverslips and incubated for 30 min at 37°C. Following incubation, coverslips were washed three times with 500 µl sterile adhesion buffer to remove non-adherent bacteria. Coverslips were air-dried and then mounted to view using epi-fluorescence microscopy. Air-drying or fixation resulted in no change in green fluorescence intensity of the bacteria.

Microplate reader detection of PAKgfp adherence to substrates

Similar experiments were performed to obtain quantitative data using flat bottom solid black 96-well tissue microplates (Corning). Wells were coated overnight with Fn or BSA as described above, washed twice with 100 µl sterile adhesion buffer, and bacteria were added at varying concentrations (2×10^8 to 7×10^8 CFU/ml) and allowed to incubate for different lengths of time (0-120 min). Following incubation, wells were washed three times with sterile adhesion buffer, and plates were read using a FLUOStar Optima microplate reader (BMG Labtech, Offenburg, Germany), with an excitation filter of 495 nm, and emission filter of 520 nm. The gain was adjusted for the most highly fluorescent well of each separate experiment, and results were reported in fluorescence units as measured by the microplate reader minus the fluorescence of the negative control (substrate coated well with no bacteria).

Bacterial association with A549 cells

Flow cytometry analysis of PAKgfp association with A549 cells in suspension

Cultures of A549 cells at 80% confluency were trypsinized (1 min treatment with 0.05% Trypsin-EDTA (Gibco) at 37°C), washed with PBS and resuspended in adhesion buffer ($2x10^6$ cells/ml). The A549 cells were mixed with equal volume of PAK*gfp* suspension containing various concentrations of bacteria (between 5×10^6 and 4×10^8 CFU/ml) and incubated for various times (0-120 min, 37°C and 5% CO_2). Following incubation, bacteria-A549 cell mixtures were centrifuged at 233 × g for 5 min to pellet the A549 cells while leaving free unattached bacteria in the supernatant, and then resuspended in sterile PBS. A549 cell viability was assessed using a Vi-Cell XR Cell Viability Analyzer (Beckman Coulter, Mississauga, ON, Canada) and was found to range between 82-93% in different experiments. The A549 cells were then analyzed

with a BD FACSCalibur flow cytometer using the BD CellQuest^{TM} Pro software (BD Biosciences, San Jose, CA) acquiring 50,000 events per trial. The instrument settings were optimized to exclude free bacteria from the forward scatter (FSC) using a FSC threshold (based on the size). The ability to detect the signal from PAK*gfp* was verified using different instrument settings specific for the bacteria's size, and by comparing the FL1 signal of PAK*gfp* to that of PAK WT (Figure 1A). Flow cytometry analysis of A549 cells incubated with PAK*gfp* was based on the geometric mean of FL1 fluorescence of the entire A549 cell population. The data were expressed as the Relative FL1 increase = $(FL1_{sample} - FL1_{control})/FL1_{control}$. A549 cells alone or A549 cells + PAK*gfp* at time 0 min served as a control. In addition, we estimated the percent of cells which shifted into a new region (R3) encompassing events of identical FSC to the original population, but with an elevated FL1 intensity (Figure 1B-C).

To test whether Fn mediates the association of PAK*gfp* with A549 cells, flow cytometry analysis was performed in the presence of exogenous Fn. In these experiments, A549 cells were pre-incubated with 10 μg/ml Fn for 15 min at 37°C and 5% CO_2 prior to addition of bacteria.

Microscopic detection of PAKgfp association with A549 cells in suspension

For visualization of A549-bacterial interactions, PAK*gfp* and A549 cells were prepared and mixed as described above, but following 90 min incubation, the cell-bacteria mixture was fixed to poly-L-lysin coated coverslips with 4% paraformaldehyde (30 min) and methanol:acetone (1:1) (10 min) to secure cells on the coverslip for viewing. Fluorescent images were acquired using a Nikon Eclipse 80i epi-fluorescent microscope with the Nikon ACT-1C acquisition program. At least three samples were tested for each experimental condition.

Association of PAKgfp with adherent A549 cells

For analysis of bacterial interactions with adherent A549 cells, we used a fluorometric plate reader-based assay. In these experiments, A549 cells were grown in black 96-well tissue culture plates. Wells were pre-treated by incubating overnight with 20 μg/ml Fn, followed by washing once with 100 μl serum-free culture medium. A549 cells were then seeded at 50,000 per well, and allowed to grow overnight until near confluency, corresponding to approximately 1×10^5 cells. Wells were washed twice with 100 μl serum-free medium, and 1×10^7 bacteria suspended in 100 μl serum free medium were added to give a ratio of 100:1 (multiplicity of infection (MOI) of 100). Following incubation at 37°C, 5% CO_2 for various times (0-120 min), the wells were washed three times with 100 μl of warm sterile adhesion buffer. The fluorescence caused by bacterial binding to A549 cells was measured using a FLUOStar Optima microplate reader (BMG Labtech) in well-scanning mode with an excitation filter of 495 nm, and emission filter of 520 nm. The average fluorescence for each well was obtained from 50 separate points measured per well.

Antibiotic internalization assay – plate count methods

Gentamicin/Polymyxin bactericidal effects

The bactericidal effects of Gn and PMx were tested by inoculating 2 ml of serum free F-12K medium containing different concentrations (ranging from 50-500 μg/ml) of the antibiotics Gn or PMx with 2 μl of overnight bacterial culture (corresponding to approximately 1.4×10^6 bacteria). Broths were visually checked 24 and 48 h later for any positive growth, and aliquots were spread on LBA plates to further test for growth. The kinetics of PMx killing were assessed by incubating PAK WT and PAK*gfp* with 100 μg/ml PMx for 30, 60, 90 and 120 min in serum free medium, then diluting it into fresh antibiotic-containing medium and incubating overnight to

test for growth. Based on the broad range of Gn concentrations used in the literature (50 to 500 μg/ml), we tested the performance of various concentrations of Gn (50, 200, 250, 300, 400 and 500 μg/ml) in the Gn internalization assay. Based on the range of commonly used PMx concentrations, we performed the assay using 10, 50, 100 and 150 μg/ml PMx.

Gentamicin exclusion assay

A549 cells were grown to near confluency in 96-well plates (approximately 100,000 cells/well) for 2 days. Two h prior to treatment with bacteria, the cells were washed 4 times with PBS to remove all traces of BCS, and serum free medium was added (pre-conditioning). Bacteria were grown overnight (37°C, 150 rpm shaking), diluted 20:1 and allowed to grow for 1 h to reach mid-log phase ($OD_{600} \sim 0.4$). Bacteria were then spun down at 3,500×g for 20 min at 4°C, and washed 2 times with PBS using the same spin conditions, resuspended in serum free medium, and adjusted to a concentration of 1×10^8 CFU/ml. Bacteria were added to pre-conditioned A549 cells at an MOI of 50:1, and incubated for 60 min, although incubation times of 5 to 90 min were also tested. An MOI of 100:1 was tested, but it was found that this high ratio of bacteria to cells resulted in detachment of some of the A549 by the end of treatment. Following incubation, all wells were washed 3 times with PBS to remove non-adherent bacteria. To measure the total amount of bacteria associated with cells (either adherent or internalized), A549 cells were detached by treatment with 30 μl trypsin for 3 min, and then lysed by addition of 70 μl Triton X-100. The entire 100 μl of liquid was serially diluted and drop plated. To measure the amount of bacteria internalized, A549 cells were washed 3 times with PBS to remove non-adherent bacteria and then incubated with 200 or 400 μg/ml Gn for 90 min. Wells were then washed 4 times with PBS to completely remove any traces of the antibiotic, and cells

were trypsinized, lysed, diluted and drop plated as described above. As a control, bacteria and Gn were added together right at the beginning, and all other steps performed as described. In addition, the amount of bacteria surviving Gn treatment was quantified by spread plating the Gn containing medium following the 90 min incubation (post-antibiotic supernatant).

Polymyxin exclusion assay

To compare the effect of PMx with that of Gn, the procedure described above was performed substituting 10 μg/ml PMx for the Gn treatment.

Polymyxin quenching internalization assay

Plate reader PMx quenching internalization assay with adherent A549 cells

A549 cells were grown for 2 days in a black 96-well plate to near confluency (100,000 cells/well). Two h prior to treatment with bacteria, the cells were washed 4 times with PBS to remove all traces of BCS, and serum free medium was added (pre-conditioning). PAK*gfp* were prepared as described above, and added to wells at an MOI of 50:1. A549 cells and PAK*gfp* were incubated at 37°C and 5% CO_2 for 60 min. Wells were washed 3 times with room temperature PBS to remove non-adherent bacteria. For analysis of internalization, PMx was added at 50 μg/ml, and incubated for 30 min, 37°C. Wells were washed 3 times with PBS to remove non-adherent bacteria. The plate was read using the FluoStar Optimum plate reader, with excitation filter 495 nm, emission filter 520 nm.

PMx quenching internalization assay with adherent A549 cells trypsinized for flow cytometry

A549 were grown in a 12-well plate for 2 d until near confluency (500,000 cells/well). PAK*gfp* were prepared and A549 cells were infected as described above, using an MOI of 50:1. Conditions tested included a) cells incubated alone, b) cells incubated with PAK*gfp* and PMx for the entire time, c) cells incubated with PAK*gfp* then treated with PMx, and d) cells incubated with PAK*gfp* then trypsinized. Following the final PBS washes, cells were trypsinized with 300 µl trypsin per well. Trypsin was quenched with 1 ml serum-containing culture medium, and cells were pelleted at 1,100 rpm for 5 min. The supernatant was removed, and the cells resuspended in PBS and used for flow cytometry

Statistics

Data were presented as mean ± SE ($n \geq 3$). F-test for equality of variance was performed on each data set, and one-tailed Student's t-tests ($\alpha = 0.05$) were performed for equal or unequal variances according to F-test results. Alternatively, for normalized data, a one sample t-test was performed comparing means to a hypothetical mean of 100 ($\alpha = 0.05$).

Results

Selection of a successful PAKgfp mutant

Following electroporation, three successfully labelled PAK*gfp* mutants that exhibited green fluorescence and Gn resistance were chosen for further analysis. API 20E biochemical test results of all three mutants were identical to PAK WT, with identification as *Pseudomonas* spp. (very good identification). Plasmid extraction analysis of the mutants demonstrated the absence of the mini-Tn7-*gfp* transposon delivery plasmid, verifying that the delivery plasmids were lost

following the transposition event (data not shown). Figure 2 illustrates the similarity in growth characteristics of the three mutants compared to PAK WT. One mutant, PAK*gfp*A was chosen for further analysis, and is hereafter referred to as PAK*gfp*. PCR results (Figure 3) show the presence of the approximately 164 bp fragment described by Lambertsen *et al.* (2004), verifying correct insertion into the chromosome. Subsequent sequencing and BLAST results verified inclusion of the 3' end of the *glmS*. The chromosomal insert was stable over 5 days of sequential sub-culturing, since green fluorescence observed each day did not diminish, and all 20 colonies grown on selective media following the test did not show growth inhibition.

Detection of PAK*gfp* adherence to immobilized substrates

PAK*gfp* binding to Fn or BSA was quantified using a microplate reader based assay. After 5 min incubation at 37°C, various concentrations of bacteria showed significantly higher levels of binding to Fn coated wells than to BSA coated wells (Figure 4A). Longer incubation showed no increase in bacteria binding to Fn, although the binding to BSA was increased (data not shown), suggesting that the specific binding to Fn occurs immediately, while non-specific binding to BSA is continuous. The fluorescent signal generated by bacteria bound to Fn-coated wells was clearly dependent on the number of PAK*gfp* added to the wells, and was approximately 3-fold higher at the concentration of $7x10^8$ CFU/ml than at $2x10^8$ CFU/ml ($p<0.005$). Although the binding to BSA was also higher at $7x10^8$ CFU/ml concentration compared to $2x10^8$ CFU/ml ($p<0.05$), a dose-dependent increase in PAK*gfp* binding to A549 was not as striking as the one for Fn (Figure 4A). In addition, higher binding of PAK*gfp* to Fn than to BSA was observed using fluorescence microscopy (Figure 4B).

Detection of PAK*gfp* association with A549 cells

Flow cytometry analysis showed that association of PAK*gfp* with A549 cells in suspension was dependent on both incubation time and bacterial concentration. For all flow cytometry data, the same trends were observed when measuring the percentage of cells in R3, or the relative increase in geometric mean of FL1 fluorescence (Figure 5A). While testing various bacterial concentrations we found that adding 1×10^8 CFU/ml of bacteria to 1×10^6 A549 cells (MOI of 100:1) showed a significant increase in both relative FL1 fluorescence and % cells in R3 after 90 min of incubation. Higher concentrations of bacteria did not show significantly more binding, while the highest concentration tested (MOI of 400:1) showed a decreased binding (Figure 5A). Therefore, the MOI ration of 100:1 was chosen for further optimization studies. Fluorescence microscopy images verified the association of PAK*gfp* with A549 cells, and also showed A549 cells that appeared to be bursting with PAK*gfp* (Figure 5B).

Based on the dose-response data, a time course experiment of PAK*gfp* binding to A549 cells was performed with an MOI of 100:1. Following incubation of the mixture of PAK*gfp* and A549 cells, both relative FL1 fluorescence and % cells in R3 increased with time. At all time points, both parameters were significantly higher than for the negative control (A549 cells incubated in adhesion buffer without bacteria, Figure 6). A time-dependent increase in bacterial binding was noted, with 60, 90 and 120 min incubation showing significantly more bacterial binding than cells incubated for 0 min ($p<0.05$). Association of PAK*gfp* with adherent A549 cells (MOI of 100:1) showed a similar trend with an increased fluorescence signal following longer incubation time (Figure 7A). At all time points, significantly higher fluorescence was detected compared to 0 min, and each consecutive time point showed significantly higher fluorescence than the previous ($p<0.05$), except for 90 and 120 minutes. Bacterial association

with adherent A549 cells was confirmed by direct visualization using fluorescence microscopy (Figure 7B). Many of the A549 cells found in areas of high bacterial localization appeared more rounded and protruded from the surface than A549 cells with low or no bacterial association.

Effect of fibronectin on PAK*gfp* association with A549 cells

To address the question whether Fn mediates *P. aeruginosa* association with lung epithelial cells, we pre-incubated suspended A549 cells with 10 μg/ml of Fn for 15 min prior to mixing them with PAK*gfp* (based on a similar experiment by Sinha *et al.* (1999). Following incubation of the bacteria-A549 mixture (MOI of 100:1), cells were analyzed by flow cytometry. Presence of exogenous Fn significantly enhanced bacterial association with A549 cells (Figure 8A). Visualization of bacteria-lung epithelial cell interactions with fluorescence microscopy confirmed these observations, showing more bacteria associated with the A549 cell surfaces, and also more A549 cells observed that appeared to be full of bacteria (Figure 8B). Interestingly, addition of the same amount of Fn to adherent A549 cells did not alter PAK*gfp* association with A549 cells at any incubation time as detected by the plate-reader assay (data not shown). Also, addition of Fn at a higher concentration (20 μg/ml) had the same effect as adding FN at 10 μg/ml (data not shown).

Gentamicin and Polymyxin-B killing effects

Fifty μg/ml Gn completely inhibited growth of PAK WT during 24 h, but not 48 h, while concentrations of 100 μg/ml to 500 μg/ml inhibited growth during 48 h. All concentrations between 100-500 μg/ml showed similar levels of extracellular bacteria killing and intracellular bacterial survival, so the concentration of 200 μg/ml was chosen to use for further studies.

Concentrations of PMx as low as 50 μg/ml showed complete growth inhibition of both PAK WT and PAK*gfp* after 24 and 48 h. For all the PMx internalization assays using the plate count method, even the lowest concentration of PMx (10 μg/ml) resulted in lower numbers of intracellular bacteria recovered than when the assay was performed with 200 μg/ml Gn (Figure 9). Because of this apparent intracellular killing ability of PMx, we chose treatment with 10 μg/ml PMx for 90 min during all PMx plate count assays. However, for the PMx quenching assays, 30 min treatment with 50 μg/ml was chosen, based on its similar killing effect, and efficient quenching of green fluorescent signal (data not shown), which is most important for these assays.

Gn/PMx exclusion assay (plate count method)

Comparison of the effects of Gn and PMx

Four Gn exclusion assays were performed using 200 μg/ml Gn, two with 400 μg/ml Gn, and three PMx exclusion assays with 10 μg/ml PMx. Associated bacteria include those adhered to and internalized into A549 cells, collected from lysed cells following incubation and washing away of excess bacteria. Internalized bacteria include only those which remain alive following antibiotic treatment because they are found within A549 cells where they are protected from the bactericidal effects of these cell-membrane impermeable antibiotics. Controls included bacteria and cells incubated together with antibiotics for the entire duration of the experiment (these background values were subtracted from experimental treatment values), and amount of bacteria surviving in the supernatant following antibiotic treatment (post-antibiotic supernatants) to verify antibiotic killing efficiency during experiments. Both concentrations of Gn showed very similar amounts of bacteria internalized (Figure 9), and the control values were similar. However, when

10 μg/ml PMx was used there were significantly fewer bacteria internalized (Figure 9). While all the antibiotics caused an approximately 5 log decrease in numbers of live bacteria in post-antibiotic supernatants, suggesting that they had similar killing efficiencies, the amount of bacteria internalized using the PMx exclusion assay was almost 2 log less than when Gn was used. When using 200 μg/ml Gn, the percentage of PAK associated (bound or internalized) with A549 cells after 60 min incubation was 12.55% (CFU/ml bacteria recovered from cells not treated with antibiotics ÷ CFU/ml bacteria added to A549 cells × 100%), while only 0.379% of these bacteria becoming internalized (CFU/ml bacteria recovered from inside A549 cells following antibiotic treatment ÷ CFU/ml bacteria associated with A549 cells × 100%). Results obtained when using 400 μg/ml Gn were similar, with 8.93% of bacteria became associated, and 0.410% of these becoming internalized.

Time Course

Using the Gn exclusion assay, PAK internalization into A549 cells was found to occur as quickly as 5 min, and to increase up to a maximum internalization after 60 min of incubation of cells with bacteria (Figure 10). Based on this time course, an incubation time of 60 min was chosen for all further studies of bacterial internalization.

Effects of Trypsin

While some groups use trypsinization followed by Triton X-100 treatment to completely remove and lyse infected cells (Zaas *et al.*, 2005), others use only Triton X-100 lysis (Plotkowski *et al.*, 1999; Gagniere & Di Martino, 2004; Leroy-Dudal *et al.*, 2004), and some do not specify. We therefore tested whether trypsin treatment had any effect on the bacterial counts. We

observed that 5 min treatment with 0.05% Trypsin-EDTA (Gibco) at 37°C prior to Triton X-100 had no effect on the amount of bacteria associated with A549 cells (Figure 11).

Effects of temperature

It has been suggested that incubating type I pneumocytes and *P. aeruginosa* at 4°C will result in only adherence of bacteria without their internalization (Zaas *et al.*, 2005). To test this hypothesis, we measured the amount of bacteria internalized after 60 min at 4°C using our standard protocols. We found that while incubation at 4°C resulted in fewer bacteria internalized, some internalization was still occurring (Figure 12). The amount of bacteria internalized was still 10^4 CFU/ml higher than the control condition of bacteria and cells incubated together with antibiotics for the entire experiment (these values are subtracted from all test conditions, and therefore not shown). Since internalization was observed as quickly as 5 min incubation, it is possible that the plates, cells and medium did not reach 4°C quickly enough to halt normal physiological processes. It is likely that pre-chilling of the plates and all reagents and completing all steps on ice would result in better inhibition of internalization. Because the conditions are inevitably different between 4°C and 37°C, and since we are mainly interested in bacterial internalization, it is preferable to conduct the experiments at 37°C. It is also interesting to note that association of PAK with A549 cells was visibly higher at 4°C than at 37°C. It is possible that chilling the bacteria to 4°C reduces their motility and results in settling out of the bacteria onto the cell surface, which would cause an increase in association.

Plate reader PMx quenching internalization assay on adherent A549 cells

Because traditional antibiotic exclusion assays are fairly laborious, and because we had possession of PAK*gfp*, we wanted to develop and optimize an assay that could measure the amount of internalized bacteria based on PMx quenching of the green fluorescent signal from extracellular PAK*gfp* while leaving intracellular bacteria fluorescent and detectable. We tested the utility of this PMx quenching internalization assay by measuring the green fluorescence of adherent cells incubated with PAK*gfp* under varying conditions. Incubation of adherent A549 with PAK*gfp* (MOI of 50:1) for 60 min caused approximately 400% increase in green fluorescence over the auto-fluorescence of the A549 cells alone (Figure 13). Following 30 min treatment with 50 μg/ml PMx, the increase in fluorescence was reduced to only 19%, which was still significantly higher than A549 cells alone ($p<0.05$). This means that of the total amount of bacteria associated with the A549 cells, approximately 4.5% are internalized.

PMx quenching internalization assay with adherent A549 cells trypsinized using flow cytometry

Flow cytometry was also tested as an alternative readout for measuring internalization based on PMx quenching. In this method, adherent cells were incubated with PAK*gfp* (MOI of 50:1) for 60 min, treated with 50 μg/ml PMx for 30 min, and then trypsinized to obtain a single cell suspension for use in flow cytometry. Cells incubated with PAK*gfp* followed by PMx treatment showed over 50% increase in fluorescence compared to cells alone or cells incubated with PAK*gfp* and PMx for the entire duration of the experiment (control) (Figure 14). Interestingly, cells which had only been trypsinized, but not treated with PMx following infection showed approximately 58% increased fluorescence, suggesting that trypsinization

effectively removes adherent extracellular bacteria, eliminating their *gfp* signal nearly as efficiently as PMx treatment.

Discussion

P. aeruginosa is a Gram-negative opportunistic pathogen ubiquitously present in the environment (water, soil and plants). Although this bacterium does not cause disease in people with normal host defences, it is the leading cause of ventilator-associated pneumonia in intensive care units (Crouch Brewer *et al.*, 1996), can cause acute life-threatening infections in immunocompromised patients and burn victims (McEachern & Campbell, 1998; Lyczak *et al.*, 2000), and is the major cause of chronic pulmonary infection in CF patients (Saiman & Siegel, 2004). Clinical outcomes of *P. aeruginosa* infection depend on the balance between host defence and bacterial virulence factors. Hence, the interactions of *P. aeruginosa* with lung epithelial cells are of major importance for understanding the pathogenesis of pulmonary disease caused by this pathogen.

P. aeruginosa express a wide range of virulence factors, such as the type III secretion system, endotoxins, exotoxins, and various enzymes, able to significantly compromise host defence and induce potent activation of inflammatory responses leading to profound tissue damage (Pollack, 1984). The mechanisms of microbial pathogenesis in *P. aeruginosa* pulmonary disease are not completely understood. Several methods have been developed to investigate how *P. aeruginosa* interact with host cells to cause infection. Traditional adhesion and invasion assays involve lysing host cells after incubation with bacteria, followed by plating and enumeration of recovered bacteria. This approach inevitably requires killing of the host cells, and incubation of the bacteria overnight before results can be obtained. Other methods include

scintillation counting of radioactive-labelled bacteria, and visualization and quantification of bacteria based on either antibody labelling or plasmid-based fluorescent labelling of the bacteria (Saiman & Prince, 1993; Feldman *et al.*, 1998; Rajan *et al.*, 2000; Pils *et al.*, 2006). The downsides of these methods include the hazards of working with radioactive probes, the potential error and variability associated with the extra step of antibody labelling, and the necessity of antibiotic use to maintain the plasmid.

In the system we have optimized, the stable chromosomal insertion of the fluorescent marker allows detection of labelled bacteria without using antibody for immunostaining. Also, adding antibiotics to select bacteria based on antibiotic resistance markers is not required. This system provides the benefits of rapid detection and screening of adherence using a microplate reader assay similar to the method described by Swanson *et al.* (2003) which uses a luminescent system. However, the methods combined in our study are superior because data can be verified by flow cytometry as well as by visualization with fluorescence microscopy. In addition, these techniques allow for rapid detection of cell-associated bacteria without killing the host cells, and can therefore be used for real-time analysis of the kinetics of host-pathogen interactions. Hence, the creation of a stable chromosomally labelled *gfp*-expressing *P. aeruginosa* PAK has provided a simple and reliable way to study the interactions between this opportunistic bacterium and its target cells. The chromosomal *gfp* labelling of *P. aeruginosa* is also an attractive research tool for studies of bacterial interaction with intracellular signalling molecules using confocal analysis. In addition, this system can be easily applied for *in vivo* studies in animal models of *P. aeruginosa* lung infection with the advantage of direct visualization of labelled bacteria in various tissues in a real-time mode, similar to the method used by Valdivia *et al.* (1996) to detect *gfp* labelled *Mycobacterium marinum* in frog spleens and livers.

Using the extracellular matrix protein Fn, we verified that the effects of exogenous substances on *P. aeruginosa* association with A549 cells could be readily detected using this system. Fn is a dimeric, modular glycoprotein that can be recognized and bound by certain microbial surface structures and host cell integrin receptors (Potts & Campbell, 1996). It has been shown for several human pathogens that Fn plays an important role in bacterial pathogenesis. For example, both *Streptococcus pyogenes* and *Staphylococcus aureus* posses Fn binding proteins that interact with the N-terminal 29-kDa domain of Fn, and exploit this mechanism to invade host cell via α5β1 integrin receptors (Schwarz-Linek *et al.*, 2004; Hauck & Ohlsen, 2006). Adding excess Fn was shown to increase *S. aureus* binding to host cells (Sinha *et al.*, 1999). Although the ability of *P. aeruginosa* to bind to Fn was recognized (Plotkowski *et al.*, 1993; Rebiere-Huet *et al.*, 2004), its significance for bacterial pathogenesis is unclear. The importance of Fn and α5β1 integrins for *P. aeruginosa* strain PAK adhesion to injured lung epithelial cells undergoing the process of reparation was demonstrated by Roger *et al.* (1999), but other studies could not confirm this observation (Gagniere & Di Martino, 2004; Leroy-Dudal *et al.*, 2004). Such discrepancies could be due to differences in bacterial strains or in experimental methods. We found that *P. aeruginosa* strain PAK specifically bound to Fn, and that the presence of exogenous Fn in a serum-free system caused an increased association of PAK*gfp* with A549 cells. In contrast, exogenous Fn did not increase bacterial association with adherent A549 cells suggesting that in this system, epithelial cells could express endogenous Fn. Indeed, A549 cells in culture are able to produce Fn and express it on the surface (Kang *et al.*, 1991). These observations emphasize the importance of using different methods for accurate data interpretation. Although we found similar time course and dose response trends for bacterial association with A549 cells in adherent culture and in suspension, we consider the

system with adherent cells biologically more relevant. Indeed, an additional substrate (Fn) was not required for the maximal association of *P. aeruginosa* with A549 cells. This emphasizes the fact that *P. aeruginosa* – lung epithelial cell interactions in adherent culture better represent *in vivo* microbial pathogenesis.

While developing the bacterial internalization assay, we observed discrepancies in the number of internalized *P. aeruginosa* depending on the antibiotic used. These discrepancies may be due to the killing efficiency of the two antibiotics. The results of bactericidal tests demonstrated that PMx is effective in killing PAK at lower concentrations than Gn (discussed in Results section: Comparison of the effects of Gn and PMx). Therefore it is possible that PMx might result in a more complete elimination of extracellular bacteria. However, Gn and PMx show similar amounts of PAK survival in post-antibiotic supernatants (Figure 9), suggesting that both treatments kill the bacteria effectively. When using 200 μg/ml Gn, our assay showed approximately 12.55% of added bacteria becoming associated with the cells, which is comparable to literature date [i.e. 8.6% PAO1 associated with Type I pneumocytes, (Zaas *et al.*, 2005)]. However, only 0.379% of these bacteria were internalized. Our results do not agree with a study by Gagniere and DiMartino (2004) that used the same A549 cells and *P. aeruginosa* PAK, since they showed only 1.1% of added bacteria adhered, while 28% of those associated bacteria were internalized, using a lower MOI of 30:1 and an incubation time of 3 h. This increased incubation time could allow more bacteria to become internalized. However, in our experiments we observed that incubation of A549 cells with *P. aeruginosa* at an MOI of 50:1 for any longer than 90 min caused many of the A549 cells to detach from the culture plate surface. Based on the fast proliferation of PAK in the culture medium, it seems hard to believe that even with an MOI of 30:1 the A549 cells would still be viable and adherent after 3 h of infection.

Because the amounts of bacteria internalized when using 400 μg/ml Gn were very similar to 200 μg/ml (Figure 9), we decided to use 200 μg/ml because it is preferable to use the lowest effective concentration. In agreement with this, most of the literature uses lower concentrations of Gn, even as low as 50 μg/ml (Zaas *et al.*, 2005).

Although PMx has been successfully used in other studies for plate count based internalization assays (Gophna *et al.*, 2001; Schaeffer & Weiss, 2001; Gophna *et al.*, 2002), we were unable to achieve reliable results using the PMx exclusion assay. We observed that PMx treatment following infection of A549 cells with PAK results in recovery of significantly fewer bacteria than Gn treatment (Figure 9). Because both antibiotics have similar killing efficiencies as demonstrated by post-antibiotic supernatant controls, we have to conclude that either PMx is crossing the cell membrane and killing intracellular bacteria, or that residual antibiotics are killing intracellular bacteria following cells lysis. It is possible that PMx is more difficult to completely remove from the medium and cells following antibiotic treatment, and that A549 cells should be washed more than 3 times with PBS prior to lysis. Because of these discrepancies, we decided not to use PMx for plate count based antibiotic exclusion assays.

On the other hand, the PMx quenching internalization assay appeared to be a fast and useful method to assess bacteria internalization into host cells. Combined results on adherent cells analyzed using both the plate reader and flow cytometer show that PMx treatment resulted in quenching of green fluorescent signal from extracellular bacteria, while leaving intracellular bacteria fluorescent, or at least leaving the intracellular *gfp* signal intact (Figure 13 and 14). Although it is possible that intracellular bacteria were no longer viable (i.e. have been killed by the PMx) their green fluorescent signal remained detectable because the gfp was retained inside the A549 cells. The plate reader data (Figure 13) shows that although PMx treatment caused a

decreased fluorescence signal, it still did not reach the level of the control. Indeed, while fluorescence increased by 400% due to total associated bacteria, the fluorescence increase due to internalized bacteria was only 19%. This means that the residual fluorescence caused by internalized bacteria was about 4.5% of the total fluorescence of associated bacteria, which agrees with the finding by Zaas *et al.* (2005) that 4.5% of total associated bacteria become internalized. The increase in fluorescence due to internalized bacteria measured by the flow cytometer was approximately 52% using PMx, and 58% using trypsinization only (Figure 14). With the flow cytometry system, we are not able to measure total bacteria associated because of the necessary trypsinization step following infection of cells with bacteria to obtain a single cell suspension. This means that the plate reader and flow cytometer results are not directly comparable. Although both systems are based on detection of green fluorescent signal, they have different detection setups and amplifications, and they are measuring cells in different forms – adherent versus suspended. Nevertheless, while only the plate reader can directly detect changes in amount of bacteria associated with cells, both systems can be used to detect the effects of inhibitors or promoters of internalization by comparing the change in fluorescence of treated and untreated cells after incubation with PAK*gfp*.

The PMx quenching internalization assay appears to be a fast and easy tool to measure PAK*gfp* internalization into host cells. Experiments can be performed in tandem using the plate reader and flow cytometer to measure the effects of various substances or treatments on bacterial internalization. Since neither of these methods give absolute numbers, it may still be beneficial to verify any results obtained using the standard Gn exclusion assay. Based on the difficulty of obtaining reliable results using PMx in place of Gn for plate count based methods, it is advisable to use the more standard and widely accepted Gn methods, with the Gn susceptible PAK WT. It

may be advantageous to use the fluorescence based PMx quenching internalization assay as an initial diagnostic tool, followed by verification with the traditional Gn based plate count method. Although we cannot directly match up numbers or percentages between the plate count and fluorescence based methods, we can compare relative effects and trends observed using both systems.

To the best of our knowledge, this is the first application of a Tn7-based transposon system for biomedical studies on *P. aeruginosa*. As we demonstrated, the stable chromosomal labelling of *P. aeruginosa* with *gfp* can be successfully used to study bacteria-host interactions using various read-out systems, such as flow cytometry, fluorescence microscopy, and fluorometric plate-reader based assays. This *gfp*-based system can now be used to investigate the mechanisms of pulmonary disease caused by *P. aeruginosa* ultimately aimed at the discovery of new therapeutic targets. In this direction, testing various inhibitors of bacterial adhesion to lung epithelial cells both *in vitro* and *in vivo* can be successfully carried out using *P. aeruginosa* chromosomally labelled with the green fluorescent protein.

Figure 1

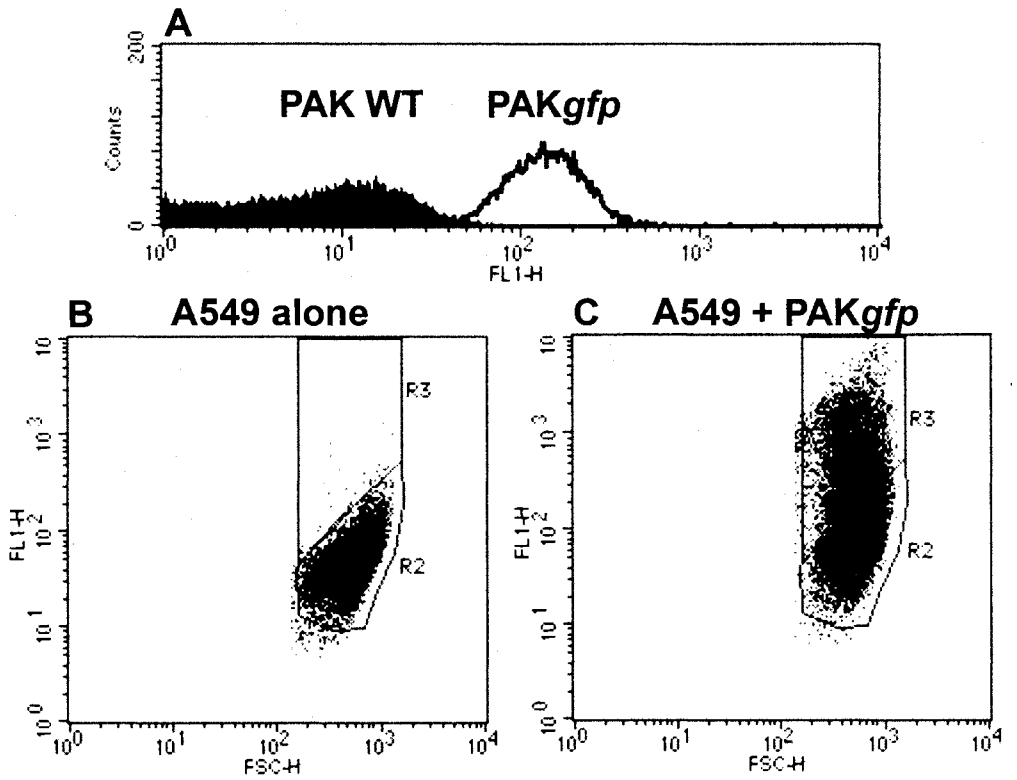


Figure 1. A) Flow cytometry discriminates PAK*gfp* from PAK WT based on fluorescence intensity (FL1). Instrument settings were optimized to detect bacteria. B) Flow cytometry on A549 cells using FSC threshold to exclude free bacteria. R2 = region where events from R1 (the main population of cells based on FSC and SSC) are displayed as FL1 versus FSC (90 min incubation in adhesion buffer). C) Following 90 min incubation of A549 cells with PAK*gfp*, a portion of the population shifts into a new region R3 comprising cells of the same size (FSC), but higher fluorescence (FL1).

Figure 2

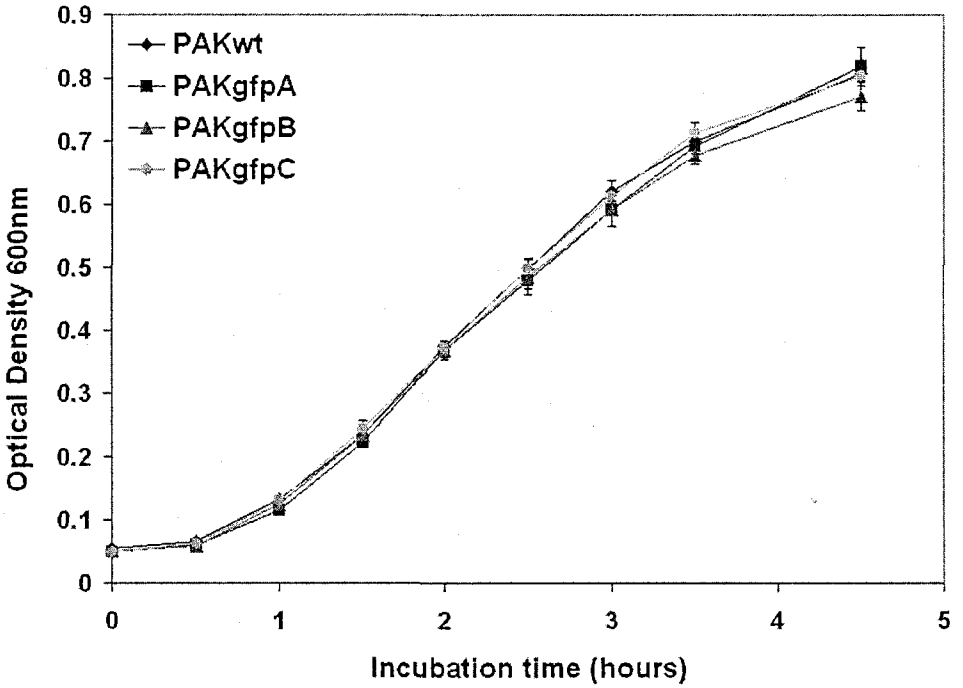


Figure 2. Growth curves for three successfully transformed mutant PAK*gfp* strains as compared to PAK WT (LB medium, 37°C). Growth was measured by monitoring optical density (at 600 nm) of each strain every 30 min until a plateau phase was reached (each strain was grown in triplicate).

Figure 3

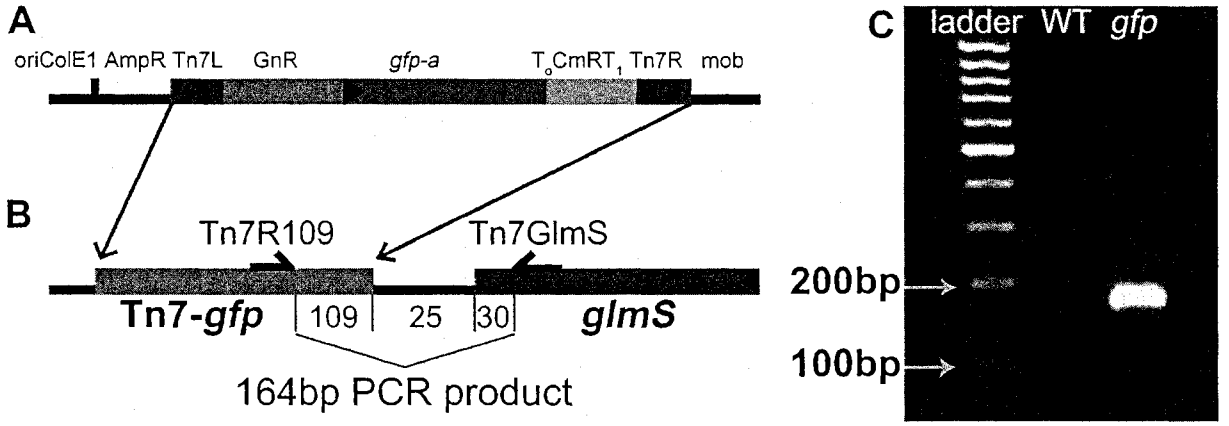


Figure 3. A) Structure of the pMiniTn7(Gm)$P_{PA1/04/03}$gfp-a transposon plasmid (Dr. Søren Molin, Technical University of Denmark, Lyngby) that was introduced into PAK. Tn7R and Tn7L = transposon flanking sequences, GnR = gentamicin resistance gene, *gfp-a* = green fluorescence protein gene under control of constitutively expressed $P_{PA1/04/03}$ promoter, T_0CmRT1 = termination sequence. B) Schematic of transposon insertion site, 25 bp downstream from *glmS*. Tn7R109 and Tn7GlmS are PCR primers used to verify correct insertion. C) One % agarose electrophoresis gel showing presence of the 164 bp PCR product in PAK*gfp*, and its absence in PAK WT (marker = 100bp ladder).

Figure 4

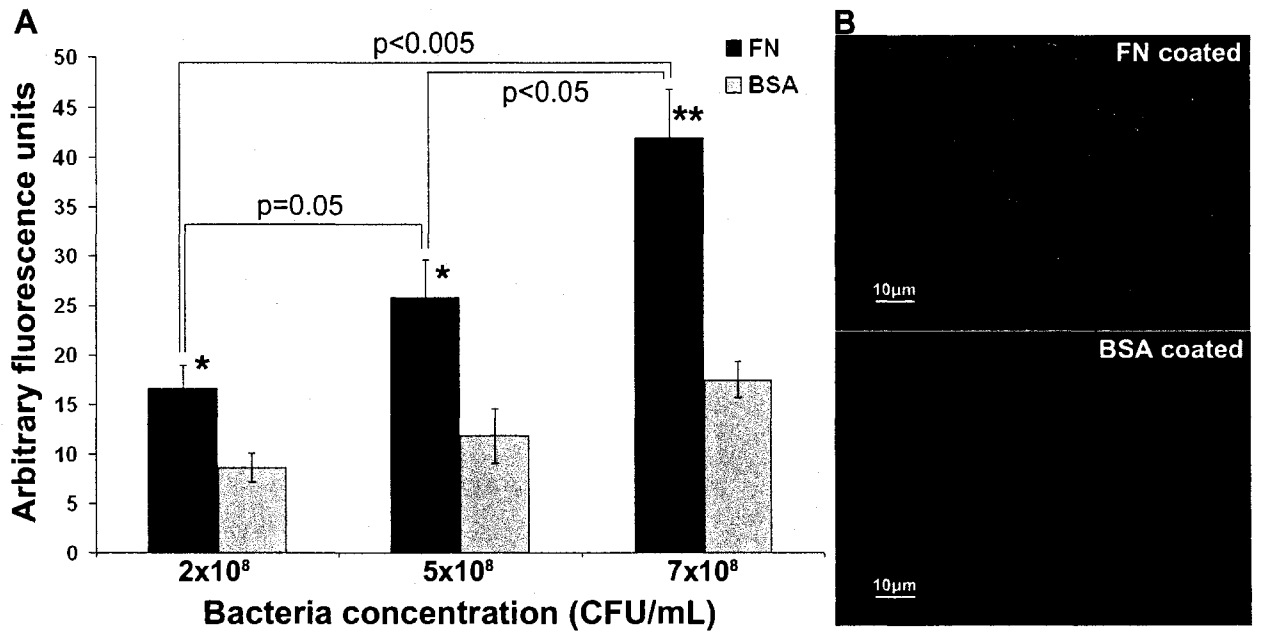


Figure 4. A) Green fluorescence as a result of PAK*gfp* binding (5 min) to Fn- (20µg/ml) or 1% BSA- coated wells as measured by a fluorometric microplate reader. At each concentration, more bacteria bound to Fn than to BSA (n = 3 independent experiments, 6 wells per treatment; *, $p<0.05$, **, $p<0.01$). B) Fluorescent microscopy images also show that more PAK*gfp* (at 2×10^8 CFU/mL) adhere to Fn- than to BSA-coated glass coverslips (magnification 1000×). Images are representative of n = 5 independent experiments.

Figure 5

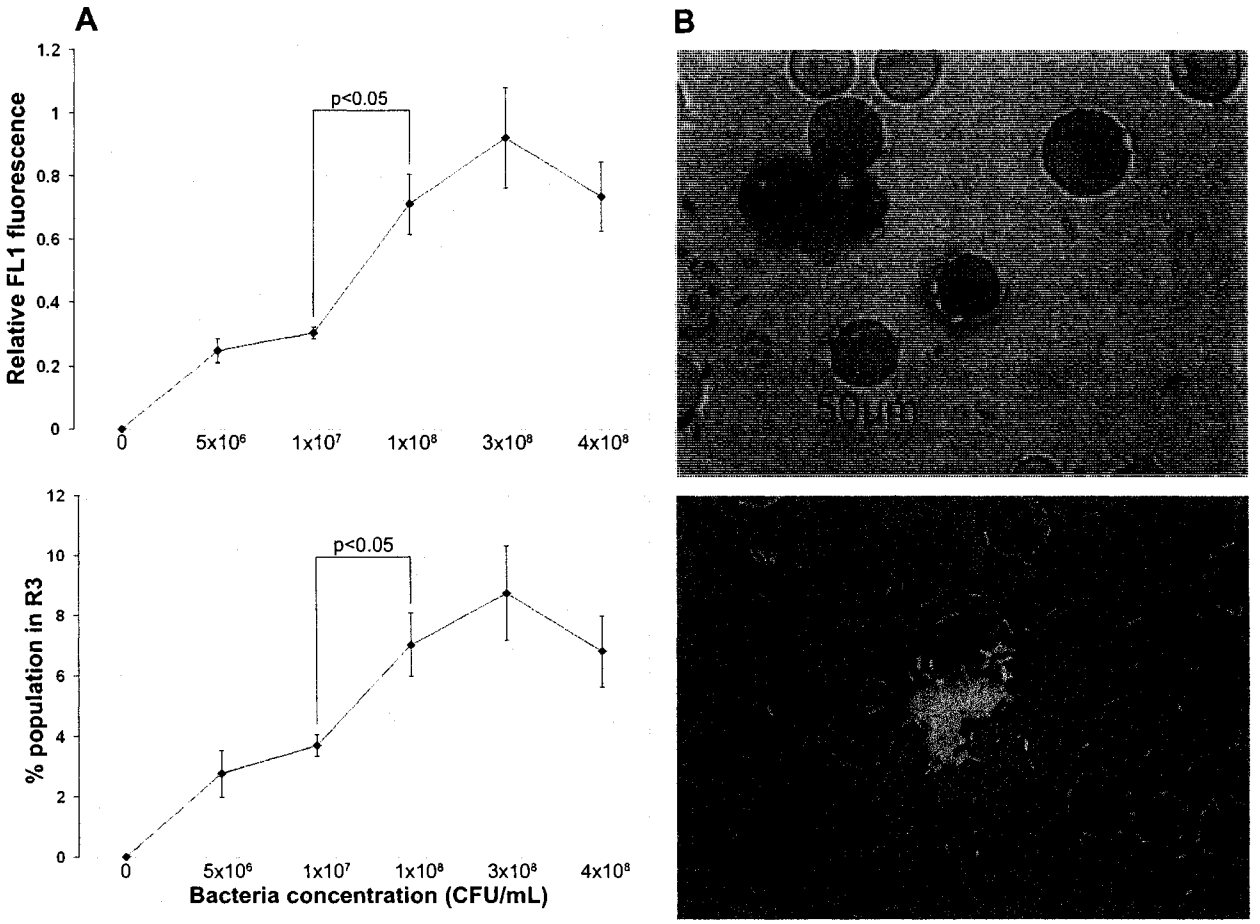


Figure 5. A) Flow cytometry analysis shows dose-dependent binding of PAK*gfp* to A549 cells (90 min incubation, n = 4 independent experiments). B) Combined phase contrast and fluorescence images of PAK*gfp* binding to A549 cells (PAK*gfp*:A549 ratio = 100:1, 90 min incubation in suspension; 1000× magnification). A549 cells appeared either with bacteria attached to surface (top) or "bursting" after bacterial invasion (bottom). Images are representative of n = 3 independent experiments.

Figure 6

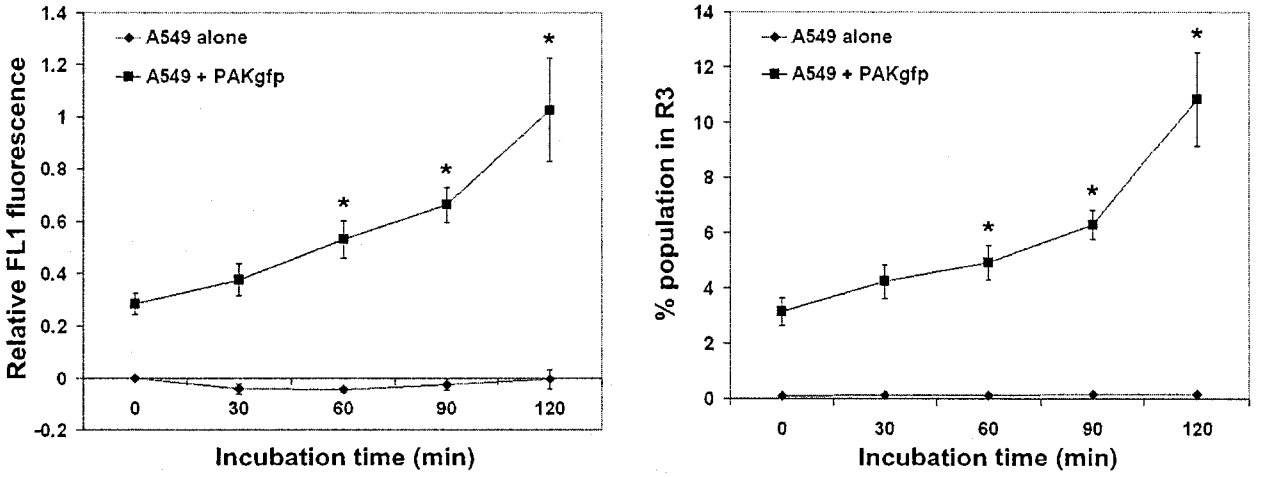


Figure 6. Flow cytometry analysis on A549 cells incubated with PAK*gfp* (MOI of 100:1) for various times. The relative FL1 increase and % R3 were higher for A549 cells incubated with bacteria than for A549 alone ($p<0.01$ for all time points). Binding of PAK*gfp* to A549 cells was significantly higher at 60, 90 and 120 min compared to 0 min (*, $p<0.05$; n = 5 independent experiments).

Figure 7

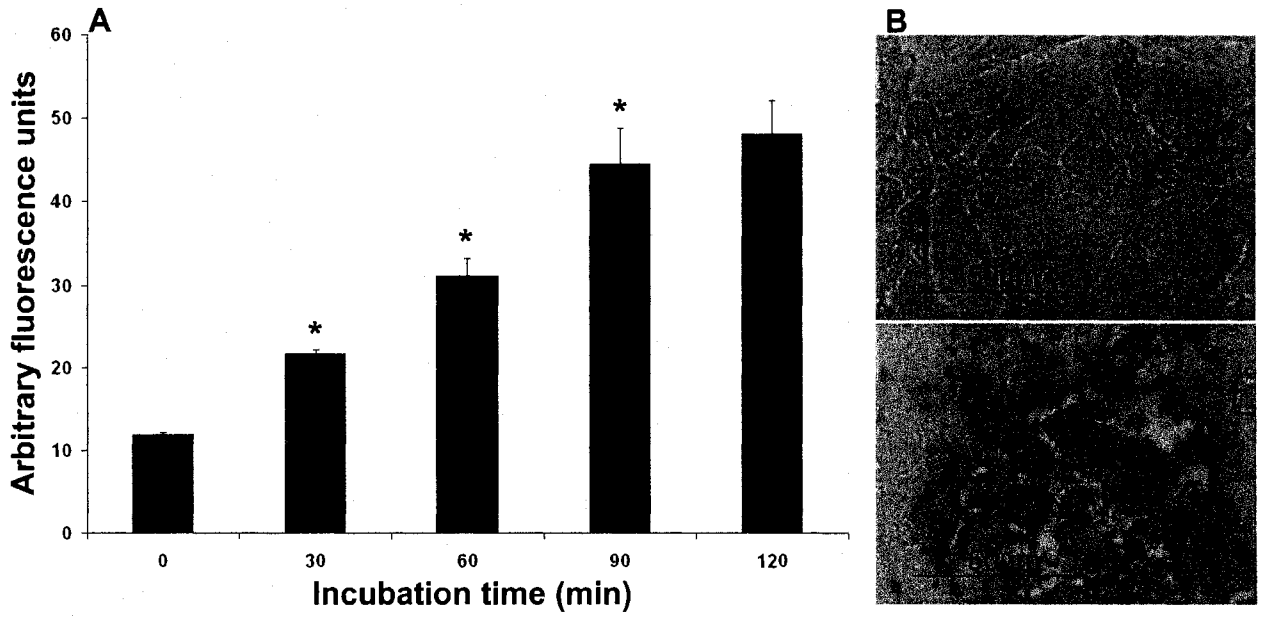


Figure 7. A) Binding of PAK*gfp* to adherent A549 as detected by a plate-reader fluorometric assay. At all time points, significantly higher fluorescence was detected compared to 0 min ($p<0.05$), and each consecutive time point showed significantly higher fluorescence that the previous one (*, $p<0.05$), except for 90 to 120 minutes ($p>0.05$) (n=3 independent experiments). B) Combined phase contrast and fluorescence images of PAK*gfp* binding to adherent cells show areas of moderate (top) or intense binding (bottom). Experiments were performed using an MOI of 100:1, and 90 min incubation (magnification 1000×).

Figure 8

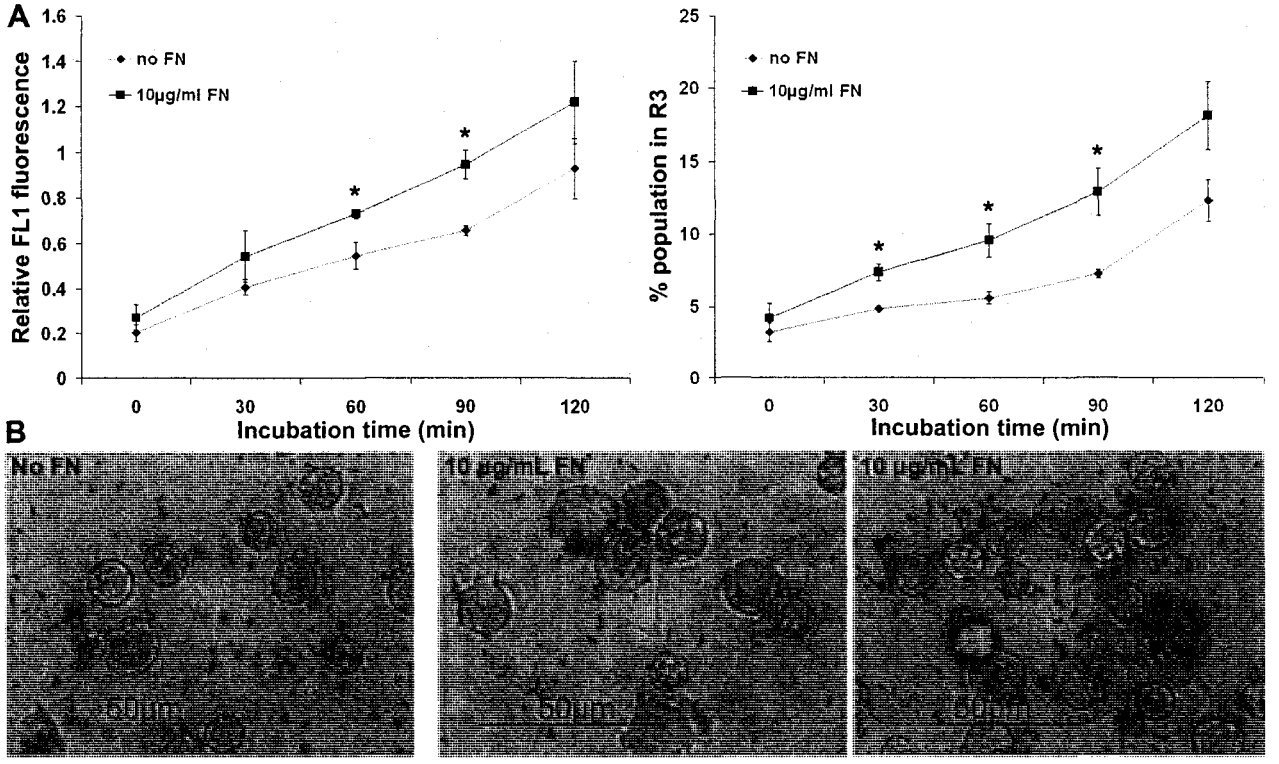


Figure 8: A) Flow cytometry analysis shows that pre-incubation of suspended A549 cells with fibronectin increases PAK*gfp* adherence, measured as both relative FL1 fluorescence and % population in R3 (PAK*gfp*:A549 ratio = 100:1, n = 3 independent trials, *, $p<0.05$). B) Combined phase contrast and green fluorescence microscopy images of cells prepared for flow cytometry shows more clustering of bacteria around the cells when FN is present (magnification 1000×, 90 min incubation). Images are representative of n = 2 independent experiments recorded by a blinded investigator.

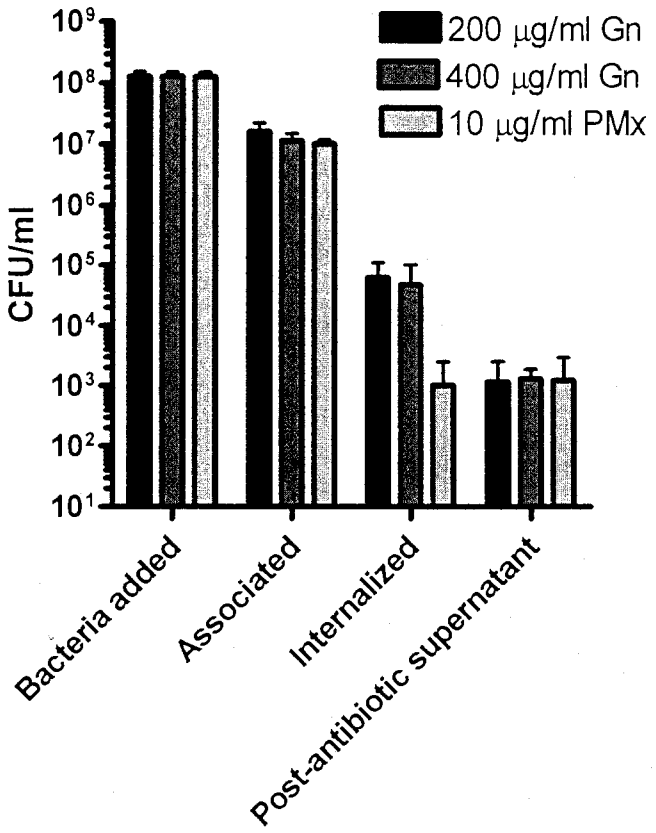


Figure 9: Gn/PMx plate count based internalization assay. PAK association with and internalization by adherent A549 cells (MOI of 50:1) was measured after 60 min incubation at 37°C. Cell associated bacteria quantified from A549 lysates following infection and washing were significantly higher than the amount of bacteria internalized using all antibiotic conditions tested (t-test, $p < 0.01$). Treatment of the PAK-A549 cell mixture with antibiotics for 90 min to kill extracellular bacteria showed that Gn at 200 or 400 μg/ml resulted in similar bacterial internalization, while PMx (10 μg/ml) treatment caused significantly less internalization than either Gn treatment (t-test, $p < 0.05$). As a control for bacterial survival during antibiotic treatment, bacteria were collected from culture supernatants following antibiotic incubation (post-antibiotic supernatants). The number of bacteria in post-antibiotic supernatants did not differ between antibiotics tested, indicating they all have similar killing efficiencies. (n=4 independent experiments for 200 μg/ml Gn, n=2 for 400 μg/ml Gn and n= 3 for 10 μg/ml PMx each performed in triplicate).

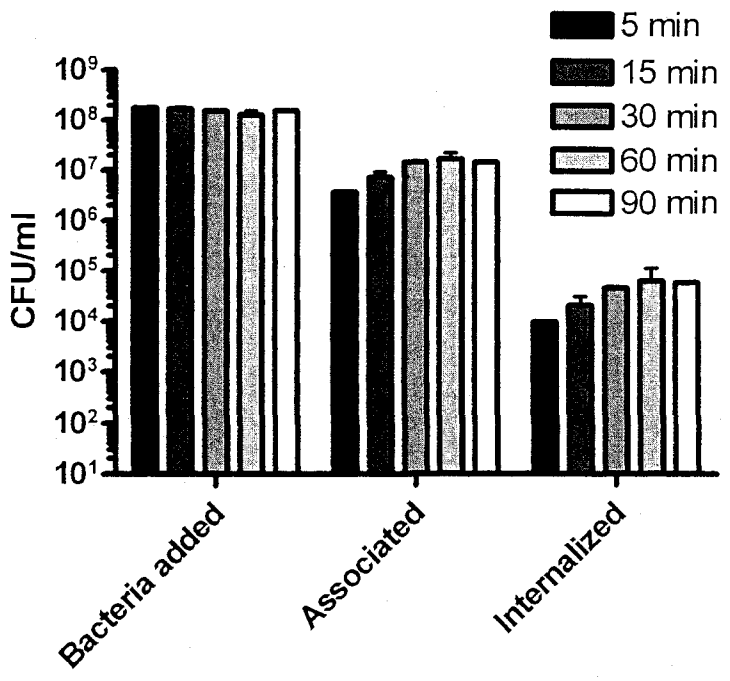


Figure 10: Gn exclusion assay time course. PAK internalization time course was investigated by performing the Gn exclusion assay with 200 μg/ml at various incubation times. Maximal association and internalization were observed at 60 min, and this time point was chosen for all subsequent experiments. (n=1 experiment for 5, 30 and 90 min, n=2 independent experiments for 15 min and n=4 independent experiments for 60 min, each performed in triplicate).

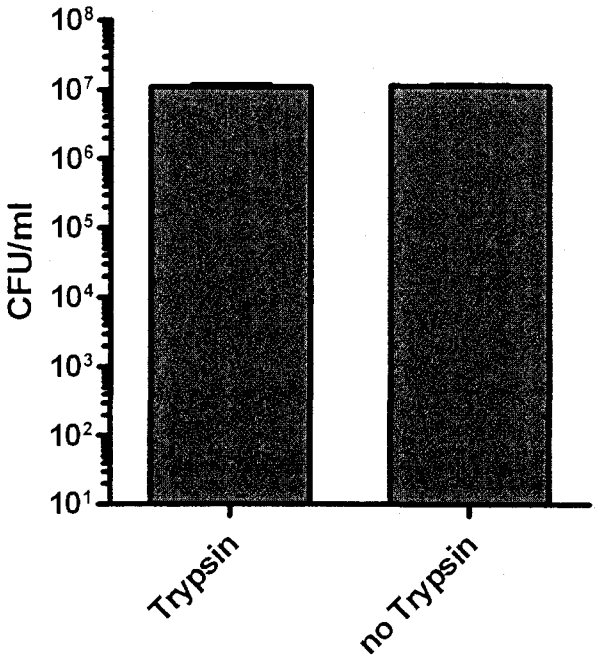


Figure 11: Effects of trypsinization on PAK association with A549 cells. Association of PAK with A549 cells (MOI of 50:1) was compared when cell lysis with Triton X-100 was preceded with trypsinization for 3 min at 37°C, and when it was not. Trypsinization did not appear to have any effect on bacterial association with A549 cells. (n=one experiment for each, performed in triplicate).

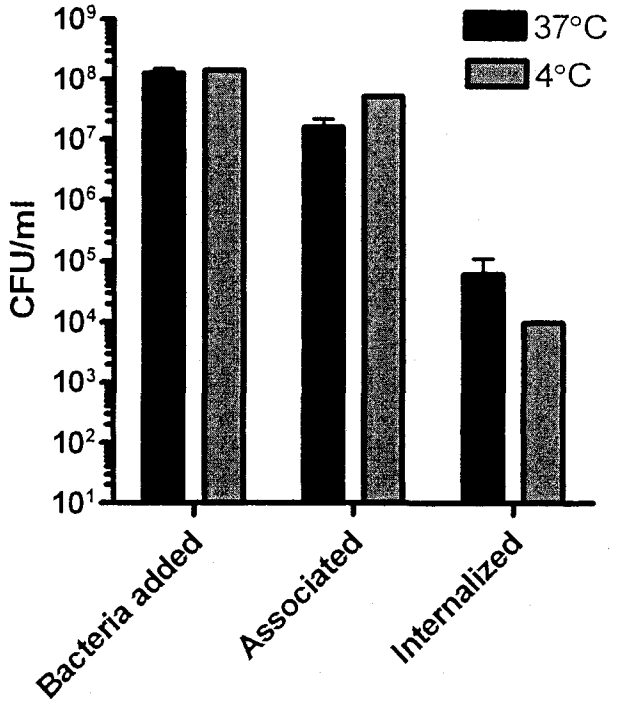


Figure 12: Effects of temperature on PAK internalization into A549 cells. Internalization of PAK into adherent A549 cells (MOI of 50:1) after 60 min was investigated at 37°C and 4°C to test the effect of decreased temperature on bacterial internalization. The Gn exclusion assay was performed at both 4°C and 37°C using 200 μg/ml Gn treatment for 90 min. Decreased internalization was observed at 4°C, but it was still 4 log higher than the control for internalization (bacteria and A549 cells incubated with antibiotic for the whole duration of the experiment, set to zero on the graph). (n=1 experiment for 4°C and n=4 independent experiments for 37°C, each performed in triplicate).

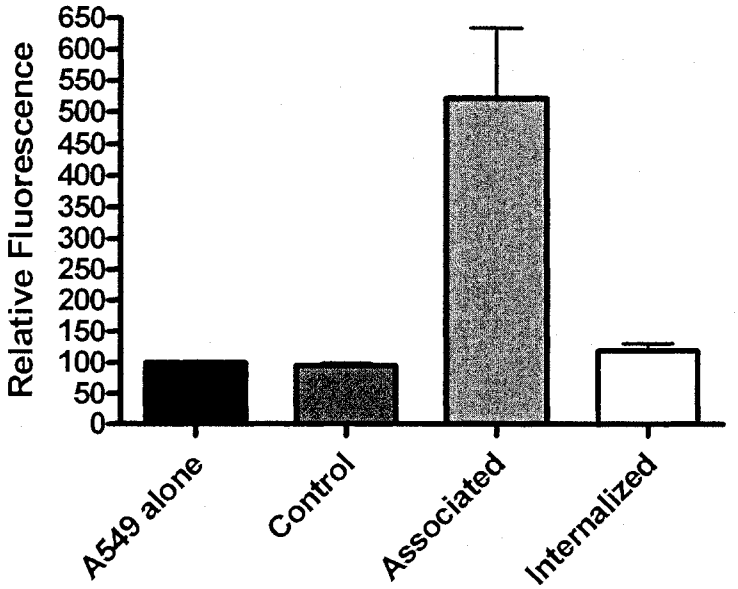


Figure 13: PMx quenching internalization assay with adherent A549 cells and plate reader. Adherent A549 cells incubated with PAK*gfp* (MOI of 50:1) for 60 min, and treated with 50 μg/ml PMx for 30 min were analyzed for increase in fluorescence compared to A549 cells alone using fluorescence based plate reader. Cells incubated with PAK*gfp* (indicating total associated bacteria) showed significantly higher fluorescence than cells alone or control (cells incubated with bacteria and PMx for the duration of the experiment), and cells incubated with PAK*gfp* and treated with PMx (representing internalized bacteria) also showed significantly higher fluorescence than cells alone (19% higher). (n=3 separate experiments, each performed in triplicate. One sample t-test comparing means to hypothetical mean of 100, $p < 0.05$).

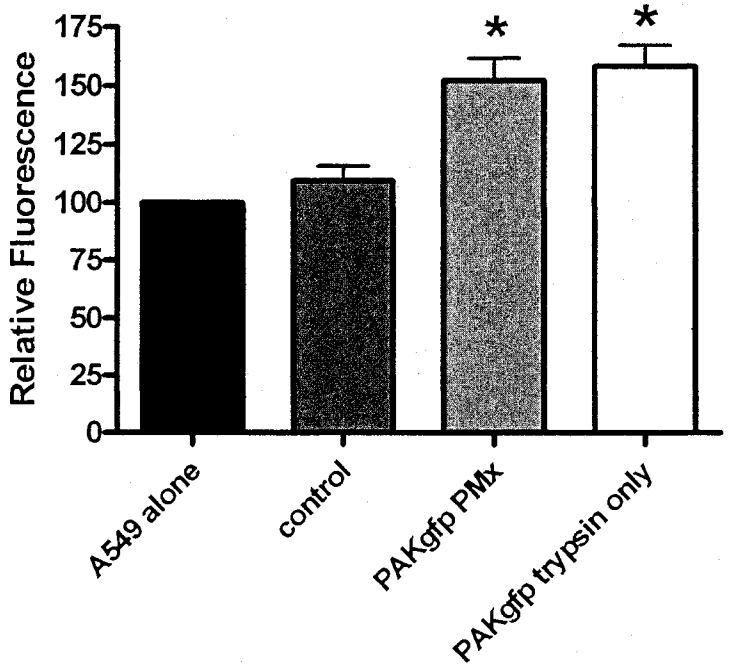


Figure 14: PMx quenching internalization assay using adherent A549 cells and flow cytometry as a readout. Adherent A549 cells incubated with PAK*gfp* (MOI of 50:1) for 60 min followed by treatment with 50 μg/ml PMx for 30 min were analyzed for increase in fluorescence over A549 cells alone using flow cytometry. The fluorescence of A549 cells alone and A549 cells incubated with PAK*gfp* and PMx for the duration of the experiment (control) were not significantly different, while cells incubated with PAK*gfp* and then treated with PMx (50 μg/ml PMx for 30 min), and cells incubated with PAK*gfp* and trypsinized without any PMx treatment (both representing internalized bacteria) showed more than 50% increase in fluorescence over cells alone and control. (n=5 independent experiments, $p < 0.05$, Student's t-test).

Chapter 2: Effect of substrates, RGD peptides, and antibodies on *P. aeruginosa* adhesion and invasion

Abstract

Several pathogens including *Staphylococcus aureus* and *Bordetella pertussis* have been shown to use lung epithelial integrins during pulmonary infection, and it has been suggested that *P. aeruginosa* also has the ability to exploit integrins, although the mechanisms and importance of these interactions with integrins are unclear. A green fluorescently labeled *P. aeruginosa* strain PAK (PAK*gfp*) was used to study the involvement of integrins in host-pathogen interactions using fluorescence based read-outs including flow cytometry and a plate reader-based assay. The ability of PAK*gfp* to bind various integrin ligands (ECM products), and the inhibitory effect of RGD-based peptides and anti-integrin antibodies on PAK*gfp* interactions with A549 lung epithelial cells were investigated. PAK*gfp* was found to preferentially bind Fibronectin and Vitronectin, the ligands for $\alpha5\beta1$ and $\alpha v\beta5$ integrins respectively, but only Fn was able to increase bacterial association with A549 cells. The RGD-based inhibitory peptides and anti-integrin antibodies did not affect PAK*gfp* interactions with A549 cells. However, *P. aeruginosa* appear to possess IgG binding capabilities, which would interfere with results obtained from antibody inhibitor studies. The ability of Fn to bind PAK*gfp* and to increase association with A549 cells suggests that integrins, particularly $\alpha5\beta1$, are involved in *P. aeruginosa* interactions with host cells, however more investigation is required before further conclusions can be made.

Introduction

Integrins

Integrins are a large family of αβ heterodimeric transmembrane receptors that interact with components of the extracellular matrix (ECM) and some cell-surface receptors. In humans there are 18 α and 8 β subunits which form 24 different heterodimers (reviewed by Arnaout *et al.*, 2005). Large extracellular domains of integrins mediate interactions with extracellular ligands, while the cytoplasmic domains mediate communications with the cytoskeleton and signaling molecules (reviewed by Luo *et al.*, 2007). Based on the crystal structure resolution of the αvβ3 integrin extracellular domain, ligand recognition is mainly mediated by a cationic binding site on the beta subunit adjacent to the exposed alpha subunit (Xiong *et al.*, 2001; Xiong *et al.*, 2002). In addition, half of the 18 α subunits contain a 200 amino acid inserted (I) domain which contributes to ligand recognition and specificity (Lee *et al.*, 1995; Luo *et al.*, 2007).

During the last decade, the role of integrins in interactions of various cells with their microenvironment has become a focus of intensive research. Recent studies on monocytes, neutrophils, platelets, fibroblasts, endothelial, and pulmonary as well as intestinal epithelial cells (EC) demonstrated integrin involvement in regulation of virtually all vital cellular functions, including cell survival, proliferation, differentiation, migration, and cytokine production (reviewed by Giancotti & Ruoslahti, 1999; Sheppard, 2003; Arnaout *et al.*, 2005). Upon binding their extracellular ligands, integrins transmit outside-in signals that regulate various cellular functions. In addition, integrins are able to provide inside-out signaling regulating the affinity of integrin binding to its ligand, and such signaling can be induced by cellular activation with chemokines or cytokines. Hence, integrins act as bidirectional signaling molecules (Arnaout *et al.*, 2005). Several signaling pathways activated by integrin engagement were identified, such as

mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3-K) pathways (Clark & Brugge, 1995; Miranti & Brugge, 2002). Integrins are involved in focal adhesion complexes comprising over 20 signaling and adaptor proteins, regulating actin cytoskeleton rearrangement and cell motility (Geiger & Bershadsky, 2001). Binding of an integrin receptor to its ligand results in large-scale conformational changes, such as separation of the cytoplasmic domains of the α and β subunits which causes cytoskeletal rearrangements and activation of downstream signaling (Kim *et al.*, 2003). According to the current concept, integrins act as specific sensors for dynamic changes in the microenvironment that occur during tissue development, inflammation, and tumorigenesis, and modulate cellular responses to these changes (Giancotti & Ruoslahti, 1999; Miranti & Brugge, 2002; Sheppard, 2003).

Integrin receptors of leukocytes are vital in both innate and adaptive immune responses. In particular, α2 integrins, such as LFA-1 (αLβ2) and Mac-1 (αMβ2), are essential for the activation of lymphocytes and for leukocyte migration during inflammatory responses. Congenital deficiency in β2 integrins (i.e. the leukocyte adhesion deficiency), is characterized by recurrent, severe bacterial infections that are eventually fatal (Bunting *et al.*, 2002). Recent studies have emphasized the importance of leukocyte integrins in the cross-talk with immunoreceptors, including T-cell receptor and Fc-receptors, for immune responses (Abram & Lowell, 2007). However, the role of epithelial integrins in innate immune and inflammatory responses in mucosal tissues, such as pulmonary epithelium, remains poorly understood.

Integrin receptors in the lung

Epithelial cells are currently recognized as primary elements generating inflammatory signals to activate other cells in the lung (Diamond *et al.*, 2000). Pulmonary epithelial cells express an array of innate immune receptors, such as toll-like receptors (TLR), as well as

cytokine, growth-factor, histamine receptors *etc* involved in the regulation of dynamic interactions of the epithelium with the environment. Integrin receptors are significantly represented in pulmonary epithelium. Eight different integrin heterodimers are expressed in airway epithelial cells, i.e. $\alpha2\beta1$, $\alpha3\beta1$, $\alpha6\beta4$, $\alpha9\beta1$, $\alpha5\beta1$, $\alpha v\beta5$, $\alpha v\beta6$, $\alpha v\beta8$ (Sheppard, 2003). These heterodimers recognize a range of ECM proteins: collagen I, tenascin C, laminins (Lmn) 5, 10, 11, osteopontin, fibronectin (Fn), vitronectin (Vn), and others (Sheppard, 2003). It is known that lung epithelial integrins are critical for maintaining epithelial integrity, repair of damaged cells, and regulation of cell differentiation and proliferation (Sheppard, 2001; Sheppard, 2003). The expression of integrin receptors in the respiratory epithelium is tightly regulated, and rapid increase in $\alpha5\beta1$ integrin level in response to injury has been demonstrated (Pilewski *et al.*, 1997). Accordingly, integrin receptor ligands such as Fn, Vn, tenascin C, and osteopontin, are rapidly induced at sites of epithelial damage or injury (Sheppard, 2003).

Despite the significant advances in the understanding the functions of pulmonary integrins, signaling pathways regulated by these receptors in the lung are still incompletely characterized. Lung epithelial integrins are known to provide co-stimulatory signals towards growth factor receptors, regulating cell survival and proliferation (reviewed by Sheppard, 2003). However, the co-stimulatory functions of pulmonary integrins appear to be wider and involve the cross-talk with other receptors. It has recently been found that $\beta1$ integrins in human bronchial epithelial cells provide co-stimulatory signals that increase TNF-induced pro-inflammatory responses (Ulanova *et al.*, 2005). Interestingly, integrin-mediated responses in these cells involved activation of the non-receptor protein tyrosine kinase Syk recently discovered in the respiratory epithelium (Ulanova *et al.*, 2005).

The role of integrin receptors in recognition of pathogenic microorganisms

Several significant human pathogens are known to utilize integrins and exploit integrin-mediated signaling to invade various types of host cells. Such mechanisms can be advantageous to the microorganisms, because the invasion of host cells often confers protection against the immune response, and may facilitate microbial growth and spreading to other cells. On the other hand, the resulting integrin-mediated signaling is potentially important for innate immune and inflammatory responses to the pathogen. As a variety of pathogens (bacteria, viruses, and fungi) bind integrins and elicit integrin-mediated signaling, it seems likely that integrins may serve as pathogen recognition receptors.

Several pathogenic bacteria are able to bind integrin receptors directly, via some specific adhesins. These are typically not respiratory pathogens, but rather those that invade other mucosal tissues such as the gastro-intestinal epithelium [*Yersinia enterocolitica, Y. pseudotuberculosis* (Isberg & Leong, 1990; Rankin *et al.*, 1992; Gustavsson *et al.*, 2002; Schmid *et al.*, 2004) and *Helicobacter pylori* (Kwok *et al.*, 2007)], or urethral epithelium [*Neisseria gonorrhoeae* (Edwards & Apicella, 2005)]. The best studied example of bacteria directly binding and exploiting integrin-mediated signaling mechanisms is the enteric pathogen *Y. pseudotuberculosis* (reviewed by Palumbo & Wang, 2006). These bacteria posses an outer membrane protein (OMP) invasin that binds to the β1 subunit of five integrin heterodimers (α3β1, α4β1, α5β1, α6β1 and αvβ1) expressed on microfold (M) cells in Peyer's patches of the small intestine (Isberg & Leong, 1990). Binding of invasin to integrin receptors leads to formation of focal adhesion complexes and subsequent activation of intracellular signaling (Gustavsson *et al.*, 2002). The resulting activation of the guanosine triphosphatase (GTPase)

Rac1 causes cytoskeletal rearrangement, mediating bacterial internalization (reviewed by Wong & Isberg, 2005).

Some bacteria have the ability to bind integrins both directly and indirectly, through an ECM ligand. For example, *Borrelia burgdorferi*, the causative agent of Lyme disease, posses a Fn-binding protein (FnBP) BBK32 (Kim *et al.*, 2004). In addition, *B. burgdorferi* has an $\alpha 3\beta 1$ integrin binding protein BBB07 which directly activates pro-inflammatory responses in human chondrocytes (Behera *et al.*, 2006; Behera *et al.*, 2007), as well as an $\alpha v\beta 3$ binding OMP P66 which mediates bacterial adhesion to host cells (Coburn & Cugini, 2003).

However, the majority of integrin-binding microorganisms interact with integrin receptors indirectly using ECM-binding proteins as a molecular bridge to engage integrin receptors. In these cases, integrin receptors recognize the common arginine-glycine-aspartate (RGD) sequence that is present in ECM proteins, such as Fn or Vn (Ruoslahti, 1996), both of which are secreted by airway epithelial cells (Kang *et al.*, 1991; Yasumitsu *et al.*, 1993). The resulting integrin-mediated signaling does not seem to depend on the type of the interactions, as both direct and indirect binding to integrins leads to tyrosine kinase phosphorylation, recruitment of adaptor molecules, and cytoskeletal rearrangement required for bacterial engulfment, as well as induction of pro-inflammatory cellular responses. However, the number of known microbial ECM-binding adhesins greatly outweighs those that bind integrins directly. Several clinically significant bacterial pathogens targeting lung epithelial integrins and the structures and mechanisms involved in these interactions are summarized in Table 1, while integrin binding structures and strategies are illustrated in Figure 1.

Table 1: Respiratory bacterial pathogens that exploit integrins or their ECM ligands during infection

Bacteria	Bacterial structures interacting with integrins	Integrins involved	Results of bacterial interactions with integrins	References
Staphylococcus aureus	FnBP A or B	$\alpha_5\beta_1$	Adhesion/invasion Activation of FAK and Src signaling ILK dependent internalization	(Sinha *et al.*, 1999; Fowler *et al.*, 2000; Massey *et al.*, 2001) (Agerer *et al.*, 2003; Fowler *et al.*, 2003; Agerer *et al.*, 2005) (Wang *et al.*, 2006a)
Group A *Streptococcus*	M1 protein PrtF1/SfbI	$\alpha_5\beta_1$	Adhesion/invasion of lung epithelial cells ILK activation Paxillin phosphorylation-dependent internalization	(Cue *et al.*, 2000; Wang *et al.*, 2006b; Wang *et al.*, 2007)
	Scl1	$\alpha2\beta1$	Adhesion/invasion of lung epithelial cells	(Caswell *et al.*, 2007)
Mycobacterium species	FAP Antigen 85B	α_5, α_v, β_1, β_3	Adhesion to and invasion of lung epithelial cells	(Bermudez & Goodman, 1996; Secott *et al.*, 2004)
Pseudomonas aeruginosa	Putative 50kDa OMP	$\alpha_5\beta_1$ or $\alpha_v\beta_5$	Adhesion to and invasion of lung epithelial cells	(Roger *et al.*, 1999; Gagniere & Di Martino, 2004; Leroy-Dudal *et al.*, 2004)
Bordetella pertussis	FHA	$\alpha5\beta1$ (lung EC) $\alpha M\beta2$ (alveolar macrophages)	Activation of lung epithelial cells inflammatory response Invasion of alveolar macrophages and induction of inflammation	(Ishibashi *et al.*, 2001; Ishibashi & Nishikawa, 2002; Ishibashi & Nishikawa, 2003) (Relman *et al.*, 1990; Perkins *et al.*, 2007)
Haemophilus influenzae	Hap	$\alpha5\beta1$, potentially $\alpha3\beta1$,$\alpha v\beta6$	Involved with TLR-4 and platelet-activating factor receptor- dependent uptake by M cells	(Fink *et al.*, 2002; Tyrer *et al.*, 2006)
Streptococcus pneumoniae	PavA FnBP	$\alpha5\beta1$?	Unclear, associated with adherence and invasion of epithelial cells	(Holmes *et al.*, 2001; Pracht *et al.*, 2005)

Abbreviations: FnBP (fibronectin binding protein), FAK (focal adhesion kinase), ILK (integrin linked kinase), FAP (fibronectin attachment protein), OMP (outer membrane protein), FHA (filamentous hemagglutinin), TLR (toll-like receptor)

Role of lung epithelial integrins in recognition of fungi and viruses

Integrins are also implicated in the pathogenesis of some fungal and viral pulmonary infections. The fungus *Pneumocystis carinii*, a major cause of acute pneumonia in AIDS patients, uses a FnBP to adhere to αv and α5 integrins (Pottratz *et al.*, 1991; Pottratz & Weir, 1995). Furthermore, *P. carinii* is able to induce upregulation of integrins, possibly enhancing its own adherence to lung epithelial cells (Pottratz *et al.*, 1994). Interestingly, some pathogenic fungi, such as *Pneumocystis* species and *Candida albicans*, possess molecules with integrin-like features that mediate fungal adhesion to Fn (Gale *et al.*, 1996; Kottom *et al.*, 2008). A novel *Pneumocystis* molecule PCINT1 with significant structural features of an integrin-like adhesion receptor has been recently characterized (Kottom *et al.*, 2008). The results of the latter study suggest an important role of this molecule in pathogen-host lung epithelial cells interactions during *Pneumocystis* pneumonia (Kottom *et al.*, 2008).

A number of viruses that infect the respiratory epithelium have been shown to use integrin receptors for both cell entry and induction of intracellular signaling important for disease pathogenesis. Some examples include members of *Adenovirus, Herpesvirus, Hantavirus, Picornavirus, Reoviridae* families (reviewed by Stewart & Nemerow, 2007). Such viruses directly bind to a variety of integrins present in the respiratory epithelium, e.g. α2β1, α3β1, α5β1, αvβ5, αvβ6 and use them as receptors to attach to the cells and enter them. The mechanisms of virus interactions with integrins and their significance for viral pathogenesis have been recently discussed in a comprehensive review by Stewart and Nemerow (2007). Several viruses, e.g. coxsackieviruses, foot-and mouth-disease viruses, human parechoviruses, and echoviruses possess a functional RGD motif in one of their capsid proteins that allow viruses to bind integrins, i.e. αvβ3 or αvβ6 (Williams *et al.*, 2004). Interactions of viruses with integrin

receptors are proven to be important in the pathogenesis of a variety of conditions ranging from acute upper respiratory tract infections and foot-and mouth-disease to highly lethal hantavirus pulmonary syndrome (Stewart & Nemerow, 2007). Recent data implicate that the severe acute respiratory syndrome (SARS) related coronavirus possesses the ability of binding to integrin I domains (Hanel *et al.*, 2006). However, it is still unclear whether integrin-mediated interactions are involved in coronavirus entry into lung epithelial cells.

Pseudomonas aeruginosa

The opportunistic Gram-negative pathogen *P. aeruginosa* causes acute life-threatening infections in immunocompromised patients. It is also the leading cause of ventilator-associated pneumonia in intensive care units and of burn wound infections with high mortality rates. *P. aeruginosa* is the major cause of chronic pulmonary infection in CF patients (Saiman & Siegel, 2004). Both integrin receptors and their ligands have been implicated in adhesion and internalization of *P. aeruginosa* in the lung epithelia. A number of studies demonstrated the ability of *P. aeruginosa* to bind Fn (Gagniere & Di Martino, 2004; Barnes *et al.*, 2008), and Vn (Leroy-Dudal *et al.*, 2004), the α5β1 and αvβ5 integrin ligands, respectively. Some papers suggest that αvβ5 and α5β1 integrins can also directly mediate *P. aeruginosa* adherence to and invasion of respiratory epithelial cells (Roger *et al.*, 1999; Gagniere & Di Martino, 2004; Leroy-Dudal *et al.*, 2004). The molecular mechanisms of such interactions have not yet been defined, although a 50 kDa OMP of *P. aeruginosa* was found associated with α5β1 integrins in respiratory epithelial cells (Roger *et al.*, 1999).

In the process of epithelial injury and repair, the expression of α5β1 receptors is increased with their redistribution from basolateral to apical sides; and respiratory epithelial cells synthesize large amounts of Fn potentially providing a basis for an enhanced adherence of *P.*

aeruginosa (Roger *et al.*, 1999). Adherence of *P. aeruginosa* to Lmn, another component of the ECM and the α3β1 integrin ligand, unmasked following epithelial injury, was also implicated in bacterial colonization of injured tissues (Plotkowski *et al.*, 1996).

Recent observations by our group have demonstrated that *P. aeruginosa* infection caused a rapid up-regulation of integrins α5, αv, β1, and β4 in A549 type II pneumocytes (Gravelle et al, unpublished data). Interestingly, this effect required live bacteria possessing intact pili and lipopolysaccharide (LPS), because heat-killed, pili-deficient, or outer-core oligosaccharide deficient *P. aeruginosa* mutants did not alter the expression of integrins (Gravelle et al, unpublished data). These findings imply that pulmonary epithelial integrins can be involved in recognition of specific microbial products of *P. aeruginosa* and hence be important in innate immune responses to this pathogen, although because of the conflicting published results, further study is required.

The role of ECM proteins in interactions of bacteria with host cells

The eight different integrin heterodimers expressed in airway epithelial cells (α2β1, α3β1, α6β4, α9β1, α5β1, αvβ5, αvβ6, αvβ8) can recognize a range of ECM proteins including collagen I, tenascin C, Lmn 5, 10, 11, osteopontin, Fn, Vn, and others (Sheppard, 2003). It is well documented that a number of bacteria can bind to various ECM products and use them to aid in infection through exploitation of integrin receptors. *S. aureus* (reviewed by Hauck & Ohlsen, 2006) and *Streptococcus sp.* (Cue *et al.*, 2000) are well known pathogens that utilize their Fn binding ability to exploit α5β1 integrins to mediate adhesion and invasion into various cell types. Other microbial pathogens including *E. coli* (Gophna *et al.*, 2002), *Aspergillus sp.* (Wasylnka & Moore, 2000) and *H. influenzae* (Fink *et al.*, 2002) are also able to bind Fn, potentially using it to

aid in their pathogenesis. In addition, *Pneumocystis carinii* is able to bind Vn and use it to increase adhesion to A549 lung epithelial cells (Limper *et al.*, 1993).

A study by Plotkowski *et al.* (1993) demonstrated that *P. aeruginosa* is able to preferentially bind to soluble cellular Fn, which is secreted by injured epithelial cells. Studies of *P. aeruginosa* binding to immobilized Fn have since verified this specific (Gagniere & Di Martino, 2004; Leroy-Dudal *et al.*, 2004) and competitive (Rebiere-Huet *et al.*, 2004) binding, although there appears to be some strain-specific variations in binding ability/affinity (Gagniere & Di Martino, 2004). However, the role of this Fn binding ability during the pathogenesis of pulmonary disease caused by *P. aeruginosa* is unclear. Anti-Fn antibodies have been shown to decrease *P. aeruginosa* adhesion to A549 lung epithelial cells (Rebiere-Huet *et al.*, 1999) and nasal epithelial cells (Roger *et al.*, 1999), although efficacy of binding inhibition may be strain dependent (Gagniere & Di Martino, 2004). *P. aeruginosa* has also been shown to bind Vn, the ligand for αvβ5 and αvβ3 integrins (Leroy-Dudal *et al.*, 2004). As demonstrated by the ability of anti-Vn antibodies to inhibit adhesion and internalization, and the ability of exogenous Vn to restore these activities, it appears that interactions of Vn with integrins may also play a role in *P. aeruginosa* internalization (Leroy-Dudal *et al.*, 2004). It has also been suggested that *P. aeruginosa* possess adhesins which bind strongly to Lmn-1 with high avidity (Plotkowski *et al.*, 1996). However, a study investigating the role of Lmn-5 has shown that this specific Lmn inhibits *P. aeruginosa* binding to ECM basal lamina substrates, possibly by competing for binding sites (Esco *et al.*, 2002). Therefore it is possible that *P. aeruginosa* reacts differently with the various Lmn types.

The role of RGD peptides in bacteria-host interactions

Often the integrins which bind ECM products do so by recognizing the common arginine-glycine-aspartate (RGD) sequence of their ligands, especially the Vn and Fn binding integrins αvβ3, αvβ5 and α5β1 (Ruoslahti, 1996). Therefore, RGD based synthetic peptides may interfere with the interactions between integrins and their ECM ligands, and the resulting functions of these integrins. Indeed, a glycine-arginine-glycine-aspartate-serine (GRGDS) synthetic peptide has been shown to inhibit platelet adhesion to Fn and Vn, but not laminin (Sheu *et al.*, 1999). In addition, RGDS peptide causes decreased macrophage adherence and pro-inflammatory signaling activation induced by LPS (Monick *et al.*, 2002). In airway smooth muscle cells, RGD peptides caused a decrease in Fn dependent cytokine release (Peng *et al.*, 2005).

Since many bacteria have been shown to bind integrins, either directly or by using integrin ECM product ligands as a bridge, the use of RGD peptides to block bacterial interactions with cells via integrins should allow for study of the involvement of integrins in bacterial pathogenesis. Studies which have used RGD peptides for analysis of bacteria-host interactions are summarized in Table 2. Several studies have successfully explored integrin mediated interactions using RGD based peptides. For example, *E. coli* internalization was inhibited by addition of RGDS peptide which interferes with the curli-fibre:Fn interactions that link these pathogens to integrins (Gophna *et al.*, 2002). In one study, a GRGDS peptide was able to significantly decrease *S. aureus* invasion of fibroblasts in a dose dependent manner compared to a control peptide (Fowler *et al.*, 2000). Although the tri-peptide RGD sequence was found to be most critical for integrin binding, it has been suggested that RGD peptides containing 5 or more amino acids are most efficient at inhibiting integrin functions, as demonstrated by optimal

inhibition of *Mycobacterium kansasi* leukocyte phagocytosis by the peptide RGDVY (Siemion & Wieczorek, 2003). Indeed, the pentapeptide GRGDSP has been shown to inhibit *B. pertussis* invasion of A549 lung epithelial cells and to decrease the resulting ICAM-1 expression (Ishibashi *et al.*, 2001; Ishibashi & Nishikawa, 2002), decrease *Klebsiella pneumoniae* mrkD mediated adherence (Huang *et al.*, 2006), and inhibit *P. aeruginosa* adhesion to nasal epithelial cells (Roger *et al.*, 1999).

However, some studies showed a less clear ability of integrin inhibition by RGD peptides. In one case, RGD peptide inhibition of *S. aureus* cellular adhesion was only observed when there was also exogenous Fn present, indicating that specific experimental conditions can greatly affect the results (Liang & Ji, 2006). The actual role of the RGD binding site, and the orientation with regards to pathogen and integrin receptor need to be considered. For example, in a study of Fn involvement in *Aspergillus* lung infections, fungal binding to Fn was not inhibited by GRGDS peptide, but the microbe was found to bind Fn at a site other than the prototypic RGD binding site (Wasylnka & Moore, 2000). Since *P. aeruginosa* interaction with Fn is also not inhibited by RGD peptides, it is possible that *P. aeruginosa* also binds Fn at another site, and that the RGD-based interactions are between Fn and integrins (Rebiere-Huet *et al.*, 2004). In contrast, some bacteria such as *Vibrio vulnificus* bind directly to immobilized RGD via their OmpU protein, such that bacterium and integrin both share (and possibly compete for) a common RGD binding site on Fn (Goo *et al.*, 2006). This also raises the possibility that RGD peptides bound to integrins could in fact function as a binding site for some bacteria, and thus result in increased adhesion in some experimental systems.

Table 2: Studies of microbial interactions with integrins using RGD based peptides

Micro-organism	Peptide inhibitor	Effects	Reference
E. coli	RGDS (20 μg/ml)	95% decreased internalization into bladder and cervical epithelial cells	(Gophna *et al.*, 2002b)
	RGES (20 μg/ml)	No effect	
Klebsiella pneumonia (MrkD genes)	GRGDSP (20 and 100 μg/ml)	Decreased adhesion to ileocecal epithelial cells (36% and 69% decrease)	(Huang *et al.*, 2006)
	GRADSP (100 μg/ml)	No effect	
Bordetella pertussis	GRGDSP (1.5 mM)	Decreased invasion of A549 and normal human derived bronchial epithelial cells and decreased ICAM-1 expression	(Ishibashi *et al.*, 2001; Ishibashi & Nishikawa, 2002)
	GRGEXP (1.5 mM)	No effect	
Vibrio vulnificus	RGD (20 μg coated per well)	Significant bacterial adhesion to immobilized RGD	(Goo *et al.*, 2006)
Mycobacterium kansasi	RGDVY and various other peptides (10, 20 and 100 μg/ml)	Decreased leukocyte phagocytosis if cells pre-inc with RGD based peptides ≥5 amino acids long	(Siemion & Wieczorek, 2003)
Staphylococcus aureus	GRGDS	Significant decreased fibroblast invasion, dose dependent	(Fowler *et al.*, 2000)
	GRGES (both 10-500 μg/ml)	Decreased invasion, but less than that caused by GRGDS	
	G4391 RGD containing peptide	Decreased cellular adhesion ONLY if exogenous Fn present	(Liang & Ji, 2006)
Pseudomonas aeruginosa	GRGDSP (100 and 1000 μg/ml)	Significantly decreased adherence to flattened dedifferentiated nasal explant outgrowth cells	(Roger *et al.*, 1999)
	GRGESP (1000 μg/ml)	No effect	
	RGD	No effect on bacterial adherence to immobilized Fn	(Rebiere-Huet *et al.*, 2004)

Amino Acid abbreviations: R = arginine, G = glycine, D = aspartate, S = serine, E = glutamic acid, P = proline, A = alanine, V = valine, X = unspecified , Y = unspecified

Effect of antibodies to integrins on bacteria-host interactions

In addition to use of RGD peptides to inhibit integrin binding by pathogens, many studies have used anti-integrin antibodies to target and block or inactivate the integrin binding sites. These inhibitors should be very specific in their blocking abilities, as they are targeted to certain integrin subunits or heterodimers. Studies using anti-integrin antibodies to investigate bacterial interactions with integrins are summarized in Table 3. In general, antibody inhibition of integrins has been successful in helping to determine integrin-mediated interactions. The importance of α5β1 integrins for cellular invasion by *B. pertussis* and Group A *Streptococcus* has been clearly shown by the use of α5, β1, or α5β1 antibodies (Ozeri *et al.*, 1998; Ishibashi *et al.*, 2001; Wang *et al.*, 2006b). Anti α5β1 and Fn antibodies have also clearly demonstrated the importance of these interactions during *S. aureus* invasion (Sinha *et al.*, 1999; Massey *et al.*, 2001). In the case of *S. aureus*, it is imperative to use SpA- mutants in such experiments, since normal *S. aureus* are able to strongly bind the Fc region of antibodies (Sinha *et al.*, 1999; Massey *et al.*, 2001). This could result in either non-specific inhibition due to bacteria becoming bound by free antibodies, or increased binding due to bacteria binding the exposed Fc region of antibodies that have bound their specific target on the cell surface. The results of using integrin antibodies to study the role of integrins in *P. aeruginosa* infections are less clear. It was originally shown that antibodies against α5, α3 and β1 decreased *P. aeruginosa* adherence to nasal epithelial cells, strongly implicating α5β1 or α3β1 integrin involvement during infection (Roger *et al.*, 1999). However, more recent studies did not confirm the effect of α5β1 antibodies on *P. aeruginosa* adhesion or invasion, while αvβ5 antibody reduced both adhesion and invasion (Leroy-Dudal *et al.*, 2004). In another study, α5β1 antibody decreased adhesion of a clinical *P. aeruginosa* strain, but not of the laboratory strain PAK, suggesting the possibility of a significant

variation amoung different *P. aeruginosa* strains (Gagniere & Di Martino, 2004). It is interesting to note that the IgG binding ability of *P. aeruginosa* has not been characterized, and it is possible that these inconsistent results could be related to this type of interference.

Table 3: Studies of microbial interactions with integrins using anti-integrin antibodies

Micro-organism	Antibody specificity	Effects	Reference
Bordetella pertussis	α2, α3, α5, α6, αv, β1, β3, α5β1 (25 μg/ml)	Anti - α5, β1, α5β1 caused significantly decreased invasion	(Ishibashi *et al.*, 2001)
	αv, α5β1 (25 μg/ml)	Anti - α5β1 caused significant reduction in ICAM-1 upregulation	(Ishibashi & Nishikawa, 2002)
Group A *Streptococcus*	β1	Decreased HeLa invasion	(Ozeri *et al.*, 1998)
	α5 and Fn	Decreased HEp-2 invasion	(Wang *et al.*, 2006a)
Staphylococcus aureus	Fn	Decreased invasion * SpA- mutants	(Sinha *et al.*, 1999)
	α5β1, control β3 (10 μg/ml) (antibody not washed away prior to addition of bacteria)	Anti - α5β1 decreased invasion * SpA- mutants	(Massey *et al.*, 2001)
Pseudomonas aeruginosa	β1, α3, α5	Decreased adherence to flattened dedifferentiated nasal explant outgrowth cells	(Roger *et al.*, 1999)
	α5β1 αvβ5	No effect Decreased adhesion and internalization	(Leroy-Dudal *et al.*, 2004)
	α5β1	Decreased adhesion of a clinical strain but not of PAK	(Gagniere & Di Martino, 2004)

* strain of *S. aureus* which lacks the surface protein SpA, which binds Fc regions of antibodies

Although the role of integrins in *P. aeruginosa* infection of lung epithelial cells has been studied with the approach of quantifying the effects of integrin inhibitors on the interactions

between the bacterium and host cells, the involvement of integrins remains unclear. In these studies, we use our *gfp* labelled *P. aeruginosa* PAK and associated fluorescence based readout techniques to test the effects of exogenous RGD peptides, anti-integrin antibodies and ECM products on integrin-dependent *P. aeruginosa* interactions with host A549 lung epithelial cells.

Materials and Methods

Role of ECM proteins in *P. aeruginosa* interactions with A549 cells

Adherence of PAKgfp to immobilized substrates

In addition to optimization experiments involving PAK*gfp* adhesion to BSA and Fn (Chapter 1), further investigations were performed to determine *P. aeruginosa* binding ability to additional substrates. Substrates tested included human plasma Vn (Sigma-Aldrich, Oakville, ON Canada), human placental Lmn (Sigma-Aldrich), and purified mouse IgG (Sigma-Aldrich). In these experiments, we also continued the testing of BSA and Fn. Experiments were performed similarly to those described in Chapter 1 (Section: Microplate reader detection of PAKgfp adherence to substrates). A substrate concentration of 10 μg/ml was chosen instead of the 20 μg/ml used during optimization, since there was no difference in bacterial binding observed (data not shown). Flat bottom solid black 96-well tissue microplate (Corning) wells were coated overnight with 100 μl substrate (10 μg/ml) at 4°C, washed once with 100 μl sterile PBS and pre-warmed for 15 min at 37°C. Next, 100 μl of PAK*gfp* at OD_{600} 1.0 (approximately 7×10^8 CFU/ml) were added and allowed to incubate for 5 min at 37°C. Five minute incubation was chosen following initial time course experiments showing that PAK*gfp* binding to Fn occurred immediately and did not increase with longer incubation, while PAK*gfp* binding to BSA increased with longer incubation time, suggesting that specific interactions between

bacteria and substrates occurs immediately, while non-specific interactions are slower, and binding effects accumulate with time (Chapter 1, Section: Results – Detection of PAK*gfp* adherence to immobilized substrates). Following incubation, wells were washed three times with sterile PBS, and plates were read using a FLUOStar Optima microplate reader (BMG Labtech, Offenburg, Germany), with an excitation filter of 495 nm, and emission filter of 520 nm. The gain was adjusted for the most highly fluorescent well of each separate experiment, and results were acquired as fluorescence units as measured by the microplate reader minus the fluorescence of the negative control (substrate coated well with no bacteria), and were reported as relative fluorescence compared to fluorescence of PAK*gfp* bound to BSA coated wells.

Effect of substrates on PAKgfp association with A549 cells

To test whether Vn, in addition to Fn, mediates association of PAK*gfp* with A549 cells, flow cytometry analysis was performed as described in Chapter 1 in the presence of exogenous Fn or Vn. In these experiments, A549 cells were pre-incubated with 10 μg/ml Fn or Vn for 15 min at 37°C and 5% CO_2 prior to addition of bacteria. In Chapter 1, a time course with Fn was performed at 0, 30, 60, 90 and 120 min. As significant differences were observed at 60 and 90 min only, additional experiments testing the effects of Vn were performed at 60 and 90 min. In these follow-up experiments, an MOI of 50:1 was used instead of 100:1, based on the further optimizations performed during the development of the PMx and Gn exclusions assays (Chapter 1).

Effect of substrates on PAK internalization into A549 cells

The effect of exogenous Fn on PAK or PAK*gfp* internalization into A549 cells was investigated using several internalization methods described in Chapter 1, including PMx quenching internalization assays with both plate reader and flow cytometry outputs, and the standard Gn internalization assay with plate counting methods. For all three of these methods, adherent A549 cells were pre-incubated for 15 min with 10 μg/ml Fn at 37°C, and then bacteria were incubated with A549 cells at an MOI of 50:1 for 60 min, 37°C, 5% CO_2.

Effect of RGD based peptides on *P. aeruginosa* interactions with A549 cells

Effect of RGD peptides on PAKgfp association with A549

Various peptides were tested for their ability to interfere with PAK*gfp* interactions with A549 cells. The peptides tested were: RGDS (Sigma-Aldrich), control scrambled SDGR (Sigma-Aldrich), and a cyclic RGD peptide called Cyclo(Arg-Gly-Asp-D-Phe-Val) (Calbiochem, San Diego CA). Peptide stocks were resuspended to stock solutions of 5 mg/ml in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5), aliquoted and stored at -20°C. Although peptide concentrations of up to 1000 μg/ml are used in the literature (Roger *et al.*, 1999), we found that this concentration significantly decreased A549 cell viability, and dramatically increased bacterial association (data not shown). Because of this, all peptide inhibition studies were performed using 500 μg/ml (final concentration) peptides, which did not cause such effects. To investigate peptide effects in a suspension system, A549 cells were prepared and analyzed as described in Chapter 1 (section: Flow cytometry analysis of PAK*gfp* adherence to A549 cells in suspension) at an MOI of 50:1, with the addition of an RGD pre-incubation step. Preliminary experiments tested pre-incubation times of 15 and 30 min, and pre-incubation temperatures of

4°C, room temperature and 37°C, but following optimization, all subsequent peptide pre-incubations were performed at room temperature for 30 min. Similarly, peptide effects on PAK*gfp* association with adherent A549 cells were analyzed as described in Chapter 1 (section: Binding of PAK*gfp* to adherent A549), with the addition of peptide pre-incubation.

Effect of RGD peptides on PAK internalization into A549 cells

To test whether RGD peptides had any effect on PAK internalization into A549 cells, the Gn exclusion assay described in Chapter 1 was performed with the addition of a 30 min peptide pre-incubation of the cells before addition of bacteria. Two separate experiments were performed, testing the RGDS and control SDRG peptides ability to inhibit PAK internalization into adherent A549 cells.

Effects of RGD peptides on PAK induced cytokine release from A549 cells

RGD peptides were investigated for their ability to inhibit cytokine release by A549 following stimulation with PAK. A549 cells were seeded in 24-well plates at 50,000 cells per well, and allowed to grow for 2 d until 80% confluency. Approximately 2 h prior to adding the bacteria, the A549 cells were washed 2 times with PBS to remove all traces of BCS, and incubated with serum free F-12 Ham medium to pre-condition. Cells were pre-incubated with RGDS or control peptide for 30 min at room temperature prior to addition of bacteria. Bacteria were prepared as described in Chapter 1 (section: Preparation of bacteria for experiments), added to A549 cells at an MOI of 50:1, and allowed to incubate for 1 h at 37°C, 5% CO_2. The bacteria were then killed by the addition of 50 μg/ml PMx, and the A549 cells were incubated for additional 17 h with the dead bacteria present. After 18 h total incubation time, the supernatants

were collected in 1.5 ml eppendorf tubes, and centrifuged at 13,000 × g for 25 min to pellet all the bacteria and debris. The cell-free supernatant were collected in 100 µl aliquots and stored at -80°C until cytokine analysis could be performed as described in Chapter 3 (section: Effects of ILK siRNA on cytokine protein production during bacterial infection) using a 4-Plex Bio-Plex assay (BioRad, Hercules CA) to measure the amount of IL-4, IL-10, IFN-γ and TNF-α.

Effect of antibodies to integrins on *P. aeruginosa* interactions with A549 cells

Effect of antibodies to integrins on PAKgfp association with adherent A549 cells

The effects of various integrin inhibitor antibodies on PAK*gfp* association with A549 cells were investigated. All antibodies used during inhibition assays were azide-free, so as not to interfere with bacteria-A549 interactions by damaging or killing the host or bacterial cells. Integrin inhibitory antibodies used included anti-β1 (clone 6S6, MAB2253A, Chemicon), 'new' anti-β1 (clone P1F6, Chemicon), and anti-αvβ5 (MAB1961Z, Chemicon). Control antibodies included isotype matched mouse IgG against limpet hemocyanin (Chemicon), isotype matched anti-CD3 (UCHTI, Calbiochem), and purified mouse IgG1 κ (BD Pharmingen). All antibody inhibition studies were performed using adherent A549 cells in 96-well plates as described in Chapter 1 (section: Binding of PAK*gfp* to adherent A549 cells). However, in these experiments A549 cells were pre-incubated with 10 µg/ml antibody for 30 min at room temperature before the addition of PAK*gfp* to the antibody containing medium at an MOI of 50-100:1. A549 cells and bacteria were then incubated for 60 min, followed by washing three times with PBS, and reading fluorescence using the plate reader.

Effect of washing away antibodies on PAKgfp association with A549 cells

To determine whether washing away any excess antibody following pre-incubation had any effect on bacterial association with A549 cells, experiments were performed as described above, except that A549 cells were washed three times with room temperature serum free medium before the incubation with bacteria. PAK*gfp* were thus incubated with A549 cells in fresh serum free medium.

Effects of antibodies to integrins on PAK internalization into A549 cells

To test whether anti-integrin antibodies have any effect on PAK internalization into A549 cells, the Gn exclusion assay described in Chapter 1 (section: Gentamicin exclusion assay) was performed with the addition of a 30 min pre-incubation of A549 cells with 10 μg/ml antibodies prior to addition of bacteria. The antibodies tested in this experiment included anti-β1 (clone 6S6), anti-αvβ5, and the control purified mouse IgG. Following 30 min pre-incubation at room temperature, the antibodies were washed away as described above prior to the addition of bacteria at MOI of 50:1 for 60 min.

Results

Role of ECM proteins in *P. aeruginosa* interactions with A549 cell

Adherence of PAKgfp to immobilized substrates

PAK*gfp* binding to various substrates was quantified using a microplate reader based assay. After 5 min incubation, bacteria showed greatly increased binding to Fn, Vn and IgG coated wells than to control BSA coated wells (Figure 2). In contrast, PAK*gfp* binding to uncoated wells or to Lmn coated wells showed a decreased binding compared to BSA (Figure 2).

Effect of substrates on PAKgfp association with A549 cells

In Chapter 1, Fn was used to show that the effects of exogenous substances on PAK*gfp* association (adherence and internalization) with A549 cells could be detected using the fluorescence based systems developed. Based on the increased association of bacteria with A549 observed when exogenous Fn was added (Chapter 1, Figure 8), we also tested the effects of another integrin ligand, Vn, on PAK*gfp* association. To assess the effects of Vn on PAK*gfp* association with A549 cells, additional flow cytometry experiments were performed comparing the effects of a 15 min pre-incubation of A549 cells in suspension with 10 μg/ml of either Fn or Vn. In agreement with the results shown in Chapter 1 (Figure 8), exogenous Fn caused an increased association of PAK*gfp* with A549 cells, after both 60 and 90 min incubation (Figure 3). However, the presence of exogenous Vn did not have any observable effect on bacterial association with A549 cells at either time point (Figure 3). (n=2 independent experiments).

Effect of substrates on PAKgfp internalization into A549 cells

The effect of exogenous Fn (10 μg/ml) on internalization of PAK*gfp* into adherent A549 cells was investigated using the plate reader based (n = 2) and flow cytometry based (n = 3) PMx quenching internalization assay, as well as the standard Gn internalization assay (n = 1). In all of these experiments, Fn did not have an effect on bacterial internalization in A549 cells (Figure 4).

Effect of RGD based peptides on *P. aeruginosa* interactions with A549 cells

Effect of RGD peptides on PAKgfp association with A549

In the initial set of peptide inhibition studies, the A549 cells used were later found to be *Mycoplasma* positive, and therefore the results from those experiments are considered separately. Flow cytometry analysis of the *Mycoplasma* contaminated A549 cells in suspension showed a significant decrease of approximately 55% in bacterial association with RGDS pre-incubated cells ($n=4$, $p<0.01$, One sample t-test), and a non-significant decrease of approximately 20% in control peptide treated cells ($n=3$) (Figure 5). The cyclic peptide did not have any significant effect on bacterial association, even though other studies have suggested that cyclic peptides posses superior inhibitory abilities over linear RGD peptides (Kim *et al.*, 2005). After obtaining new A549 cells that were *Mycoplasma* negative, the same experiments were repeated. Flow cytometry analysis of the *Mycoplasma*-free A549 cells incubated with PAK*gfp* in suspension showed that RGDS pre-treatment did not cause decreased association, while there was a tendency for the control peptide to decrease association (Figure 6). Subsequent peptide inhibition studies with *Mycoplasma*-free A549 cells incubated with PAK*gfp* under adherent conditions showed that both active RGDS and control peptide appeared to reduce bacterial association with A549 cells, but they were not significantly different from each other (Figure 6).

Effect of RGD peptides on PAK internalization into A549 cells

The ability of RGDS peptide to alter PAK internalization into A549 cells was investigated using the standard Gn internalization assay following pre-incubation of cells with either active RGDS or control scrambled peptide for 30 min at room temperature. Neither the active RGDS nor the control scrambled peptide had any effect on bacterial internalization into

A549 cells (Figure 7). The effects of peptides on association were not measured in this assay based on the inability of RGDS to inhibit bacterial association in the previous experiments.

Effect of RGD peptides on PAK induced cytokine release by A549 cells

The ability of RGDS peptide to alter the cytokine response profile of A549 cells stimulated with PAK was investigated by measuring the TNF-α protein levels released by A549 cells following 18 h incubation with PAK, with or without peptide pre-treatment. RGDS peptide was observed to have no effect on the production and release of the pro-inflammatory cytokine TNF-α from A549 cells, since after 18 h total stimulation time with PAK (1 h alive, 17 h antibiotic killed), neither the control nor the active peptide had any significant effect on TNF-α production (Figure 8). However, the amount of TNF-α produced from all the bacterial stimulated treatments was significantly higher than from non-bacterial stimulated cells, verifying that treatment of A549 cells with PAK does in fact result in the induction of pro-inflammatory responses. ($n=3$, $p<0.05$, Student's t-test).

Effect of antibodies to integrins on *P. aeruginosa* interactions with A549 cells

Effect of antibodies to integrins on PAK association with A549 cells

Pre-incubation of adherent A549 cells with various control or anti-integrin antibodies was performed to either block integrin function or binding site availability in order to quantify the effects this may have on bacterial association with A549 cells using the fluorescence microplate reader assay. While anti-integrin β1 and αvβ5 antibodies showed a significant decrease in bacterial association with adherent A549 cells, the isotype controls also showed a similar reduction in association (Figure 9). In light of the observed ability of PAK to preferentially bind

IgG, the effects of adding purified IgG as a control were investigated, and it also appears to be able to decrease bacterial association with A549 cells by approximately 50% (Figure 9). This raises the concern that PAK*gfp* may non-specifically bind and aggregate with the free, unbound antibodies. Thus, the effect of removing these antibodies by washing prior to adding bacteria was tested. Figure 10 shows that washing away the excess antibody results in reversing the inhibitory effects of both the controls and the active anti-integrin antibodies.

Effect of antibodies to integrins on PAK internalization into A549 cells

The ability of anti-integrin antibodies to inhibit PAK internalization into adherent A549 cells was investigated using the Gn exclusion assay. Pre-incubation of cells with 10 μg/ml of control IgG, β1 or αvβ5 antibodies, followed by washing, did not have any effect on bacterial association or internalization into A549 cells (Figure 11). While the results are only representative of one experiment performed in triplicate, the negative outcome, and the negative results obtained from the other antibody and peptide experiments discouraged further investigation into the use of antibodies as inhibitors to study the role of integrins in host-pathogen interactions.

Discussion

The ability of microbes to bind ECM ligands of integrins and exploit them during infection is an important virulence mechanism of several pathogens, including *Staphylococcus aureus* and Group A *Streptococcus* (Myhre & Kuusela, 1983; Hauck & Ohlsen, 2006). Based on previous studies that have suggested a potential role for Fn and α5β1 (Roger *et al.*, 1999) as well as Vn and αvβ5 (Leroy-Dudal *et al.*, 2004) during *P. aeruginosa* infection, we chose to

investigate the ability of PAK*gfp* to adhere to these ECM products. In addition, the α6β4 ligand Lmn, control BSA and uncoated surface were also investigated for their ability to bind PAK*gfp*. Our findings agreed with previous findings that *P. aeruginosa* binds preferentially to Fn and Vn over control uncoated or BSA coated wells (Figure 2) (Plotkowski *et al.*, 1993; Rebiere-Huet *et al.*, 1999; Gagniere & Di Martino, 2004; Leroy-Dudal *et al.*, 2004). However, we observed that PAK*gfp* did not bind preferentially to Lmn, suggesting that interactions with α6β4 integrin may not be involved during the infectious process. To investigate the involvement of Fn and Vn binding in *P. aeruginosa* infection, we quantified the effects of adding exogenous substrate on PAK*gfp* binding to suspended A549 cells, similar to a method used by Sinha *et al.*, (1999) who investigated the importance of these substrates for *S. aureus* cellular invasion. We observed that exogenous Fn, but not Vn, resulted in increased binding of PAK*gfp* to A549 cells. In these experiments, trypsinized cells in suspension were used because trypsin cleaves the ECM products from the cells, leaving them with 'empty' integrins without bound ligands (Sinha *et al.*, 1999). Since Fn increased bacterial association with cells, it seems likely that Fn must be present and bound to its integrin receptor to mediate effective bacterial binding to A549 cells (Figure 3). Since excess Vn does not increase bacterial association with A549 cells, the Vn binding ability of PAK*gfp* is apparently not involved in the host-pathogen interactions. However, it is possible that the expression of αvβ5 is not high enough during the early stages of infection to demonstrate an observable effect, while α5β1 shows slightly higher basal expression, and may be more readily available during the earliest stages of infection (Gravelle et al, unpublished). A549 cells can produce Vn under culture conditions (Yasumitsu *et al.*, 1993), and the interaction of this substrate with its receptors can be disrupted by EDTA, which suggests that it behaves in a

similar fashion to Fn, and that the results obtained should be comparable (Limper *et al.*, 1993). It therefore seems likely that Vn is not important for PAK interactions with A549 cells.

Although Fn was able to clearly increase association of PAK*gfp* with A549 cells, it was not able to increase internalization of the bacteria into A549 cells (Figure 4). These results were surprising based on the ability of PAK*gfp* to internalize into A549 cells (as demonstrated in Chapter 1). Also, Fn has been shown to be important for the internalization of other bacteria that exploit integrins, especially *S. aureus* (Sinha *et al.*, 1999). It is possible that a time course with shorter infection lengths would have allowed for detection of the effects of these substrates, based on the observations that PAK*gfp* binds Fn very strongly after only 5 min (discussed in Chapter 1, Results section: Detection of PAK*gfp* adherence to immobilized substrates), and that PAK*gfp* become rapidly internalized into A549 cells (Chapter 1, Figure 10). If the Fn dependent internalization was indeed occurring as quickly as 5 min, it is possible that with longer incubation, bacterial internalization by exploitation of other cellular machinery may mask the effects of Fn dependent internalization. Binding of bacteria to integrin receptors can result in activation of several cellular signaling pathways, including FAK and paxillin dependent actin cytoskeleton rearrangement, resulting in bacterial internalization (Cue *et al.*, 2000; Wang *et al.*, 2006a). However, this is not the only pathway activated by integrin engagement, and it is possible that *P. aeruginosa* interaction with integrins via Fn is more involved in the induction of pro-inflammatory signaling cascades (Dedhar, 2000). In addition, it is possible that invasion does not play as significant a role during pathogenesis for *P. aeruginosa* as it does for other integrin utilizing pathogens.

To further investigate the role of both $\alpha5\beta1$ and $\alpha v\beta5$ integrins during *P. aeruginosa* infection of A549 cells, we tested the effects of blocking peptides and integrin antibodies on *P.*

aeruginosa interactions with A549 cells. RGD-based peptides were chosen based on studies showing effective RGD peptide inhibition of bacterial association with or internalization into epithelial cells for *P. aeruginosa, S. aureus,* and *B. pertussis* (Roger *et al.*, 1999; Fowler *et al.*, 2000; Ishibashi *et al.*, 2001; Ishibashi & Nishikawa, 2002; Liang & Ji, 2006). We originally observed that RGD peptides caused over 50% decrease in bacterial association with A549 cells compared to control peptide as measured by flow cytometry (Figure 5). However, the cells used in these experiments were later discovered to be *Mycoplasma* positive. Subsequent experiments using *Mycoplasma* free A549 cells did not follow the same trend, with RGD causing no change in PAK*gfp* association with suspended or adherent A549 cells (Figure 6). In addition, both RGD and control peptides were unable to influence PAK internalization into these *Mycoplasma* free A549 cells (Figure 7), and they did not produce any measurable differences in cytokine response of infected cells (Figure 8). These results could possibly be explained by the observations that certain pathogens including Group A *Streptococcus* (Klenk *et al.*, 2005; Wang *et al.*, 2006b) can upregulate both mRNA and protein expression of αv integrins following bacterial infection of cells. Indeed, we have also observed in our lab that infection of A549 cells with PAK results in increased mRNA and surface protein expression of various integrin subunits including α5, αv, β1 and β4 (Gravelle et al, unpublished data). If cells infected with *Mycoplasma* behaved in a similar fashion and possessed higher basal levels of integrin expression than non-contaminated cells, it is possible that RGD peptides were able to inhibit bacterial interactions with cells in these experiments because there were more integrins present for the bacteria to interact with, and thus more opportunities for the peptides to block these interactions. Since the ability of the peptides to inhibit bacterial interactions with integrins may be weak, the effects might only be measurable under circumstances with high integrin levels. Unfortunately, we were unable to characterize the

integrin expression profile of the *Mycoplasma* infected cells due to our laboratory policies inhibiting further culturing of contaminated cells once they are identified.

The inability of RGD peptides to block bacterial interactions with integrins could also be due to some of the physical properties of the peptides themselves. Since these peptides are only composed of a few amino acids, they are inherently very small, and while they block the RGD binding site of integrins, there may still be other interactions which they cannot interfere with (Ruoslahti, 1996). Beyond the simple presence of the RGD sequence, other conformation details of RGD peptides, including length of peptide, composition of additional amino acids, and cyclization all greatly influence the inhibitory ability of the peptide (Siemion & Wieczorek, 2003; Kim *et al.*, 2005). In addition, the RGD peptide must bind a very specific site of integrins which is only exposed while the integrin is in an 'open', conformationally active state (Luo *et al.*, 2003). The conformation of an integrin can be altered by the activation state of the cell (Sims *et al.*, 1991), as well as the presence of various divalent cations including calcium, magnesium and manganese (Shimizu & Mobley, 1993). It is known that *P. aeruginosa* posses many other virulence factors which aid in adhesion and invasion including pili, flagella and LPS, and it is possible that these interactions may mask any effects of blocking integrins because it is just one of many possible ways that these bacteria can interact with host cells. Indeed, activation of both integrins and TLR have been observed to be necessary for complete cellular responses to bacterial LPS, emphasizing the importance of receptor cross-talk, and the complicated nature of cellular responses (Perera *et al.*, 2001; Monick *et al.*, 2002; Wang *et al.*, 2003).

The interpretation of our results obtained using anti-integrin antibodies requires careful consideration. Unlike the peptides which may have variable efficacies, inconsistencies and possible non-specific effects, the monoclonal antibodies should exhibit entirely specific and

efficient blocking of integrins. Figure 9 shows that active anti-integrin antibodies, isotype control antibodies and purified IgG all caused a significant decrease in PAK*gfp* association to adherent A549 cells. The ability of PAK*gfp* to bind to immobilized IgG and also the ability of IgG to inhibit bacterial binding to cells suggests that *P. aeruginosa* may possess Fc binding ability similar to that of *S. aureus*. Indeed, during antibody inhibitor experiments with *S. aureus*, it is common practice to use SpA mutants that cannot bind to the Fc region of antibodies (Sinha *et al.*, 1999; Massey *et al.*, 2001). To the best of our knowledge, *P. aeruginosa* binding to Fc region of antibodies has not yet been investigated or characterized. If PAK*gfp* were able to bind Fc regions, it is possible that the bacterium is binding to free antibody, becoming 'coated' with antibody and sequestered in aggregates away from the cells. This would non-specifically decrease the number of bacteria actually interacting with the cells. In support of this theory, after repeating the antibody inhibitor experiments with an additional step to wash away excess antibody prior to adding bacteria, the inhibitory ability of all mAb and IgG was abolished (Figure 10). Interestingly, while there is some published literature showing that anti-α5β1 and αvβ5 significantly decrease adhesion and invasion of certain *P. aeruginosa* strains, neither of these papers use isotype controls to confirm the specificity of the antibody effects (Gagniere & Di Martino, 2004; Leroy-Dudal *et al.*, 2004), and thus they do not exclude the possibility that *P. aeruginosa* was interacting non-specifically with the Fc regions of the antibodies.

However, even with washing excess antibody away, we still did not see any effect on either bacterial association with A549 cells (Figure 10) or internalization into A549 cells (Figure 11). One possibility is that integrins are not involved in *P. aeruginosa* interactions with these cells. However, it is also possible that the integrin blocking effects of antibodies are difficult to detect because of the other multiple interactions between the bacteria and host cells discussed

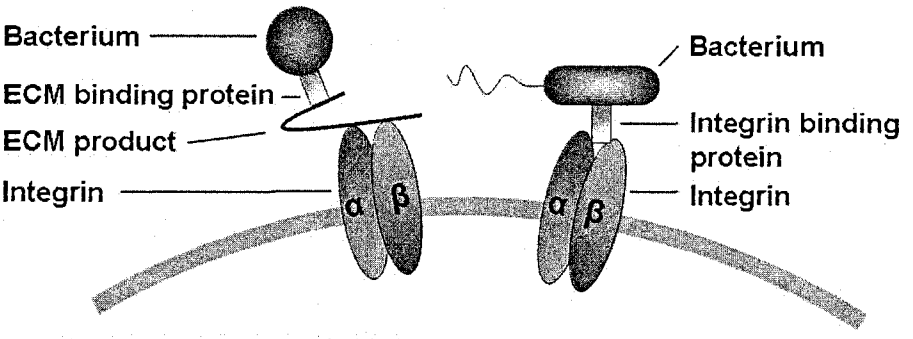
above. In addition, if *P. aeruginosa* can bind to immunoglobulin Fc regions, then antibodies which are bound to integrins on the cell surface would be able to bind bacteria at their exposed Fc regions. In this case, bacteria would not be directly associated with the integrin receptors on the cells, but they would still be detected as cell associated. It is also possible that integrin inhibition is not important for association of the bacterium with the host cells, but rather may affect the subsequent signaling activation. In addition, the antibody inhibitor studies were performed with adherent cells, which produce ECM products and may have them bound to their integrin receptors (Kang *et al.*, 1991; Yasumitsu *et al.*, 1993). If this is the case, and the bacteria are binding to the ECM products as opposed to binding directly to integrins, then it is possible that integrin antibodies would not be effective because the bacteria's binding sites are already attached to the integrins and not affected by the antibodies. Indeed, anti-Fn and anti-Vn antibodies have been shown to inhibit *P. aeruginosa* interactions with lung epithelial cells, although in these experiments, again, isotype controls were not used to verify the specificity of these interactions (Rebiere-Huet *et al.*, 1999; Gagniere & Di Martino, 2004; Leroy-Dudal *et al.*, 2004).

Our data showing that PAK*gfp* binds preferentially to the integrin ligands Fn and Vn, and that Fn can mediate bacterial association with cells suggests that integrins, particularly $\alpha5\beta1$, may have an important role in *P. aeruginosa* pathogenesis. However, subsequent studies using both peptide and antibody integrin inhibitory assays gave less clear results. The compounds used are described to be specifically targeted to inhibit integrins, but there appear to be many interfering variables that complicate this theoretically simple system. The small peptide size, effects of peptide conformation, integrin conformation, levels of integrin expression, and other bacterial virulence strategies may all contribute to confusing the results from such experiments.

The observed ability of PAK*gfp* to bind IgG, and the inhibitory effects of non-specific IgG suggest that *P. aeruginosa* posses Fc binding capabilities, which may interfere with all antibody based assays. Although our results suggest that blocking integrin receptors is not important for adhesion, internalization or cytokine production, we cannot confidently make this conclusion in light of the technical difficulties we discovered with the inhibitory assays used. It is interesting to note that these assays are frequently used by other groups who also have obtained conflicting results. While initial observations of *P. aeruginosa* interactions with nasal epithelial cells showed both RGD and anti-α5 or β1 antibody inhibition of adherence (Roger *et al.*, 1999), another study found that anti-αvβ5 but not α5β1 antibodies decreased adhesion and invasion of *P. aeruginosa* to lung epithelial cells (Leroy-Dudal *et al.*, 2004), while yet another study found strain-specific effects of anti-α5β1 antibodies on adhesion (Gagniere & Di Martino, 2004). This collection of inconsistent data in the literature could therefore be a result of the imperfections inherent in the inhibitory assays used. It is therefore prudent to approach the problem from the side of the host cells, and instead of trying to block or inhibit integrins that are already present, to knock down protein expression of the integrins or the key essential signaling molecules and determine their role in internalization and inflammation.

Aside from the microbial factors that may be affecting our results, we now recognize that it is extremely important to understand the integrin expression profile of the host cells being used. Since the basal level of integrin expression is low on the A549 cells we used (Gravelle et al, manuscript in preparation), we do not feel confident in concluding that integrins are not important, since it is possible that if integrins were more highly expressed that the results of integrin inhibitors would be more clear. To investigate this further, it would be beneficial to repeat the peptide and antibody assays using a cell line that shows higher basal integrin

expression, such as the human mucoepidermoid pulmonary carcinoma cell line NCI-H292, which has been characterized in our laboratory to express high basal levels of integrins (Gravelle S., unpublished data).



Microbes using ECM products to bind integrins

Microbe	ECM binding protein	ECM product	Integrin
Staphylococcus aureus	FnBP	Fn	α5β1
Group A *Streptococcus*	M1 protein, PrtF1/SfbI	Fn	α5β1
Pseudomonas aeruginosa	Putative OMP	Fn, Vn	α5β1, αvβ5
Streptococcus pneumoniae	PavA	Fn	α5β1
Haemophilus influenzae	unknown	Fn	α5β1
Mycobacterium	FAP, Antigen 85B	Vn? Fn?	αv, β1
Pneumocystis carinii	FnBP	Fn	αv, β5

Microbes binding directly to integrins

Microbe	Integrin binding protein	Integrin
Bordetella pertussis	FHA	α5β1
Group A *Streptococcus*	Scl1 (proposed)	α2β1
Viruses (ie: coxsackievirus)	RGD containing capsid proteins	α2β1, α3β1, α5β1, αvβ5, αvβ6

Figure 1: Bacterial interactions with host respiratory epithelial cell integrins.

Microorganisms can interact with lung epithelial integrins either directly via integrin-binding proteins or indirectly by using an extracellular matrix protein such as Fn or Vn as a molecular bridge to engage these receptors.

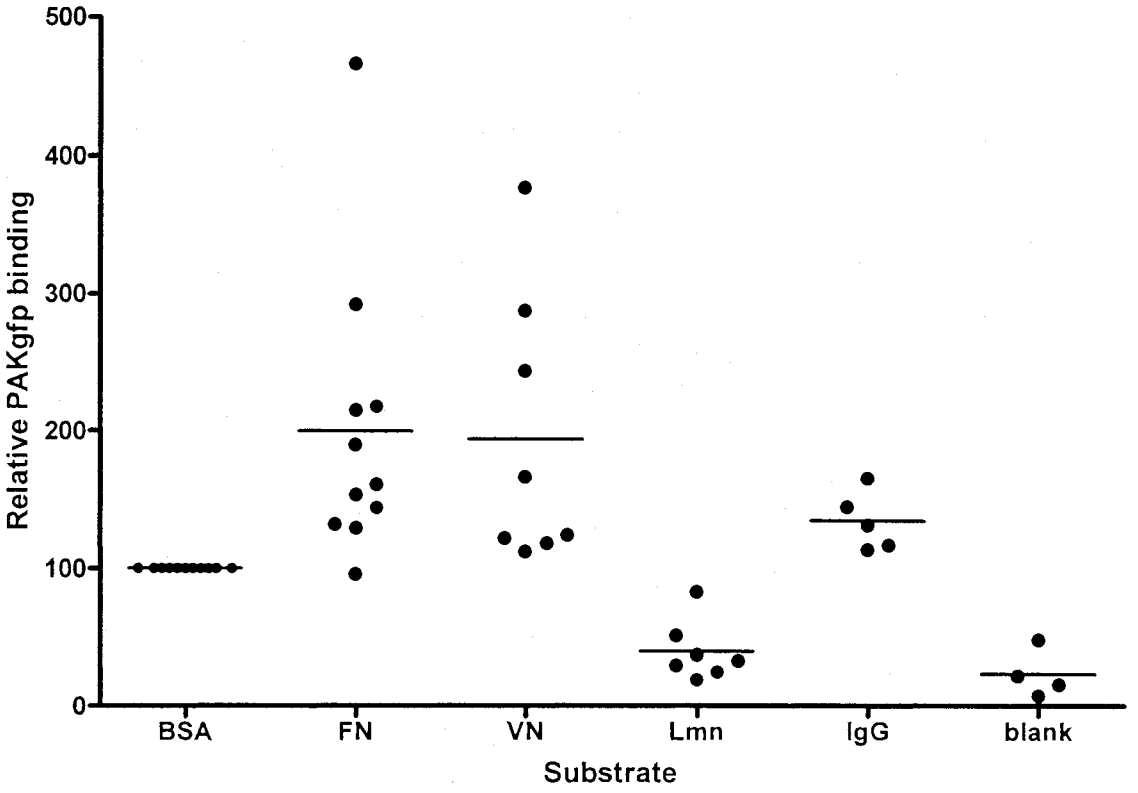


Figure 2: PAKgfp binding to immobilized substrates. PAK*gfp* binding to substrate coated wells (10 μg/ml) was quantified using a microplate reader following 5 min incubation at 37°C. Dots show the results of each individual experiment, while horizontal bars indicate the mean of all experiments. Fn, Vn and IgG all showed increased PAK*gfp* binding compared to BSA, while Lmn and blank (non-coated) wells showed less binding.

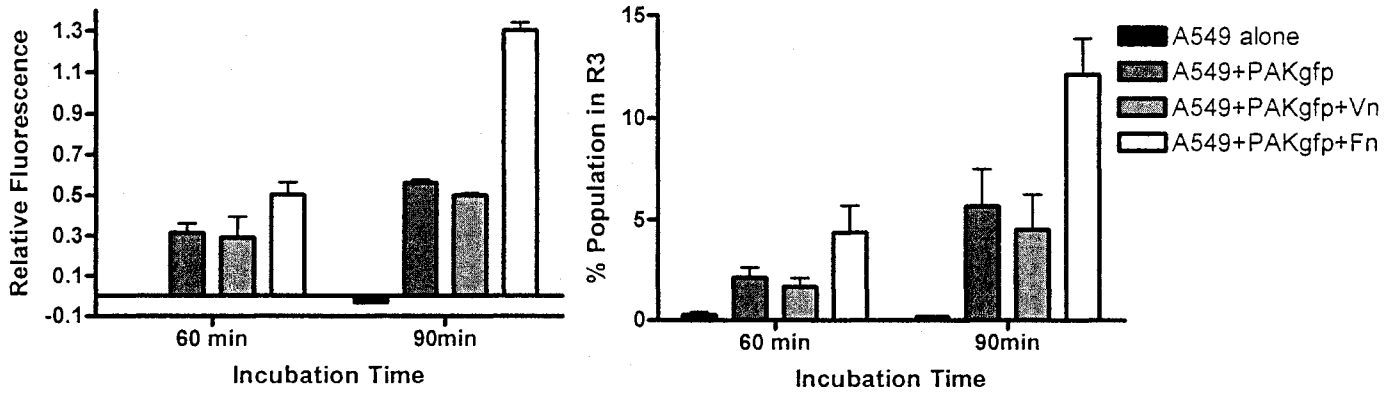


Figure 3: Effects of Fn and Vn on PAK*gfp* association with A549 cells: Flow cytometry analysis of suspended A549 cells incubated with PAK*gfp* (MOI of 50:1) shows that pre-incubation of A549 cells with Fn (10 μg/ml) increases PAK*gfp* association, measured as both relative FL1 fluorescence and % population in R3 at both 60 and 90 min incubation, while pre-incubation with Vn (10 μg/ml) had no affect on bacterial association with A549 cells. (n = 2 independent trials).

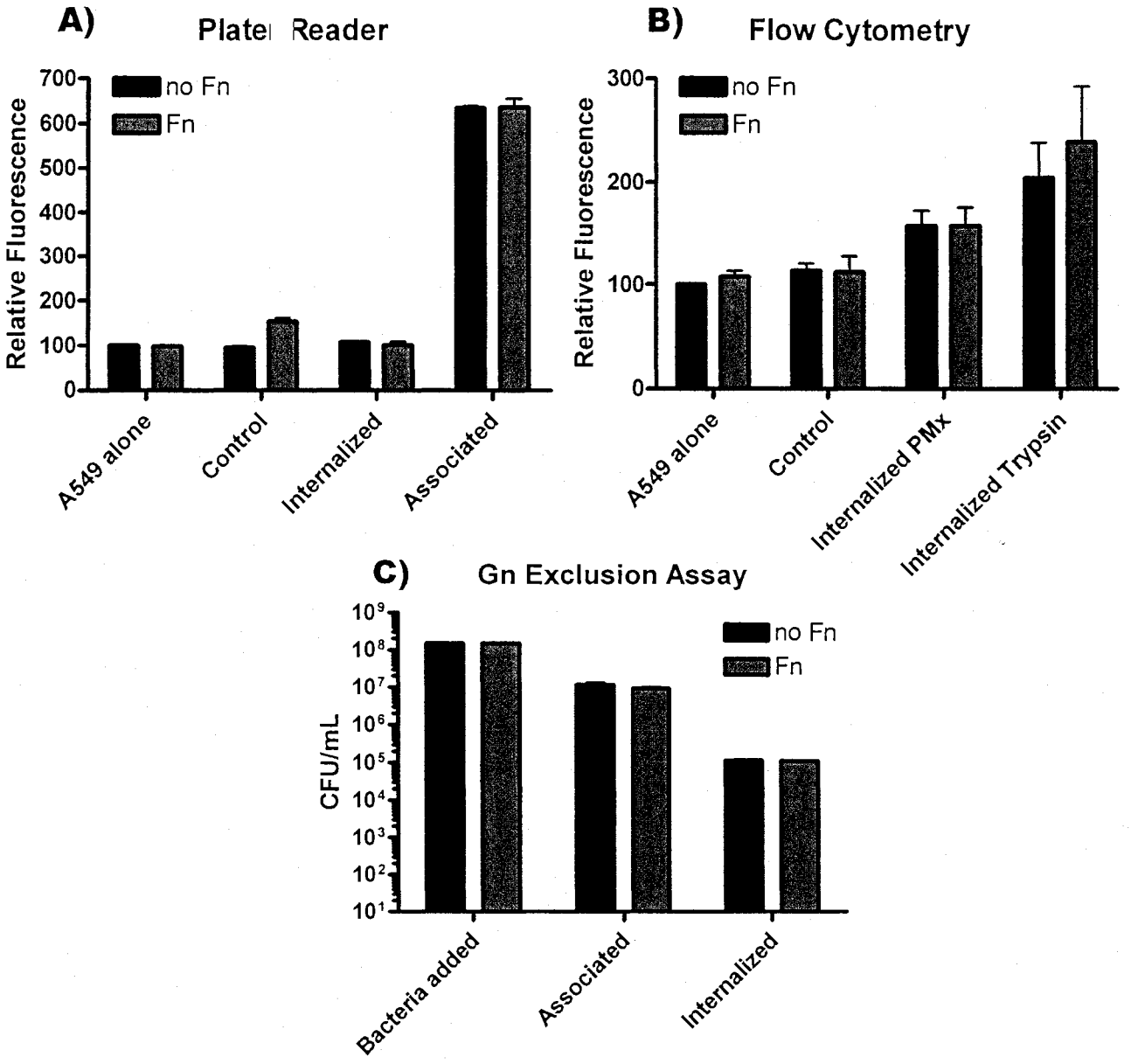


Figure 4: Effects of Fn on PAKgfp internalization. A) Plate reader based PMx quenching internalization assay showed that exogenous Fn (10 μg/ml, 15 min pre-incubation) had no significant effect on PAK*gfp* internalization into adherent A549 cells (n=2). B) Flow cytometry based PMx quenching internalization assay also did not show any effect of exogenous Fn on PAK*gfp* internalization (n=3). C) In agreement with these results, standard Gn exclusion assay did not show any effect of exogenous Fn on PAK internalization (n=1 independent experiment performed in triplicate). 'Control' includes cells and bacteria incubated in presence of antibiotic for the entire experiment, 'Associated' includes bacteria adherent to or internalized into A549 cells, while 'Internalized' is bacteria surviving inside A549 cells following antibiotic treatment. The association of PAK*gfp* with adherent A549 was also unaffected by Fn, in agreement with observations from Chapter 1.

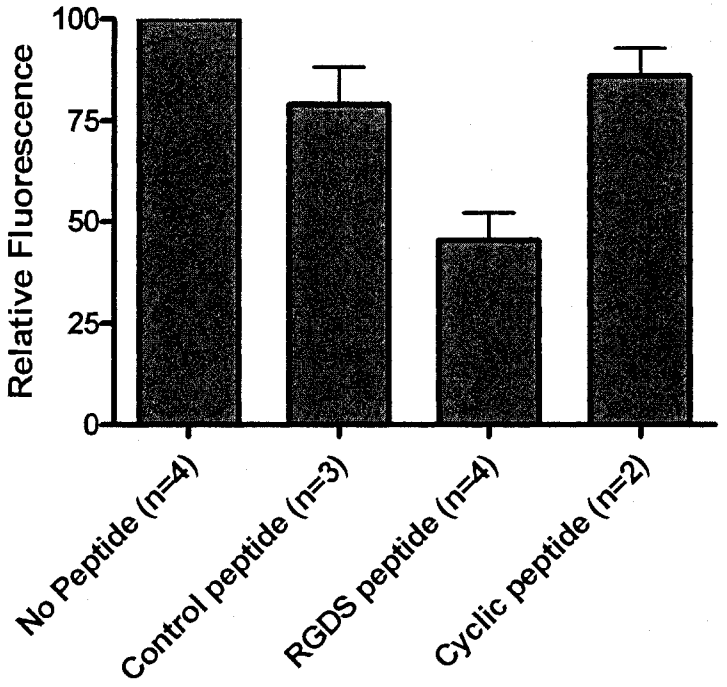


Figure 5: The effect of peptides on PAK*gfp* association with A549 cells infected with *Mycoplasma*. Thirty min pre-incubation of A549 cells with RGDS peptide (500 μg/ml) at room temperature caused a significant decrease in PAK*gfp* association with *Mycoplasma* contaminated A549 cells in suspension following 60 min infection ($p<0.01$), while both control scrambled peptide and cyclic RGD peptide did not (flow cytometry analysis). (One sample t-test, n indicated in figure).

	Relative fluorescence	
	Suspension A549 (n=5)	Adherent A549 (n=3)
No peptide	100	100
control peptide	69	79
RGDS peptide	100	84

Figure 6: Peptide effects on PAK*gfp* association with *Mycoplasma*-free A549. 30 min pre-incubation of uncontaminated A549 cells with RGDS peptide (500 μg/ml) did not significantly decrease PAK*gfp* association with either A549 cells in suspension or adherent conditions following 60 min infection (one sample t-test).

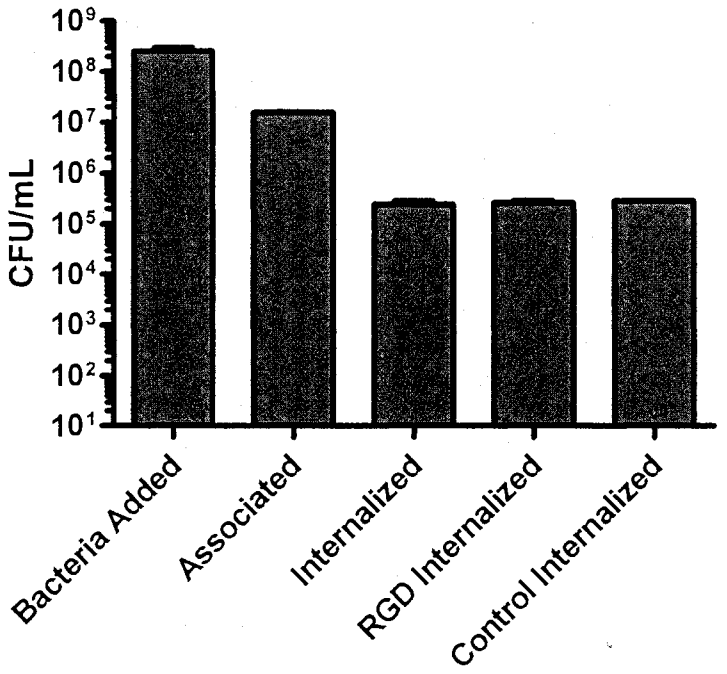


Figure 7: Effects of RGD peptide on PAK internalization into A549 cells. 30 min pre-incubation of adherent A549 cells with active RGD or control scrambled peptide (both at 500 μg/ml) did not inhibit PAK internalization during 60 min infection, as measured using the Gn exclusion assay (n = 2 separate experiments, each performed in triplicate).

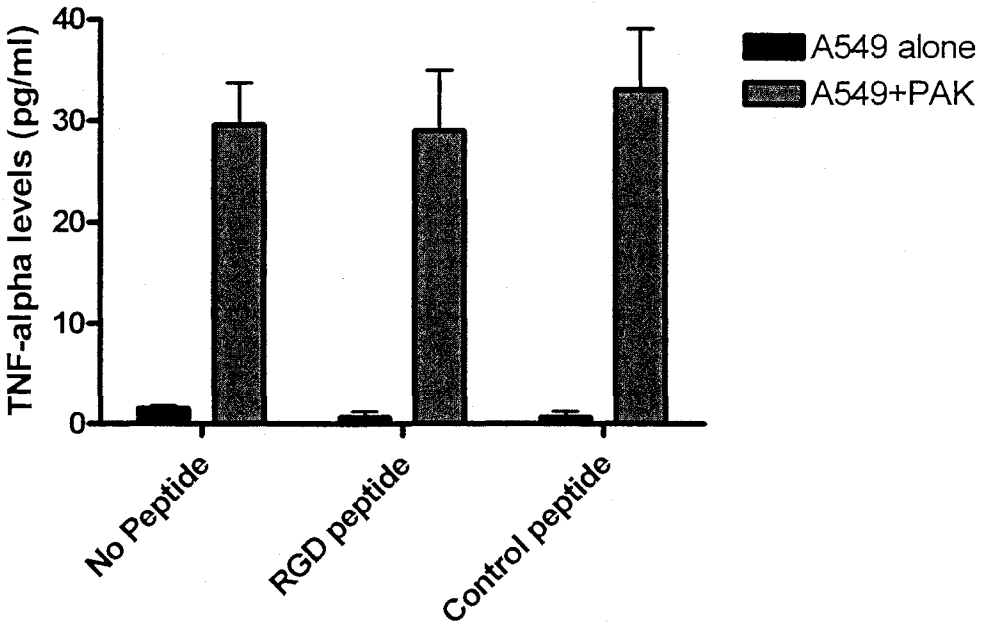


Figure 8: Effects of RGD peptide on PAK induction of TNF-α release from A549 cells. TNF-α released by adherent A549 incubated with PAK for 18 h (live bacteria for 1 h, antibiotic killed bacteria for 17 h) was not affected by the presence of either RGD or control peptide (500 μg/ml). In all experiments, stimulation of A549 cells with bacteria caused significant TNF-α release (n=3 separate experiments, $p<0.05$, Student's t-test).

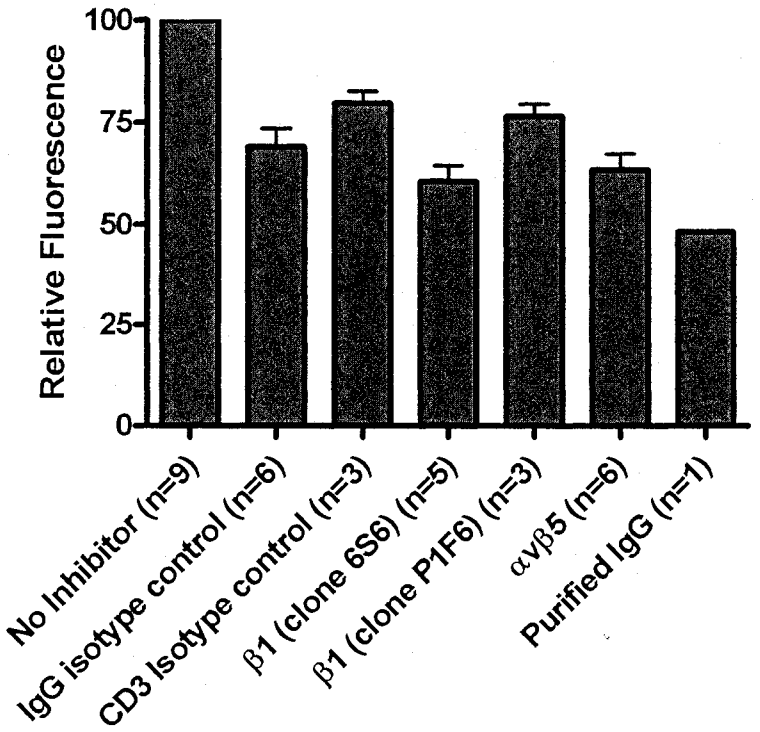


Figure 9: Effect of antibodies on association of PAK*gfp* with adherent A549 cells. Pre-incubation of adherent A549 cells for 30 min with active anti-integrin antibodies or isotype controls (all at 10 μg/ml) resulted in significantly decreased PAK*gfp* association with A549 cells following 60 min infection at an MOI of 50:1 using the fluorescence plate reader based assay (n as specified in figure, $p<0.05$, One sample t-test). Interestingly, purified IgG also caused inhibition of PAK*gfp* association with A549 cells, although no statistics were performed because n=1.

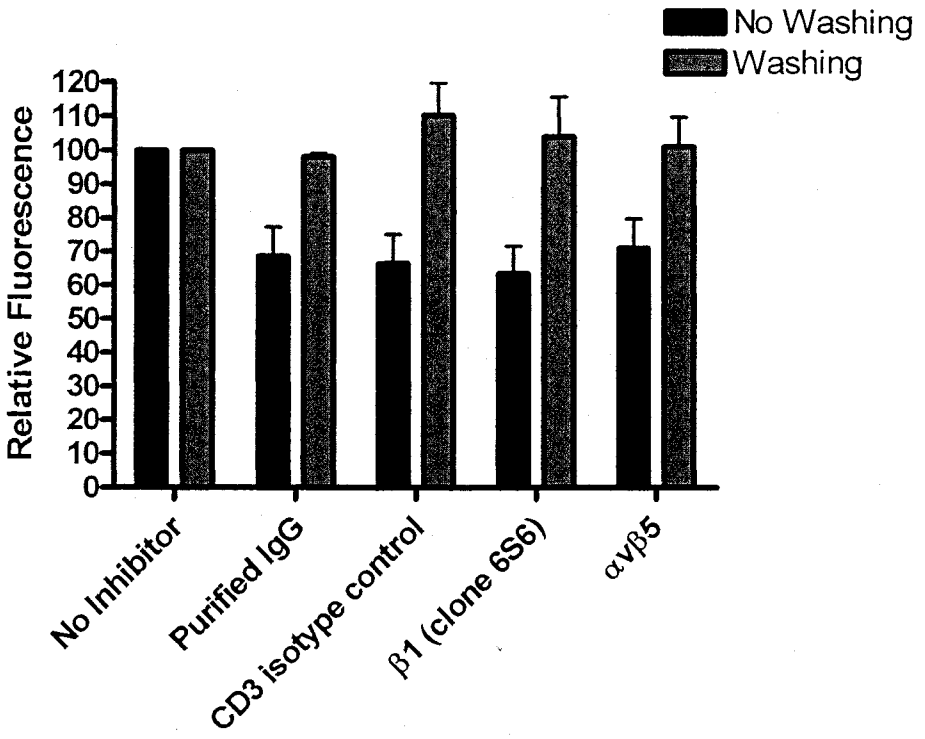


Figure 10: Effects of washing antibodies away prior to incubation with PAK*gfp*. Incubation of PAK*gfp* with adherent A549 cells (MOI of 50:1) for 60 min in the presence of exogenous antibody (10 μg/ml) shows a significantly decreased bacterial association due to the presence of either purified IgG, isotype controls, or active anti-integrin antibodies using the fluorescence plate reader based assay. In contrast, washing the excess antibody away following the 30 min pre-incubation and prior to addition of bacteria removes this inhibitory effect of all the controls and active antibodies (n=3 separate experiments, $p<0.05$, One sample t-test) .

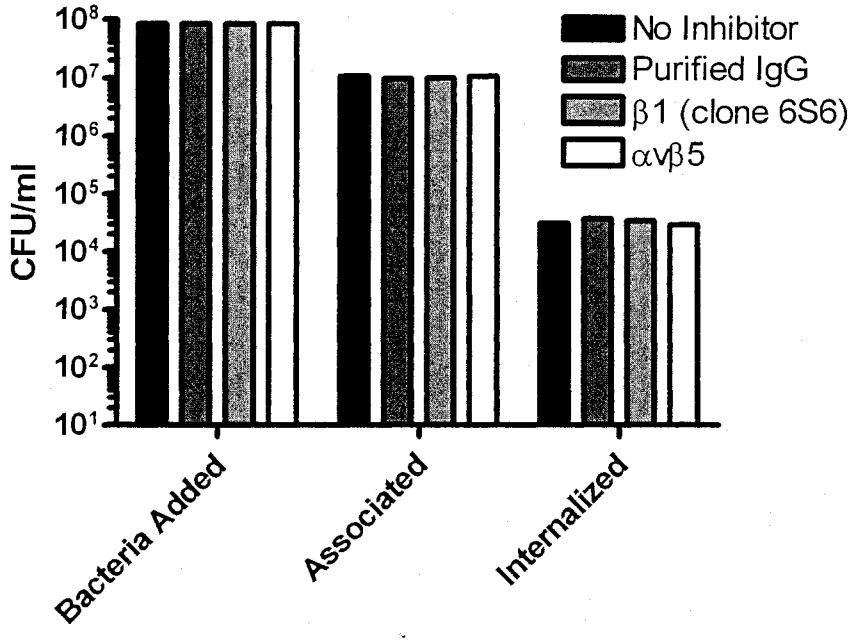


Figure 11: Effects of anti-integrin antibodies on PAK internalization into adherent A549 cells. Pre-treatment of adherent A549 cells with 10 μg/ml anti-integrin β1, αvβ5, or control IgG for 30 min, followed by washing antibodies away did not affect PAK association with or internalization into A549 cells during 60 min infection at MOI of 50:1 using the Gn exclusion assay. (n = 1 experiment performed in triplicate).

Chapter 3: The role of epithelial cell responses in bacteria-host interactions

Abstract

Investigating host-bacterial interactions from the perspective of cellular responses such as cytokine production affords insight into both bacterial and host cell aspects involved in the infectious process. Using siRNA knockdown of the αv integrin subunit and integrin linked kinase (ILK) we have investigated the importance of these proteins for *P. aeruginosa* PAK interactions with A549 lung epithelial cells, specifically stimulation of cytokine release by these cells. In addition, the importance of the bacterial structures flagella, pili and LPS for the cytokine response of A549 cells was quantified using the isogenic PAK mutants deficient for these virulence factors, PAK fliC, PAK NP and PAK rmlC, respectively. The response of A549 cells to clinical *P. aeruginosa* isolates from CF patients was also investigated to determine the effect of mutations these bacteria may acquire during chronic lung colonization. ILK was found to be involved with cytokine release from A549 cells in response to PAK infection, but not important for bacterial association or internalization into A549 cells. In addition, PAK fliC and PAK rmlC elicited a reduced cytokine release from A549 cells compared to PAK WT. Clinical isolates of *P. aeruginosa* were observed to induce much less cytokine release from A549 cells than PAK WT, and mucoid variants tended to induce less cytokine release than non-mucoid variants of the same strain.

Introduction

Cellular responses to *P. aeruginosa* infection

During bacterial infection, host cells react by mounting an innate immune response which involves secretion of various cytokines and chemokines, resulting in attraction, activation and survival of immune effector cells. Several cytokines play important roles during bacterial infection. Interleukin (IL)-1 is a highly inflammatory, multi-functional cytokine that exists in two forms, IL-1α and IL-1β, with biologically identical roles including induction of cytokine upregulation from nearly every cell type through nuclear factor (NF)-κB and activator protein (AP)-1 transcription factor activation (Muegge *et al.*, 1989). While IL-1β is readily released from activated cells, IL-1α is found in a membrane associated biologically active form (Kurt-Jones *et al.*, 1985), and is not normally found in circulation or in any body fluids, unless it has been released from dying cells or cleaved by calpain (Wakabayashi *et al.*, 1991; Watanabe & Kobayashi, 1994). IL-6 is a potent pro-inflammatory cytokine with multiple roles, including activation of B cells to produce immunoglobulins (Hirano *et al.*, 1984; Teranishi *et al.*, 1984), T cell activation, growth and differentiation (reviewed Van Snick, 1990; Houssiau & Van Snick, 1992), and neutrophil stimulation (Dalrymple *et al.*, 1995). IL-8 is a leukocyte attracting chemokine that recruits neutrophils to the site of infection (Pease & Sabroe, 2002). Tumour necrosis factor (TNF)-α is important for a normal response to infection, as it is critical for activation of both AP-1 and NF-κB transcription factors which induce transcription of important cell survival and pro-inflammatory genes, including intracellular adhesion molecule-1 (ICAM-1) (Bradley, 2008). However, excessive TNF-α response can be quite harmful to a host, and thus regulation of this cytokine is critical. Granulocyte macrophage colony stimulating factor (GM-CSF) has many important effects on various immune cells. GM-CSF increases the antimicrobial

activity of neutrophils by increasing phagocytosis, release of lysozyme and chemotactic factors, recruiting complement molecules, and increasing Fc-receptor expression (reviewed by Ruef & Coleman, 1990; Jones, 1994). GM-CSF stimulation of monocytes results in their differentiation into mature macrophages with antigen presenting capabilities, and induces release of TNF-α, IL-1α and β, IL-6, and IL-8 (reviewed by Jones, 1996). In the case of dendritic cells, GM-CSF is the major stimulant for the differentiation, viability and function, and is thus extremely important in antigen presentation (Witmer-Pack *et al.*, 1987).

Patients with chronic *Pseudomonas aeruginosa* infections, particularly those with cystic fibrosis (CF) have elevated levels of the leukocyte attracting chemokine IL-8 in their lungs (Richman-Eisenstat *et al.*, 1993). Indeed, certain *P. aeruginosa* exoproducts (Massion *et al.*, 1994) such as PA autoinducer (DiMango *et al.*, 1995), nitrite reductase (Mori *et al.*, 1999) and pyocyanin (Denning *et al.*, 1998), as well as bacterial structures such as pili and flagella (DiMango *et al.*, 1995) cause an increased expression of IL-8 in airway epithelial cells *in vitro*. In addition to IL-8, *P. aeruginosa* stimulates the release of the potent pro-inflammatory cytokines IL-6 (Kube *et al.*, 2001), IL-1β and TNF-α (Tseng *et al.*, 2006), as well as activates the transcriptional regulator of all these genes, NF-κB (DiMango *et al.*, 1998; Mori *et al.*, 1999).

While *P. aeruginosa* can stimulate lung epithelial cells to produce and release cytokines, two of these pro-inflammatory cytokines, TNF-α and IL-1β, subsequently act together to further increase the activation state of the cells. TNF-α is important for a normal response to infection (Bradley, 2008), and IL-1β is also important for normal host responses to *P. aeruginosa* infections, since patients with CF have lower levels of IL-1β released during the course of their chronic infections (Reiniger *et al.*, 2007). Working together or separately, TNF-α and IL-1β can activate NF-κB and AP-1 (Muegge *et al.*, 1989; Tseng *et al.*, 2006; Bradley, 2008), further

increase levels of IL-8 (Kube *et al.*, 2001; Perez & Davis, 2004; Wu *et al.*, 2005), IL-6 (Kube *et al.*, 2001; Perez & Davis, 2004), IL-1β and TNF-α (Kube *et al.*, 2001; Perez & Davis, 2004; Vos *et al.*, 2005), and the leukocyte chemoattractant and growth factor GM-CSF (Kube *et al.*, 2001). These cytokines thus act in a positive feedback loop, amplifying the pro-inflammatory responses of cells being infected with pathogens such as *P. aeruginosa*.

Although it is clear that multiple bacterial products and pathogen-host interactions can result in the activation of pro-inflammatory responses and cytokine release, there is some evidence that integrins may play a possible role in these processes as well. The respiratory pathogen *Bordetella pertussis* has been shown to activate NF-κB and to upregulate ICAM-1 expression in an α5β1 integrin dependent fashion (Ishibashi & Nishikawa, 2002; Ishibashi & Nishikawa, 2003). The bacterium *Yersinia enterocolitica* is able to induce IL-8 secretion from epithelial cells through the binding of the integrin β1 subunit (Schmid *et al.*, 2004). It has been clearly and elegantly demonstrated that *Borrelia burgdorferi* can induce expression of pro-inflammatory mediators using the integrin α3β1 to induce a toll like receptor (TLR) independent inflammatory response in human chondrocytes (Behera *et al.*, 2006a). In addition, β1 integrins in human bronchial epithelial cells provide co-stimulatory signals that increase TNF-α induced pro-inflammatory responses (Ulanova *et al.*, 2005). Based on such observations made on other bacteria and cell types, it is possible that *P. aeruginosa* may also cause inflammatory responses in lung epithelial cells using integrins as signaling mediators.

Small interfering RNA (siRNA)

The concept of post-transcriptional gene silencing (PTGS) was first suggested based on observations that transgenic petunia plants over-expressing the gene for purple pigment

production actually exhibited loss of pigment production (Napoli *et al.*, 1990). After intensive studies in many plant, fungi and nematode models, it was discovered that the presence of excess double stranded RNA (dsRNA), composed of both the sense and anti-sense strands specific to a particular gene sequence resulted in silencing of that gene's product (Fire *et al.*, 1998; Hamilton & Baulcombe, 1999). PTGS using small interfering RNA (siRNA) sequences of 21 nucleotides in length was found to effectively silence gene expression in cultured mammalian cells (Elbashir *et al.*, 2001) and even *in vivo* using a mouse model (McCaffrey *et al.*, 2002).

The exact mechanisms of RNA interference (RNAi) are now well understood, and have been reviewed extensively and thoroughly by several authors (Tuschl, 2001; Hannon, 2002; Agrawal *et al.*, 2003; Dykxhoorn & Lieberman, 2005). To briefly summarize, dsRNA that is 21-25 nucleotides in length is introduced into a cell and recognized and cleaved into two short fragments of approximately 12 nucleotides in length by an RNaseIII type enzyme called Dicer (Agrawal *et al.*, 2003). These short dsRNA sequences are then separated into single stranded RNAs, and they associate with the RNA-induced silencing complex (RISC) which includes Argonaute protein family members. This RISC complex is targeted to the specific mRNA sequence matching the single stranded siRNA contained in the RISC complex, and the mRNA is then degraded by RISC, resulting in gene silencing (Agrawal *et al.*, 2003). Although RNAi has likely evolved as a defense mechanism of cells to protect against foreign DNA such as viral DNA during an infection, it is now used extensively as an important biological tool to research the functions and involvement of various gene products in cellular processes, and more recently is being tested as a therapeutic target (Dykxhoorn & Lieberman, 2005).

siRNA knockdown of integrins

siRNA has been used to study the role of various integrin subunits in several contexts. Studies using integrin siRNA are summarized in Table 1. In general, an siRNA experimental setup involves a transfection period usually ranging from 24 to 72 h, during which time the siRNA duplex is introduced into the cells by a lipid-based transfection reagent. It is good practice to utilize several controls, including non-targeting scrambled siRNA, empty transfection reagent, multiple functional readouts (for mRNA and protein), and 'rescue' experiments to restore gene function if possible (Editorial, 2003). It is also important to use the lowest concentration of siRNA possible, as increasing concentrations often result in non-specific off-target effects (Editorial, 2003).

siRNA knockdown of integrins has not been extensively utilized in literature. The usefulness of siRNA knockdown depends on the reduction in levels of the integrin surface protein so that the functional role of the protein can be examined. Although some of the studies outlined in Table 1 reported successful knockdown of integrin protein (Zhou *et al.*, 2004; Andjilani *et al.*, 2005; Cao *et al.*, 2006; Liu *et al.*, 2007; Singh *et al.*, 2007; Lv *et al.*, 2008), many did not report protein levels. Indeed, during their study of α8 integrin knockdown, Zargham and Thibault (2005) reported that the half life of the α8 subunit protein was 20 h. This could greatly affect the efficiency of siRNA knockdown of integrins because even if the amount of mRNA is successfully reduced, the long half-life of the protein means that the functional knockdown is very difficult to achieve. Most studies that have had successful integrin protein knockdown have used primary cells, which typically grow much slower than immortalized cells lines, and would therefore be easier to culture for the prolonged transfection periods required for integrin protein knockdown.

Both α5β1 and αvβ5 integrins were of interest to our study based on their Fn and Vn ligand binding respectively. While our results suggested that α5β1 integrin may be the most important for *P. aeruginosa* infection of lung epithelial cells, there have been no reports of successful α5 siRNA knockdown, and β1 knockdown exhibited variable results (Wang *et al.*, 2005; Singh *et al.*, 2007). Experiments using αv siRNA on the other hand have shown successful mRNA and protein knockdown of up to 100% (Zhou *et al.*, 2004; Cao *et al.*, 2006). In particular, the experiments by Cao *et al.* (2006) were very clearly described, utilizing proper controls, multiple readouts, and the custom αv siRNA sequence they used was provided. For these reasons, we chose to target αv for siRNA knockdown in our cells. It should be noted that there is no published data showing attempted or successful integrin knockdown using the A549 cell line.

Table 1: Summary of published integrin siRNA knockdown experiments

Integrin subunit	Cell type	siRNA concentration	Transfection time	mRNA knockdown	Protein knockdown	Ref
β1	HeLa cervical epithelial cell line Normal human skin fibroblasts	360 pmol per transfection	48 h	80% Not measured	None 75%	(Singh *et al.*, 2007)
	Primary human pancreatic islet cells	60 nM	72 h		40%	(Wang *et al.*, 2005)
β3	CV-1 African green monkey fibroblast cell line	100 nM	72 h	Not quantified	Not quantified	(Bergh *et al.*, 2005)
β4	Primary mouse neurons	40, 60, and 80 nM	24-72 h		100% at 72 h	(Lv *et al.*, 2008)
	Human umbilical vascular endothelial cells (primary)	20 nM	48 h		70%	(Liu *et al.*, 2007)
α3	Primary human chondrocyte cells	100 nm	24 h	71%		(Behera *et al.*, 2006b)
α6	Human testicular embryonic carcinoma cell line NCCIT	10 nM	96 h total; 2 transfections 1st at 0 h 2nd at 72 h		70%	(Andjilani *et al.*, 2005)
	MCF-7 breast cancer cell line MSS-derived cells	not specified	24 h	70%		(Cariati *et al.*, 2008)
α8	primary vascular smooth muscle cells	not specified	24 h 48h 72h	not specified decreased increased	decreased 50% increased	(Zargham *et al.*, 2007)
αv	MDA-MB-435 breast cancer cell line	10 nM	48 h	80%	75-80% surface protein	(Cao *et al.*, 2006)
	primary human and rat Hepatic stellate cells	5 nM 10 nM	24 h 72 h	90-100%	 100%	(Zhou *et al.*, 2004)
	CV-1 African green monkey fibroblast cell line	100 nM	72 h	good reduction, not quantified		(Bergh *et al.*, 2005)

siRNA knockdown of integrin linked kinase (ILK)

Integrin linked kinase (ILK) is a serine-threonine protein kinase that was first described as a coupling molecule to connect β1 and β3 integrins to the actin cytoskeleton, that has autophosphorylation abilities and also can phosphorylate integrin β subunits (Hannigan *et al.*, 1996). Upon integrin activation, ILK acts as a structural and catalytic component in actin

cytoskeleton rearrangement in a phosphoinositol-3-kinase (PI3K) dependent fashion (Persad & Dedhar, 2003). ILK links β subunits to actin through various ILK binding proteins including focal adhesion kinase (FAK), paxillin as well as affixin, which is a substrate of ILK directly involved in actin cytoskeleton rearrangement (Wu & Dedhar, 2001). ILK is found co-localized with β integrins in focal adhesion complexes (Li *et al.*, 1999), structurally connecting integrins to these complexes through linking to focal adhesion proteins like PINCH (Persad & Dedhar, 2003). Since integrin binding to extracellular matrix (ECM) products such as Fn results in their clustering at focal adhesion complexes (Pankov & Yamada, 2002), and since Fn binding is critical for ILK activation (Delcommenne *et al.*, 1998), it is in these focal adhesion complexes where ILK signaling takes place. ILK has been shown to be essential for the internalization of Group A *Streptococcus* into human larynx and cervical epithelial cells (Wang *et al.*, 2006a). The authors found that bacteria bound to Fn induced integrin clustering, and subsequent ILK activation via PI3K and phosphatidylinositol 3-kinase (PIP3), which resulted in paxillin and FAK activation and rearrangement of the actin cytoskeleton leading to bacterial internalization (Wang *et al.*, 2006a). The authors also showed that integrin dependent invasion of *S. aureus* and *Yersinia spp.* required activated ILK, suggesting that ILK may have a universal role in mediating bacterial invasion into non-phagocytic cells (Wang *et al.*, 2006a). A study of *P. aeruginosa* PAK found that internalization was dependent on PI3K activation, and also on protein kinase B/Akt (PKB/Akt) activation (Kierbel *et al.*, 2005), both of which are directly involved in ILK mediated signaling (PKB/Akt involvement with ILK is described below).

In addition to its role in cytoskeletal rearrangement, ILK is also able to activate several cell signaling pathways in a PI3K dependent fashion (Dedhar, 2000). Indeed, binding of integrins to their ECM products results in regulation of gene expression through activation the

transcription factors AP-1 and NF-κB (Dedhar, 2000). Of particular note, ILK is able to phosphorylate and activate PKB/Akt (Delcommenne *et al.*, 1998; Lynch *et al.*, 1999; Persad *et al.*, 2001), and to phosphorylate and inhibit glycogen synthase kinase-3 (GSK-3) (Delcommenne *et al.*, 1998; Troussard *et al.*, 2003), both of which result in increased activity of the transcription factor AP-1 through c-Jun binding to AP-1 promoter sequences (Dedhar, 2000; Troussard *et al.*, 2003). This is of interest because AP-1, acting in concert with NF-κB, can induce increased production of pro-inflammatory cytokines including IL-8 (Mukaida *et al.*, 1990; Wu *et al.*, 2005), and many other cytokines and chemokines. In addition, ILK-induced AP-1 activation has been directly linked with increased expression of matrix metalloproteinase (MMP)-9 (Troussard *et al.*, 2000). A study by Behera *et al.* (2006) clearly demonstrated a TLR independent, β3-dependent route for *Borrelia burgdorferi* induction of various pro-inflammatory responses including increased production of matrix metalloproteinases, various interleukins, TNF-α, regulated upon activation, normal T cell expressed and secreted (RANTES) and more. These responses may therefore be a result of ILK activation of AP-1 induced by bacterial interactions with β3 integrin subunit.

siRNA knockdown of ILK has been successfully used in several different experimental systems, as summarized in Table 2. Knockdown of ILK is a useful tool to study the role of either β1 or β3 integrins, since ILK directly mediates the structural and signaling outcomes of the engagement of these subunits (Wu & Dedhar, 2001). Although there are several commercially available pre-synthesized ILK siRNA duplexes, most studies custom order one of two ILK targeting siRNAs designed by Troussard *et al.* (2003). The two siRNA duplexes include ILK-FSF (nt 1176-1194), which targets the kinase domain of the ILK mRNA (sense 5'UGUCAAGUUCUCUUUCCAAUGdTT), or ILK-H (nt 741-759), which targets the plexstrin

homology domain of ILK (sense 5'CCUGACGAAGCUCAACGAGAAdTT) (Troussard *et al.*, 2003). While both sequences have been used with success, ILK-H is more common, and we have therefore chosen this sequence for our ILK knockdown studies. In addition, a study on the role of ILK in cellular responses to Group A *Streptococcus* has used ILK-H siRNA to successfully knockdown ILK, which resulted in a 60% decrease in bacterial internalization into human larynx epithelial cells (Wang *et al.*, 2006a).

Table 2: Experiments using ILK siRNA

Cell type	siRNA concentration	Transfection time	mRNA knockdown	Protein knockdown	Ref
HEK-293 human embryonic kidney cell line	100 nM	4 days	unspecified	'significant'	(Troussard *et al.*, 2003)
PC3 prostate carcinoma cell line	25, 50 and 100 nM	4 days	unspecified	'significant'	(Tan *et al.*, 2004)
Bovine aortic endothelial cells	20-50 nM	24 h or 48 h with 2nd transfection at 24 h	unspecified	70-80%	(Vouret-Craviari *et al.*, 2004)
Pancreatic ductal adenocarcinoma cell lines PANC1, MIAPaCa2, Capan2	unspecified	48 h	unspecified	80%	(Duxbury *et al.*, 2005)
Human lung fibroblast CCL-210 cell line	100 nM	24 h	almost 100%	decreased, not as much as mRNA	(Nho *et al.*, 2005)
Primary ovarian surface epithelium	unspecified	4 days	unspecified	'significant'	(Ahmed *et al.*, 2006)
Multiple glioblastoma cell lines	120 nM	48 h	unspecified	'significant'	(Edwards *et al.*, 2006)
Human glomerular mesangial cells	50 nM	48 h	65%	'decreased'	(Ohnishi *et al.*, 2006)
Hep-2 human larynx epithelial cell line	80 nM	72 h	unspecified	'significant'	(Wang *et al.*, 2006b)
Aortic vascular smooth muscle cells	250 pmol per transfection	24 h	unspecified	60%	(Dwivedi *et al.*, 2008)
Human umbilical vein endothelial cells	20 nM	48 h 72 h	unspecified	70% 90%	(Joshi *et al.*, 2007)
SKOV3 human ovarian carcinoma cell line	100 nM	48 h	'significant'	'successful' for 52kDa protein by not 59kDa protein	(Lin *et al.*, 2007)

PAK mutants and *P. aeruginosa* clinical isolates

P. aeruginosa is known to posses several structures involved in the pathogenesis of epithelial infection, namely flagella, pili and lipopolysaccharide (LPS). *P. aeruginosa* normally express one polar flagellum that has the conserved structure of most Gram-negative bacteria (Prince, 2006). Isolated *P. aeruginosa* flagellin is able to induce local inflammatory responses in mouse models (Feldman *et al.*, 1998), and pro-inflammatory cytokine release in cell culture (Tseng *et al.*, 2006) without requirement for other bacterial factors. *P. aeruginosa* mutants lacking flagella induce lower levels of IL-8, TNF-α and IL-1β, IL-6 and MMP-7 production from epithelial cells (DiMango *et al.*, 1995; Kube *et al.*, 2001; Lopez-Boado *et al.*, 2001; Hybiske *et al.*, 2004; Tseng *et al.*, 2006), and induce an overall diminished and localized inflammatory response in a mouse model of respiratory infection (Feldman *et al.*, 1998). Flagella activate pro-inflammatory responses primarily by binding to and activating TLR5 (Hayashi *et al.*, 2001), which is constitutively expressed on the lung epithelial surface (Adamo *et al.*, 2004). In addition to TLR5, flagella are able to bind the glycoprotein asialoGM1, which is found in lipid rafts in association with TLR2, and this can result in NF-κB activation via TLR2 in addition to TLR5 (Adamo *et al.*, 2004).

P. aeruginosa pili are approximately 1000-4000 nm long with a diameter of 5.2 nm, and are composed of a single structural monomer called pilin (Keizer *et al.*, 2001). Pili are extremely important for *P. aeruginosa* adhesion to cells, as demonstrated by the fact that purified pili and anti-pilus antibodies inhibit non-mucoid *P. aeruginosa* adhesion to tracheal epithelial cells (Ramphal *et al.*, 1984; Doig *et al.*, 1988). In addition, non-piliated (NP) PAK also exhibit decreased bacterial binding to A549 lung epithelial cells (Chi *et al.*, 1991), emphasizing the importance of this structure in adherence. The major cellular structure that pili bind to is

asialoGM1 (de Bentzmann *et al.*, 1996), and exogenous pili competitively block the binding of *P. aeruginosa* PAK or PAO to asialoGM1 (Baker *et al.*, 1990). Based on the close structural association of asialoGM1 with TLR2, pili dependent binding does have some influence on inflammatory responses. Indeed, a decreased binding of NP mutants to asialoGM1 results in lower activation of TLR2, and therefore decreased production of the inflammatory cytokine IL-8 (DiMango *et al.*, 1995; Ratner *et al.*, 2001). Various *P. aeruginosa* non-piliated mutants were observed to be significantly less virulent than their wild-type counterparts in a mouse model of respiratory infection (Tang *et al.*, 1995), while PAK NP was found to be approximately half as virulent as PAK WT, but still twice as virulent as the non-flagellated mutant PAK fliC (Feldman *et al.*, 1998).

LPS is the major structural and functional outer membrane component in *P. aeruginosa* (Kropinski *et al.*, 1985). It consists of an endotoxic lipid A (6 long-chain fatty acids anchored in the bacterial outer membrane), LPS core (composed of ketodeoxyoctonate, heptose, hexosamine and hexoses), and immune-reactive O antigen polysaccharide side chains (Goldberg & Pier, 1996). *P. aeruginosa* LPS causes a less potent and more sustained lung inflammation than LPS from *E. coli*, stimulating epithelial cells to release various pro-inflammatory cytokines (Koyama *et al.*, 1999), although the reactivity of the LPS has been found to vary between strains, and even within strains cultured under different conditions. PAK was found to elicit a more rigorous pro-inflammatory response when there were higher levels of hexa-aceylated lipid A members (Ernst *et al.*, 2003). Increased acelyation of lipid A could be induced by low magnesium culture medium (8 μM) to produce these more stimulatory LPS structures (Ernst *et al.*, 1999). The main receptor for LPS is TLR4 (in association with CD-14), and TLR4 deficient mice exhibit hypo-responsiveness to LPS stimulation (Poltorak *et al.*, 1998; Hoshino *et al.*, 1999). It has been

observed that TLR4 deficient mice exhibit decreased pro-inflammatory cytokine production, but maintain normal clearance of the *P. aeruginosa* (Ramphal *et al.*, 2005; Skerrett *et al.*, 2007). Most studies investigating the role of LPS involve addition of purified LPS to cellular or animal models and measuring the outcomes. Variable results have been reported using such cell stimulation experiments: some groups have reported pro-inflammatory responses to *P. aeruginosa* LPS including IL-8 and β-defensin-2 production (Armstrong *et al.*, 2004; Guillot *et al.*, 2004; MacRedmond *et al.*, 2005), while others found no effect of LPS on cellular responses (DiMango *et al.*, 1995; Ratner *et al.*, 2001).

To study the role of each structure in bacterial pathogenesis, it is helpful to use bacterial mutants lacking these structures. There are two well characterized PAK mutants which lack functional flagella (fliC) or pili (NP) that have been used extensively to study the role of these structures during infection (Saiman & Prince, 1993; DiMango *et al.*, 1995; Feldman *et al.*, 1998; Lillehoj *et al.*, 2002; Hybiske *et al.*, 2004; Tseng *et al.*, 2006). PAK NP has had the *pilA* pilin encoding gene removed by homologous recombination and replaced with a tetracycline resistance gene containing cassette (Saiman & Prince, 1993). PAK fliC is non-motile due to removal of the *fliC* flagellin encoding gene by homologous recombination with a gentamicin resistance gene containing cassette (Feldman *et al.*, 1998).

LPS mutants, however, are much more difficult to create since LPS is such an integral molecule for bacterial structure. Although it is impossible to knockout the entire LPS structure, a PAK mutant has been created which lacks the L-Rhamnose component of the LPS core oligosaccharide. These PAK rmlC mutants therefore synthesize incomplete or truncated core oligosaccharide regions that are not able to accept O-antigens (Rahim *et al.*, 2000). While such

bacteria would maintain structural integrity of the outer membrane, they lack the immune-reactive O-antigens.

While these PAK laboratory strains have been intentionally modified to produce specific mutants for study, there also exists a vast array of naturally occurring *P. aeruginosa* mutants that can be found in patients with chronic lung infections. In the case of cystic fibrosis (CF) patients, the lungs can be colonized for years by a single strain of *P. aeruginosa*, which manages to mutate and survive through various courses of antibiotic treatments, changing lung conditions, and constant immune responses (Oliver *et al.*, 2000). Indeed, in one study of 128 *P. aeruginosa* isolates from 30 different CF patients, 36% of the isolates were found to be hypermutable, while none of the *P. aeruginosa* strains isolated from 75 acute pneumonia patients were hypermutable (Oliver *et al.*, 2000). A more recent study looking at CF patients sputum isolates over the course of 25 years has shown that hypermutable strains develop following approximately 5 years of colonization, and that during this course of 25 years, over 54% of CF patients will acquire mutated *P. aeruginosa* (Ciofu *et al.*, 2005). Even more interesting, the authors of this study have linked the increase in mutation rate to the chronic oxidative stress produced by continuous polymorphonuclear (PMN) cell activity in the CF lung (Ciofu *et al.*, 2005). The ability to rapidly mutate in response to conditions in the lungs during infection would result in selection of strains that can best survive the onslaught of antibiotic treatments and immune effector cells, resulting in accumulation of bacterial strains that become increasingly more difficult to clear. Other changes that can occur to *P. aeruginosa* in the CF lung over time include conversion from non-mucoid to the alginate over-producing mucoid phenotype associated with poorer prognosis in CF patients (Speert *et al.*, 1990; Mathee *et al.*, 1999), loss of biofilm formation ability by

persistent non-muciod strains (Lee *et al.*, 2005), and gain of antibiotic resistance (Oliver *et al.*, 2000; Ciofu *et al.*, 2005).

While we have been unable to find a firm role for integrins in *P. aeruginosa* interactions with lung epithelial cells by investigating the physical interactions between the bacterium and integrins (association and internalization), it is still possible that the cellular signaling functions of integrins are linked to bacterial interaction or activation of these receptors. In these studies we investigated the cellular responses of A549 cells to *P. aeruginosa* in an integrin dependent fashion by knockdown of integrin or ILK protein, and also investigated the role of bacterial phenotype and virulence factors in activating cellular responses.

Materials and Methods

siRNA knockdown

Preparing siRNA

Twenty nmol lyophilized αv integrin siRNA purchased from Dharmacon (Lafayette, CO), or custom ILK-H siRNA (targeting the sequence corresponding to the plekstrin homology domain nucleotides 741-759 'AACCTGACGAAGCTCAACGAGAA' of human ILK) purchased from Qiagen (Missisauga, ON, Canada) were resuspended to 20 μM by the addition of 1 ml siRNA dilution buffer (Qiagen). The solution was mixed for 20 min at 30°C with shaking. Aliquots were then stored at -80°C. Five nmol control (non-targeting siRNA) was also purchased from Dharmacon, and resuspended in 250 μl RNA dilution buffer to a final concentration of 20 μM as described above.

siRNA transfection protocol optimization

For transfections carried out when cells were at a high (80%) confluency, A549 cells were seeded in 12-well plates (Corning) at 70,000 cells per well, and allowed to grow for 2 d at 37°C, 5% CO_2 until confluency reached approximately 80%. Cells were then transfected for 24, 48 or 72 h with either 10 nM or 100 nM siRNA as described below. For transfections longer than 24 h, 1 ml of F-12 Ham medium (Sigma-Aldrich) with 10% bovine calf serum (BCS) was added to the transfection medium at 24 h post-transfection, and cells were left untouched for the remaining incubation time. For transfections carried out while cells were at a low confluency, A549 cells were seeded in 12-well plates at 55,000 cells per well, and allowed to grow for one day to reach approximately 30% confluency before the first transfection occurred. Cells were then transfected at time 0 h, followed by a second transfection at 24 h, and left for up to 48 h total. All transfections were carried out according to the protocols recommended by Dharmacon. Briefly, the 20 μM stock siRNA was diluted to a 2 μM solution using siRNA dilution buffer (Qiagen). For 10 nM transfections, one tube was prepared containing 5 μl of 2 μM stock siRNA diluted in 95 μl Opti-MEM 1 Reduced Serum transfection medium (Gibco, Burlington, ON, Canada) per well to be transfected. For 100 nM transfections, 50 μl of 2 μM stock siRNA was diluted in 50 μl Opti-MEM per well. Another tube was prepared containing 2.5 μl DharmaFECT 1 Transfection Reagent (Dharmacon) and 97.5 μl Opti-MEM per well. Both tubes were incubated for 5 min at room temperature. Following this, 100 μl of the DharmaFECT mixture was added to each 100 μl of siRNA mixture, and the solution was incubated for 20 min at room temperature. Opti-MEM (800μl) was added to each 200 μl of this reaction mixture to make the final transfection mixture. Cells were washed twice with PBS, and 1 ml of the transfection mixture was added per well. Controls included untransfected cells incubated in Opti-MEM

medium, untransfected cells incubated in Opti-MEM medium containing DharmaFECT, or cells transfected with control non-silencing siRNA. The volumes described are for 12-well plate transfections, and all reagent volumes and concentrations were adjusted for each type of experiment according to vessel size and cell numbers.

Verifying transfection efficiency

The efficiency of RNA delivery was measured using a stable FITC labelled Block IT Fluorescent Oligo (Invitrogen) during transfections of cells at high confluency at 24 and 48 h using both 10 and 100 nM concentrations of siRNA. Following transfection, A549 cells were trypsinized with 200 µl 0.05% trypsin-EDTA (Gibco), washed once with PBS, and resuspended in PBS for use in flow cytometry. Transfected cells were recognized by their green fluorescence signal as measured using the FL-1 channel. A marker in FL-1 was set to contain 95.5% of the untransfected control cells, and any cells falling above this marker were considered to be successfully transfected.

Measuring αv integrin knockdown following siRNA treatment using flow cytometry

The success of αv siRNA in reducing cell surface expression of αv protein was quantified using flow cytometry. Following transfection, A549 cells were trypsinized with 200 µl 0.05% trypsin-EDTA for 2 min, and then quenched with 800 µl serum-containing F-12 Ham medium. Cells were pelleted at 500×g for 5 min, resuspended in 1 ml PBS, and pelleted again. Cells were then resuspended in 1 ml PBS with 0.1% BSA, and mouse anti-human CD51 PE conjugated anti-αv integrin antibody (Chemicon International, now Millipore, Billerica, MA) was added at a dilution of 40:1. Alternatively, PE conjugated mouse IgG_1 isotype control (BD-Biosciences, San

Hose, CA) was added at the same dilution. Cells were incubated with antibody for 1 h at room temperature in the dark. Following incubation, cells were washed twice with 1 ml PBS, and finally resuspended in 500 µl PBS to be analyzed with a BD FACSCalibur flow cytometer using the BD CellQuest™ Pro software (BD Biosciences) acquiring 20,000 events per trial. Cells were measured using FL2 channel to detect the PE signal. Results are reported as percentage change in fluorescence of αv siRNA treated cells from untreated cells stained for αv integrins.

Measuring ILK knockdown following siRNA treatment using qRT-PCR

Since ILK is an intracellular protein, it cannot be easily measured using flow cytometry on unpermeabilized cells. Therefore, ILK expression was quantified by measuring changes in mRNA using quantitative real time (qRT)-PCR. Following transfection, total RNA was extracted from cells with an Aurum Total RNA Mini-Kit (Biorad), following the manufacturer's protocols. Extreme care was taken to maintain RNase free supplies, reagents and working area for all steps from RNA extraction to PCR. cDNA was synthesized from the RNA using the First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON, Canada), also following the manufacturer's protocol. Maximal volume of input RNA was used (10 µl per reaction) to obtain sufficient amount of cDNA. Following synthesis, cDNA concentration was quantified by obtaining OD_{260} readings using a UV-1700 PharmaSpec UV-Visible spectrophotometer (Shimadzu, Guelph, ON, Canada). Briefly, cDNA was diluted 50:1 in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5), and OD_{260} readings were obtained using a Hellma 1 mm gap quartz cuvette (Hellma USA, Plainview, NY). The concentration of cDNA was calculated using the following equation:

Concentration DNA (µg/ml) = OD_{260} reading × dilution factor × 50 µg/ml DNA/ml

For qRT-PCR reactions, cDNA was diluted to 1 μg/μl. In addition to ILK, expression levels of β-actin were tested in parallel for each sample to ensure equal cDNA input values, and to normalize the results. A master mix was prepared for each gene of interest, containing 9 μl DNase free water, 9 μl iQ SYBR Green Supermix (Biorad) and 1 μl of primer per well. The PCR Primer Human ACTB primer for β-actin, and Human ILK primer were both purchased from SuperArray (Frederick, MD). Nineteen μl of master mix was distributed into each well of a 96-well PCR plate (Biorad), and 1 μl of the appropriate 1 μg/μl cDNA was added per well. In addition, non-template controls (NTC) consisting of master mix with no cDNA were run in triplicate for each master mix solution to ensure that none of the solutions were contaminated. The qRT-PCR reaction was carried out with a IQ5 Multicolor Real-Time PCR Detection System with iCycler PCR thermocycler (BioRad), and data collected and analyzed using Biorad IQ5 Standard Edition Software. A two-step PCR program with 95°C 3 min activation, followed by 40 cycles of 95°C for 10 s and 55°C for 30 s was used for all reactions. A melt curve was always performed following the PCR to ensure specificity of amplification. All results were normalized to the β-actin control and expressed as ΔΔCT values, which indicate 'fold differences' in mRNA expression between control and treated cells.

Effects of siRNA on bacterial interactions with host cells

Effects of ILK siRNA on PAKgfp association with A549 cells

To measure the effect of ILK knockdown on PAK*gfp* adherence to and invasion of A549 cells, cells were seeded, prepared, and infected with PAK*gfp* as described in Chapter 1 (section: Plate reader PMx quenching internalization assay with adherent A549 cells), following 48 h ILK siRNA treatment described above (adjusted to a total volume of 100 μl per well for 96 well

plates). Bacteria associated and internalized were discriminated from each other as described in Chapter 1 (section: Plate reader PMx quenching internalization assay with adherent A549 cells). The effects of ILK siRNA on PAK*gfp* internalization were also quantified using flow cytometry as described in Chapter 1 (section: PMx quenching internalization assay on adherent A549 cells trypsinized using flow cytometry). In this case, cells were grown in 6-well plates, and ILK siRNA procedures were adjusted for a total volume of 1 ml per well.

Effects of siRNA on cellular responses during bacterial infection

Effects of siRNA on TNF-α mRNA expression during bacterial infection

The effects of ILK siRNA on PAK induced cellular responses was measured by quantifying the levels of TNF-α mRNA, as well as the amount of secreted pro-inflammatory cytokines. To determine the infection period that resulted in maximal TNF-α mRNA production, an infection time course was performed. A549 cells were seeded in 6-well plates at 400,000 cells/well, and allowed to grow for 2 d to approximately 80% confluency. PAK WT was prepared as described in Chapter 1 (section: Preparation of bacteria for experiments), and cells were infected at an MOI of 50:1. After 1 h incubation, 50 μg/ml PMx was added to kill bacteria, and A549 cells together with dead bacteria were incubated further for a total of 4, 6, 8, 12, 16, 20 or 24 h. At each time point, RNA was extracted, cDNA was synthesized as described above, and qRT-PCR was performed for TNF-α mRNA expression using a TNF-α primer (SuperArray), and the procedures described above (section: Measuring ILK knockdown following siRNA treatment using qRT-PCR).

Effects of ILK siRNA on cytokine release during bacterial infection

The effect of ILK siRNA on cytokine release by A549 cells in response to *P. aeruginosa* infection was measured using a Bio-Plex Suspension Bead array system (Biorad). For all cytokine harvesting experiments, A549 cells were seeded into 24-well plates at 50,000 cells/well in 500 µl F-12 Ham medium with 10% BCS, and allowed to grow for 2 d. Cells were transfected with 10 nM ILK siRNA or empty DharmaFECT transfection reagent for 48 h as described above (section: siRNA transfection protocol optimization). Following siRNA treatment, cells were infected with PAK WT at an MOI of 50:1. Cells and bacteria were incubated for 1 h at 37°C, 5% CO_2, and then bacteria were killed with 50 µg/ml PMx. Cells and dead bacteria were incubated together for a further 17 h at 37°C, 5% CO_2. Supernatants were collected into 1.5 ml eppendorf tubes, and centrifuged at 13,000 × g for 25 min to pellet the bacteria, and the clear supernatant was aliquoted into 100 µl volumes and stored at -80°C until analysis. Cytokines measured included IL-1β, IL-6, IL-8, GM-CSF and TNF-α from the Bioplex Human Cytokine Group I 5-Plex Assay (Biorad), and IL-1α from a Bioplex Human Cytokine Group II 1-Plex Assay (Biorad). The microplate, buffer solutions and PE-fluorescent antibody were obtained from a Bio-Plex Reagent Kit (Biorad), while standards and detection antibodies accompanied each cytokine plex (Biorad). All preparation and assay steps were performed according to the manufacturer's protocols. Samples from n=3 separate experiments were run in duplicate for each experimental condition. Treatments tested included cells incubated without transfection medium or siRNA, cells incubated with DharmFECT transfection reagent only, and cells transfected with 10 nM ILK siRNA and DharmaFECT, each tested with and without PAK WT infection for 18 h. Standards were prepared for high photomultiplier tube (PMT) fluorescence amplification settings, based on the expectation of low levels of cytokine production.

Effect of bacterial structures or inter-strain variability on cellular responses

Similar experiments were performed to determine the cytokine response profile of A549 cells to both PAK mutants and to clinical *P. aeruginosa* isolates. The PAK mutants tested included the non-flagellated PAK fliC, non-piliated PAK NP (both strains kindly donated by Alice Prince, Columbia University, NY), and the LPS outer core oligosaccharide mutant PAK rmlC (kindly donated by Joseph Lam, University of Guelph, ON). The clinical *P. aeruginosa* isolates were collected by Dr Niels Høiby (University of Copenhagen) from CF patients at various stages during the disease progress. We investigated 3 sets of paired isolates, one mucoid (identified as A) and one non-mucoid (identified as B) of the same strain, obtained from the same patient at different times. The isolate pairs were 8503A/02 (mucoid) and 8503B/02 (non-mucoid), 1395A/03 (mucoid) and 1395B/03 (non-mucoid), 75887A/01 (mucoid) and 75887B/01 (non-mucoid).

A549 cells were seeded at 70,000 cells/well in 24-well plates and allowed to grow for 2 d to approximately 80% confluency. As described in the previous section, cells were prepared and infected with either PAK mutants or clinical isolates (prepared as described in Chapter 1, section: Preparation of bacteria for experiments) at an MOI 50:1, and supernatants were harvested as described above in the previous section. Samples were also analyzed as described above using the Bio-Plex Suspension Bead array system.

Results

siRNA knockdown

Transfection efficiency

The suitability of the transfection procedure when cells were at high confluency was verified by measuring the transfection efficiency achieved using this protocol with FITC-labeled non-silencing siRNA. Treatment with both 10 and 100 nM siRNA was tested for uptake and retention efficiency at 24 and 48 h post-transfection. The percentage of cells containing the FITC siRNA was very high for all treatments, ranging from 88-99% (Figure 1). These numbers are highly satisfactory for transfection efficiency, based on the literature (Wang *et al.*, 2005; Zargham & Thibault, 2005).

Effects of αv integrin siRNA on surface protein expression in A549 cells

Although the FITC siRNA indicated that the transfection protocol was indeed efficient in delivering the siRNA into the cells, the surface expression of αv following transfection was not reduced. In fact, surface αv expression was increased for both 10 and 100 nM concentrations of siRNA, at 24, 48 and 72 h transfection times compared to non-siRNA treated control cells (Figure 2). The αv expression appeared to increase with longer siRNA treatments. Because of the unsuccessful knockdown of αv integrin using transfection with A549 cells at a high confluency growth stage, transfection of A549 cells at a low confluency (30%) was tested, followed by a second transfection at 24 h, and further incubation to 48 h total. This new protocol also increased αv surface expression when both 10 and 100 nM siRNA concentration were used (Figure 3).

Effects of ILK siRNA on ILK mRNA expression in A549 cells

Because of the unsuccessful knockdown of αv integrin, siRNA knockdown of the β integrin linked enzyme ILK was attempted. Transfection of A549 cells at high confluency, and transfection at low confluency (with a second transfection 24 h later) were tested to ascertain optimal knockdown methods. For the transfections performed at high cell confluency, all transfection lengths and both siRNA concentrations (10 and 100 nM) showed a great decrease in ILK mRNA expression as measured by qRT-PCR (Figure 4). Results were normalized to the housekeeping gene β-actin for each separate sample. The treatment condition with the lowest ILK expression was set to a value of 1, and the other conditions were expressed as fold increases over this value. From the conditions tested, 48 h transfection with 10 nM ILK siRNA showed the largest decrease in ILK expression of approximately 19 fold. Transfection of A549 cells at low confluency with ILK siRNA also showed significant decrease in ILK expression (approximately 11 fold decrease) (Figure 5). Because it was the lowest dose of siRNA that resulted in the largest reduction in expression, transfection of A549 cells at high confluency for 48 h with 10 nM ILK siRNA was chosen for all subsequent experiments. While it is good practice to always use the lowest effective dose of siRNA possible to reduce off-target effects, the presence of excess siRNA can actually result in decreased specific knockdown, due to the activation of non-specific dsRNA responses by host cells including effector proteins like 2-5-oligoadenylate synthase and protein kinase dsRNA dependent that will use up some of the specific siRNA for non-specific knockdown. In addition, transfection of A549 cells with 10 nM control non-silencing siRNA did not affect ILK mRNA expression (data not shown).

Effects of ILK siRNA on PAK*gfp* interactions with A549 cells

Effects of ILK siRNA on PAKgfp association with A549 cells

PAK*gfp* association with adherent A549 cells following ILK knockdown was quantified using the PMx quenching internalization assay described in Chapter 1. Forty-eight h long transfection with 10 nM ILK siRNA had no effect on the amount of bacteria associated with A549 cells (adhered or internalized) compared to untreated A549 cells or A549 cells treated with transfection reagent only (Figure 6). Results are expressed as relative fluorescence compared to untreated cells set to 100, and were obtained from n=2 separate experiments.

Effects of ILK siRNA on PAKgfp internalization into A549 cells

The PMx quenching internalization assay was used to measure the effect of ILK knockdown on PAK*gfp* internalization into adherent A549 cells using both the plate reader and flow cytometer readouts. Results from both assays show that ILK siRNA treatment had no effect on PAK*gfp* internalization (Figure 7 and Figure 8). In agreement, flow cytometer data did not show any difference in internalization between the control transfection reagent only and ILK siRNA treated cells (Figure 8). It appeared that both the transfection reagent and siRNA slightly increased PAK*gfp* internalization.

Effects of ILK siRNA on cellular responses during bacterial infection

Effects of ILK siRNA on pro-inflammatory cytokine protein production during bacterial infection

Time course of qRT-PCR data for TNF-α mRNA expression following PAK infection shows maximal TNF-α mRNA production at 8 h post-infection (Figure 9). For measurement of

cytokine protein production, cells must be incubated for longer than 8 h to allow this up-regulated mRNA to translate into protein, and it is common practice to incubate bacteria and cells for up to 24 h before measuring cytokine protein responses (Kube *et al.*, 2001). Stimulation of A549 cells with PAK WT for 18 h (1 h alive, 17 h anti-biotic killed) resulted in significantly higher release of all cytokines compared to unstimulated cells, indicating that PAK is able to induce a very strong inflammatory response (Figure 10 and 11). The treatment of A549 cells with 10 nM of ILK siRNA appeared to decrease the ability of these cells to produce pro-inflammatory cytokines during *P. aeruginosa* infection ($n=3$, $p<0.05$, One sample t-test) (Figure 10 and 11). Figure 10 shows the actual data in pg/ml protein detected, with each of the three separate experiments represented by a different symbol (circle, square or diamond). Figure 11 shows the data normalized within each experiment, setting PAK stimulated untransfected cells to 100, and expressing the other values relative to that. Treatment of cells with the transfection reagent appeared to have some inhibitory effect on cytokine response to *P. aeruginosa* infection, as DharmaFECT caused a significant decrease in IL-1α, IL-1β, IL-6, GM-CSF and TNF-α compared to untreated cells (Figure 11). However, for all cytokines except IL-1α, 10 nM ILK siRNA treatment caused significantly less cytokine release than the DharmaFECT control, indicating that ILK siRNA treatment does indeed inhibit the cytokine response of A549 cells during PAK infection (Figure 11). ($n=3$, one-sample t-test, $p<0.05$).

Effect of bacterial virulence factors and inter-strain variability on cellular responses

Cytokine production by A549 cells following infection with mutant strains of P. aeruginosa PAK

The amount of cytokines released by A549 cells in response to infection with *P. aeruginosa* PAK mutants fliC, NP and rmlC was different from the response to PAK WT, and also different among each other (Figure 12 and 13). Figure 12 shows the absolute levels of the cytokines IL-1α, IL-1β, IL-6, GM-CSF and TNF-α in cell culture supernatants (pg/mL), with each of n=2 separate experiments represented by a different symbol (square or triangle). Figure 13 shows the data normalized within each experiment, setting the response of PAK WT stimulated cells to 100, and expressing the other values relative to that. While statistics cannot be performed on this data because of n=2, there are several trends that can be observed. In general, both PAK fliC and rmlC mutants caused lower release of all cytokines measured, while PAK NP caused either similar or higher cytokine release than PAK WT infection (Figure 13).

Cytokine production by A549 cells following infection with clinical isolates of P. aeruginosa

The cytokine responses of A549 cells to infection with the three pairs of *P. aeruginosa* clinical isolates were different from the responses to PAK WT, and were also slightly variable from each other (Figure 14-18). While statistics cannot be performed on this data because of n=2, there are several trends which can be observed. The data for A549 cells alone and for cells infected with PAK WT are shown pooled from all experiments. Since siRNA experiments involved pre-incubation in serum-free Opti-MEM prior to infection, these data are circled to distinguish them. In general, the clinical isolates caused much less cytokine release by A549 cells compared to PAK WT (Figure 14-18). There did not appear to be any clear trend amongst

the isolates regarding IL-1α release (Figure 14). The mucoid member of the 8503 pair (8503A) appeared to cause less IL-1β, IL-6, GM-CSF and TNF-α release than its non-mucoid counterpart (Figure 15-18). The mucoid 1395A was observed to cause less IL-1β and GM-CSF release than the non-mucoid 1395B, while the difference between the two was not noticeable for IL-6 and TNF-α (Figure 15-18). The matched pair 75887A and B did not appear to differ for any of the cytokines produced, as even though the means may appear to be different, there was an overlap in the measurements (Figure 15-18).

Discussion

We used several approaches to target various aspects of host-pathogen interactions to investigate the cellular responses of epithelial cells to *P. aeruginosa* infection. Knockdown of integrin expression was attempted in order to determine the importance of integrins in bacterial interaction with cells, and in activation of cellular responses. Due to the difficulty of integrin knockdown, we next targeted the β integrin linked signaling molecule ILK for silencing, in order to disrupt the functions of activated integrins. To determine the relative importance of the virulence factors flagella, pili and LPS, we investigated the cellular responses of A549 cells to *P. aeruginosa* mutants lacking each of these structures. Finally, we investigated the ability of clinical isolates of *P. aeruginosa* obtained from CF patients to determine the effect that mutations acquired during chronic colonization may have on cellular responses to these bacteria. The data gathered from this set of studies has provided some insightful observations into the role of integrins, other pattern recognition receptors, and bacterial structures in eliciting host cell responses to infection.

Our initial attempts to study the role of integrins by removing integrin proteins from A549 cells with siRNA were unsuccessful due to the inability to knockdown integrin protein expression (Figure 2 and 3). While our protocols and choice of target integrin αv was based on a previous study which had success with integrin knockdown (Cao *et al.*, 2006), we were unable to reproduce their results. Success of siRNA induced gene silencing is known to depend on transfection efficiency, which can be affected by the transfection reagent and transfection time (Zhao *et al.*, 2008). Since we obtained extremely high transfection rates using a control FITC-labeled siRNA (up to 99% for 72 h), we cannot attribute the unsuccessful αv knockdown to low transfection efficiency (Figure 1). For effective gene silencing, the siRNA sequence must be properly designed to specifically target the gene of interest without causing any off-target effects with other genes (Saetrom & Snove, 2004; Huppi *et al.*, 2005). To avoid the difficulties associated with siRNA design, we used the same sequence described by Cao *et al.* (2006), which had shown efficient and specific αv knockdown in a breast cancer cell line. Because of this demonstrated efficiency of the specific siRNA sequence that we used, it is unlikely that targeting efficiency is responsible for the unsuccessful knockdown. RNAi knockdown efficiency can also depend on the cell type used, as some cell types are more easily transfected than others. The study by Cao *et al.* (2006) showed an efficient αv knockdown in the breast cancer cell line MDA-MB-435, which could have different basal levels of integrin expression, and may not respond the same way as A549 cells to siRNA treatment. Although we cannot discount that the difference in cell type is to blame for the inefficient knockdown, A549 cells are generally considered to be an easy cell type to use for siRNA gene silencing. Many companies including Dharmcon and SantaCruz often use A549 cells to optimize protocols and test controls for siRNA.

The most likely explanation for the unsuccessful αv knockdown is that the integrin protein target is very stable with a slow turnover rate (half life of approximately 20 h), making it a difficult target for silencing at a post-transcriptional level (Zargham & Thibault, 2005). Since we observed an increased surface αv protein expression as a result of siRNA transfection, it is possible that in addition to the existing surface αv subunits not being knocked down, the intracellular αv subunits may actually become localized to the surface. In our group, we have observed that infection of A549 cells with PAK results in increased surface expression of αv, α5, β1 and β4 subunits (Gravelle et al, manuscript in preparation), so it is possible that the stress of culturing the cells under transfection conditions (serum free medium in the presence of lipid-based transfection reagent) also causes a re-distribution of integrins in a similar fashion. It would have been beneficial to quantify the total expression of αv integrins by performing immunostaining and flow cytometry on permeabilized A549 cells for basal expression and following siRNA, and also to quantify the αv mRNA expression response to siRNA treatment, however due to time constraints we decided to move away from integrin siRNA. Regardless of the explanation, we were unable to achieve successful αv knockdown using siRNA, so an alternative approach was needed to target integrin based signaling and cellular responses.

ILK is a serine-threonine protein kinase that connects β1 and β3 integrin subunits to focal adhesion proteins including FAK and paxillin to mediate integrin activation dependent cytoskeletal rearrangement and cell signaling pathways, both occurring in a PI3K dependent fashion (Dedhar, 2000; Persad & Dedhar, 2003). Because of the intimate connection of ILK to these β integrins and their functions, knockdown of ILK can be expected to block the functions of these activated integrins, acting as a suitable alternative to siRNA knockdown of integrins themselves. In addition to the suitability of ILK for mimicking integrin knockdown, ILK siRNA

knockdown has been successfully achieved in the literature (see Table 2). The half life of ILK is approximately 8 h (Aoyagi *et al.*, 2005), which should make ILK much easier to knockdown than integrins. Indeed, using the same protocols optimized for αv siRNA knockdown, we were able to show 11-19 fold decreases in ILK mRNA expression, with maximal knockdown at 48 h after transfection with 10 nM ILK siRNA (Figure 4). Although it is preferable to measure protein levels after knockdown, ILK is an intracellular protein, and thus cannot be measured easily with flow cytometry on unpermeabilized cells. We therefore chose to measure the knockdown efficiency using qRT-PCR. Although we did not directly investigate protein knockdown, most studies that did this showed good protein knockdown efficiency using protocols similar to ours (See Table 2). Using the optimized conditions of 48 h transfection of A549 cells at high confluency with 10 nM ILK siRNA, we tested the effects of ILK knockdown on *P. aeruginosa* association with and internalization into A549 cells, and also in bacterial induced cytokine release by A549 cells.

ILK knockdown did not appear to affect PAK association with or internalization into A549 cells (Figures 6-8). The lack of effect of ILK knockdown on bacterial adhesion can be explained by the fact that ILK is an intracellular protein connected to the cytoplasmic domain of either β1 or β3 integrins (Hannigan *et al.*, 1996), which should not affect the ability of bacteria to interact with the extracellular portions of integrins. However, ILK PTGS could be expected to affect internalization based on the importance of integrins for internalization of other bacteria including *S. aureus* (Fowler *et al.*, 2000), *Yersinia spp.* (Isberg *et al.*, 2000), and the demonstration of 60% decreased internalization of Group A *Streptococcus* into human larynx epithelial cells because of ILK knockdown (Wang *et al.*, 2006a). This group proposed that GAS uptake involved bacterial induced integrin clustering in focal adhesions, leading to PI3K

dependent activation of ILK which then interacts with focal adhesion proteins such as FAK and paxillin to induce cytoskeletal rearrangement and bacterial ingestion (Wang *et al.*, 2006a). Indeed, the close relation of ILK with proteins interacting with the actin cytoskeleton such as paxillin, FAK and affixin support this model (Wu & Dedhar, 2001). Although this group also investigated the role of ILK in internalization of *S. aureus* and *Yersinia*, they did not address *P. aeruginosa*, so it is not known whether the same ILK dependent internalization exists for *P. aeruginosa*. However, it has been suggested that integrins are important for *P. aeruginosa* internalization into host cells (Gagniere & Di Martino, 2004; Leroy-Dudal *et al.*, 2004), and that PAK internalization is dependent on PI3K and PKB/Akt (Kierbel *et al.*, 2005), which suggests an involvement of ILK mediation signaling. However, the direct role of ILK has not been investigated in *P. aeruginosa* internalization, so it is possible that these bacteria exploit another signaling pathway, either in place of or in addition to ILK mediated internalization.

To study the role of integrins in pro-inflammatory responses of A549 cells to *P. aeruginosa* infection, we investigated the production of 6 cytokines involved in cellular responses to pathogens. IL-8 is of interest because it is a potent leukocyte attractant (Pease & Sabroe, 2002), and IL-6 is able to activate many immune cells attracted to the site of infection, including B cells (Hirano *et al.*, 1984), T cells (Houssiau & Van Snick, 1992) and neutrophils (Dalrymple *et al.*, 1995). GM-CSF is of interest because of its ability to stimulate growth, activation, and differentiation of various leukocytes including neutrophils, monocytes and dendritic cells (Witmer-Pack *et al.*, 1987; Jones, 1994; Jones, 1996). IL-1α, IL-1β and TNF-α are important for cellular responses to infection because they all act to induce production of pro-inflammatory cytokines in various cell types, acting to propagate the inflammatory signal cascades during infection, (Muegge *et al.*, 1989; Bradley, 2008), and IL-1α and β have been

shown to activate endothelial cells to help recruit inflammatory cells to the lung during *P. aeruginosa* infection (Lin *et al.*, 2002).

P. aeruginosa caused increased expression of all 6 cytokines measured (IL-1α, IL-1β, IL-6, GM-CSF, TNF-α and IL-8) by A549 lung epithelial cells during infection (Figure 10-18). However, IL-8 levels were so high that the amount of IL-8 released from PAK stimulated A549 cells fell outside the range of the standard curve (data not shown), and thus could not be included in our analysis and will not be considered in our discussion. The absolute values of cytokines released also varied between the other 5 cytokines measured, with IL-1β being the lowest and IL-6 being the most highly released cytokines (Figure 10). Since all the cytokines investigated can be transcriptionally up-regulated by both NF-κB and AP-1 transcription factors, it is unclear as to why they show such different levels of release from PAK infected cells. These results are novel in that they represent the first report of release of these 5 cytokines from A549 cells stimulated with PAK, and they validate that the infection protocols we developed are indeed sufficient to induce detectable cellular responses.

We found that down-regulation of ILK using siRNA caused decreased cytokine production in A549 cells stimulated with *P. aeruginosa* PAK. While treatment with transfection reagent alone also caused a decrease in cytokine release, ILK siRNA caused a significantly larger decrease in all cytokines except IL-1α (Figure 11). However, two out of three ILK siRNA experiments showed the same trend of decreased IL-1α release due to ILK siRNA, but one experiment showed an increase, which raises the mean for that treatment and may be the reason that there appears to be no effect of siRNA for this cytokine. While ILK has been indirectly implicated in *P. aeruginosa* internalization (based on PI3K and PKB/Akt dependent internalization) (Kierbel *et al.*, 2005), our results are the first observations linking *P. aeruginosa*

activation of pro-inflammatory responses with ILK. These results suggest that a β integrin subunit is involved in mediating the pro-inflammatory response of cells to *P. aeruginosa* infection. Based on the Fn binding ability of PAK observed in Chapter 2 (Figure 2), it is likely that β1 is responsible for the ILK-mediated cytokine production during *P. aeruginosa* infection. Indeed, ECM product binding and subsequent integrin clustering are the first steps necessary for ILK activation (Delcommenne *et al.*, 1998; Pankov & Yamada, 2002). A similar study investigating the role of β3 integrin in *Borrelia burgdorferi* infection of human chondrocytes found that β3 siRNA treatment resulted in significantly decreased levels of MMP-1, MMP-3, IL-1β, TNF-α, IL-6, IL-8, monocytes chemotactic protein-1, RANTES and IL-10 mRNA levels during bacterial infection (Behera *et al.*, 2006). In this study the authors concluded that this signaling occurred in a TLR-independent, β3 dependent manner (Behera *et al.*, 2006). In addition, studies of *Bordetella pertussis* infection of respiratory epithelial cells suggests that this bacterium induces TNF-α and ICAM-1 production in an α5β1 dependent manner (Ishibashi & Nishikawa, 2002; Ishibashi & Nishikawa, 2003). Considering these studies, our current results, and the known involvement of ILK with signaling activation (Dedhar, 2000), it is possible that bacterial induced TLR-independent, integrin and ILK-dependent cellular responses occur through activation of AP-1 and NF-κB, which result in increased transcription of various pro-inflammatory cytokines (Wu & Dedhar, 2001; Bradley, 2008). It has been shown that *P. aeruginosa* is able to induce both AP-1 and NF-κB activation, and subsequently increase IL-8 production in tracheal epithelial cells (Wu *et al.*, 2005). As discussed earlier, TNF-α and IL-1β are able to act in an autocrine fashion to further increase expression of pro-inflammatory cytokines, and it has been suggested that AP-1 in addition to NF-κB activation is critical for this TNF-α-IL-1β induced activation of IL-8 (Mukaida *et al.*, 1990). The fact that we did not observe

complete reduction of cytokine release due to ILK silencing suggests that these β integrin dependent signaling pathways are not the only signaling mechanism which activates cells, and it is most likely that TLR are also involved in causing cytokine release from A549 cells.

Isogenic PAK mutants lacking bacterial components that are known TLR ligands were used to determine the importance of TLR signaling in *P. aeruginosa* induced cytokine production by A549 cells. Non-flagellated PAK fliC was observed to induce less cytokine release than PAK WT (Figures 12-13), suggesting that flagella are important for induction of host cell inflammatory responses. This result was expected, as previous studies using PAK fliC showed reduced NF-κB activation in stimulated epithelial cells compared to PAK WT stimulated cells (Hybiske *et al.*, 2004; Zhang *et al.*, 2005; Tseng *et al.*, 2006). In addition, PAK fliC was observed to cause decreased IL-6, IL-1β and TNF-α production compared to PAK WT (Hybiske *et al.*, 2004). Motile, flagellated *P. aeruginosa* have also been observed to increase IL-8 and NF-κB in A549 cells, while non-motile *P. aeruginosa* (non-flagellated) strains did not (Cobb *et al.*, 2004). The decrease in cytokine production is most likely due to the reduced ability of these bacteria to stimulate epithelial cells via TLR5 on the epithelial surface (Adamo *et al.*, 2004). However, even though flagella are considered to be a major immunostimulatory molecule of *P. aeruginosa*, it is interesting to note that lack of flagella did not completely eliminate cytokine responses, indicating that other bacterial products, or other cellular signaling pathways (such as β integrin ILK activation discussed above) are important for cellular stimulation.

The cytokine release by A549 cells induced by non-piliated PAK NP was found to be similar or even higher compared to the cellular responses to PAK WT (Figure 13), suggesting that pili are not necessary for the inflammatory response to *P. aeruginosa*. This is in agreement with most studies that implicate the extreme importance of pili for adhesion to cells, but not for

inflammation (Ramphal *et al.*, 1984; Doig *et al.*, 1988; Chi *et al.*, 1991), although some studies also suggested a role of pili in inflammation due to the proximity of asialoGM1 with TLR2 (DiMango *et al.*, 1995; Feldman *et al.*, 1998; Ratner *et al.*, 2001). We observed that in some cases PAK NP induced more cytokine release from A549 cells than PAK WT (Figure 13), suggesting that bacteria without pili may be more immunogenic than those with pili. The discrepancy in cytokine release could be due to the fact that PAK NP experience decreased internalization into A549 cells (Chi 1991), which would leave more bacteria in the extracellular space, potentially causing increased stimulation of other pattern recognition receptors such as TLR5 activation by flagella.

The mutant that caused the most dramatic reduction in cytokine release was the LPS core oligosaccharide deficient strain PAK rmlC. These results are interesting, as previous studies have suggested that *P. aeruginosa* LPS does not stimulate cytokine release (DiMango *et al.*, 1995; Ratner *et al.*, 2001). In addition, the main pattern recognition receptor for LPS is TLR4, and although primary alveolar type II cells express functional TLR4 (Armstrong *et al.*, 2004), we have observed in our laboratory that there is no surface TLR4 protein expression on A549 cells, and therefore no receptor to mediate signaling by LPS (Werner J., unpublished data). In agreement, Guillot *et al.* (2004) observed that A549 do not express surface TLR4. However, they found that the protein was expressed intracellularly, and that A549 responded to *P. aeruginosa* LPS despite the intracellular TLR4 localization. Contrary to these findings, surface TLR4 was detected on A549 cells in a study by MacRedmond *et al.* (2005), and it was also found that these cells responded directly to *P. aeruginosa* LPS by producing β-defensin (MacRedmond *et al.*, 2005). These discrepancies in TLR4 expression and location may be due to the passage number of the A549 cells, as we have observed in our laboratory that cells at a

high passage number exhibit much lower expression of surface integrin proteins than cells with a low passage number (Gravelle G., unpublished data), and this may also be the case with TLR expression. Regardless of the lack of TLR4 surface expression, we have not ruled out the possibility that LPS can stimulate a pro-inflammatory response through intracellular TLR4, which would mean that the rmlC mutant indeed elicited decreased cytokine release because it lacks LPS.

The PAK rmlC mutation affects the core oligosaccharide of LPS and consequently O chain attachment, while not affecting the lipid A portion of LPS. This is a concern with our findings because lipid A is primarily responsible for stimulating TLR4 (Pier, 2007), and thus the rmlC mutant should still be able to stimulate TLR4. In addition, it should be noted that the PAK rmlC mutant appeared phenotypically different from PAK WT and other mutants, exhibiting a more rounded shape and non-motile behaviour. Since this strain has not been characterized or used for studies of infection, it is possible that mutation in core oligosaccharide could cause other structural problems for the bacterium, such as altering the attachment or function of flagella or pili.

The clinical isolates used in this study were donated by Niels Høiby from a collection of 141 *P. aeruginosa* clinical isolates taken from 11 chronically infected CF patients between 1973 and 1999 (Ciofu *et al.*, 2005; Lee *et al.*, 2005). The six isolates we investigated were paired isolates from the same patient and of the same genotype taken over time, one exhibiting non-mucoid phenotype (designated 'B') and the other a mucoid phenotype (designated 'A'). As these clinical isolates exhibited many phenotypic changes and hypermutable characteristics acquired over time during chronic infection (Ciofu *et al.*, 2005), we were interested in the effect such changes would have on the pro-inflammatory responses from lung epithelial cells during

infection with these bacteria. We observed that while PAK WT caused dramatic increases in cytokine release by A549 cells, all 6 clinical isolates of *P. aeruginosa* from chronically infected CF patients had lost much of this ability to induce inflammatory responses (Figures 14-18). The decreased cytokine stimulation ability of the clinical isolates is very exciting, as it connects previously observed phenotypic changes that occur in *P. aeruginosa* during chronic colonization, including loss of swimming, twitching and swarming motilities (Lee *et al.*, 2005), conversion to mucoidy (Speert *et al.*, 1990) and the emergence of antibiotic resistance (Oliver *et al.*, 2000; Ciofu *et al.*, 2005) with a decreased induction of cellular responses. These changes in bacterial behaviour have been attributed to the gain of a hypermutable phenotype, which allows the bacterium to acquire mutations at an elevated rate, selecting bacteria that are better suited to survive under conditions of the activated immune system and intense antibiotic treatment (Oliver *et al.*, 2000; Ciofu *et al.*, 2005). Indeed, the hypermutable phenotype may actually be a result of the oxidative stress conditions caused by chronic activation of immune effector cells in the CF lung (Ciofu *et al.*, 2005). In addition, it has been observed that almost 40% of *P. aeruginosa* isolates from CF patients had lost the ability to express flagellin, while almost no environmental or clinical isolate from non-CF patients were non-motile (Mahenthiralingam *et al.*, 1994). This loss of flagella could also contribute to a decreased activation of cytokine release, given the observed importance of flagella for cytokine release during PAK infection of A549 (Figure 13). Whatever the reasons for induction of hypermutable *P. aeruginosa* during the course of chronic infection are, our results indicate that once these bacteria have established themselves in the CF lung, they lose much of their immune-stimulatory ability, making it even more difficult to clear the infection.

In addition to the differences observed between PAK WT and the clinical isolates, we also found that two out of three isolate pairs exhibited a trend towards higher cytokine production by the non-mucoid (B) variant over the mucoid (A) member of a paired set of isolates. Indeed, with the isolate pair 8503A and B we observed higher IL-1β, IL-6, GM-CSF and TNF-α production by cells stimulated with the non-mucoid 8503B than cells stimulated with 8503A (Figure 15-19, approximate increase in protein levels: 20% IL-1β, 93% IL-6, 133% GM-CSF, 40% TNF-α). In addition, the non-mucoid isolate 1395B induced higher levels of IL-1β and GM-CSF than its mucoid counterpart 1395A (Figure 15 and 17, approximate increase in protein levels: 9.4% IL-1β, 53% GM-CSF). It is well known that many *P. aeruginosa* strains that chronically colonize CF patients change from non-mucoid to alginate over-producing mucoid phenotype several years after colonization (Speert *et al.*, 1990; Govan & Deretic, 1996; Mathee *et al.*, 1999). This phenotype is associated with a poor prognosis for the CF patient and increased survival for the bacterium, mainly attributable to the ability of alginate to protect the bacterium from phagocytosis and recognition by antibodies, to aid in biofilm formation (Govan & Deretic, 1996), to confer protection against reactive oxygen species, and to confer antibiotic resistance (Bjarnsholt *et al.*, 2005). Indeed, when mice were infected with either the non-mucoid laboratory strain PAO1 or its alginate over-producing mucoid derivative, the mucoid PAO1 was found to be less efficiently cleared from the lungs, and to induce more severe lung damage (Song *et al.*, 2003). The reduced ability of these mucoid bacteria to induce cytokine production from epithelial cells may therefore decrease the intensity and prolong the duration of the inflammatory response, which may contribute to the inefficient clearance and increased damage during chronic infection.

The observed difference in cytokine production by A549 cells in response to the laboratory strain PAK and the *P. aeruginosa* clinical isolates, and between mucoid and non-mucoid pairs of isolates emphasizes the importance of experimental design. Indeed, if the purpose of a study is to reproduce a model of CF infection, then it would be wise to use clinical *P. aeruginosa* isolates as opposed to laboratory strains. A recent study of the role of TLR5 activation during *P. aeruginosa* infections during CF used 151 clinical isolates to test whether they retained the ability to activate TLR5, and they found that 75% of isolates were still able to activate TLR5 (Blohmke *et al.*, 2008). While much care has been taken to use CF appropriate cell lines in laboratories of CF lung infections, it is clear that using correctly matched CF clinical isolates of *P. aeruginosa* is equally important for accurate representation of CF conditions.

By investigating the interactions of *P. aeruginosa* with A549 cells from the perspective of cell mediated signaling and activation, we have found several factors that are important for host-pathogen interactions. The ability of *P. aeruginosa* to activate pro-inflammatory cytokine release from A549 lung epithelial cells would contribute to activation of an immune response and recruitment of immune effector cells to the lungs *in vivo*. We have made the novel observation that induction of cytokine production is partially dependent on β integrin subunit-mediated activation of the signaling molecule ILK, since ILK gene silencing resulted in less cytokine release. Considering the ability of Fn to bind PAK and to cause increased bacterial association with A549 cells (Chapter 1), these results suggest that β1 integrin (particularly α5β1) do indeed have a role in the pathogenesis of *P. aeruginosa* pulmonary infections. In addition to integrin and ILK dependent cellular activation, flagella and LPS appear to be involved in *P. aeruginosa* induced cytokine release, most likely through TLR5 and TLR4 activation respectively. However, we did not directly investigate the role of TLR, and it is possible that the

flagella and LPS dependent cellular responses were due to some interaction of these structures with integrins. It is also very likely that the observed cross-talk between integrins and TLR could be working together to influence the reduced cytokine response of A549 cells infected with *P. aeruginosa* (Monick *et al.*, 2002). Indeed, since neither ILK knockdown nor PAK TLR ligand mutants cause 100% reduction in cytokine signal activation, it is likely that the two pathways work in parallel to elicit pro-inflammatory cellular responses. In addition, clinical isolates of *P. aeruginosa* with hypermutable phenotypes appear to have lost much of their immuno-stimulatory structures or abilities, as they caused drastically less cytokine release than the laboratory strain PAK.

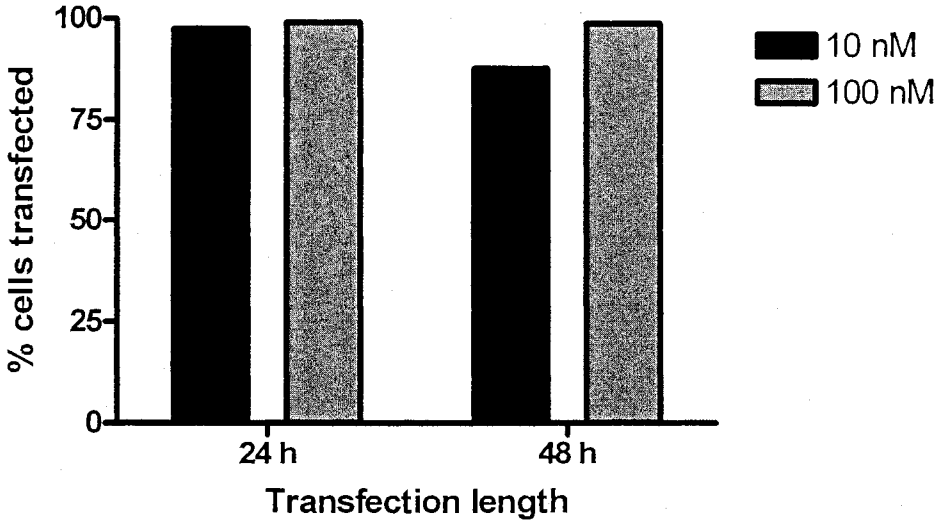


Figure 1: Transfection efficiency measured using FITC labeled siRNA. Efficiency of transfecting A549 cells at high confluency growth stage (80%) with a single siRNA treatment was verified by flow cytometric analysis of A549 cells transfected with FITC labeled non-silencing control siRNA. Successfully transfected cells were distinguished by their increase in green fluorescence detected in the FL1 channel over untransfected control cells. All transfection conditions tested showed very high levels of efficiency, ranging from 88-99%.

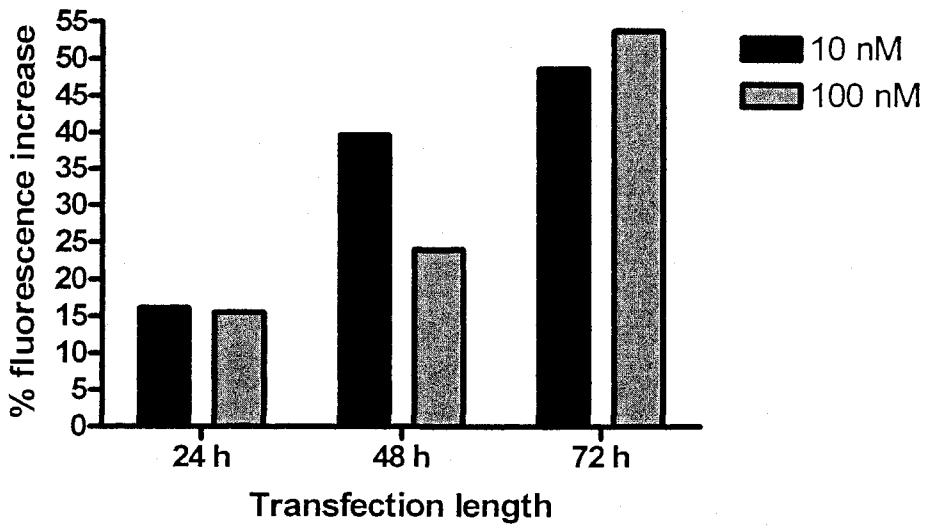


Figure 2: Effects of αv siRNA on surface expression of αv integrin protein using A549 cells at high confluency. Transfection of A549 cells with a single αv siRNA treatment at high confluency growth stage (80% confluent) resulted in increased surface αv integrin expression under every condition tested, compared to control non-transfected cells (set to 0 %).

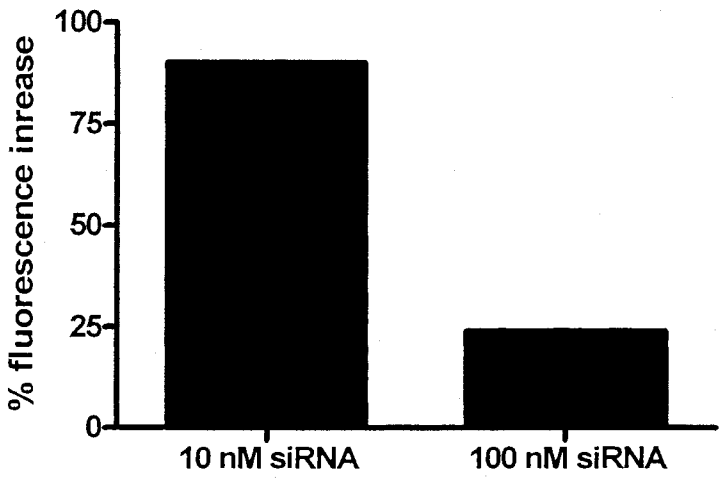


Figure 3: Effects of αv siRNA on surface expression of αv protein using A549 cells at low confluency. When A549 cells were transfected at a lower confluency growth stage (30%), followed by a second transfection 24 h later and incubated for a total of 48 h, there was an increase in αv surface protein compared to control non-transfected cells (set to 0%).

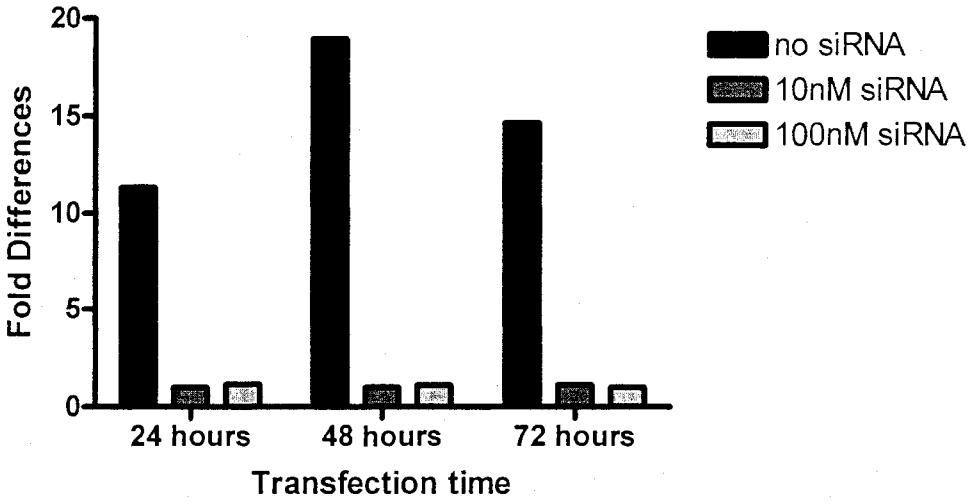


Figure 4: Effect of ILK siRNA on ILK mRNA expression of A549 cells transfected at high confluency. Single transfection of A549 cells at high confluency growth stage (80%) with ILK siRNA resulted in great reduction of mRNA expression under all conditions tested. ΔΔCt values, or 'Fold differences' in ILK mRNA compared to control non-transfected cells ranged from 11 to 19 fold. Transfection with 10 nM siRNA for 48 h showed the greatest decrease in expression of 19 fold, and was chosen for further studies.

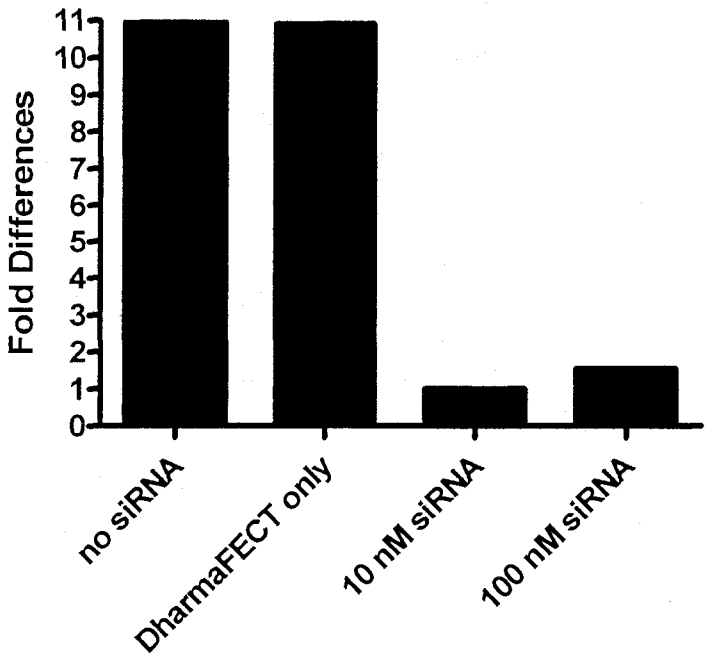


Figure 5: Effect of ILK siRNA on ILK mRNA expression of A549 cells transfected at low confluency. To ensure that the most efficient siRNA knockdown was achieved, A549 cells were also treated with ILK siRNA transfection at low confluency (30%), and then transfected again 24 h later and incubated for a total of 48 h. Using this protocol, there were large reductions in ILK mRNA, with approximately 11 fold difference between 10 nM ILK siRNA and non-treated cells.

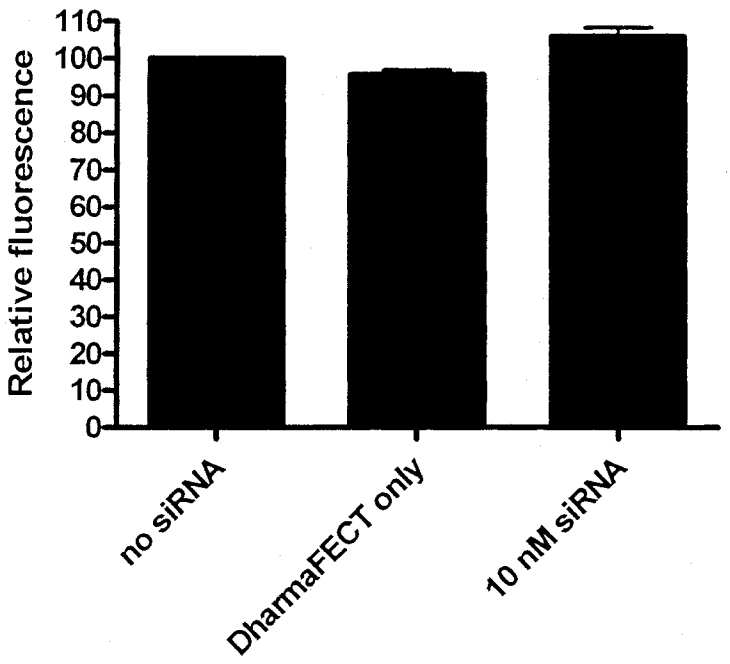


Figure 6: Effects of ILK siRNA on PAK*gfp* association with A549 cells. Treatment of A549 cells at high confluency growth stage with 10 nM ILK siRNA for 48 h did not have any affect on the amount of PAK*gfp* associated with adherent A549 cells (adhered or internalized), as determined by the amount of fluorescent signal emitted from bacteria associated with cells measured using a fluorometric plate reader. Fluorescence of PAK*gfp* stimulated control DharmaFECT and active 10 nM siRNA treated cells were not significantly different from non-siRNA treated cells ($n=2$, $p<0.05$, one sample t-test).

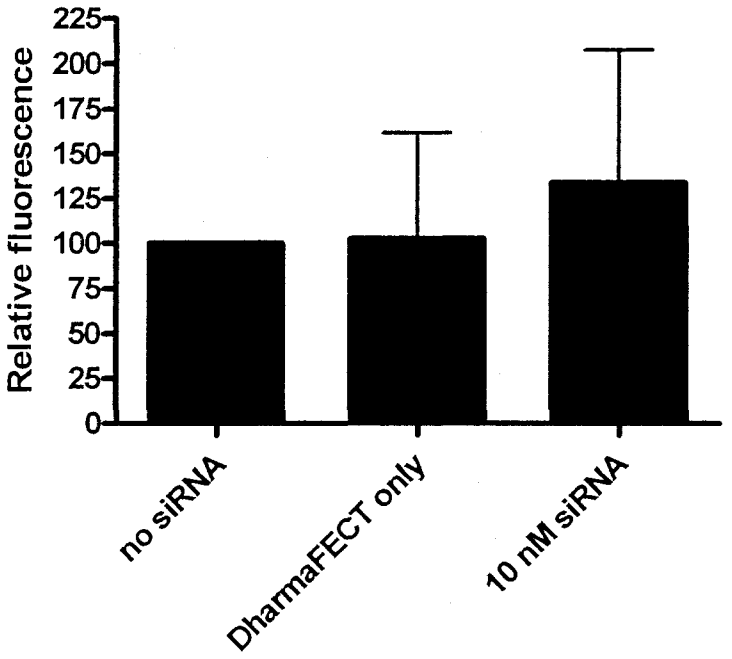


Figure 7: Effects of ILK siRNA on PAK*gfp* internalization into A549 cells (plate reader). Treatment of A549 cells at high confluency growth stage with 10 nM ILK siRNA for 48 h did not have any affect on the amount of PAK*gfp* internalized into adherent A549 cells, as determined by the amount of fluorescent signal emitted from bacteria internalized by cells following PMx treatment (10 μg/ml, 30 min) as measured by fluorometric plate reader. Neither control DharmaFECT treatment nor active 10 nM siRNA caused changes in amount of bacteria internalized into A549 cells (n=2).

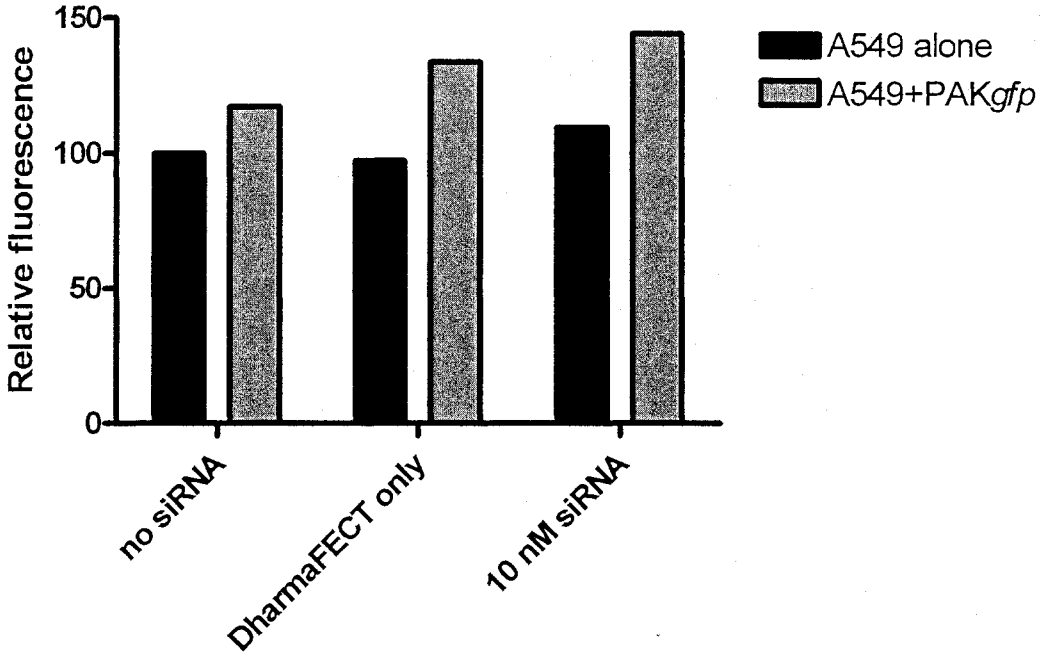


Figure 8: Effects of ILK siRNA on PAK*gfp* internalization into A549 (flow cytometer). Treatment of A549 cells at high confluency growth stage with 10 nM ILK siRNA for 48 h did not have any effect on the amount of PAK*gfp* internalized into adherent A549 cells, as determined by the amount of fluorescent signal emitted from bacteria internalized by cells following PMx treatment (10 μg/ml, 30 min) as measured by flow cytometry. Neither control DharmaFECT treatment nor active 10 nM siRNA appeared to decrease the amount of bacteria internalized into A549 cells (n=1).

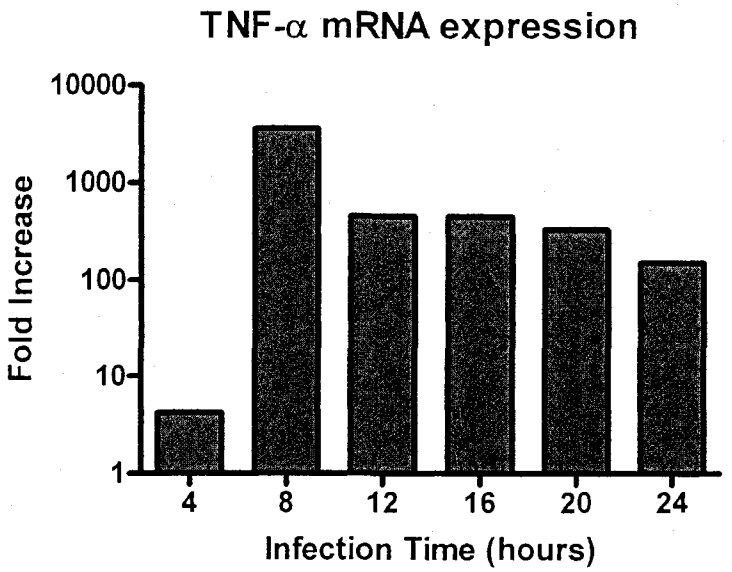


Figure 9: Time course of PAK induced TNF-α mRNA expression by A549 cells. Infection of A549 cells with PAK WT at an MOI of 50:1 for varying lengths of time results in increased expression of TNF-α mRNA compared to non-stimulated cells detected using qRT-PCR. Maximum TNF-α mRNA levels of approximately 3,000 fold higher than non-stimulated cells were observed at 8 h post infection.

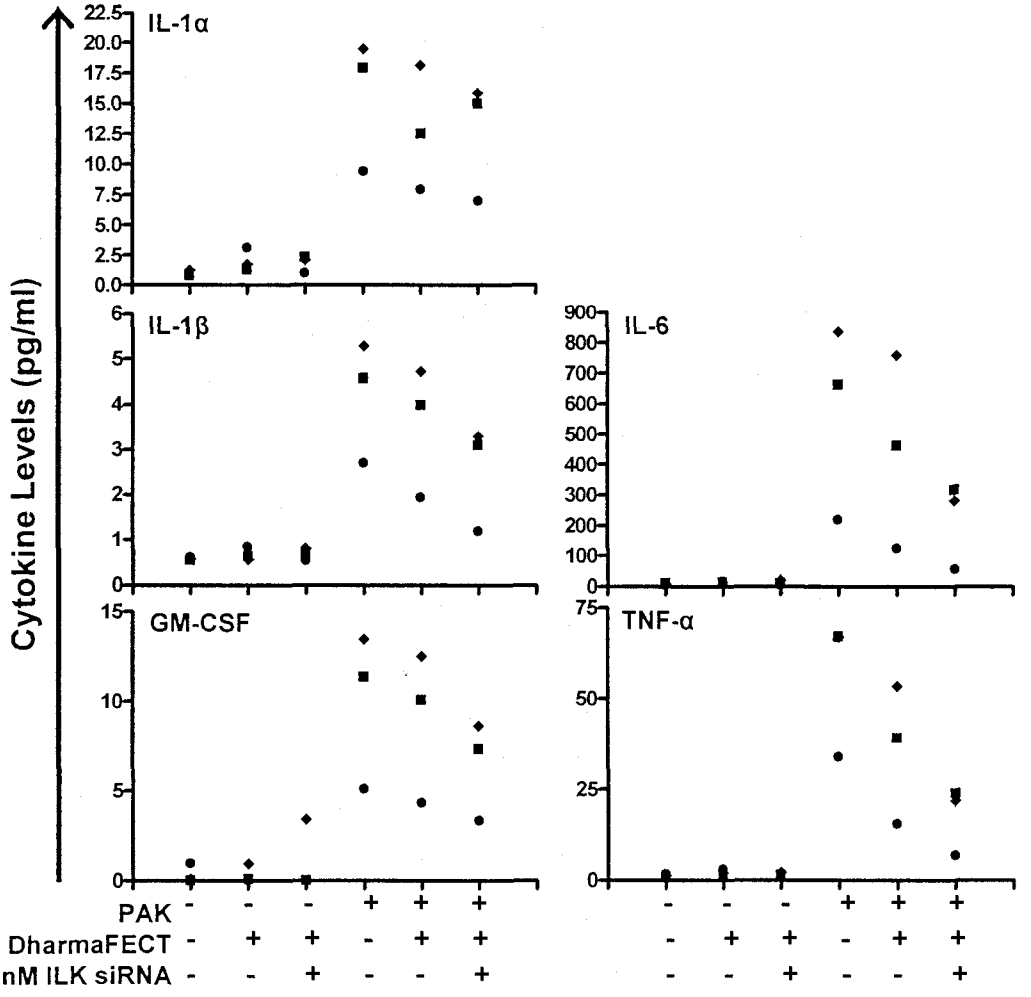


Figure 10: Effects of ILK siRNA on cytokine levels during PAK stimulation of A549 cells (absolute values). Stimulation of A549 cells with PAK WT at an MOI of 50:1 for 18 h resulted in increased production of IL-α, IL-1β, IL-6, GM-CSF and TNF-α compared to cells not stimulated with PAK. Pre-treatment with 10 nM ILK siRNA for 48 h prior to PAK stimulation resulted in a general decrease in pro-inflammatory cytokine release. Control treatment with DharmaFECT transfection reagent for 48 h prior to infection also caused some decrease in cytokine release, but not as much as ILK siRNA treated cells. Each set of circles, squares and diamonds represent results from 3 independent experiments. Values are shown as absolute levels of cytokines in culture supernatants (pg/ml).

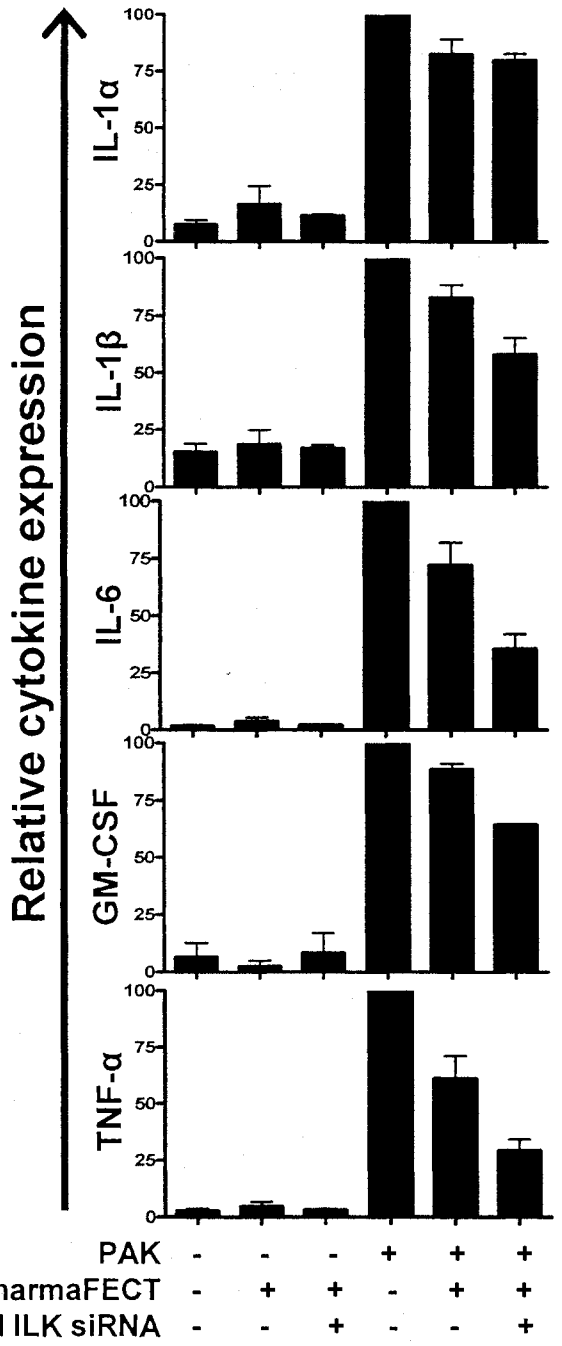


Figure 11: Effects of ILK siRNA on cytokine protein levels during PAK stimulation of A549 (normalized values). Stimulation of A549 with PAK WT at an MOI of 50:1 for 18 h resulted in increased production of IL-α, IL-1β, IL-6, GM-CSF and TNF-α compared to cells not stimulated with PAK. Control DharmaFECT treatment for 48 h prior to PAK stimulation caused significantly less release of all 5 cytokines measured, however, 10 nM ILK siRNA treatment for 48 h prior to PAK stimulation caused significantly less cytokine release than the DharmaFECT controls for all cytokines except IL-1α ($n=3$, $p<0.05$, One sample t-test).

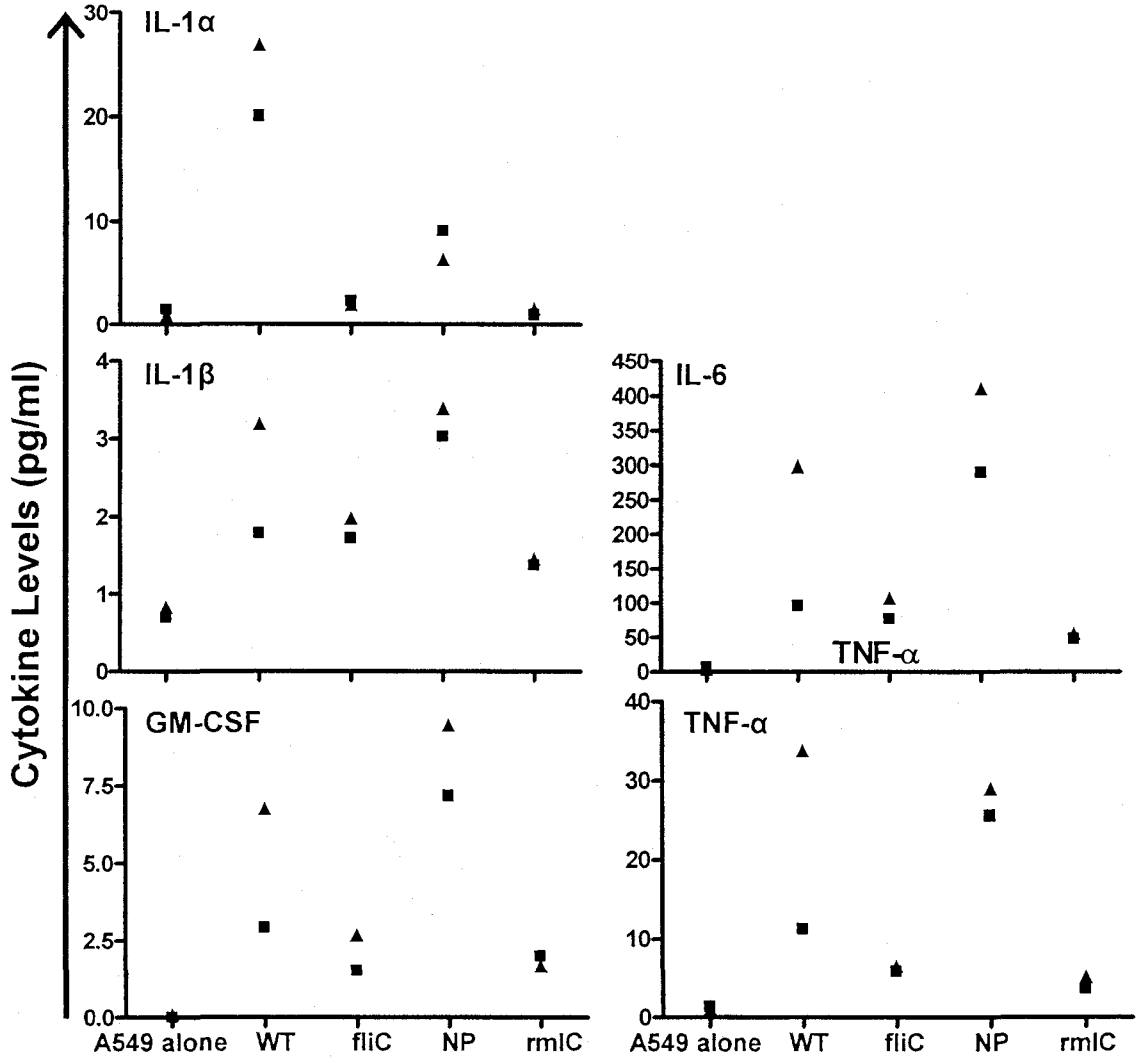


Figure 12: Cytokine response of A549 to infection with PAK mutants (absolute values). Stimulation of A549 cells for 18 h with PAK WT, fliC, NP or rmlC mutants at an MOI of 50:1 resulted in variable levels of cytokine release from cells. In general, PAK fliC and rmlC caused less cytokine release than PAK WT, while PAK NP caused similar or higher levels of cytokine release. Each set squares or triangles represent results from 2 independent experiments. Values are shown as absolute levels of cytokines in culture supernatants (pg/ml).

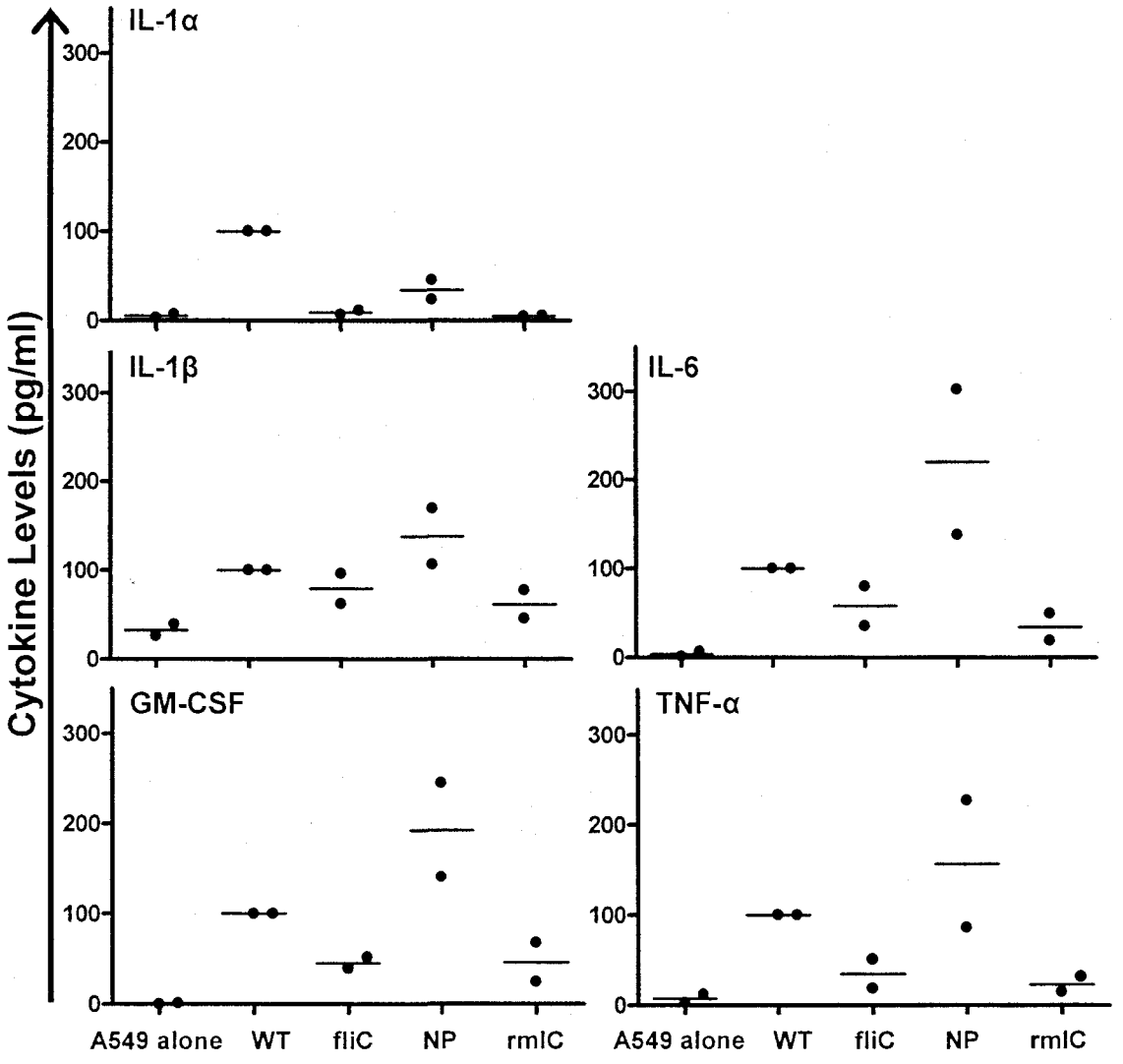


Figure 13: Cytokine response of A549 to PAK mutant infection (normalized values). Stimulation of A549 cells for 18 h with PAK WT, fliC, NP or rmlC mutants at an MOI of 50:1 resulted in variable levels of cytokine release from cells. Data is shown with PAK WT stimulated cells from each experiment normalized to 100, and the other values expressed relative to PAK WT. In general, PAK fliC and PAK rmlC elicited less cytokine release from cells than PAK WT, while PAK NP caused similar or higher levels of cytokine release. Dots represent separate measurements from n=2 separate experiments, and horizontal bars indicate the mean value.

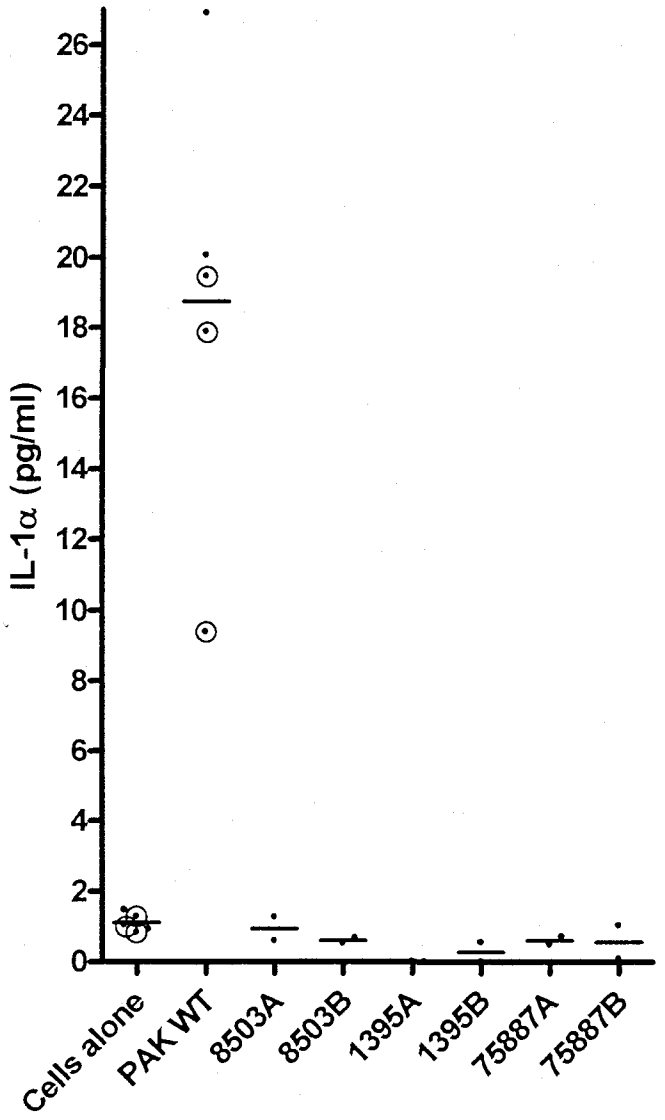


Figure 14: IL-1α production by A549 cells infected with clinical isolates of *P. aeruginosa*. Stimulation of A549 cells for 18 h with PAK WT or paired *P. aeruginosa* clinical isolates resulted in variable IL-1α expression by A549 cells. PAK WT caused greater levels of IL-1α protein expression than any of the isolates. IL-1α levels did not appear to vary between the different isolates. Dots represent values from n=2 independent experiments for each isolate and horizontal bars represent means. Dots with circles indicate experiments during which cells were pre-treated for 48 h under siRNA control conditions (serum free OptiMEM) prior to infection. Values are shown as absolute cytokine levels in culture supernatant (pg/ml).

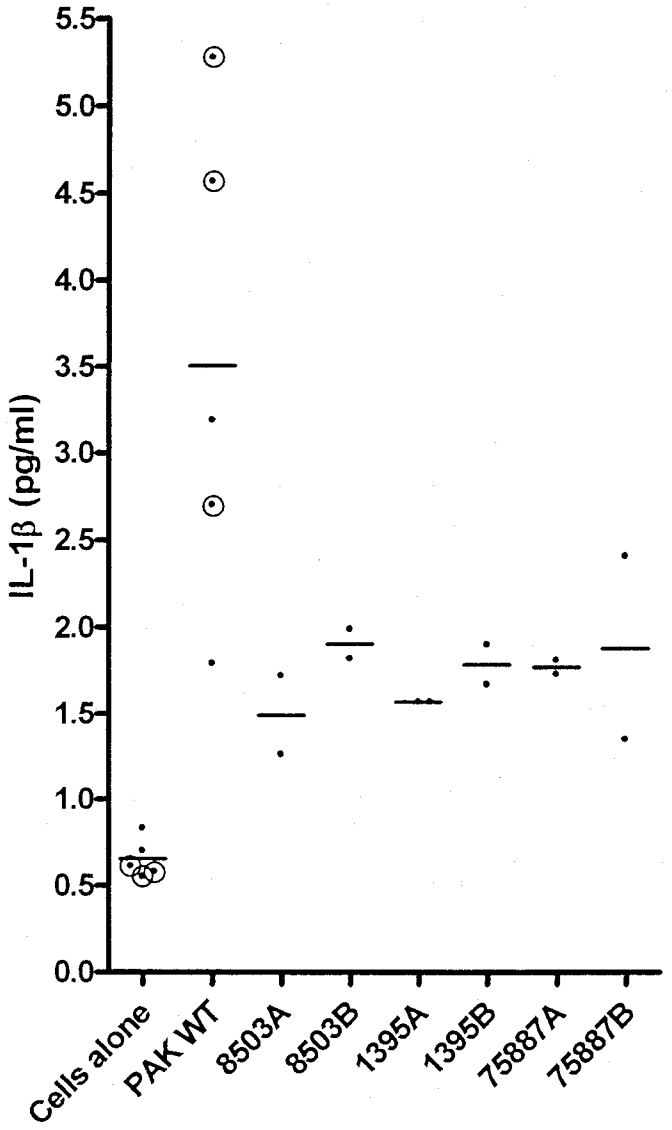


Figure 15: IL-1β production by A549 cells infected with clinical isolates of *P. aeruginosa*. Stimulation of A549 cells for 18 h with PAK WT or paired *P. aeruginosa* clinical isolates resulted in variable IL-1β expression by A549 cells. PAK WT caused greater levels of IL-1β protein expression than any of the isolates. IL-1β levels did vary somewhat between the isolates, with the mucoid 8503A and 1395A stimulating less IL-1β protein than their non-mucoid pair 8503B or 1395B respectively. Dots represent values from n=2 independent experiments for each isolate and horizontal bars represent means. Dots with circles indicate experiments during which cells were pre-treated for 48 h under siRNA control conditions (serum free OptiMEM) prior to infection. Values are shown as absolute cytokine levels in culture supernatant (pg/ml).

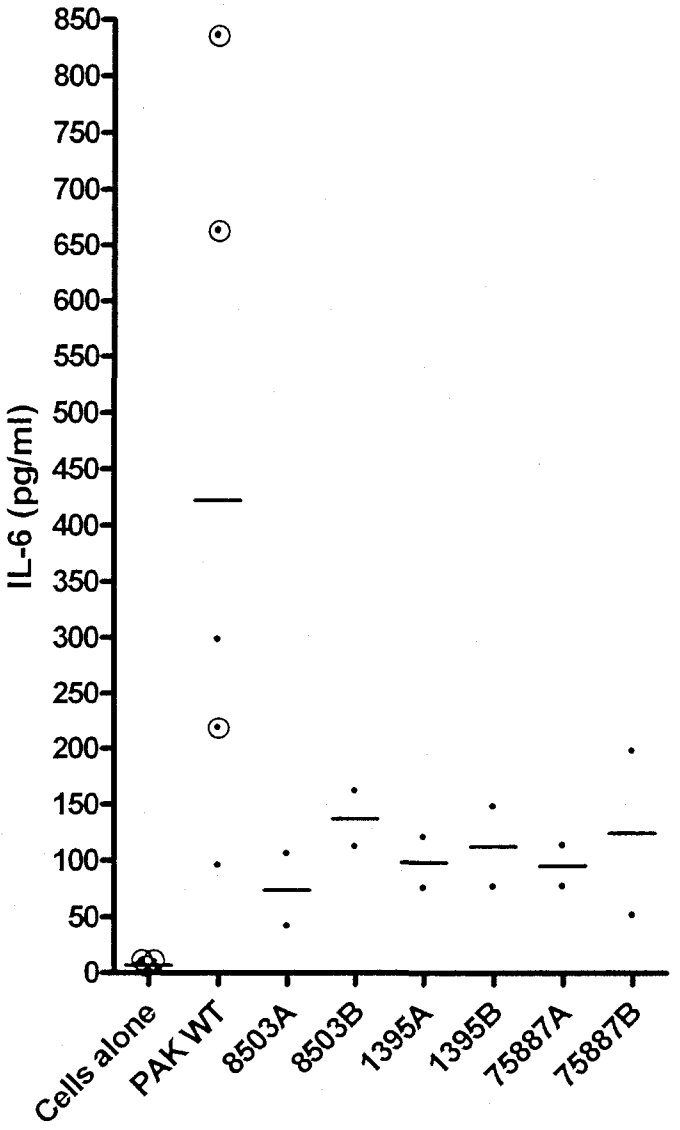


Figure 16: IL-6 production by A549 cells infected with clinical isolates of *P. aeruginosa*. Stimulation of A549 cells for 18 h with PAK WT or paired *P. aeruginosa* clinical isolates resulted in variable IL-6 expression by A549 cells. PAK WT caused greater levels of IL-6 protein expression than any of the isolates. IL-6 levels did vary somewhat between the isolates, with the mucoid 8503A stimulating less IL-6 protein than its non-mucoid pair 8503B. Dots represent values from n=2 independent experiments for each isolate and horizontal bars represent means. Dots with circles indicate experiments during which cells were pre-treated for 48 h under siRNA control conditions (serum free OptiMEM) prior to infection. Values are shown as absolute cytokine levels in culture supernatant (pg/ml).

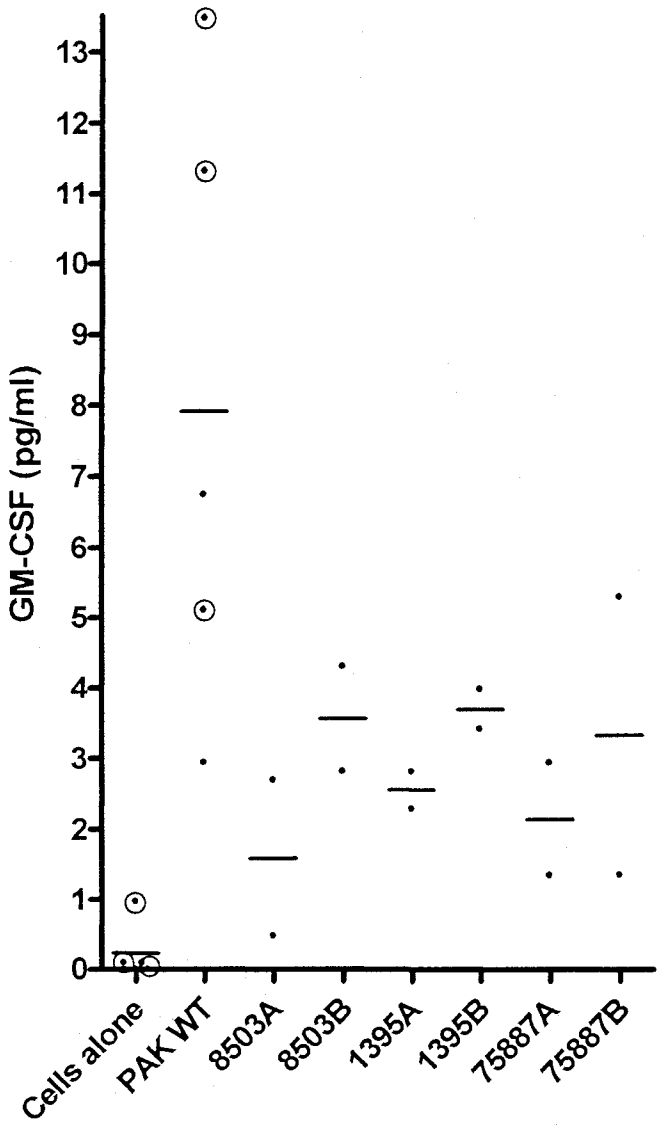


Figure 17: GM-CSF production by A549 cells infected with clinical isolates of *P. aeruginosa*. Stimulation of A549 cells for 18 h with PAK WT or paired *P. aeruginosa* clinical isolates resulted in variable GM-CSF expression by A549 cells. PAK WT caused greater levels of GM-CSF protein expression than any of the isolates. GM-CSF levels did vary somewhat between the isolates, with the mucoid 8503A and 1395A stimulating less IL-GM-CSF protein than their non-mucoid pair 8503B or 1395B respectively. Dots represent values from n=2 independent experiments for each isolate and horizontal bars represent means. Dots with circles indicate experiments during which cells were pre-treated for 48 h under siRNA control conditions (serum free OptiMEM) prior to infection. Values are shown as absolute cytokine levels in culture supernatant (pg/ml).

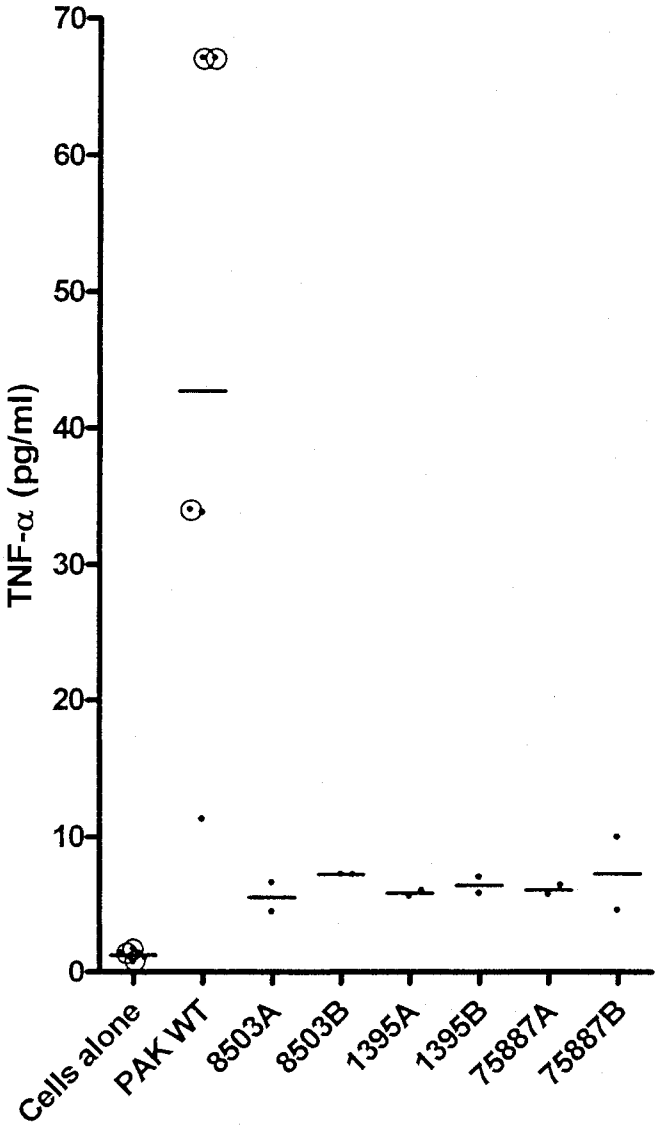


Figure 18: TNF-α production by A549 cells infected with clinical isolates of *P. aeruginosa*. Stimulation of A549 cells for 18 h with PAK WT or paired *P. aeruginosa* clinical isolates resulted in variable TNF-α expression by A549 cells. PAK WT caused greater levels of TNF-α protein expression than any of the isolates. TNF-α levels did vary somewhat between the isolates, with the mucoid 8503A stimulating less TNF-α protein than its non-mucoid pair 8503B. Dots represent values from n=2 independent experiments for each isolate and horizontal bars represent means. Dots with circles indicate experiments during which cells were pre-treated for 48 h under siRNA control conditions (serum free OptiMEM) prior to infection. Values are shown as absolute cytokine levels in culture supernatant (pg/ml).

Conclusions

The ultimate goal of these studies was to develop and optimize a set of methods which could be used to explore host-pathogen interactions in a model of lung infection by the bacterium *P. aeruginosa*, and to use the systems to specifically investigate the role of integrins during bacterial interactions with lung epithelial cells. We chose to genomically label a strain of *P. aeruginosa* PAK with a green fluorescent protein gene so that interactions of this bacterium with host cells could be easily and reliably measured using multiple fluorescence based read-outs including flow cytometry, plate reader based assay, and fluorescence microscopy. The successful labelling and optimizing of this PAK*gfp* based system has provided us with a unique ability to measure bacterial association with and internalization into A549 lung epithelial cells, and to target specific pathogen and host mechanisms to measure any changes and determine their involvement during pathogenesis.

Once the *gfp* based system was developed and optimized, we were able to use it to investigate whether lung epithelial integrin receptors are involved in *P. aeruginosa* interactions with A549 cells. Integrin blocking RGD based peptides were found to not have any effect on any PAK*gfp* interactions with A549 cells (association, internalization, cytokine release), unless the A549 cells were infected with *Mycoplasma*. Although we cannot make any firm conclusions about the importance of integrins from these studies with peptides, it did alert us that the activation status of host cells (i.e. already infected with another bacterium or not) can drastically interfere with the experimental systems. Indeed, we suspect that the contaminated cells possessed higher basal expression of surface integrins, which may explain why inhibitor effects were only detectable when using these cells. We also could not draw any firm conclusions about the importance of integrins during bacterial host interactions using anti-integrin antibodies, but

the observations from this 'failure' were very valuable. We discovered that *P. aeruginosa* is able to bind to IgG, suggesting that this bacterium may posses Fc region IgG binding capabilities similar to *S. aureus* (Sinha *et al.*, 1999). Because of this IgG binding ability, we believe that during antibody inhibitor studies *P. aeruginosa* is able to bind to both unbound and cell-associated antibodies in the experimental system, and thus the effects of antibodies cannot be truly observed. IgG binding could help explain the conflicting results on the effects of anti-integrin antibodies during *P. aeruginosa* infection of epithelial cells reported in the literature, suggesting that antibodies cannot be reliably used to study such interactions (Roger *et al.*, 1999; Gagniere & Di Martino, 2004; Leroy-Dudal *et al.*, 2004). However, we have observed that PAK*gfp* can bind preferentially to the α5β1 integrin ligand Fn and to the αvβ5 integrin ligand Vn, and that Fn increases the ability of PAK*gfp* to associate with A549 cells. These results suggest that integrins may indeed play a role in *P. aeruginosa* interactions with lung epithelial cells, and warrant further investigation using cellular-based methods of integrin manipulation.

By targeting ILK (the signaling molecule directly downstream of some β integrin subunits) for siRNA knockdown we found that integrin responses relayed through ILK are important for *P. aeruginosa* interactions with host epithelial cells (cellular response pathway summarized in Figure 1). ILK knockdown was found to significantly decrease the production of pro-inflammatory cytokines by A549 cells due to PAK stimulation, but was not found to affect either PAK*gfp* association with or internalization into A549 cells. This suggests that the inability of RGD peptides and anti-integrin antibodies to inhibit bacterial association or internalization into host cells may have been valid observations, and that integrins are truly not important for the physical interactions of *P. aeruginosa* with lung epithelial cells under normal circumstances. Again, while peptides were observed to inhibit PAK*gfp* association with *Mycoplasma* infected

A549 cells, it is possible that if integrins are present at higher levels then they may be able to contribute significantly to bacterial association and internalization, but when there are not many surface integrins expressed (i.e. in the *Mycoplasma*-free A549 cells), that there are too few integrins present to have any measurable effect. Indeed, the most well-controlled high quality study of integrin involvement with *P. aeruginosa* infection was performed using a model of damaged and repairing airway epithelium, which is known to have a different integrin expression profile than normal airway epithelium (Roger *et al.*, 1999). However, even if integrins are not present at high enough levels to contribute to bacterial association and internalization, it is quite feasible that they could contribute significantly to cellular signaling in response to bacteria, due to the nature of signal transduction cascades, which can amplify a very small stimulus to result in a robust cellular response.

By investigating the ability of isogenic PAK mutants lacking different TLR ligands, we observed that flagella and LPS were very involved with cytokine release from A549 cells. However, neither PAK mutants nor ILK gene silencing resulted in complete reduction of cytokine response by A549 cells, suggesting that both TLR and integrins can be involved in activating epithelial cells during *P. aeruginosa* infection. It is possible that the cross-talk between TLR and integrins are important for a complete cellular cytokine response during infection as has been previously suggested using different experimental models (Perera *et al.*, 2001; Monick *et al.*, 2002; Wang *et al.*, 2003). Alternatively, it is possible that flagella and LPS can bind to and directly activate integrins as well as TLR, although the integrin binding structure(s) of *P. aeruginosa* have not yet been clearly identified (Roger *et al.*, 1999).

Finally, we have made some novel and exciting findings regarding the ability of *P. aeruginosa* clinical isolates taken from chronically infected CF patients to induce cytokine

release from A549 cells. Infection with all clinical isolates resulted in much lower cytokine release from A549 cells compared to PAK WT, and mucoid isolates tended to cause less cytokine release than their non-mucoid counterparts. Since these isolates have been shown to possess many mutations (Ciofu *et al.*, 2005; Lee *et al.*, 2005), and bacteria which chronically colonize the lung often become less immunogenic over time (Mahenthiralingam *et al.*, 1994; Govan & Deretic, 1996), the environment of the chronically infected lung may select for reduction in immunostimulatory surface structures which are necessary for activating cytokine responses and subsequently mounting an efficient immune response to clear the infection. Indeed, if cytokine production by lung epithelial cells is lower resulting in less immune cell recruitment and activation, then this is beneficial for the bacterium. Since integrins appear not to be important for *P. aeruginosa* association or internalization into host cells, less activation of integrins (corresponding to less cytokine production) should be beneficial to the *P. aeruginosa* in establishing long term colonization of the lung (such as occurs with CF), as it would result in decreased clearance by immune cells. However, during acute infection, increased integrin activation (and cytokine release) causing a more vigorous immune response resulting in epithelial damage may provide opportunities for *P. aeruginosa* to disseminate to different tissues. Therefore, the importance of integrins for the pathogenesis of *P. aeruginosa* pulmonary infections may depend very much on the context and clinical setting under which the infection occurs.

Since cellular signaling responses to *P. aeruginosa* involve the activation of the β integrin subunit linked signaling molecule ILK, we conclude that *P. aeruginosa* induction of cellular responses occurs in a β integrin dependent fashion (proposed model in Figure 1). Based on the ability of Fn to bind *P. aeruginosa* and to increase association with lung epithelial cells

when endogenous Fn is not present, this ILK activation is likely achieved by bacterial activation of the integrin α5β1. While *P. aeruginosa* binding to integrins may not normally contribute significantly to association or internalization, the Fn binding ability is crucial to activating ILK signaling, since the first step to signal activation is ECM binding of integrins which causes clustering in lipid rafts (Delcommenne *et al.*, 1998; Pankov & Yamada, 2002), resulting in PI3K dependent activation of ILK signaling. In addition to integrins, we have observed that cellular responses of A549 cells to *P. aeruginosa* infection can depend on the presence of TLR ligands and also on the phenotype of the *P. aeruginosa* strain interacting with the lung epithelium. While we are suggesting that integrins play an important role in *P. aeruginosa* host-pathogen interactions, it is important to keep in mind the opportunistic nature of this pathogen, and that these interactions with integrins are one of many different modes that *P. aeruginosa* utilizes to exploit host cells during pulmonary infections.

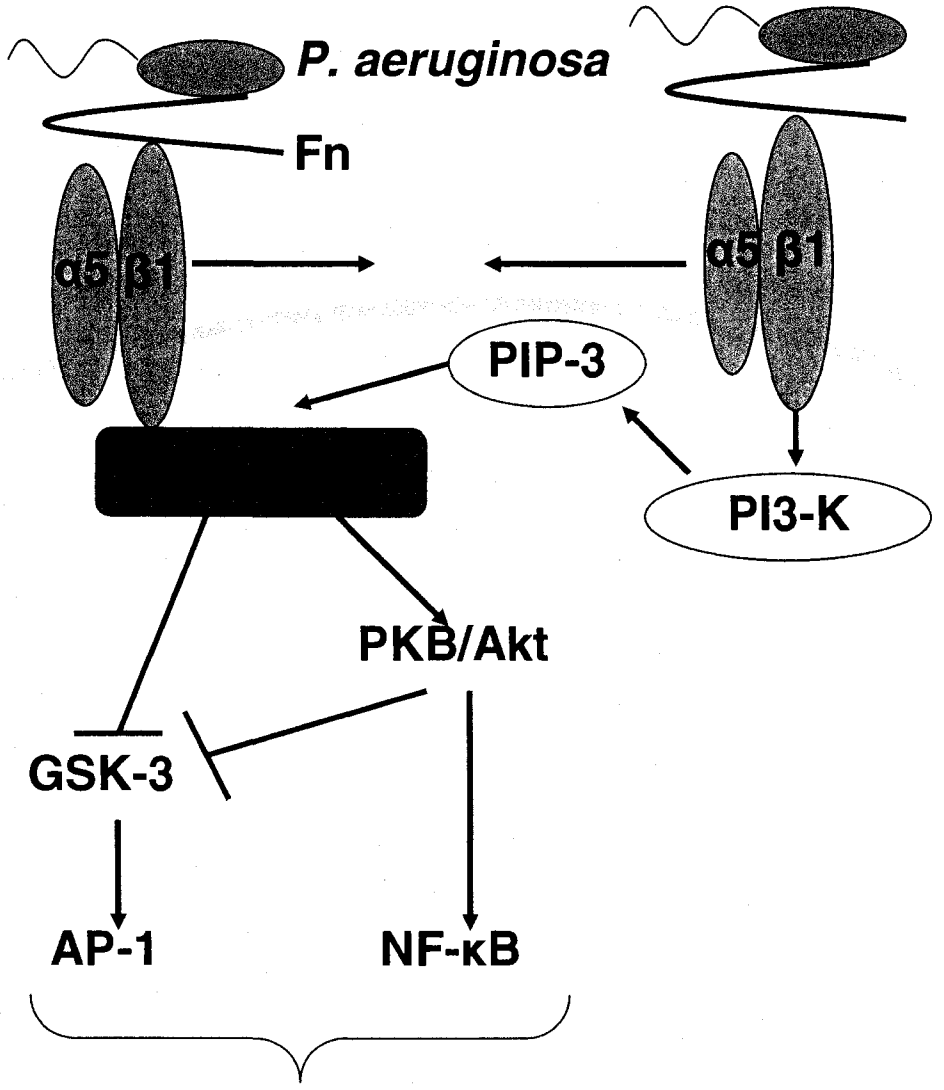


Figure 1: Proposed model of integrin involvement in *P. aeruginosa* activation of lung epithelial cells. Based on the results of our studies, we propose that *P. aeruginosa* uses Fn to bind to α5β1 integrins, resulting in their clustering into focal adhesion complexes and subsequent activation of the integrin linked signaling molecule ILK. ILK activation results in induction of pro-inflammatory cytokine responses to the bacterium, but not to bacterial internalization. There is also likely cross-talk with other host signaling receptors, including TLR, which contributes to the cellular responses to *P. aeruginosa*, and the extent of activation of all these signaling pathways can depend on the phenotype of the specific strain of *P. aeruginosa*.

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