**Pseudomonas aeruginosa** accentuates epithelial to mesenchymal transition in the airway

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Abstract

Epithelial to mesenchymal transition has been implicated in the dysregulated epithelial wound repair that contributes to obliterative bronchiolitis after lung transplant. Acquisition of *Pseudomonas aeruginosa* in the transplanted airway has been shown to be a risk factor for the development of obliterative bronchiolitis. We investigated the potential of *Pseudomonas aeruginosa* to drive epithelial to mesenchymal transition in primary bronchial epithelial cells isolated from lung transplant recipients. Changes in the expression of epithelial and mesenchymal markers was assessed in cells challenged with clinical isolates of *Pseudomonas aeruginosa*, or co-cultured with *Pseudomonas aeruginosa* activated monocytic cells (THP-1) in the presence or absence of TGF-β1. *Pseudomonas aeruginosa* did not drive or accentuate TGF-β1 driven epithelial to mesenchymal transition directly. Co-culturing *Pseudomonas aeruginosa* activated THP-1 cells with primary bronchial epithelial cells did not drive epithelial to mesenchymal transition. However, co-culturing *Pseudomonas aeruginosa* activated THP-1 cells with primary bronchial epithelial cells significantly accentuated TGF-β1 driven epithelial to mesenchymal transition. *Pseudomonas aeruginosa*, via the activation of monocytic cells, can accentuate TGF-β1 driven epithelial to mesenchymal transition. These *in vitro* observations may help explain the *in vivo* clinical observation of a link between acquisition of *Pseudomonas aeruginosa* and an increased risk of developing obliterative bronchiolitis.

Key words
Bronchiolitis Obliterans Syndrome, Lung Transplantation, Macrophages, Obliterative Bronchiolitis, TGF-β1.
Introduction

Over the last 25 years lung transplantation has evolved from an experimental intervention to an accepted therapeutic option for selected patients with end stage lung diseases. To date approximately 28,000 lung transplants have been reported to the international registry and although early outcomes have significantly improved, long term survival remains disappointing, limited to a median of approximately 5 years. The major cause of late allograft loss is due to the development of bronchiolitis obliterans syndrome (BOS)[1].

The pathological lesion of BOS is obliterative bronchiolitis (OB) which is characterised by inflammation and fibrosis in small and medium sized airways leading to airflow obstruction[2]. The pathogenesis of the disease remains unclear, but it is hypothesised that repeated epithelial injury from both alloimmune and non-alloimmune mechanisms, and particularly a failure to re-establish an intact epithelium, contribute to fibrotic repair in the airway and disease progression[2-4]. Recently, several groups have suggested a potential role for epithelial to mesenchymal transition (EMT) in the pathogenesis of OB [5-8]. EMT is a process by which an epithelial cell alters both its phenotype and function to that of a mesenchymal cell including acquiring the ability to produce matrix metalloproteinases (MMPs) and deposit extra cellular matrix (ECM) [9].

Transforming growth factor-β1 (TGF-β1) is regarded as the ‘master switch’ for fibrosis in several organs including the lung [6, 10-14] and has been shown to be elevated in the bronchoalveolar lavage (BAL) of patients with BOS [15-17]. TGF-β1 is a pleiotropic molecule and its ability to drive EMT is dependent on both amount of activated growth factor
and the microenvironment in which it acts. There is established evidence that acute inflammatory cytokines are elevated in BAL of patients with BOS including IL-8, TNFα and IL-1β [18, 19]. Our group has recently shown that TNFα and IL-1β accentuate TGF-β1 driven EMT and causes dysregulated wound repair of injured epithelium suggesting that a pro-inflammatory microenvironment may play a role in airway remodelling [5].

The cause of the insult responsible for injuring the epithelium is likely to be multifactorial including oxidative stress[20], gastroesophageal reflux disease (GERD)[21], and infections with viruses or bacteria [22-24]. Recurrent bacterial infections are common in the transplanted lung and may contribute to both airway epithelial injury and repeated activation of the innate immune system. We have previously shown evidence that acquisition of *Pseudomonas aeruginosa* in the post-transplant lung is associated with an increased risk of BOS[22]. However, how low grade infection with *Pseudomonas aeruginosa* leads to the airway remodelling characteristic of OB remains to be determined.

Based on these observations we hypothesised that low grade infection with *Pseudomonas aeruginosa* in the transplanted lung may contribute to the airway remodelling characteristic of OB by driving or accentuating EMT.
Materials and Methods

This study was performed in accordance with approval from the Local Research Ethics Committee and informed written consent from all study patients.

Cell Culture

Primary bronchial epithelial cells (PBEC) from stable lung transplant recipients were isolated by airway brushing at bronchoscopy as previously described [25]. Cells were cultured in Small Airway Growth Media (Lonza) on collagen coated flasks. A549 cells were maintained in DMEM (Sigma). THP-1 cells (0.5 x 10^6/cells/ml) and alveolar macrophages isolated from the BAL of stable lung transplant recipients were cultured in RPMI-1640 Media (Sigma).

Co-culture experiments

THP-1 cells (1 x 10^6/ml) activated with *Pseudomonas aeruginosa* lysate (12.5µl/ml) were added to PBEC ± TGF-β1 (10ng/ml) for 72 hours and EMT assessed (Figure 5). THP-1 cells (1 x 10^6/ml) were activated with *Pseudomonas aeruginosa* lysate (12.5µl/ml) for 6 hours and then either the THP-1 cells themselves or the conditioned media from THP-1 cells was added to PBEC cells ± TGF-β1 (10ng/ml) for 72 hours and EMT assessed (Figure 6-8).

*Pseudomonas aeruginosa* whole cell lysate preparation
*Pseudomonas aeruginosa* whole cell lysates were prepared from a reference strain (NCTC10662) and nine clinical isolates from our local repository of post-transplant patients (A-I). Strains were grown overnight on 1% horse blood agar plates, harvested into PBS and standardised to 0.2 at an optical density of 600nm. Bacterial suspensions were disrupted (using a Branson Digital Sonifier at an amplitude of 10% for 3 minutes on ice) and incubated with deoxyribonuclease II (200μg/ml) at 37°C for one hour. Lysates were then treated with Proteinase K (2mg/ml) at 60°C for 2 hours, boiled for 20 minutes (inactivating Proteinase K) and stored at -80°C prior to use.

**Flow Cytometry**

Following treatment, cells were pelleted and resuspended in 300μl Phenol Red negative RPMI media (Invitrogen). Cells were incubated at 37°C for 30 minutes and 30μl of propidium iodide (1mg/ml) added prior to assessment of cell viability.

**ELISA**

Cytokine levels in supernatants were measured by sandwich ELISA performed with commercially available matched antibody pairs. The following cytokines were measured: IL-8, IL-1β and TNFα (R&D Systems, UK).

**Western Blotting**

Protein concentrations were determined using the BCA protein assay kit (Perbio).
Total cell lysates (10μg) were separated on 4-12% bis-Tris gels (Invitrogen) and electrophoretically blotted onto HyBond-P Polyvinylidene difluoride (Amersham). Membranes were incubated with primary antibodies and detected with HRP-labelled IgG conjugates (Abcam). Antibody complexes were visualised using SuperSignal West Pico chemiluminescent kit (Perbio). Results are normalised to β-tubulin as appropriate.

**TCA Protein Precipitation**

Trichloroacetic acid (TCA - 100% w/v) was added to culture media at a 1:4 ratio and incubated at 4°C for 10 minutes. The protein precipitate was pelleted by centrifuging at 14000xg for 5 minutes. The protein pellet was washed twice in 200μl of cold acetone and dried by heating to 95°C for 10 minutes. The pellet was resuspended and separated under denaturing conditions by SDS-PAGE. Membranes were incubated with primary antibodies and detected as previously described.

**Immunoflourescence**

Cells fixed in 4% paraformaldehyde were incubated with primary antibodies and detected using appropriate fluorochrome-linked secondary antibodies. DAPI was used as a nuclear counterstain. Images acquired using a LSM 510 laser scanning confocal microscope (x63 magnification).

**Statistical Analysis**
The response of cells from each subject to a range of treatments were assessed and compared to untreated control cells. Changes in protein expression (relative band density) or protein secretion were quantified relative to untreated controls and expressed as mean ± standard error of the mean (SEM).

The significance of differences between groups was assessed by a one way analysis of variance (ANOVA) using SPSS 14.0. Differences with a p-value of <0.05 were considered statistically significant.
Results

Characterisation of patient study group

Cultures of PBEC were obtained from six (2M/4F) lung transplant recipients with stable allograft function between 3 and 48 months post transplant. No subjects showed any evidence of acute rejection (grade A2 or above by ISHLT classification), or infection and were BOS stage 0 (>90% baseline FEV1) at the time of sampling.

Investigating the effect of clinical isolates of *Pseudomonas aeruginosa* on primary bronchial epithelial cells from lung transplant patients

PBEC stimulated with *Pseudomonas aeruginosa* lysate show no significant reduction in cell viability compared to untreated controls (p>0.05, n=3) (Figure 1Ai) and demonstrated a dose dependant increase in the release of IL-8 up to 50μl/ml (p<0.05, n=3) after which no significant increase was observed (p>0.05, n=3) (Figure 1Aii).

Stimulating PBEC with *Pseudomonas aeruginosa* lysate or *Escherichia coli* (055) lipopolysaccharide (LPS - Sigma) had no significant effect on epithelial phenotype (p>0.05, n=6). In contrast treatment with TGF-β1 (10ng/ml) significantly reduced the expression of the epithelial marker E-cadherin, increased the expression of the mesenchymal markers vimentin and fibronectin and increased the deposition of fibronectin (p<0.05, n=6) consistent with the induction of EMT. Stimulation with *Pseudomonas aeruginosa* lysate in the presence of TGF-β1 had no significant effect on protein expression or fibronectin deposition compared to TGF-β1 alone (p>0.05, n=6) (Figure 1B). These results suggest that although
Pseudomonas aeruginosa lysate provide pathogen associated molecular patterns (PAMP) to PBEC, as seen by the increased IL-8 secretion, it does not drive EMT by acting on epithelial cells directly alone or in synergy with TGF-β1.

**Cytokine release from THP-1 cells and alveolar macrophages induced by clinical isolates of Pseudomonas aeruginosa**

As *Pseudomonas aeruginosa* does not directly affect EMT we proceeded to determine if it could act indirectly via activation of immune cells and the generation of a pro-inflammatory microenvironment. Treatment of the THP-1 monocytic cell line with 12.5µl/ml of *Pseudomonas aeruginosa* lysate induced a significant increase in the release of IL-8 (Figure 2B), IL-1β (Figure 2C) and TNFα (Figure 2D) (p<0.05, n=3) while maintaining cell viability above 80%. Concentrations above 12.5µl/ml of *Pseudomonas aeruginosa* lysate induced little additional cytokine release and compromised cell viability. We therefore used a *Pseudomonas aeruginosa* lysate concentration of 12.5µl/ml in future experiments.

We compared the relative pro-inflammatory cytokine release from THP-1 cells in response to a laboratory reference strain of *Pseudomonas aeruginosa* (NCTC10662) and nine clinical transplant isolates of *Pseudomonas aeruginosa* (A-I). Stimulation with clinical isolates of *Pseudomonas aeruginosa* induced a significantly greater secretion of IL-8 (68815pg/ml ± 4498 vs 45194pg/ml ± 2345, Figure 3A), IL-1β (1294pg/ml ± 83 vs 780pg/ml ± 20, Figure 3B) and TNFα (2739pg/ml ± 280 vs 1202 pg/ml ± 20, Figure 3C) than a laboratory reference strain. Furthermore, pre-treatment of THP-1 cells with an anti-CD14 antibody (400ng/ml) blocked LPS-induced TNFα release (p<0.01, n=3), returning levels to those comparable to control (p=0.69, n=3) (Figure 3D). However, the anti-CD14 antibody (400ng/ml) was able to inhibit only 39 ± 3.6% (p<0.01, n=3) of TNFα release from THP-1 cells stimulated with
Pseudomonas aeruginosa lysate, suggesting Pseudomonas aeruginosa is acting on multiple toll-like receptors (TLR) and providing us with a more representative model to that seen during infection in vivo. Hereafter, strain G was used as a representative Pseudomonas aeruginosa clinical isolate.

To confirm the suitability of the THP-1 monocytic cell line as a surrogate macrophage model in our co-culture system it was important to compare the secretion of cytokines in response to activation with Pseudomonas aeruginosa between THP-1 cells and alveolar macrophages isolated from the BAL of stable lung transplant recipients (Figure 4). IL-8 secretion was slightly higher in THP-1 cells compared to alveolar macrophages (116 ± 4 ng/ml vs 91 ± 14 ng/ml) (p=0.02, n=3). However we found no significant difference in the levels of TNFα (3898 ± 31 pg/ml vs 3283 ± 372 pg/ml) and IL-1β (1309 ± 118 pg/ml vs 1425 ± 48 pg/ml) secreted from THP-1 cells and alveolar macrophages respectively (p<0.05, n=3). We therefore proceeded to use THP-1 cells in all future experiments.

THP-1 cells stimulated with clinical isolates of Pseudomonas aeruginosa accentuate TGF-β1 driven EMT

Pseudomonas aeruginosa activated THP-1 cells were co-cultured with epithelial cells to investigate the indirect effects of Pseudomonas aeruginosa on EMT. Untreated cells maintain the classical ‘cobblestone’ appearance of epithelial cells, express high levels of E-cadherin and express little to no vimentin or fibronectin. Co-culturing cells with untreated THP-1 cells or Pseudomonas aeruginosa activated THP-1 cells had no effect on morphology or EMT marker expression compared to control (p>0.05, n=6). TGF-β1 treatment promoted a loss in cell to cell contact and elongation of cells, downregulation of E-cadherin expression and an increase in vimentin and fibronectin expression (p<0.05, n=6). Co-culturing cells
with untreated THP-1 cells in the presence of TGF-β1 had no additional effect on EMT marker expression compared to TGF-β1 alone (p>0.05, n=6). However, co-culturing cells with *Pseudomonas aeruginosa* activated THP-1 cells in the presence of TGF-β1 induced a more dramatic change in cell phenotype and significantly accentuates the change in EMT marker expression seen with TGF-β1 alone (p<0.05, n=6) (Figure 5). The results suggest that *Pseudomonas aeruginosa* can accentuate EMT in an environment with elevated TGF-β1, such as that seen in the transplanted lung, but is unable to drive EMT in the absence of TGF-β1.

**Soluble products released from *Pseudomonas aeruginosa* activated THP-1 cells are responsible for accentuation of TGF-β1 driven EMT**

To further understand how *Pseudomonas aeruginosa* activated THP-1 cells accentuate TGF-β1 driven EMT, the relative contribution of membrane bound factors expressed on the surface of THP-1 cells and secreted factors from THP-1 cells on TGF-β1 driven EMT were investigated (Figure 6). Co-culturing PBEC with untreated THP-1 cells or *Pseudomonas aeruginosa* activated THP-1 cells had no effect on EMT marker expression compared to untreated control cells (p>0.05, n=6). Similarly, media from untreated THP-1 cells or media from *Pseudomonas aeruginosa* activated THP-1 cells had no effect on protein expression compared to control (p>0.05, n=6). The results suggest that the secretory products or membrane bound factors from untreated or *Pseudomonas aeruginosa* activated THP-1 cells do not drive EMT in the absence of TGF-β1.

In contrast, treatment with TGF-β1 downregulated the expression of the epithelial markers cytokeratin-19 and E-cadherin and increased the expression of vimentin and fibronectin (p<0.05, n=6). Addition of untreated THP-1 cells or *Pseudomonas aeruginosa* activated
THP-1 cells had no significant effect on EMT marker expression compared to TGF-β1 alone (p>0.05, n=6).

However, treatment with media from *Pseudomonas aeruginosa* activated THP-1 cells in the presence of TGF-β1 significantly accentuates the change in EMT marker expression seen with TGF-β1 alone (p<0.05, n=6). The results suggest that secretory products released from *Pseudomonas aeruginosa* activated THP-1 cells, not membrane bound factors on the surface of THP-1 cells, are responsible for accentuating TGF-β1 driven EMT in our co-culture model (Figures 7 & 8).

This observation was further confirmed by co-culturing epithelial cells with *Pseudomonas aeruginosa* activated THP-1 cells that were separated from the epithelial cells in a cell culture insert (Corning). This technique allows only the secreted products released from THP-1 cells to impact on the epithelial cells (Supplementary Figure E1). The results confirmed that the secreted products released from *Pseudomonas aeruginosa* activated THP-1 cells can accentuate TGF-β1 driven EMT (Supplementary Figure E2).
Discussion

Viral and bacterial infections, which are common in the transplanted lung, can cause injury to the airway epithelium and activation of the pulmonary innate immune system. Such insults are believed to be important in driving crosstalk with alloimmune mechanisms and contributing to aberrant epithelial repair in the development of BOS. In particular, our group and others have shown that acquisition of the gram negative opportunistic bacteria *Pseudomonas aeruginosa* in the transplanted airway is associated with an increased risk of developing BOS [22-24]. In this study we explore the mechanism by which this organism might drive aberrant epithelial repair in the airway. Our data have demonstrated that clinical isolates of *Pseudomonas aeruginosa* cultured from the transplanted lung can, via the action of secretory products from activated immune cells, accentuate TGF-β1 driven EMT in airway epithelial cells isolated from lung transplant recipients. These *in vitro* findings may help explain the clinical observation that acquisition of *Pseudomonas aeruginosa* in the transplanted airway is associated with an increased risk of developing BOS.

It is recognised that repeated sub-clinical injury and persistent inflammation in airway epithelium coinciding with defective regeneration will favour excessive fibroproliferation and obliteration of small and medium sized airways in OB [26]. In clinical practice, BOS is often first diagnosed (or progresses more rapidly) following a bacterial or viral infection and these sources of acute inflammation may be very important in accentuating the processes already driving chronic lung allograft dysfunction. Our group has previously shown that TNFα and IL-1β can cause dysregulated wound repair of injured epithelium by accentuating TGF-β1 driven EMT demonstrating the link between acute inflammation and epithelial remodelling
Previous data from Vos and colleagues, suggests that even transient infection with 
*Pseudomonas aeruginosa* in lung transplant recipients is associated with a significant 
increase in inflammation [27]. We therefore hypothesised that acquisition of *Pseudomonas 
aeruginosa* in the transplanted lung might produce sufficient inflammatory stimulus to 
accentuate dysregulated repair via EMT in the airway epithelium.

Our data shows that *Pseudomonas aeruginosa* does not drive EMT directly but can 
accentuate TGF-β1 driven EMT indirectly via activation of innate immune cells. Airway 
epithelial cells are able to respond to the presence of micro-organisms by expression of TLRs 
on their surface yet these cells commonly show a hyporesponsiveness to TLR ligands [28], 
whereas pulmonary innate immune cells show a much more vigorous inflammatory response 
to TLR ligands. When activated, lung macrophages produce a multitude of growth factors 
and cytokines including proteins that are present in elevated levels in the BAL of patients 
with BOS [18, 19] such as IL-8, TNFα and IL-1β [29]. There is growing interest in the role 
of the macrophage as an effector cell in allograft injury and fibrosis. In the murine 
heterotopic tracheal transplant model depletion of recipient macrophages significantly 
abrogates obliteration of the transplanted airway [30]. In this study we used the THP-1 
monocytic cell line as a model for lung innate immune cells as the use of human 
macrophages isolated from either healthy controls or stable lung transplant patients was not 
feasible due to the large number of cells required for the co-culture work. Importantly, as 
part of the validation of our model, we showed that undifferentiated THP-1 cells show a 
similar inflammatory response to *Pseudomonas aeruginosa* as alveolar macrophages isolated 
from the BAL of stable lung transplant recipients (Figure 4). It is also well established that 
THP-1 cells can be differentiated using phorbol myristate acetate into adherent macrophage
like cells [31]. However, we required the THP-1 cells to remain in suspension to perform our co-culture experiments and therefore used undifferentiated cells.

Another important aspect of our model was the use of clinical isolates of *Pseudomonas aeruginosa*. The whole bacterial lysates used in this study contain multiple PAMP that signal via different TLRs and provides a more clinically relevant stimulus. This is further demonstrated by the observation that clinical isolates of *Pseudomonas aeruginosa* induce a greater inflammatory response than the laboratory reference strain (NCTC10662).

The observations noted above suggest that targeting persistence of *Pseudomonas aeruginosa* or its inflammatory consequences by limiting innate immune activation in the transplant lung may be a potential therapy to limit dysregulated epithelial repair. Our group and others have shown that azithromycin treatment can reverse the decline in lung function in some patients with BOS [32-35]. Azithromycin does not have any significant anti-*pseudomonal* activity but has been shown to exhibit extensive inhibition of the quorum-sensing systems, diminished virulence factor production and impairment of the oxidative stress response [36-38]. However recent data suggests that although azithromycin exhibits activity against *Pseudomonas aeruginosa* biofilms, resistant mutants are readily selected and tailored anti-*pseudomonal* therapies may be required to eradicate infection in patients receiving azithromycin treatment [39]. This suggests that azithromycin is more likely to be exerting its benefits *via* its anti-inflammatory actions than by a direct action on *Pseudomonas aeruginosa* colonisation.

Finally, it remains unclear in the literature if *Pseudomonas aeruginosa* can be a primary driver of airway remodelling in the transplanted lung or is in fact accelerating an already...
established process. The data we present in this paper suggests that an elevated background level of TGF-β1 is required for *Pseudomonas aeruginosa* to have its accentuating effects on EMT. This supports the hypothesis that *Pseudomonas aeruginosa* may be having an accentuating effect on an already smouldering alloimmune injury to the airway epithelium.

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References


Figure Legends

Figure 1

Effect of *Pseudomonas aeruginosa* on primary bronchial epithelial cells

A) Stimulation of primary bronchial epithelial cells (PBEC) with *Pseudomonas aeruginosa* lysate (10, 50 or 100 μl/ml) had no affect on cell viability (i) (p>0.05, n=3) and induced a dose dependant increase in the secretion of IL-8 with a maximal response at 50μl/ml (ii).

*p<0.05, n=3.

B) Stimulation of PBEC with *Pseudomonas aeruginosa* lysate (50μl/ml) or E-coli derived lipopolysaccharide (LPS) (10μg/ml) had no affect on protein expression or fibronectin deposition compared to control (p>0.05, n=4). Treatment with TGF-β1 (10ng/ml) downregulated E-cadherin expression, increased fibronectin and vimentin expression and increased fibronectin deposition compared to control cells (p<0.05, n=4). Stimulation with *Pseudomonas aeruginosa* lysate in the presence of TGF-β1 had no additional effect on protein expression or fibronectin deposition compared to TGF-β1 alone (p>0.05, n=4). β-tubulin is shown as a loading control.

Figure 2

Cytokine release by THP-1 cells stimulated with *Pseudomonas aeruginosa*

THP-1 cells (1 x 10⁶/ml) were stimulated with *Pseudomonas aeruginosa* lysate (at 0, 12.5, 25, 50, 100, 200 or 400μl/ml) for 24 hours (n=3). Cell viability was assessed by PI staining and the production of TNFα, IL-8 and IL-1β analysed by ELISA. A) Results showed a dose
dependant decrease in cell viability from 96% in control to 72% at a concentration of 400μl/ml of *Pseudomonas aeruginosa* lysate. B) *Pseudomonas aeruginosa* lysate induced a dose-dependent increase in the release of IL-8. C + D) *Pseudomonas aeruginosa* lysate induced a dose-dependent increase in IL-1β and TNFα release up to a concentration of 50μl/ml after which no further increase was observed.

**Figure 3**

*Cytokine production by THP-1 cells stimulated with clinical isolates of Pseudomonas aeruginosa taken from patients with BOS*

THP-1 cells (1x10^6/ml) were stimulated with clinical isolates of *Pseudomonas aeruginosa* lysates (A-I) and a lab reference strain of *Pseudomonas aeruginosa* lysate (NCTC10662) (all 12.5μl/ml) for 24 hours (n=3) and the producing of TNFα, IL-8 and IL-1β analysed by ELISA. All clinical isolates of *Pseudomonas aeruginosa* induced a significantly greater release of TNFα (A), IL-8 (B) and IL-1β (C) than the lab reference strain of *Pseudomonas aeruginosa* (p<0.05, n=3).

D) THP-1 cells were pre-treated with anti-CD14 antibody (at 100, 200 or 400ng/ml) and stimulated with either E-coli derived LPS (100ng/ml) or *Pseudomonas aeruginosa* lysate (12.5μl/ml) for 24 hours and the production of TNFα analysed by ELISA (n=3). Anti-CD14 antibody affects a dose-dependent blocking effect on LPS stimulated TNFα release, returning it to control levels. There was a significant reduction in the *Pseudomonas aeruginosa* induced TNFα release with the anti-CD14 antibody but even at high doses TNFα release did not return to control levels.

**Figure 4**
Comparative assessment of cytokine release from THP-1 cells and alveolar macrophages stimulated with clinical isolates of *Pseudomonas aeruginosa*

A) THP-1 cells and alveolar macrophages isolated from the bronchoalveolar lavage of stable lung transplant patients (both 1x10^6/ml) were stimulated with *Pseudomonas aeruginosa* lysate (12.5µl/ml) for 24 hours and the secretion of TNFα (i), IL-8 (ii) and IL-1β (iii) assessed by ELISA (n=3). The secretion of all markers was similar between THP-1 cells and alveolar macrophages.

B) Alveolar macrophages (1 x 10^6/ml) were stimulated with *Pseudomonas aeruginosa* lysate (at 0, 12.5, 25 or 50µl/ml) for 24 hours (n=3) and the production of TNFα analysed by ELISA. *Pseudomonas aeruginosa* lysate induced a dose-dependent increase in the release of TNFα.

**Figure 5**

**Accentuation of TGF-β1 driven epithelial to mesenchymal transition by THP-1 cells stimulated with clinical isolates of *Pseudomonas aeruginosa***

A) Treatment with TGF-β1 (10ng/ml) promotes a loss of cell to cell contact and elongation of A549 cells (ii). Co-culturing with *Pseudomonas aeruginosa* activated THP-1 cells in the presence of TGF-β1 induces a dramatic change in cell phenotype to spindle shape myofibroblast like cells (iv). *Pseudomonas aeruginosa* activated THP-1 cells had little to no effect on epithelial morphology in the absence of TGF-β1 (iii).

B) Untreated PBEC express high levels of E-cadherin and little to no vimentin or fibronectin. Co-culture PBEC with untreated THP-1 cells or *Pseudomonas aeruginosa* activated THP-1 cells has no effect on protein expression compared to control (p>0.05, n=6). Treatment with
TGF-β1 (10ng/ml) downregulated E-cadherin expression and increased vimentin and fibronectin expression compared to control (p<0.05, n=6). Co-culture PBEC with untreated THP-1 cells in the presence of TGF-β1 had no effect on protein expression compared to TGF-β1 alone (p>0.05, n=6). However, co-culture PBEC with *Pseudomonas aeruginosa* activated THP-1 cells in the presence of TGF-β1 further downregulate E-cadherin expression and increase vimentin and fibronectin expression compared to TGF-β1 alone (p<0.05, n=6).

**Figure 6**

**Investigation of the effect of secretory vs membrane bound products from THP-1 cells on EMT**

THP-1 cells (1x10⁶ cells/ml) were incubated with *Pseudomonas aeruginosa* lysate (12.5µl/ml) to activate the cells. After 6 hours the *Pseudomonas aeruginosa* activated THP-1 cells were pelleted, re-suspended in fresh media, and added to PBEC in the presence or absence of TGFβ1 (10ng/ml) allowing us to investigate the effect of membrane bound factors on the surface of THP-1 cells on EMT. The supernatant from the *Pseudomonas aeruginosa* activated THP-1 cells was also added to PBEC in the presence or absence of TGFβ1 allowing us to investigate the effect of soluble products released from THP-1 cells on EMT.

**Figure 7**

**Secretory products from THP-1 cells activated with Pseudomonas aeruginosa lysates are responsible for driving EMT**

Untreated PBEC maintained the classic ‘cobblestone’ morphology characteristic of epithelial cells, express high levels of cytokeratin 19 (i) and do not express vimentin (vii) or fibronectin.
Cells treated with TGF-β1 (10ng/ml) alone begin to lose cell-cell contact, downregulated cytokeratin-19 expression (ii) and increase vimentin (viii) and fibronectin (xiv) expression. Addition of media from untreated THP-1 cells, untreated THP-1 cells or Pseudomonas aeruginosa activated THP-1 cells had no affect on cell phenotype or protein expression (iii, ix, xv; v, xi, xvii and vi, xii, xviii respectively) compared to TGF-β1 alone. Addition of media from Pseudomonas aeruginosa activated THP-1 cells accentuated the change in cell phenotype and protein expression (iv, x, xvi) compared to TGF-β1 alone. Images were acquired on a Leica confocal microscope (×63 magnification).

**Figure 8**

Accentuation of TGF-β1 driven EMT in PBEC is mediated by the soluble products released from activated THP-1 cells

A) Untreated PBEC express little to no vimentin or fibronectin and express high levels of E-cadherin. Media from untreated THP-1 cells, media from Pseudomonas aeruginosa activated THP-1 cells, untreated THP-1 cells or Pseudomonas aeruginosa activated THP-1 cells had no effect on EMT marker expression compared to control (p>0.05, n=6). Treatment with TGF-β1 (10ng/ml) downregulated E-cadherin expression and increased vimentin and fibronectin expression compared to control (p<0.05, n=6). Addition of untreated THP-1 cells or Pseudomonas aeruginosa activated THP-1 cells had no significant effect on EMT marker expression compared to TGF-β1 alone (p>0.05, n=6). Addition of media from untreated THP-1 cells increased fibronectin expression (p<0.05, n=6) but had no significant effect on the expression of E-cadherin and vimentin compared to TGF-β1 alone (p>0.05, n=6). Addition of media from Pseudomonas aeruginosa activated THP-1 cells further
downregulate E-cadherin expression and increase vimentin and fibronectin expression compared to TGF-β1 alone (p<0.05, n=6).

B) Changes in protein expression (relative band density) were quantified and expressed as mean ± standard error of the mean (n=6). *p<0.05, n=6.
Figure 1

A) (i) Cell Viability (%)

Concentration of PA lysate / ml

- 0 µl
- 10 µl
- 50 µl
- 100 µl

A) (ii) IL-8 concentration (pg/ml)

Concentration of PA lysate / ml

- 0 µl
- 10 µl
- 50 µl
- 100 µl

B) PA lysate
LPS
TGF-β1

- - - - + +

E-Cadherin
Fibronectin
Vimentin
β-Tubulin
Fibronectin TCA

120 kDa
220 kDa
42 kDa
55 kDa
220 kDa
Figure 2
Figure 3
Figure 4
Figure 5

A) i) Control  ii) TGFβ1  iii) PA activatedTHP-1  iv) TGFβ1 + PA activatedTHP-1

B) TGF-β1  THP-1  PA lysate

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<tr>
<td>Vimentin</td>
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<td>β-Tubulin</td>
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Figure 6
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<tr>
<td>Vimentin</td>
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</tr>
<tr>
<td>Fibronectin</td>
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Figure 7
Figure 8: Analysis of protein expression using Western blotting and densitometry. A) Results for E-cadherin, Fibronectin, Vimentin, and β-Tubulin. B) Graphical representation showing relative band density for E-cadherin, Fibronectin, and Vimentin under different treatment conditions: Control, TGF-β1, TGF-β1 + THP-1, TGF-β1 + PA activated THP-1.