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Up-regulation of integrin expression in lung adenocarcinoma cells caused by bacterial infection: *in vitro* study

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Integrins are a large family of adhesion receptors that are known to be key signaling molecules in both physiological and pathological processes. Previous studies have demonstrated that the expression of integrin receptors in the pulmonary epithelium can change under various pathological conditions, such as injury, inflammation, or malignant transformation. We hypothesize that integrin expression can be altered by stimulation of lung epithelial cells with an opportunistic bacterial pathogen *Pseudomonas aeruginosa*. Using the A549 adenocarcinoma cell line that expressed a low level of several integrin subunits we have demonstrated that *P. aeruginosa* infection *in vitro* caused a rapid up-regulation of α5, αv, β1, and β4 integrins at both the mRNA and protein level. Neither heat-killed *P. aeruginosa* strain PAK nor its live isogenic mutants lacking pili or lipopolysaccharide (LPS) core oligosaccharide showed any effect on integrin expression in A549 cells as compared to the use of the wild-type PAK strain. These results establish that up-regulation of integrin expression is dependent on the internalization of live bacteria possessing intact pili and LPS. Gene silencing of integrin-linked kinase in A549 cells caused a significant decrease in the release of pro-inflammatory cytokines in response to *P. aeruginosa* stimulation. Although further studies are warranted towards understanding the precise role of integrin receptors in prominent inflammation caused by *P. aeruginosa*, our findings suggest a possibility of using specific integrin inhibitors for therapy of pulmonary inflammatory conditions caused by pathogenic micro-organisms.

Keywords: inflammation, integrin linked kinase, *Pseudomonas aeruginosa*, epithelial cell receptors

Introduction

Integrins are a large family of αβ heterodimeric transmembrane adhesion receptors that mediate cellular interactions with the extracellular matrix (ECM) and other cells in the micro-environment. Following ligand recognition, integrins undergo clustering and conformational changes that result in recruitment of a number of intracellular signaling molecules. This is followed by activation of several signaling cascades, and consequently regulates vital cellular functions, such as proliferation, differentiation, migration, cytokine release, *etc*. (reviewed by Arnaout *et al*.[1]). Eight different αβ integrin heterodimers are expressed in normal lung epithelial cells, *i.e*. α2β1, α3β1, α6β4, α9β1, α5β1, αvβ5, αvβ6, and αvβ8. These receptors recognize an array of ECM proteins: collagen I, tenascin C, laminins 5, 10, 11, osteopontin, fibronectin, vitronectin, *etc*.[2] It has been established that lung integrins are critical for tissue development, maintaining epithelial integrity, repair of damaged tissue, and regulation of inflammatory responses and tissue remodeling.[2]

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Previous studies have demonstrated that the expression of integrin receptors in the pulmonary epithelium can change under various pathological conditions, such as injury, inflammation, or malignant transformation.[3–5] Some pathogenic micro-organisms (*e.g. Streptococcus pyogenes* and *Pneumocystis carinii*) are able to increase integrin expression in infected respiratory epithelial cells, and the resulting events have been implicated in microbial pathogenesis.[6,7] However, specific mechanisms underlying the effects of pathogens on integrin expression as well as the functional consequences of integrin alterations for the pulmonary epithelium are poorly understood.

The opportunistic Gram-negative pathogen *Pseudomonas aeruginosa* causes severe pulmonary infections in immunocompromized patients. It is the leading cause of ventilator-associated pneumonia in intensive care units with high mortality rates.[8] *Pseudomonas aeruginosa* frequently causes pulmonary infections in lung cancer patients, especially in those with neutropenia as a result of chemotherapy.[9] *Pseudomonas aeruginosa* is the major cause of chronic pulmonary infection in cystic fibrosis patients that determines the overall prognosis of this genetic disease,[10] as well as a significant cause of exacerbations of chronic obstructive pulmonary disease (COPD).[11]

In this study, we address the question of whether an infection with *P. aeruginosa* can cause an alteration in the expression of integrins in lung epithelial cells. We have used an *in vitro* model of a lung adenocarcinoma A549 cell line that expressed a low level of several integrin subunits. We found that *P. aeruginosa* infection caused rapid transcriptional up-regulation of integrins α5, αv, β1, β4 that was dependent on the internalization of live virulent, piliated, Lipopolysaccharide-expressing bacteria into A549 cells. Moreover, gene silencing of integrin linked kinase (ILK) caused a significant decrease in the release of pro-inflammatory cytokines in response to *P. aeruginosa* infection.

Materials and Methods

Cell line and bacterial strains

The A549 human lung adenocarcinoma cell line (ATCC # CCL-185) at the passage numbers 75–85 was used in this study. A549 cells were maintained in DMEM (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% heat-inactivated FBS (SAFC Biosciences, Lenexa, KS, USA) and 1% L-glutamine (Gibco, Carlsbad, CA, USA) without antibiotics at 37°C with 5% CO_2.

Pseudomonas aeruginosa strain PAK (kindly provided by Dr R.J. Irvin, University of Alberta, Edmonton, described by Pasloske *et al.*[12]), and the isogenic *P. aeruginosa* PAK mutants PAK NP (pili deficient), PAK *fliC* (flagella deficient; generously donated by Dr A.S. Prince, Columbia University, New York, USA) and PAK *rmlC* (isogenic knockout LPS mutant with truncated core oligosaccharide[13]) were maintained on sterile Luria Burtani (LB) medium (Fischer Scientific, Fair Lawn, NJ, USA) with 1% agar (LBA).

Preparation of P. aeruginosa *for experiments*

Pseudomonas aeruginosa cultures were grown for 18 h in sterile LB medium on a shaking platform at 100 rpm, and diluted by a factor of 20 into fresh sterile LB medium. Cultures were allowed to grow for approximately 1 h, until mid-log phase when optical density at 600 nm (OD_{600}) reached 0.4–0.45 and then centrifuged at 3500 *g* for 20 min at 4°C. Bacteria were washed twice in sterile PBS (pH 7.4). Following the final resuspension, bacteria were diluted to an OD_{600} of 0.45 in sterile DMEM that corresponded approximately to 2×10^8 CFU/ml, as determined by serial dilutions and drop plating on LBA. From this stock, bacteria were added to A549 cells at a multiplicity of infection (MOI) of 100 : 1, and actual numbers of bacteria added were verified by serial dilutions and drop plating on LBA. To kill *P. aeruginosa* by heating, a suspension of bacteria at 2×10^8 CFU/ml was heated in a 60°C water bath for 45 min. Killing efficiency was verified by the inability of bacteria to grow on LBA spread plates following overnight incubation at 37°C.

Purification of P. aeruginosa *pili and flagella*

Purification of *P. aeruginosa* flagella and pili was performed following protocols kindly provided by Dr R. Irvin (University of Alberta, Edmonton, AB, Canada) and Dr L. Burrows (McMaster University, Hamilton, ON, Canada), respectively.

To isolate flagella, *P. aeruginosa* were grown overnight in LB medium as described above. Bacteria were centrifuged (10,000 *g* for 20 min), then resuspended in sterile PBS containing 10 mM $MgCl_2$. Flagella were sheared off by blending bacteria in a Waring blender for 20 s and the resulting loss of motility was confirmed by light microscopy. The cells were sedimented by centrifugation at 12,000 *g*, and then flagella-containing supernatant was collected. Flagella were then pelleted by ultracentrifugation (100,000 *g* for 30 min at 4°C) and resuspended in PBS containing 10 mM $MgCl_2$.

To isolate pili, *P. aeruginosa* PAK *fliC*, a mutant strain defective in flagella synthesis, was cultured on an LBA plate for 18 h, then scraped off the plate and

deposited into 15 ml of sterile PBS. The bacteria were then vortexed 3 times in 15 s bursts to shear the pili. Cells were pelleted, and the supernatant containing the pili transferred to a new tube, and centrifuged at 10,000 *g* to remove any remaining debris. The supernatant was again transferred, followed by addition of 1/10 vol each of 5 M NaCl and 30% polyethylene glycol. The mixture was incubated on ice for 60 min, and then centrifuged at 10,000 *g* for 30 min at 4°C to collect the pili.

Purity of the isolated products was assessed by SDS-PAGE and Coomassie Blue staining. A single band corresponding to a molecular weight of 45 kDa was detected in the flagella preparation, and a single band of molecular mass 15 kDa was detected in the pili preparation.

Stimulation of A549 cells with P. aeruginosa

Bacterial stimulation of A549 cells in suspension

Cultures of A549 cells at 85% confluency were trypsinized (1 min treatment with 0.05% Trypsin-EDTA (Gibco) at 37°C), washed with PBS, counted using a Vi-Cell XR Cell Viability Analyzer (Beckman Coulter, Mississauga, ON, Canada), and resuspended in sterile serum-free DMEM (2×10^6 cells/ml). The A549 cells were mixed with an equal volume of *P. aeruginosa* suspension containing 2×10^8 CFU/ml (MOI 100 : 1), and incubated for 1, 2, or 4 h at 37°C and 5% CO_2 with occasional agitation. 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 PBS. A549 cell viability was assessed using a Vi-Cell XR Cell Viability Analyzer and was found to range between 82–93% after the longest bacterial stimulation in different experiments.

Bacterial stimulation of adherent A549 cells

A549 cells were grown in 6-well plates for 2 d until they reached near 85% confluency, corresponding approximately to 1×10^6 cells per well. Wells were washed twice with sterile PBS, and prepared bacteria in serum-free DMEM were added at an MOI of 100 : 1. Following incubation for 1, 2, or 4 h at 37°C, 5% CO_2, the cells were washed three times with PBS and trypsinized to achieve a single-cell suspension. Cells were centrifuged at 233 *g* for 5 min and then resuspended in PBS to proceed with immunostaining and flow cytometry analysis, or with RNA extraction and real-time PCR. For 24-h long stimulation, a similar procedure was done with the exception that, following the first 4 h incubation, 50 μg/ml polymyxin B (Sigma-Aldrich) was added.

To inhibit cytoskeleton re-arrangement, adherent A549 cells were exposed to 5 μg/ml cytochalasin B (Sigma-Aldrich) in serum-free DMEM for 24 h at 37°C and 5% CO_2. Following pre-treatment, cells were washed twice with PBS, and stimulation with *P. aeruginosa* was performed, as described above.

Internalization assay

Pseudomonas aeruginosa uptake by A549 cells was assessed using gentamicin exclusion assay. Confluent cultures of A549 cells in 96-well plates were exposed to *P. aeruginosa* (50 μl of bacterial suspension/well at a MOI of 100 : 1). After 1 h at 37°C, supernatants were removed and cultures were incubated for 90 min with 200 μg/ml gentamicin (Sigma-Aldrich) in serum-free medium to kill extracellular bacteria. Antibiotic-containing medium was removed; cells were washed 2 times with PBS to remove the antibiotic and lysed with sterile PBS containing 0.1% Triton X-100. Dilution series and drop plates were made to quantify viable bacteria.

Flow cytometry analysis

A549 cells were exposed to *P. aeruginosa* suspended in serum-free DMEM at a MOI of 100 : 1 for 1, 2, 4, or 24 h at 37°C and 5% CO_2. Alternatively, A549 cells were exposed to 10 μg/ml of *P. aeruginosa* LPS purified by gel-permeation chromatography (Sigma-Aldrich), purified pili, or purified flagella for 4 h at 37°C and 5% CO_2. Treatment of cells with isolated bacterial products alone for 4 h was found to have no effect on cell viability which was 97% in all experiments. The expression of integrin subunits α5, αv, β1, and β4, as well as ICAM-1 was determined using indirect immunostaining. Cell suspension (100 μl), containing 1×10^5 cells, was incubated with 10 μg/ml of mAb against integrin αv (anti-CD51, mouse IgG1, clone 13C2, Southern Biotech, Birmingham, AL, USA), β1 (anti-CD29, mouse IgG1, clone 12G10, AbD Serotec, Raleigh, NC, USA), α5 (anti-CD49e, mouse IgG1, clone IIA1), β4 (anti-CD104, mouse IgG1, clone 450-9D), or ICAM-1 (anti-CD54, mouse IgG1, clone HA58) (Biosciences, San Jose, CA, USA) at 4°C for 1 h in the dark. Cells were then washed twice with PBS and incubated with FITC-conjugated anti-mouse IgG at 4°C for 1 h in the dark. Mouse IgG1, an anti-CD3 mAb (BD Biosciences), was used as an isotype control. After washing, the cells were resuspended in 100 μl of PBS and analyzed by flow cytometry using a FACSCalibur with CELLQuest Pro software (BD Biosciences). The expression of integrin subunits

was presented as relative mean fluorescence intensity (MFI) calculated using the following equation:

$$\text{Relative fluorescence} = \frac{(\text{antibody MFI} - \text{isotype control MFI})}{(\text{isotype control MFI})} \quad (1)$$

Treatment of A549 cells with siRNA

Eighty percent confluent A549 cells were transfected with either 10 nM or 100 nM custom ILK-H siRNA (targeting the sequence corresponding to the plekstrin homology domain nucleotides 741–759 'AACCTGACGAAGCTCAACGAGAA' of human ILK; Qiagen, Mississauga, ON, Canada). Previous studies have demonstrated highly efficient and specific ILK gene silencing in human respiratory epithelial cells using this siRNA.[14] The transfection procedure was carried out for 24, 48, or 72 h according to the protocol recommended by Dharmacon, Inc. (Chicago, IL, USA) using Opti-MEM Reduced Serum transfection medium (Gibco, Burlington, ON, Canada) and DharmaFECT Transfection Reagent (Dharmacon). Controls included cells incubated in Opti-MEM medium, cells incubated in Opti-MEM medium containing DharmaFECT, and cells transfected with non-silencing siRNA (Qiagen).

The efficiency of RNA delivery was assessed using a stable FITC-labeled Block IT Fluorescent Oligo (Invitrogen Canada Inc, Burlington, ON, Canada) 24 h and 48 h post-transfection using both 10 nM and 100 nM concentrations of siRNA. In these experiments, following transfection, A549 cells were trypsinized, washed with PBS, and resuspended in PBS for flow cytometry analysis. Transfected cells were recognized by their green fluorescence signal. 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. The percentage of cells containing FITC labeled siRNA ranged between 88–99% in different experiments; the highest transfection efficiency was detected at 48 h with 10 nM siRNA.

Quantitative real-time PCR

Adherent A549 cells at 85% confluency were exposed to *P. aeruginosa* suspended in serum-free DMEM at a MOI of 100 : 1 for 1, 2, or 4 h at 37°C and 5% CO_2. After stimulation, cells were washed twice with PBS, and RNA was extracted using the Ultraclean RNA isolation kit (MO Bio Laboratories, Carlsbad, CA, USA) according to the supplier's instructions. One microgram of total RNA was reverse transcribed using the First Strand cDNA Synthesis kit (Fermentas Life Sciences, Burlington, ON, Canada). Polymerase chain reaction was performed using the Chromo4 Real Time PCR detector thermal cycler (Bio-Rad Laboratories, Mississauga, ON, Canada), with each reaction containing 5 ng of reverse-transcribed RNA in 25 µl, RT2 SYBR Green PCR Master Mix (Superarray, Frederick, MD, USA), and commercially available primers for integrin subunits α5, αv, β1, and β4, as well as the housekeeping gene GAPDH (Superarray). Polymerase chain reaction was also performed to measure the knockdown efficiency for ILK using an ILK-specific primer (Superarray) and to measure the expression of Jun (AP-1) mRNA using a Superarray primer. The polymerase chain reaction was performed as follows: 95°C for 15 min, 40 cycles of 95°C for 30 s, 55°C for 30 s, plate read, 72°C for 30 s, plate read, and 72°C for 2 min, followed by a melting curve from 57°C to 95°C, with plate reads every 1°C. The cycle threshold (C_T) at which amplification entered the exponential phase was determined and this number was used as an indicator of the amount of target RNA in each sample. The cycle threshold was used to compare relative amounts of different transcripts. To account for differences in the amount of total RNA, the results obtained for each integrin subunit were normalized to GAPDH expression levels from the same sample, yielding relative values for the expression of each subunit. This was presented as ΔC_T, and relative fold expression of each gene was calculated using the $\Delta\Delta C_T$ method using Equation 2:

$$2^{-[\Delta C_T(\text{stimulated A549}) - \Delta C_T(\text{A549 alone})]} \quad (2)$$

Cytokine assay

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 (Bio-Rad). In these experiments, A549 cells were seeded into 24-well plates at 50,000 cells/well in 500 µl DMEM with 10% FBS, and allowed to grow for 2 d. Cells were transfected with 10 nM ILK siRNA using DharmaFECT transfection reagent, or with the transfection reagent alone for 48 h as described above. Following siRNA treatment, cells were infected with *P. aeruginosa* PAK wild-type at a MOI of 50 : 1. A lower MOI than in previous experiments was selected as it was found to yield optimal cellular activation over longer incubation times. Cells and bacteria were incubated for 1 h at 37°C, 5% CO_2, and then bacteria were killed by adding 50 µg/ml polymyxin B. A549 cells and dead bacteria were incubated together for further 17 h at 37°C, 5% CO_2. Supernatants were collected and centrifuged at 13,000 *g* for 25 min (4°C) to pellet the bacteria, and the clear supernatants were stored in aliquots at −80°C

until analysis. The levels of IL-1β, IL-6, GM-CSF and TNF-α were measured using a Bio-Plex Human Cytokine Reagent Kit with a Multiplex Biomolecular Analyzer (Bio-Rad) according to the manufacturer's protocols. Samples from three independent experiments were run in duplicate. Treatments tested included A549 cells incubated without transfection reagent or siRNA, cells incubated with DharmFECT transfection reagent alone, and cells transfected with 10 nM ILK siRNA and DharmaFECT, each tested with and without *P. aeruginosa* infection. Detection levels of cytokines were 0.2–3200 pg/ml.

Statistical analysis

Data were expressed as mean ± SEM for n separate experiments. For studies where $n > 3$, statistical analysis was performed using Mann–Whitney's test, and for studies where $n = 3$, statistical analysis was performed using Student's t-test. P-values < 0.05 were considered significant.

RESULTS

The expression of multiple integrin subunits in A549 cells is up-regulated in response to P. aeruginosa *infection*

To address the question of whether *P. aeruginosa* infection can alter the expression of integrin receptors in lung epithelial cells, we used the A549 cell line at passage numbers 75–85 that had low basal levels of α5, αv, β1, and β4 integrin surface expression (Fig. 1). We focused on these four subunits because they are involved in all αβ integrin heterodimers expressed in normal lung epithelial cells.[2] A549 cells were stimulated with *P. aeruginosa* strain PAK for 1, 2, or 4 h, and the surface expression of α5, αv, β1, and β4 integrin subunits was examined using flow cytometry analysis. We used both adherent A549 cells as a more physiologically-relevant model of bacterial infection (Fig. 1A,C), and A549 cells in suspension to confirm the findings (Fig. 1B). In both systems, the surface expression of α5 and β1 integrins was significantly up-regulated after 2 h of *P. aeruginosa* infection. In contrast, an increase in surface expression of αv and β4 integrins occurred after 4-h stimulation. Following prolonged stimulation (24 h), the expression of α5 integrin was still higher in infected cells compared to uninfected ones (M ± SD: 2.53 ± 0.57 versus 0.83 ± 0.91; $P < 0.05$), although the expression of other integrin subunits was similar to control, *i.e.* αv, 9.5 ± 3.15 versus 9.1 ± 2.9; β1, 2.5 ± 0.3 versus 2.4 ± 0.3; β4, 2.9 ± 0.5 versus 2.2 ± 1.0 (Fig. 1D). To determine whether this increase in integrin surface expression was due to transcriptional up-regulation, we performed real-time PCR (Fig. 2). We found that α5 and β1 mRNA was constitutively expressed and significantly increased between 1 h and 2–4 h bacterial stimulation, in accordance with the surface protein up-regulation. Conversely, αv and β4 mRNA was below the detection threshold in both unstimulated cells and after 1 h of stimulation, but showed an increase between 2–4 h. Hence, the results show that the infection of A549 cells with *P. aeruginosa* caused a rapid up-regulation of both mRNA and surface protein expression of multiple integrin subunits.

Integrin up-regulation in A549 cells requires internalization of live bacteria

It has been established that, in the process of pulmonary infection, *P. aeruginosa* is internalized by epithelial cells.[15] To address the question of whether bacterial internalization was necessary for the effect of *P. aeruginosa* on integrin expression, A549 cells were pre-treated with cytochalasin B (5 μg/ml) for 24 h. This concentration of cytochalasin B was found to inhibit bacterial internalization;[16] in our experiments, it did not cause a decrease in cell viability below 95%. Following cytochalasin B pre-treatment, adherent A549 cells were infected with *P. aeruginosa* for 4 h, then immunostained with antibodies to integrins α5, αv, β1, and β4 and analyzed using flow cytometry. The cytochalasin B pre-treatment completely abolished *P. aeruginosa* induced up-regulation of all integrin subunits (Fig. 3A), and caused significant decrease in bacterial internalization measured with the gentamicin exclusion assay (Fig. 3B). These results indicate that the ability to internalize bacteria is required for the up-regulation of integrin expression by A549 cells.

Based on these findings, we hypothesized that interactions of live *P. aeruginosa* with A549 cells were essential for the up-regulation of integrin expression. To test this hypothesis, we stimulated adherent A549 cells with heat-killed *P. aeruginosa* for 1, 2, and 4 h using the same experimental conditions as in the case of infection with live bacteria. The adhesion molecule ICAM-1 was used as a positive control because it is known to become rapidly up-regulated in epithelial cells upon *P. aeruginosa* stimulation via activation of NF-κB.[17] Heat-killed *P. aeruginosa* did not cause up-regulation of any integrin subunit studied (Fig. 3C) although it did induce a significant increase in the expression of ICAM-1 (Fig. 3D). Collectively, the results indicate that internalization of live bacteria is essential for up-regulation of integrins in A549 cells caused by *P. aeruginosa* infection.

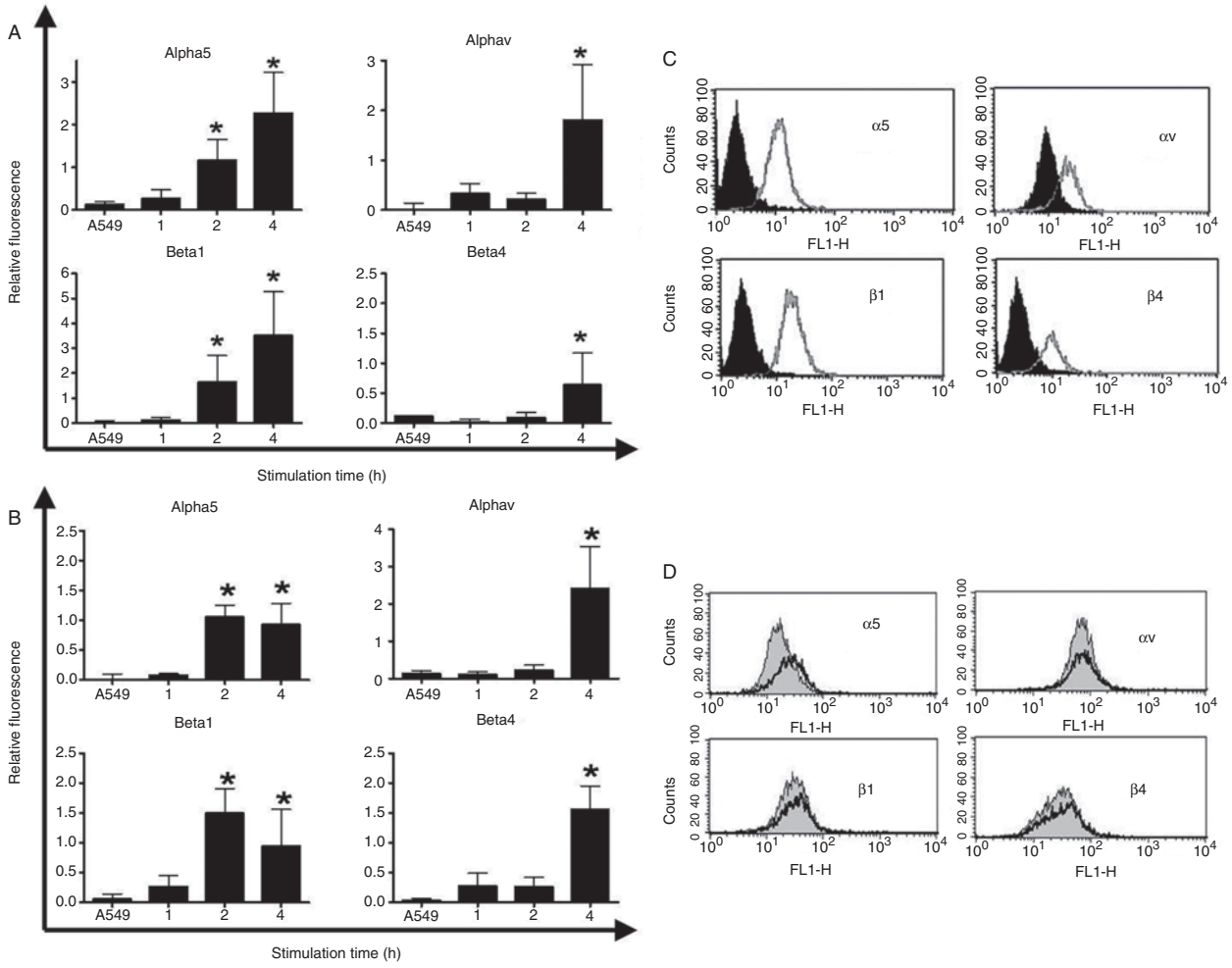

Fig. 1. *Pseudomonas aeruginosa* stimulation induces rapid up-regulation of integrin cell surface expression in A549 cells. As described in Materials and Methods, adherent A549 cells (A) or A549 cells in suspension (B) were stimulated with live *P. aeruginosa* strain PAK in mid-log phase at a MOI of 100 : 1 for 1, 2, or 4 h and immunostained for α5, αv, β1, and β4 integrin subunits, followed by flow cytometry analysis. The results are expressed as relative fluorescence. (C) Original histograms of one representative experiment showing integrin expression in adherent unstimulated A549 cells (filled histograms) and following 4 h stimulation with *P. aeruginosa* (empty histograms). $n = 4$, $^*P < 0.05$, Mann–Whitney U-test, compared with unstimulated cells. (D) As described in Materials and Methods, integrin expression was assessed in adherent A549 cells following long-term stimulation with *P. aeruginosa*. Integrin expression in unstimulated A549 cells (filled histograms) and following 24 h stimulation with *P. aeruginosa* (empty histograms) of one representative experiment out of three independent trials is shown.

Specific bacterial structures are required for up-regulation of integrin expression

It is well established that pili, flagella, and LPS are the major virulence factors of *P. aeruginosa*, mediating bacterial adhesion and the resulting epithelial cellular responses (*i.e.* inflammation). We hypothesized that these virulence factors may be required for the up-regulation of integrins. To test this hypothesis, we used live isogenic mutants of *P. aeruginosa* deficient in pili (PAK NP),[18,19] flagella (PAK *fliC*),[20] or LPS outer core oligosaccharide (PAK *rmlC*)[13] to infect adherent A549 cells for 4 h, followed by immunostaining and flow cytometry analysis of α5, αv, β1, and β4 integrin surface expression. Neither the pili- nor the LPS-deficient mutant of *P. aeruginosa* was able to induce up-regulation of integrin expression although the flagella-deficient mutant produced an effect similar to that of the wild-type bacteria (Fig. 4A). These results indicate that pili and the outer core oligosaccharide of LPS, both of which have been shown to participate in *P. aeruginosa* adhesion to epithelial cells,[21] are required to induce up-regulation of integrins, whereas the presence of flagella was not essential for this effect. Nevertheless, all the mutant strains were able to up-regulate expression of ICAM-1, indicating cellular activation and induction of pro-inflammatory responses to bacterial stimulation had occurred (Fig. 4B). An increase in ICAM-1 expression following infection with the flagella-deficient strain appeared to be lower than following infection with

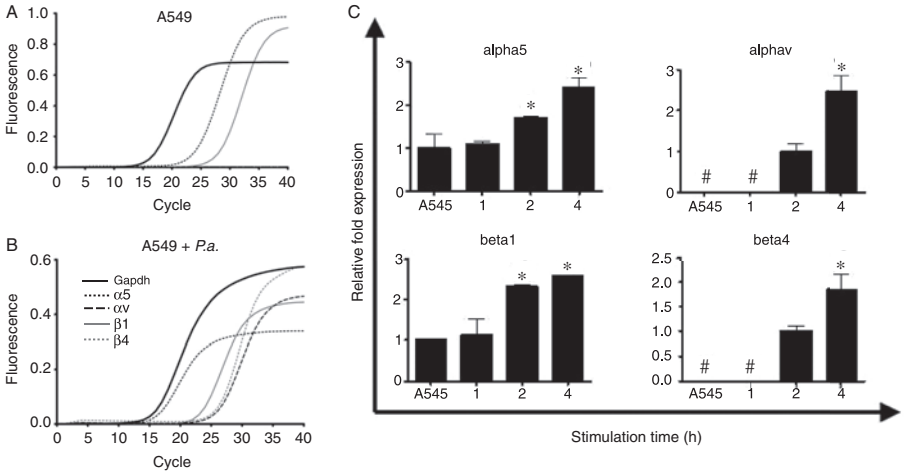

Fig. 2. *Pseudomonas aeruginosa* stimulation induces up-regulation of integrin mRNA expression in A549 cells. As described in Materials and Methods, adherent A549 cells were stimulated with live *P. aeruginosa* strain PAK in mid-log phase at a MOI of 100 : 1 for 1, 2, or 4 h. After stimulation, A549 cells were trypsinized, washed, and total RNA was isolated. Then, mRNA was reverse transcribed to cDNA, and qPCR was performed. GAPDH served as the housekeeping gene. Representative cycle curves illustrate the amplification of α5, αv, β1, and β4 integrin mRNA in (A) unstimulated A549 cells at t = 0 and (B) A549 cells stimulated with *P. aeruginosa* at t = 4 h. Note that in unstimulated cells, the expression levels of αv and β4 were not detectable. (C) Relative fold induction of each integrin subunit. Expression of α5, αv, β1, and β4 integrin subunit mRNA was calculated, using GAPDH as a housekeeping gene, and relative fold expression of each subunit was assessed by calculating the $\Delta\Delta C_T$ value. $n = 3$, * $P < 0.05$, Student's *t*-test, compared to unstimulated cells; #below detection level.

pili- or LPS-deficient mutants corroborating previous data on the dominant role of flagella in *P. aeruginosa* induced inflammatory responses.[22]

Isolated pili or LPS of P. aeruginosa *are unable to induce integrin up-regulation in A549 cells*

Given that the pili- and LPS-deficient *P. aeruginosa* mutants were unable to induce up-regulation of integrins in infected A549 cells, we hypothesized that isolated pili and LPS would induce this effect. Adherent A549 cells were treated for 4 h with isolated pili, isolated LPS, as well as a combination of pili and LPS. In these experiments, we used concentrations of pili and LPS (10 μg/ml) which have been shown by other authors to activate NF-κB and the resulting release of pro-inflammatory cytokines.[23] None of the treatments resulted in an up-regulation of β1 integrin surface expression (Fig. 5A). Nevertheless, stimulation of A549 cells with isolated *P. aeruginosa* pili caused an increase in surface expression of ICAM-1, whereas isolated LPS had no effect (Fig. 5B). As expected, stimulation of A549 cells with isolated flagella in concentrations of 1–100 μg/ml did not alter integrin expression, although it did cause a significant increase in ICAM-1 expression within the first 4 h of stimulation (data not shown).

Based on these findings, we hypothesized that mutant strains or heat-killed bacteria could still be rescued by co-stimulation with isolated bacterial products to induce up-regulation of integrins. We addressed this question by stimulating adherent A549 cells with the following combinations: pili-deficient mutant and isolated pili, heat-killed wild-type bacteria and isolated pili, LPS-deficient mutant and isolated LPS, or heat-killed wild-type bacteria and isolated LPS. Interestingly, none of these treatments resulted in an increase in β1 integrin surface expression (Fig. 5C). When combined, these findings indicate that the up-regulation of integrin receptors in A549 cells caused by *P. aeruginosa* requires the internalization of live bacteria that possess pili and intact LPS.

ILK gene silencing leads to a decreased release of pro-inflammatory cytokines by infected A549 cells

An increase in integrin expression caused by *P. aeruginosa* suggests a possibility of the activation of integrin-mediated signaling. To address the role of such signaling in cellular responses to *P. aeruginosa* infection, we applied siRNA-induced gene silencing of the serine–threonine protein kinase ILK, a critical molecule directly involved in initiation and propagation of β1 integrin dependent signaling.[24]

A single transfection of A549 cells with either 10 nM or 100 nM siRNA resulted in a great reduction in ILK mRNA expression following 24, 48, and 72 h post-transfection as detected by real-time PCR. Differences in $\Delta\Delta C_T$ values in ILK mRNA between siRNA transfected and non-transfected cells ranged from 11–19-fold (Fig. 6A). As transfection with 10 nM siRNA for 48 h

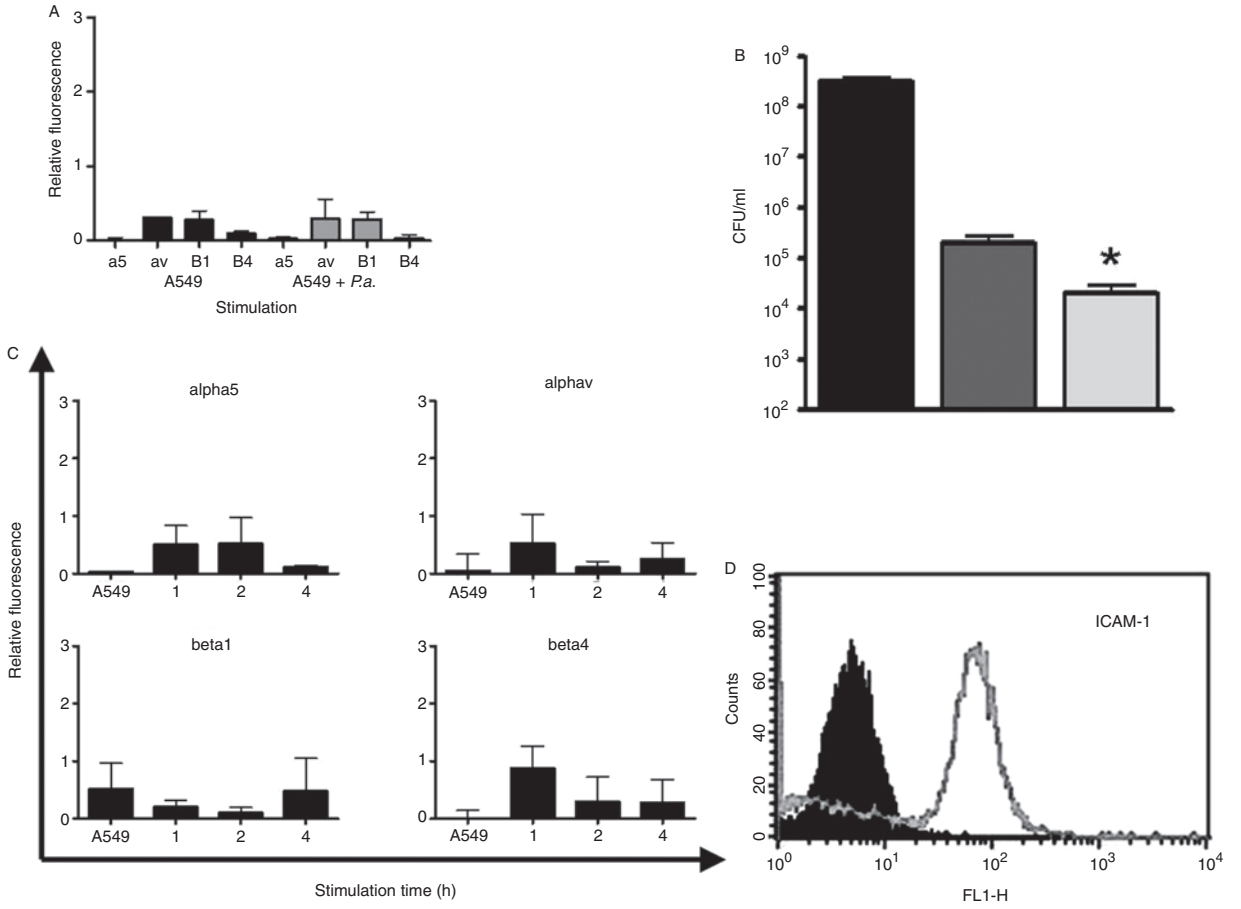

Fig. 3. Internalization of live *P. aeruginosa* is required for up-regulation of integrins in A549 cells. (A) Adherent A549 cells were pre-treated with 5 μg/ml cytochalasin B for 24 h. Following pre-treatment, A549 cells were stimulated with live *P. aeruginosa* strain PAK in mid-log phase at a MOI of 100 : 1 for 1, 2, or 4 h. Immunostaining for α5, αv, β1, and β4 integrin subunits was performed followed by flow cytometry analysis. The results are expressed as relative fluorescence. (B) Internalization of live *P. aeruginosa* (dark gray bar) significantly decreased following pre-treatment of adherent A549 cells with cytochalasin B (light gray bar) as detected by gentamicin exclusion assay (black bar: number of bacteria added to A549 cells at a MOI of 100 : 1). $*P < 0.05$, $n = 2$ independent experiments performed in triplicates. (C) *Pseudomonas aeruginosa* were killed by heating bacteria to 60°C for 45 min, then used to stimulate adherent A549 cells at a MOI of 100 : 1 for 1, 2, or 4 h. Flow cytometry analysis of integrin expression is shown. (D) Adherent A549 cells were treated with heat-killed bacteria as described above, immunostained for ICAM-1 and subjected to flow cytometry analysis. Original histograms show ICAM-1 expression (one representative experiment) in adherent unstimulated A549 cells (filled histogram) and following 4-h stimulation with heat-killed bacteria (empty histogram). Flow cytometry results represent three independent experiments.

showed the greatest decrease in ILK mRNA (19-fold), these conditions were used for further studies. To confirm specificity of ILK gene silencing, we have tested mRNA expression of an unrelated gene, AP-1 (Jun) in cells transfected with siRNA. The ILK siRNA did not have any effect on Jun expression in A549 cells under the same conditions that caused significant ILK gene silencing (Fig. 6B).

Following siRNA treatment, A549 cells were stimulated with live wild-type *P. aeruginosa* strain PAK for 1 h, then antibiotics were added, and A549 cells together with killed bacteria were incubated for another 17 h. Following 18-h bacterial stimulation, cytokine release was assessed using an immunoassay. Based on preliminary experiments, a MOI of 50 : 1 of bacteria to A549 cells appeared to be optimal for prolonged stimulation of cells. As expected, stimulation of A549 cells with *P. aeruginosa* caused a release of large amounts of cytokines IL-1β, IL-6, TNF-α, and GM-CSF (Fig. 6C) indicating the induction of potent inflammatory responses that corroborates previous findings by other groups. Integrin linked kinase gene silencing caused a significant decrease in the cytokine release although the transfection reagent alone also decreased the cytokine levels (Fig. 6C). However, in all cases, the effect of siRNA was significantly stronger compared to the transfection reagent (Fig. 6C). Although the effect of ILK gene silencing accounted for ~30–60% decrease in cytokine levels, this treatment did not completely abrogate the cytokine release. These data suggest that

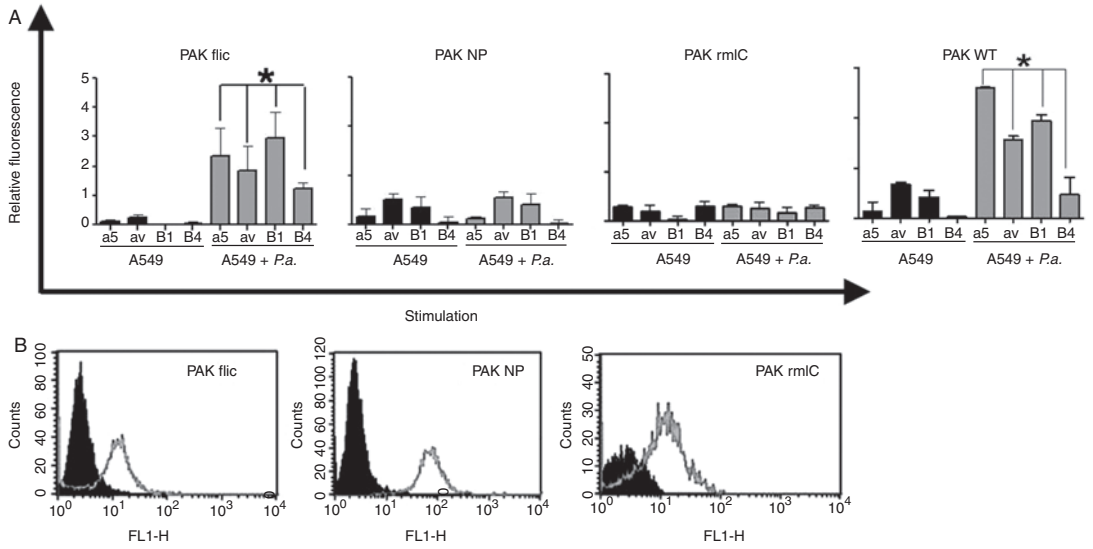

Fig. 4. Pili- or LPS-deficient *P. aeruginosa* mutants are unable to induce up-regulation of integrins. (A) Adherent A549 cells were stimulated with indicated mutant strains of *P. aeruginosa* lacking major virulence factors (flagella, pili, or the outer core oligosaccharide of LPS), or with wild-type strain for 4 h, immunostained for α5, αv, β1, and β4 integrins, and subjected to flow cytometry analysis. The results are expressed as relative fluorescence. (B) Adherent A549 cells were infected with indicated mutant strains, immunostained for ICAM-1 and subjected to flow cytometry analysis. Original histograms show ICAM-1 expression (one representative experiment) in adherent unstimulated A549 cells (filled histograms) and following 4-h stimulation with the indicated mutant strains (empty histograms). $n = 4$ (*fliC*), $n = 3$ (wild-type), $^*P < 0.05$, compared to unstimulated cells; $n = 2$ (NP and *rmlC*).

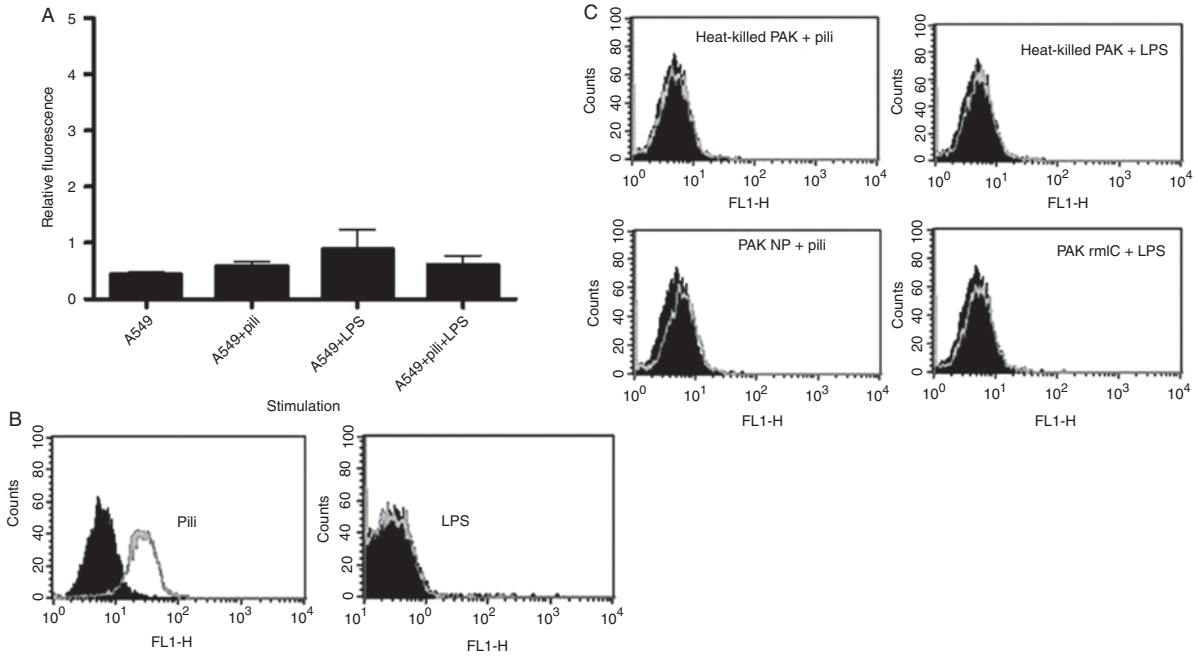

Fig. 5. Isolated pili and LPS, either alone or combined with mutant or heat-killed *P. aeruginosa*, have no effect on integrin expression. (A) Adherent A549 cells were stimulated with pili, LPS, or a combination of both at a concentration of 10 μg/ml for 4 h, then immunostained for β1 integrin and subjected to flow cytometry analysis. The results are expressed as relative fluorescence. (B) Adherent A549 cells were stimulated with pili or LPS at 10 μg/ml, immunostained for ICAM-1 and subjected to flow cytometry analysis. Original histograms show ICAM-1 expression in adherent unstimulated A549 cells (filled histograms) and following 4-h stimulation with indicated bacterial products (empty histograms; one representative experiment). (C) Adherent A549 cells were treated with a combination of 10 μg/ml of pili or LPS and either heat-killed *P. aeruginosa* or the indicated live mutant strain at a MOI of 100 : 1. After stimulation, immunostaining was performed for β1 integrin, followed by flow cytometry analysis. Data are presented as original histograms showing integrin expression in adherent unstimulated A549 cells (filled histograms) and following 4-h stimulation (empty histograms). One representative experiment out of two independent experiments is shown.

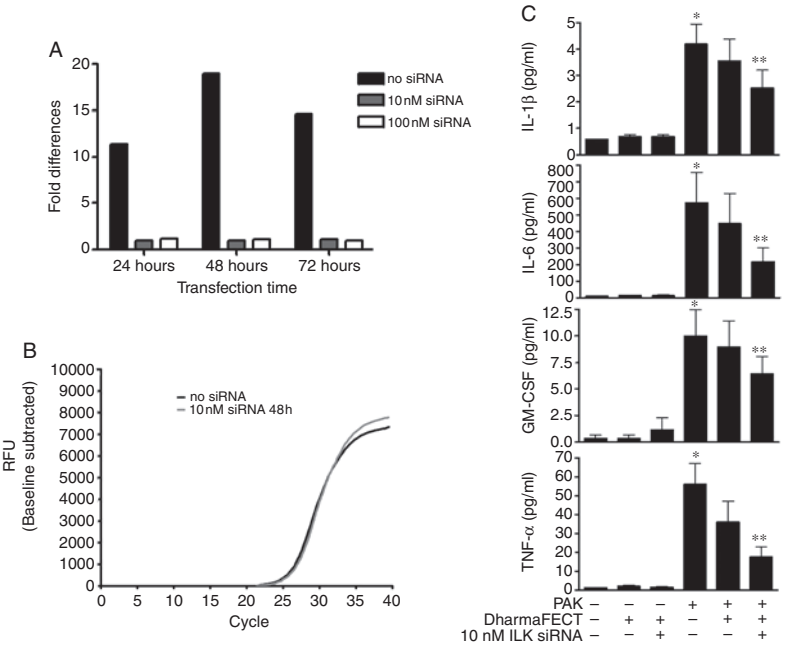

Fig. 6. Integrin linked kinase gene silencing leads to a decreased release of pro-inflammatory cytokines by *P. aeruginosa* infected A549 cells. (A) Single transfection of A549 cells with ILK siRNA at both 10 nM (gray bar) and 100 nM (white bar) resulted in great reduction of ILK mRNA expression. $\Delta\Delta C_T$ values, or 'Fold differences' in ILK mRNA compared to control non-transfected cells ranged from 11–19-fold. (B) Representative cycle curves confirm the specificity of ILK directed siRNA. The specific mRNA expression of a non-targeted gene Jun does not differ significantly in samples transfected with and without ILK siRNA (10 nM). (C) Stimulation of A549 cells with *P. aeruginosa* resulted in significant release of IL-1β, IL-6, GM-CSF and TNF-α compared to cells not stimulated with bacteria (*$P < 0.05$). Pre-treatment with 10 nM ILK siRNA for 48 h prior to bacterial stimulation caused decreased release of all cytokines compared to the control treatment with DharmaFECT (**$P < 0.05$). Three independent experiments.

integrin-mediated signaling can be, at least partially, responsible for inflammatory responses of lung epithelial cells to *P. aeruginosa* infection.

Discussion

The hallmark of *P. aeruginosa* caused disease is the severe inflammatory response of an infected tissue. The pathogen is capable of producing a wide range of virulence factors, including flagella, pili, LPS, alginate, pyocyanin, type III secretion system, and several potent proteases (reviewed by Lau *et al.*[25]). During *P. aeruginosa* pulmonary infection, host–pathogen interactions are mediated by a variety of cellular receptors expressed by epithelial cells, including members of the Toll-like receptor (TLR) family, the membrane glycosphingolipid asialoGM1, and others.[22] Such interactions lead to prominent inflammatory responses characterized by the activation of transcription factors (*i.e.* NF-κB and AP-1), resulting in release of pro-inflammatory mediators, recruitment of activated neutrophils, and severe tissue damage eventually causing lung failure.[26]

Some *in vitro* studies have indicated that integrins α5β1 and αvβ5 as well as their ligands fibronectin and vitronectin can be involved in adhesion of *P. aeruginosa* to pulmonary epithelial cells.[27–29] In this study, we addressed the question of whether integrin expression may be altered by interaction with these bacteria. Indeed, we observed that infection of A549 lung adenocarcinoma cells with *P. aeruginosa* led to a rapid up-regulation of integrin subunits α5, αv, β1, and β4, at both mRNA and protein levels. Interestingly, the effect of bacteria on integrins was clearly dependent on the basal level of their expression. In this study, we have used A549 at higher passage numbers (75–85) that expressed low levels of all integrin subunits studied. However, at early passage numbers (10–20), the basal surface protein expression of the same integrin subunits was much higher. No further increase occurred upon *P. aeruginosa* infection although a 1.7-fold increase in β1 integrin mRNA expression was detected by real-time PCR analysis (S. Gravelle, unpublished observations). Precise mechanisms underlying the changes in integrin expression upon prolonged culture of this cell line are unknown, although such findings are not unexpected considering general genetic instability of cancer cells. Indeed, A549 cells are known to carry a mutation at the 12th codon of K-ras proto-oncogene[30] that causes constitutive activation of ras that, in turn, is involved in the activation of a number of signaling molecules (*i.e.* MAP kinase cascade).

To determine which bacterial factors were responsible for integrin up-regulation during *P. aeruginosa*

infection, we used cytochalasin B to inhibit the capability of A549 cells to internalize bacteria, *P. aeruginosa* mutants lacking specific virulence factors, heat-killed wild-type bacteria, as well as isolated pili, LPS, and flagella of *P. aeruginosa*. It is known that pili, LPS, and flagella are critical virulence factors of *P. aeruginosa* responsible for both epithelial cell invasion and induction of pro-inflammatory signaling.[19,20,31] Our experiments demonstrated that up-regulation of integrins was dependent on the internalization of live bacteria, as well as on the presence of bacteria-associated pili and LPS. Interestingly, isolated pili and LPS did not substitute for the effect of live piliated, Lipopolysaccharide-expressing *P. aeruginosa* even when combined with heat-killed bacteria, or with live mutants lacking pili or LPS, indicating that these structures were essential, but not sufficient, for the observed effect. Nevertheless, pili retained functional capabilities to induce pro-inflammatory cellular responses (*i.e.* up-regulation of ICAM-1 expression). In contrast, LPS at a concentration of 10 μg/ml did not cause ICAM-1 up-regulation in A549 cells. It has been estimated that 1 μg LPS corresponds to 10^9 *P. aeruginosa* bacterial cells.[32] Considering that we have stimulated 10^6 A549 cells with 10^8 *P. aeruginosa* in a 1-ml volume, the cells would be exposed to 0.1 μg/ml of LPS which is 100-times lower than the dose of purified LPS. Therefore, the lack of A549 cell response to this large LPS dose could be due to the fact that A549 cells do not express TLR4 on the surface (Guillot *et al.*[33] and our unpublished observations), rather than the dose was insufficient. In preliminary experiments, we tested lower doses of LPS (0.1 μg/ml and 1 μg/ml) and those did not induce ICAM-1 expression in A549 cells either (unpublished observations).

Our findings suggest that signaling pathways mediating the effect of bacteria on integrin expression can be initiated as a result of direct interactions of live piliated, Lipopolysaccharide-expressing *P. aeruginosa* with epithelial cell plasma membrane. Studies by others have also demonstrated that adhesion of *P. aeruginosa* to A549 cells mediated by pili and LPS was essential for the expression of genes involved in cellular responses to bacteria, such as the transcription factor interferon regulatory factor 1.[32] Both pili and LPS have been shown to be capable of binding to the glycosphingolipid asialoGM1.[19,21] Internalization of non-piliated strains of *P. aeruginosa* by epithelial cells as well as resulting NF-κB activation are greatly decreased.[15,19] In our experiments, internalization of heat-killed *P. aeruginosa* by epithelial cells could also be significantly impaired because pili become destroyed during the heat-killing process.[34]

Undoubtedly, interactions of live *P. aeruginosa* possessing intact pili and LPS with epithelial cells involve complex cellular receptor networks. It is known that TLRs form an integrated network among themselves and other receptors, *i.e.* asialoGM1, such as TLR2–TLR4, TLR2–TLR5, TLR2–asialoGM1, *etc.*[35] We hypothesize that transcriptional up-regulation of α5, αv, β1, and β4 integrin subunits in A549 cells by *P. aeruginosa* infection is mediated by clusters of several receptors (*i.e.* TLRs and asialoGM1), that are organized along with lipid rafts on cellular plasma membrane. It is interesting that this process is independent of flagella which are an important virulence factor of *P. aeruginosa* capable of inducing potent pro-inflammatory responses via activation of TLR5.[22,36]

Our findings that siRNA induced ILK gene silencing leads to down-regulation of cytokine release corroborate previous observations that the transcription factors NF-κB and AP-1 regulating pro-inflammatory molecule gene expression are among downstream targets of ILK.[24] However, to the best of our knowledge, this is the first evidence of the involvement of ILK in inflammatory responses induced by *P. aeruginosa*. The role of ILK in bacterial infections is poorly understood although this molecule was shown to be important for the invasion of epithelial cells by *Streptococcus* pyogenes.[14] It remains to be established whether ILK involvement in cellular responses to *P. aeruginosa* is unique to the A549 cancer cell line, or represents a more general mechanism. Considering that integrin-mediated signaling was found to be essential in inflammatory cellular responses to some pathogenic micro-organisms, such as *Bordetella pertussis* and *Yersinia pseudotuberculosis*,[37,38] the involvement of ILK in antibacterial defense may represent a general biological phenomenon. The significance of ILK involvement in cellular responses to *P. aeruginosa* infection warrants further investigation.

Activation of NF-κB triggered by ILK may represent a critical pathway initiated by *P. aeruginosa* infection. Indeed, our data indicate that ILK gene silencing inhibits release of cytokines IL-1β, IL-6, TNF-α, and GM-CSF that are known to be regulated by NF-κB at the transcription level.[39] Hence, our findings suggest that integrin-mediated signaling is involved in pro-inflammatory cytokine production induced by *P. aeruginosa* infection. This idea is supported by our previous observations that integrins provide co-stimulatory signals towards inflammatory responses of bronchial epithelial cells stimulated with TNF.[40]

It has been previously demonstrated that some other microbial pathogens (i.e. *Streptococcus pyogenes*, *Pneumocystis carinii*, and *Helicobacter pylori*) are able to induce up-regulation of epithelial integrin expression during the infectious process.[6,7,41] We have found that *P. aeruginosa* infection causes rapid increase in α5, αv, β1, and β4 integrin subunits in A549 cells and, although precise mechanisms behind this effect require further studies, it appears that internalization of live piliated

LPS-expressing bacteria is essential. Our findings suggest the following hypothetical model. *Pseudomonas aeruginosa* infection can promote adhesion of A549 cells to the ECM via up-regulating integrin receptor expression. Clustering integrins upon binding their ligands is known to initiate several signaling cascades. As a central molecule in integrin-mediated signaling, ILK can be recruited to the cytoplasmic domain of β-subunit and, upon its activation, regulates downstream pathways including ones leading to an increased pro-inflammatory cytokine production. Although further studies are warranted towards understanding the precise role of integrin receptors in prominent inflammation caused by *P. aeruginosa*, the discovery of the requirement of bacterial internalization suggests a possibility of using specific integrin inhibitors for therapy of pulmonary inflammatory conditions caused by pathogenic micro-organisms.

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