Transforming Growth Factor- β 1 (TGF- β 1) Driven Epithelial to Mesenchymal Transition (EMT) is Accentuated by Tumour Necrosis Factor α (TNF α) *via* Crosstalk Between the SMAD and NF- κ B Pathways

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Abstract Epithelial to mesenchymal transition (EMT) is a process by which an epithelial cell alters its phenotype to that of a mesenchymal cell and plays a critical role in embryonic development, tumour invasion and metastasis and tissue fibrosis. Transforming growth factor- β 1 (TGF- β 1) continues to be regarded as the key growth factor involved in driving EMT however recently tumour necrosis factor α (TNF α) has been demonstrated to accentuate TGF- β 1 driven EMT. In this study we investigate how various signalling pathways contribute to this accentuated effect. A549 cells were treated with TGF- β 1 (10 ng/ml), TNF α (20 ng/ml) or a combination of both for 72 h and EMT assessed. The effect of selective inhibition of the SMAD, MAPK and NF- κ B pathways on EMT was assessed. A549 cells treated with TGF- β 1 downregulate the expression of epithelial markers, increase the expression of mesenchymal markers, secrete matrix-metalloproteinases and become invasive. Significantly, TGF- β 1 driven EMT is accentuated by co-treatment with TNF α . SMAD 3 inhibition attenuated

TGF- β 1 driven EMT but has no effect on the accentuation effect of TNF α . However, inhibiting IKK β blocked both TGF- β 1 driven EMT and the accentuating action of TNF α . Inhibiting p38 and ERK signalling had no effect on EMT. TNF α accentuates TGF- β 1 driven EMT in A549 cells *via* a SMAD 2/3 independent mechanism involving the NF- κ B pathway independent of p38 and ERK 1/2 activation.

Keywords Epithelial to mesenchymal transition · Cancer · Fibrosis · Microenvironment · Transforming growth factor- β 1 · Tumour necrosis factor α

Introduction

Epithelial to mesenchymal transition (EMT) has been postulated to play an important role in cellular trans-differentiation during embryonic development, tumour invasion and metastasis and in tissue fibrosis [1]. EMT is a process by which an epithelial cell alters its phenotype to that of a mesenchymal cell in response to external stressors or specific growth factors. This process involves the down regulation of epithelial markers such as cytokeratin and the adherens proteins E-cadherin and ZO-1 and the up regulation of mesenchymal markers including vimentin, fibronectin and α -smooth muscle actin (α -SMA). Additionally cells undergoing EMT gain functional characteristics of mesenchymal cells such as acquiring invasive potential and the ability to secrete matrix metalloproteinases (MMP's) and extra cellular matrix (ECM) proteins [2–4].

Growth factors capable of regulating EMT include transforming growth factor- β 1 (TGF- β 1), epithelial growth factor, fibroblast growth factor and hepatocyte growth factor [5–8]. However, it is TGF- β 1 that continues to be

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regarded as the key growth factor involved in driving EMT and has been implicated in driving EMT in multiple organs including the lung, kidney and liver [9].

TGF- β 1 has been shown to signal primarily *via* heteromeric complexes of type II and type I serine/threonine kinase receptors which activate the mothers against decapentaplegic homolog (SMAD) signalling pathway [10–12]. However, the activity of TGF- β 1 is not exclusive to the SMAD signalling pathway and can regulate, and be regulated by, other signalling pathways. For example TGF- β 1 has been shown to activate extracellular signal-regulated kinases (ERK), p38 and c-Jun N-terminal kinases (JNK) mitogen activated protein kinases (MAPKs) [11, 13]. Therefore the downstream effects of TGF- β 1 should not be considered solely as a consequence of the SMAD signalling pathway but as a result of multiple signalling pathways acting to modulate the effects of TGF- β 1 signalling. Conversely, the activation of the MAPK signalling pathway is not exclusive to TGF- β 1 and may be activated by other extracellular stimuli including cell-matrix interactions and cytokines such as TNF α [14].

There are conflicting reports in the literature regarding the ability of TNF α to drive EMT in the absence of TGF- β 1 with some groups reporting positive findings [15, 16] and others negative findings [3, 17–19]. However, there is compelling evidence that TNF α is able to accentuate TGF- β 1 driven EMT in a range of cell types [17, 19, 20]. For example it has previously shown that TNF α can accentuate TGF- β 1 driven EMT in primary bronchial epithelial cells causing dysregulated wound repair of the injured lung epithelium [21] and that TNF α can increase metastatic potential in human colonic epithelial organoid models of colon cancer by accentuating EMT [22]. These results suggest that a pro-inflammatory microenvironment rich in TNF α may play an important role in modulating EMT in both tissue fibrosis and cancer *in vivo*. However the signalling pathways involved in this synergistic effect remain ill-defined and further research is required to identify potential therapeutic targets to block or even reverse EMT.

In this study we aim to comprehensively assess the ability of TNF α to drive or accentuate EMT in the A549 lung adenocarcinoma epithelial cell line and to investigate how various signalling pathways contribute to this accentuated effect.

Materials and Methods

Cell Culture

A549 and THP-1 cells were acquired from ATCC / LGC Promochem (Middlesex, UK). A549 were maintained in DMEM and THP-1 cells were maintained in RPMI (both

Sigma). Media was supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were grown at 37°C in a humidified 95% air / 5% CO₂ incubator.

Co-culture Experiments

THP-1 cells (1×10^6 /ml) were added to A549 cells $\pm$ TGF- β 1 (10 ng/ml) in the presence or absence of *Pseudomonas aeruginosa* whole cell lysate (12.5 μ l/ml) for 72 h and EMT assessed.

Pseudomonas aeruginosa Whole Cell Lysate Preparation

Pseudomonas aeruginosa whole cell lysate was prepared from a laboratory reference strain (NCTC10662) as previously described [23, 24]. Briefly, the strain was grown overnight on 1% horse blood agar plates, harvested into PBS and standardised to 0.2 at an optical density of 600 nm. Bacterial suspensions were disrupted (using a Branson Digital Sonifier at an amplitude of 10% for 3 min on ice) and incubated with deoxyribonuclease II (200 μ g/ml) at 37°C for 1 h. The lysate was then treated with Proteinase K (2 mg/ml) at 60°C for 2 h, boiled for 20 min (inactivating Proteinase K) and stored at -80°C prior to use.

Immunofluorescence

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 Leica TCS-SP-2UV laser scanning confocal microscope ($\times 63$ magnification).

Fig. 1 TNF α accelerates and accentuated TGF- β 1 driven EMT in A549 cells. **a** Treatment of A549 cells with TGF- β 1 (10 ng/ml) downregulated E-cadherin expression and increased fibronectin and vimentin expression compared to control cells ($p < 0.05$, $n = 4$). Alone TNF α (20 ng/ml) had no effect on EMT marker expression compared to control cells ($p > 0.05$, $n = 4$). However, co-treatment with TGF- β 1 and TNF α significantly accentuated the change in EMT marker expression compared to TGF- β 1 alone ($p < 0.05$, $n = 4$). All results are normalised to β -tubulin as a loading control. **b** A549 cells were incubated with TGF- β 1 (10 ng/ml) or TGF- β 1 (10 ng/ml) + TNF α (20 ng/ml) for 0–72 h and fibronectin expression detected by immunoblotting. TGF- β 1 treated cells show little or no fibronectin expression until 72 h post treatment. TGF- β 1 and TNF α treated cells show an increase in fibronectin expression at 24 h which was dramatically increased by 72 h. **c** Untreated cells and cells treated with TNF α alone retained the morphological appearance of epithelial cells, express a high level of cytokeratin 19 but do not express α -SMA (i, v, ix and iii, vii, xi). TGF- β 1 treated cells show a loss of cell to cell attachments, a decrease in cytokeratin 19 expression and an increase in α -SMA expression (ii, vi, x). TGF- β 1 + TNF α treated cells show a bi-polar phenotype, further decreased cytokeratin 19 expression and increased α -SMA expression (iv, viii, xii). Images acquired on a Leica confocal microscope ($\times 63$ magnification)

Western Blotting

Protein concentrations were determined using the BCA protein assay kit (Perbio).

Total cell lysates (10 µg) 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.

Gelatin Zymography

To assay for pro-MMP-2 and pro-MMP-9 secretion conditioned media from cells treated as indicated were separated on an 8% SDS-PAGE gel containing 0.1% gelatin. Following electrophoresis, gels were incubated in 2.5% (v/v) Triton X-100 for 30 min and then overnight in developing buffer (50 mM Tris-HCl, 0.2 M NaCl, 5 mM CaCl_2) at 37°C. Gels were stained with Coomassie blue stain (40% methanol, 10% acetic acid, 0.05% Coomassie blue) and destained (40% methanol, 10% acetic acid) until the desired contrast was achieved.

Invasion Assay

The invasive potential of cells was evaluated by assessing penetration of Matrigel coated filters. Briefly, cells were treated with TGF- β 1 (10 ng/ml), TNF α (20 ng/ml) or a combination of both for 72 h, 1×10^4 cells loaded in the invasion chamber and incubated for 24 h at 37°C. The number of invasive cells was determined following Diff-Quik (Dade Behring, UK) staining and scored at bright field microscopy.

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 min. The protein precipitate was pelleted by centrifuging at $14,000 \times g$ for 5 min. The protein pellet was washed twice in 200 µl of cold acetone and dried by heating to 95°C for 10 min. The pellet was resuspended and separated under denaturing conditions by SDS-PAGE. Membranes were incubated with primary antibodies and detected as previously described.

Statistical Analysis

The response of cells to a range of treatments were assessed and compared to untreated control cells. Changes in protein expression and protein secretion (relative band density) and numbers of invasive cells were quantified relative to

untreated controls and expressed as mean $\pm$ 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

Investigating the Effect of TNF α on TGF- β 1 Driven EMT in Lung Carcinoma (A549) Cells

Untreated A549 cells maintain uniform cobblestone morphology with intimate cell-cell contact, express high levels of the epithelial markers cytokeratin 19 and E-cadherin and express little or none of the mesenchymal markers vimentin, fibronectin or α -SMA. TGF- β 1 (10 ng/ml) treatment promoted a loss of cell to cell contact that was associated with a decreased expression of cytokeratin 19 and E-cadherin ($31\% \pm 6\%$) and an increased expression of α -SMA, vimentin ($57\% \pm 11\%$) and fibronectin ($135\% \pm 18\%$) ($p < 0.05$, $n = 4$). Treating the cells with TNF α (20 ng/ml) alone had no significant effect on either cell phenotype or protein expression compared to control cells ($p > 0.05$, $n = 4$). However, co-treatment of the cells with TGF- β 1 and TNF α accentuated both the change in cell phenotype, with cells adopting a bi-polar spindle like shape characteristic of fibroblasts, and the change in EMT marker expression (E-cadherin ($65\% \pm 3\%$), vimentin ($152\% \pm 37\%$), fibronectin ($466\% \pm 18\%$)) compared to cells treated with TGF- β 1 alone ($p < 0.05$, $n = 4$) (Fig. 1a, c).

To investigate the effect of TNF α on the kinetics of TGF- β 1 driven EMT, cells were treated with TGF- β 1 alone or with TGF- β 1 + TNF α for a time course of 2 to 72 h and the expression of fibronectin assessed (Fig. 1b). Cells treated with TGF- β 1 alone express little to no fibronectin until 72 h post treatment. However, cells stimulated with TGF- β 1 + TNF α begin to express fibronectin as early as 24 h and show a greatly increased expression at 72 h compared to TGF- β 1 alone. The results suggest that both the kinetics and magnitude of TGF- β 1 driven EMT can be accentuated by TNF α .

In order to confirm that cells have undergone complete EMT it is important to investigate both phenotypic and functional changes. The effect of TGF- β 1 and TNF α alone or in combination on invasive potential of cells and on the secretion of MMP's and ECM proteins was investigated (Fig. 2). The ability of A549 cells to penetrate Matrigel coated Millipore filters was assessed as a measure of cell invasiveness. A small number of non-stimulated cells spontaneously penetrated the filters (263 ± 47) but this number was increased when the cells were treated with

Fig. 2 Modulation of the mesenchymal-like properties of A549 cells by TGF- β 1 and TNF α . **a** A549 cells (1×10^4) were incubated with TGF- β 1 (10 ng/ml), TNF α (20 ng/ml) or TGF- β 1 + TNF α for 24 h and the number of invasive cells assayed. Untreated cells show little to no invasion. Treatment of cells with TGF- β 1 or TNF α alone induced an increase in the number of invasive cells with some cells adopting a bipolar phenotype ($n=4$, $p<0.05$). Co-treatment with TGF- β 1 + TNF α induces an increased number of invasive cells compared to treatment with TGF- β 1 or TNF α alone with the majority of cells adopting a bi-polar phenotype characteristic of fibroblasts ($n=4$, $p<0.05$). **b** Co-treatment of A549 cells with TGF- β 1 (10 ng/ml) and TNF α (20 ng/ml) for 24 h induces pro-MMP-9 production

whereas treatment with TGF- β 1 or TNF α alone had no effect. After 72 h TGF- β 1 increases the production of both pro-MMP-2 and pro-MMP-9 ($n=4$, $p<0.05$). However, co-treatment of the cells with TGF- β 1 and TNF α dramatically accentuates this ($n=4$, $p<0.05$). **c** Extra cellular matrix secretion from A549 cells was measured following treatment with TGF- β 1 (10 ng/ml), TNF α (20 ng/ml) or TGF- β 1 + TNF α for 72 h. Untreated cells do not secrete collagen I or fibronectin however treatment with TGF- β 1 induces the secretion of collagen I and fibronectin ($n=4$, $p<0.05$). Co-treatment with TGF- β 1 + TNF α had no effect on fibronectin secretion ($n=4$, $p>0.05$) but significantly inhibited collagen secretion compared to treatment with TGF- β 1 alone ($n=4$, $p<0.05$)

TGF- β 1 (1108 ± 146) or TNF α (974 ± 212) ($p < 0.01$, $n = 4$). Furthermore, when the cells were treated with TGF- β 1 + TNF α a further approximately 3 fold increase in invasive cells was seen when compared to TGF- β 1 alone (2919 ± 388 , $n = 4$, $p = 0.04$). It was also noted that the majority of migrating cells in the co-treated group had a marked fibroblastic-like morphology (Fig. 2a).

Untreated cells or cells treated with TGF- β 1 or TNF α for 24 h secrete very little to no pro-MMP2 or pro-MMP9. However, cells co-treated with TGF- β 1 + TNF α secrete detectable levels of pro-MMP-9. At 72 h cells treated with TGF- β 1 or TNF α secrete pro-MMP-2 and pro-MMP-9 and the expression is further accentuated when the cells are co-treated with TGF- β 1 + TNF α ($p < 0.05$, $n = 4$) (Fig. 2b).

A549 cells left unstimulated or treated with TNF α alone do not secrete either collagen I or fibronectin into the extracellular environment. However, cells stimulated with TGF- β 1 secrete collagen I and fibronectin ($p < 0.05$, $n = 4$), consistent with cells demonstrating functional evidence of EMT. Interestingly, the addition of TNF α ameliorates the TGF- β 1 driven secretion of collagen I ($68\% \pm 13\%$, $p = 0.03$, $n = 4$) while having no significant effect on the secretion of fibronectin ($p > 0.05$, $n = 4$) (Fig. 2c).

TNF α Accentuates TGF- β 1 Driven EMT by a SMAD Independent Pathway Involving IKK β

It has been previously shown that TGF- β 1 driven EMT involves signalling *via* the phosphorylation of SMAD 2/3 and its subsequent translocation to the nuclei [11, 12]. We therefore investigated if TNF α was exerting an accentuating effect on EMT *via* modulation of SMAD 2/3 signalling (Fig. 3a, bi). Untreated cells show a uniform distribution of SMAD 2/3 throughout the cell with little phosphorylated SMAD 2/3 in the nuclei. As expected, TGF- β 1 treatment promotes an increase in SMAD 2/3 phosphorylation and translocation to the nuclei within 30 min of treatment. However, treatment of the cells with TNF α alone or in combination with TGF- β 1 does not increase SMAD 2/3 phosphorylation or translocation to the nucleus compared to control or TGF- β 1 alone suggesting that TNF α is not accentuating EMT by direct modulation of SMAD 2/3 signalling.

Another signalling pathway activated by TNF α that has been proposed to be important in EMT is the MAPK pathway and in particular p38 [25, 26]. The role of p38 MAPK in the accentuating effect of TNF α was therefore investigated (Fig. 3a, bii). Untreated cells maintain a baseline level of p38 phosphorylation that is increased by treatment with TGF- β 1 or TNF α alone. Significantly, co-treatment of the cells with TGF- β 1 + TNF α showed an additive effect compared to either

treatment alone identifying p38 as a potential candidate for the synergy observed between TGF- β 1 and TNF α in EMT. In addition, a similar additive effect in response to co-treatment was observed in the phosphorylation of ERK1/2 (data not shown).

We proceeded to investigate the relative contribution of SMAD2/3, p38 and ERK1/2 signalling to TGF- β 1-driven, TNF α -accentuated EMT using selective inhibition. Cells were pre-treated for 1 h with either SIS3 (SMAD3i-10 μ M), SB203580 (p38i-10 μ M) or PD98059 (ERK 1/2i-10 μ M) before treatment with TGF- β 1 (10 ng/ml) or TGF- β 1 + TNF α (20 ng/ml) and EMT assessed. Pre-treatment with SMAD3i significantly inhibits the change in cell phenotype and EMT marker expression seen in cells treated with TGF- β 1 alone (E-cadherin $46\% \pm 9\%$; vimentin $41\% \pm 19\%$; fibronectin $58\% \pm 19\%$; $p < 0.05$, $n = 3$). In addition, SMAD3i also reduced the accentuation of TGF- β 1 driven EMT induced by TNF α (E-cadherin $52\% \pm 10\%$; vimentin $169\% \pm 24\%$; fibronectin $67\% \pm 19\%$; pro-MMP-9 $96\% \pm 3\%$ $p < 0.05$, $n = 3$). However, an accentuation effect was still observed suggesting that an alternative pathway is involved in this phenomenon (Figs. 4, and 5a). In contrast, we found no significant effect of pre-treatment with p38i or ERK 1/2i (data not shown) on cell phenotype or protein expression in cells driven to EMT by TGF- β 1 alone or in combination with TNF α ($p > 0.05$, $n = 3$). However in contrast, both p38i and ERK 1/2i inhibited the secretion of pro-MMP 9 ($93\% \pm 1\%$; $p < 0.05$, $n = 3$) from cells undergoing EMT and may be critically involved in the pathway regulating MMP transcription or secretion (Figs. 4, and 5b).

In light of a lack of effect on EMT from inhibition of the MAPK pathway we proceeded to investigate an alternative pathway activated by TNF α , the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) pathway [27, 28]. Cells were pre-treated with inhibitors against the inhibitor of nuclear factor kappa-B kinase (IKK β) subunit of IKK (IKK β 4i or IKK β 6i) and EMT assessed (Figs. 4,

Fig. 3 TNF α accentuates TGF- β 1 driven EMT *via* a SMAD independent pathway in A549 cells. **a** Untreated A549 cells show diffused cellular staining of SMAD 2/3 (i) and p38 (ix) throughout the cell with little to no phosphorylated SMAD 2/3 (v) or p38 (xiii). TNF α (20 ng/ml for 30 mins) treated cells show increased phosphorylation of p38 (xv) compared to untreated cells although no effect on SMAD2/3 localisation (iii) or phosphorylation (vii) was observed. TGF- β 1 (10 ng/ml for 30 mins) treatment promoted an increase in the phosphorylation of SMAD 2/3 (vi) and p38 (xiv) and initiated the translocation of SMAD 2/3 (ii) to the nuclei. Co-treatment of the cells with TGF- β 1 + TNF α increased p38 phosphorylation (xvi) compared to treatment with TGF- β 1 or TNF α alone but had no effect on the phosphorylation (viii) or localisation (iv) of SMAD 2/3. Images acquired on a Leica confocal microscope ($\times 63$ magnification). **b** Western blots for phosphorylated SMAD 2/3 (i) and phosphorylated p38 (ii) confirming the immunofluorescence images in A). Total SMAD 2/3 and p38 are included to confirm that no change in total protein was observed

Fig. 4 Identification of potential targets to block EMT. Untreated cells (i) and cells treated with TNF α (20 ng/ml) (ix) show a uniform cobblestone appearance and clear cell-cell attachment which was not affected by SIS3 (ii and x) (SMAD3i-10 μ M), SB203580 (iii and xi) (p38i-10 μ M) or IKK β inhibitor 4 (iv and xii) (IKK β i(4)-10 μ M). TGF- β 1 (10 ng/ml) treated cells show a decrease in cell-cell contact and adopt a more elongated phenotype (v). Pre-treatment with the

SMAD3i (vi) and IKK β i(4) (viii), but not p38i (vii), reduced the TGF- β 1 induced change in cell phenotype. Co-treatment of the cells with TGF- β 1 + TNF α (xiii) accentuated the change in phenotype compared to TGF- β 1 alone. This change in phenotype was inhibited by pre-treatment with IKK β i(4) (xvi) but not by pre-treatment with SMAD3i (xiv) and p38i (xv)

and 5c). Pre-treatment with IKK β i significantly inhibited the change in cell phenotype and EMT marker expression seen in cells treated with TGF- β 1 alone (IKK β 6i ; E-cadherin 67% $\pm$ 19%; vimentin 78% $\pm$ 19%; fibronectin 168% $\pm$ 43%; pro-MMP-9 22% $\pm$ 6% p <0.05, n =3; IKK β 4i ;

E-cadherin 34% $\pm$ 13%; vimentin 68% $\pm$ 22%; fibronectin 94% $\pm$ 12%; pro-MMP-9 111% $\pm$ 16% p <0.05, n =3). In addition, IKK β i also reduced the accentuation of TGF- β 1 driven EMT induced by TNF α (IKK β 6i ; E-cadherin 30% $\pm$ 10%; vimentin 61% $\pm$ 27%; fibronectin 108% $\pm$ 24%;

Fig. 5 TNF α accentuates TGF- β 1 driven EMT by a SMAD independent pathway involving IKK β . Treatment of A549 cells with TGF- β 1 (10 ng/ml) downregulated E-cadherin expression, increased fibronectin and vimentin expression and increased pro-MMP-9 secretion compared to control cells. Co-treatment with TGF- β 1 and TNF α significantly accentuated the change in EMT marker expression compared to TGF- β 1 alone ($p < 0.05$, $n = 4$). **a** Pre-treatment of the cells with the SMAD3i (10 μ M) significantly attenuated both TGF- β 1

driven EMT and TNF α accentuated TGF- β 1 driven EMT ($p < 0.05$, $n = 4$). **b** Pre-treatment of the cells with the p38i (10 μ M) had no significant effect on either TGF- β 1 driven EMT or the accentuating effect of TNF α on TGF- β 1 driven EMT ($p > 0.05$, $n = 4$). **c** Pre-treatment of the cells with the IKK β i(4) or IKK β i(6) (both at 10 μ M) significantly attenuated both TGF- β 1 driven and TNF α accentuated TGF- β 1 driven EMT ($p < 0.05$, $n = 4$). All results are normalised to β -tubulin as a loading control

pro-MMP-9 $59\% \pm 10\%$ $p < 0.05$, $n = 3$; IKK β 4i; E-cadherin $12\% \pm 4\%$; vimentin $60\% \pm 37\%$; fibronectin $83\% \pm 14\%$; pro-MMP-9 $95\% \pm 13\%$ $p < 0.05$, $n = 3$) identifying NF- κ B signalling as a critical pathway involved in TGF- β 1 driven EMT as well as the accentuating effect of TNF α .

Activated Macrophages Can Accentuate TGF- β 1 Driven EMT in Lung Carcinoma (A549) Cells

Upon activation, macrophages release a wide range of cytokines and growth factors including TNF α [29]. We therefore

Fig. 6 Activated macrophages can accentuate TGF- β 1 driven EMT. Untreated cells and cells co-cultured with unstimulated THP-1 cells (M Φ) retain the morphological appearance of epithelial cells, express high levels of cytokeratin 19 and E-cadherin and express little to no vimentin or fibronectin. Co-culturing cells with *Pseudomonas aeruginosa* activated THP-1 cells had little effect on EMT marker expression compared to control cells. TGF- β 1 treated cells show a loss of cell to cell attachments, a decrease in cytokeratin 19 and E-cadherin expression and an increase in the expression of vimentin and fibronectin ($p < 0.05$, $n = 4$). Co-culturing cells with unstimulated THP-1 cells in the presence of TGF- β 1 had no significant affect on EMT marker expression compared to TGF- β 1 alone ($p > 0.05$, $n = 4$). However, co-culturing cells with *Pseudomonas aeruginosa* activated THP-1 cells in the presence of TGF- β 1 significantly accentuated the change in cell phenotype and EMT marker expression compared to TGF- β 1 alone ($p < 0.05$, $n = 4$). Images acquired on a Leica confocal microscope ($\times 63$ magnification). All results are normalised to β -tubulin as a loading control

proceeded to investigate the potential of a macrophages-like cell line (THP-1) to drive or accentuate EMT in lung carcinoma cells (Fig. 6). Co-culturing A549 cells with unstimulated THP-1 cells or with *Pseudomonas aeruginosa* activated THP-1 cells had little to no effect on cell phenotype or EMT marker expression compared to control cells ($p > 0.05$, $n = 4$). Furthermore, co-culturing A549 cells with unstimulated THP-1 cells in the presence of TGF- β 1 had no significant affect on EMT marker expression compared to TGF- β 1 alone ($p > 0.05$, $n = 4$). In contrast, co-culturing A549 cells with *Pseudomonas aeruginosa* activated THP-1 cells in the presence of TGF- β 1 significantly accentuated the change in cell phenotype and EMT marker expression compared to TGF- β 1 alone ($p < 0.05$, $n = 4$). The results suggest that THP-1 cells alone do not drive EMT in lung carcinoma cells but that activated THP-1 cells can accentuate TGF- β 1 driven EMT.

Discussion

EMT is believed to play an important role in cellular trans-differentiation during embryonic development, tumor invasion and metastasis and tissue fibrosis [1]. The pleiotropic growth factor TGF- β 1 has been postulated as a ‘master switch’ regulating EMT and has been demonstrated to signal primarily *via* the SMAD 2/3 pathway [10–12]. However, as TGF- β 1 is a multifunctional molecule that regulates several critical homeostatic cellular functions including proliferation and apoptosis it is unlikely that a non-specific blockade will be a viable therapeutic option. It has been demonstrated that several growth factors and cytokines can modulate EMT independently or co-operatively with TGF- β 1 [5–8]. Recently TNF α has been shown to accentuate TGF- β 1 driven EMT in a range of cell types highlighting a potential link between EMT and inflammation [17–23]. However, the mechanisms responsible have been ill-defined and further studies are required to elucidate the signalling pathways

involved so potential therapeutic targets to limit or even reverse inflammatory driven EMT can be identified. In this study we evaluated the role of TNF α on TGF- β 1 driven EMT in the A549 human adenocarcinoma epithelial cell line and investigated the effect of inhibiting the SMAD, MAPK and NF- κ B signalling pathways on EMT.

The demonstration of complete EMT requires evidence that cells have undergone both phenotypic (changed morphology, lost or downregulated expression of epithelial markers, gained expression of mesenchymal markers) and functional (increased invasive potential, secretion of MMP’s and ECM proteins) changes consistent with transition to a mesenchymal cell. This more thorough demonstration of EMT allows more robust conclusions to be drawn than in studies that have used a single marker of EMT. We have shown that TNF α accentuates phenotypic and some functional markers (invasive potential and MMP secretion) of EMT compared to TGF- β 1 alone suggesting that a pro-inflammatory tumour microenvironment rich in TNF α can accentuate TGF- β 1 driven EMT. In contrast, TNF α ameliorates the TGF- β 1 driven secretion of collagen I. This result is in agreement with previous studies showing that TNF α can inhibit collagen production both *in vivo* and *in vitro* [30–32]. Both TGF- β 1 and TNF α have been shown to be elevated in lung cancer [33–35] and raises the intriguing possibility that TNF α accentuated EMT could promote tumour metastasis by a combination of the above functional changes; by increasing the secretion of MMP’s to degrade ECM proteins, by inhibiting the secretion of new ECM proteins and by increasing cell invasion and migration.

Furthermore, TGF- β 1 has been shown to be elevated in the airways of patients with idiopathic pulmonary fibrosis (IPF), bronchiolitis obliterans syndrome (BOS) and chronic obstructive pulmonary disease (COPD) [9, 36–40]. Interestingly, it is well recognised clinically that exacerbations in patients with chronic fibrotic lung disease often lead to an increased rate of disease progression and these exacerbations are commonly associated with an increase in the level of pro-inflammatory cytokines including TNF α [41]. It is possible that this increased level of TNF α , coupled with the already elevated levels of TGF- β 1, could drive EMT and consequently increase the number of mesenchymal cells in the lung and contributing to disease pathogenesis.

We evaluated the signalling pathways involved in the accentuating action of TNF α on TGF- β 1 driven EMT. Previous studies have shown that TGF- β 1 drives EMT predominantly *via* the SMAD 2/3 signalling pathway [10–12, 17, 18, 20]. However, TNF α had no effect on the phosphorylation or localisation of SMAD 2/3 suggesting that TNF α was not accentuating EMT by potentiating TGF- β 1 induced SMAD 2/3 phosphorylation and may be proceeding *via* an alternative pathway. The activity of TGF- β 1 is not exclusive to the SMAD signalling pathway and can regulate other signalling pathways including those

that can also be activated by $\text{TNF}\alpha$ such as $\text{NF-}\kappa\text{B}$, ERK and p38 MAPK [11, 13, 27, 28, 42–45]. However, in agreement with previous reports, inhibition of p38 or ERK-1 had no effect on TGF- β 1 driven or $\text{TNF}\alpha$ accentuated EMT [17, 18]. In contrast inhibiting the IKK β subunit of $\text{NF-}\kappa\text{B}$ significantly reduced both TGF- β 1 driven and $\text{TNF}\alpha$ accentuated EMT. The above results suggest an important link between inflammatory driven EMT and $\text{NF-}\kappa\text{B}$ activation. Indeed it has already been demonstrated that $\text{NF-}\kappa\text{B}$ is essential for EMT and metastasis in breast cancer progression [46–48] further highlighting $\text{NF-}\kappa\text{B}$ as a potential therapeutic target for blocking EMT in fibrosis and cancer.

Although many different inflammatory cells are capable of increasing $\text{TNF}\alpha$ levels in the lung microenvironment, alveolar macrophages are likely to play a crucial role in initiating an inflammatory response *via* the production of a multitude of pro-inflammatory cytokines and growth factors [29]. Our results have shown that culturing activated macrophages with A549 cells in the absence of TGF- β 1 does not drive EMT however culturing activated macrophages with A549 cells in the presence of TGF- β 1 accentuates TGF- β 1 driven EMT. The results are in agreement with previously published reports demonstrating that conditioned media from activated tumour associated macrophages (TAMs) induced EMT and stimulated the migratory and invasive activities of hepatoma cells [49]. Furthermore, decreasing the number of TAMs markedly suppressed tumour growth and metastasis in breast cancer [50] suggesting that targeting TAMs in lung cancer may limit EMT and provide a novel therapeutic target.

In summary, our data provides a detailed assessment of the ability of $\text{TNF}\alpha$ to accentuate TGF- β 1 driven EMT in the A549 human lung adenocarcinoma epithelial cell line. This accentuating effect occurs *via* a SMAD 2/3 independent mechanism involving the $\text{NF-}\kappa\text{B}$ pathway independent of p38 and ERK 1/2 activation. The potential role of $\text{NF-}\kappa\text{B}$, as well as signalling molecules both downstream and upstream of $\text{NF-}\kappa\text{B}$, require further investigation to identify possible therapeutic targets that may inhibit EMT in cancer and fibrosis.

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