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TGF-β-induced epithelial-mesenchymal transition of A549 lung adenocarcinoma cells is enhanced by pro-inflammatory cytokines derived from RAW 264.7 macrophage cells

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Cancer cells undergo epithelial-mesenchymal transition (EMT) during invasion and metastasis. Although transforming growth factor-β (TGF-β) and pro-inflammatory cytokines have been implicated in EMT, the underlying molecular mechanisms remain to be elucidated. Here, we studied the effects of proinflammatory cytokines derived from the mouse macrophage cell line RAW 264.7 on TGF-β-induced EMT in A549 lung cancer cells. Co-culture and treatment with conditioned medium of RAW 264.7 cells enhanced a subset of TGF-β-induced EMT phenotypes in A549 cells, including changes in cell morphology and induction of mesenchymal marker expression. These effects were increased by the treatment of RAW 264.7 cells with lipopolysaccharide, which also induced the expression of various proinflammatory cytokines, including TNF-α and IL-1β. The effects of conditioned medium of RAW 264.7 cells were partially inhibited by a TNF-α neutralizing antibody. Dehydroxy methyl epoxyquinomicin, a selective inhibitor of NFκB, partially inhibited the enhancement of fibronectin expression by TGF-β, TNF-α, and IL-1β, but not of N-cadherin expression. Effects of other pharmacological inhibitors also suggested complex regulatory mechanisms of the TGF-β-induced EMT phenotype by TNF-α stimulation. These findings provide direct evidence of the effects of RAW 264.7-derived TNF-α on TGF-β-induced EMT in A549 cells, which is transduced in part by NFκB signalling.

Keywords: EMT/lung adenocarcinoma/NFκB/TGF-β/TNF-α.

Abbreviations: δEF1, delta-crystallin/E2-box factor 1; DHMEQ, dehydroxy methyl epoxyquinomicin; EMT, epithelial-mesenchymal transition; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMGA2, high mobility group AT-hook 2; ICAM-1, intercellular adhesion molecule-1; Id, inhibitor of differentiation; IL, interleukin; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; RT, reverse transcription; SIP1, Smad interacting protein-1; siRNA, small interfering RNA; TβR, TGF-β receptor; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α; TTF-1, thyroid transcription factor-1.

Cytokines of the transforming growth factor-β (TGF-β) family have multiple roles in development and diseases (*1–3*). TGF-β inhibits the proliferation of normal epithelial cells, but cancer cells often evade this control. Furthermore, TGF-β induces epithelial–mesenchymal transition (EMT) in cancer cells, enabling the cells to become motile and invasive (*2*, *4–7*). Since cancer cells are subjected to numerous extracellular stimulations *in vivo*, elucidating the roles of these factors on TGF-β-induced EMT is important for developing cancer treatments.

TGF-β binds to the TGF-β type II receptor (TβRII) on the cell membrane, forming a complex with the type I TGF-β receptor (TβRI) and activating it by phosphorylation (*8*, *9*). The intracellular signalling pathway of the TGF-β family is primarily induced by Smad family proteins. The receptor complex phosphorylates Smad2 and Smad3 on their C-terminal SSXS motifs, resulting in hetero-oligomer formation with Smad4, followed by translocation to the nucleus where they act as transcription factors.

The mechanism of TGF-β-induced EMT has been intensively examined, and each phenotype of EMT was found to be regulated by distinct regulatory factors. For example, expression of E-cadherin mRNA was suppressed by TGF-β via the induction of transcription factors Snail, Slug, high-mobility group AT-hook 2 (HMGA2), delta-crystallin/E2-box factor 1 (δEF1) and Smad interacting protein-1 (SIP1) (*10*, *11*). E12/E47 also represses E-cadherin when inhibitor of differentiation (Id) proteins are downregulated by TGF-β (*12*). Complex formation between Smads and Snail has been reported to be important for E-cadherin regulation by TGF-β (*13*). In contrast, the regulatory mechanisms involved in the expression of mesenchymal markers fibronectin and N-cadherin is poorly understood, and is generally not regulated by the

above factors (*11*, *14*). Induction of α-smooth muscle actin by TGF-β is reported to be induced by nuclear translocation of myocardin family proteins (*15*, *16*). We previously found that thyroid transcription factor-1 (TTF-1) is expressed in the lung epithelium and inhibits TGF-β-induced EMT in A549 lung adenocarcinoma cells, suggesting that TTF-1 is an intrinsic inhibitor of TGF-β-induced EMT (*17*).

Extracellular signals other than TGF-β have been shown to induce EMT in a variety of cells with or without the cooperation of TGF-β (*18*). FGF-2 cooperates with TGF-β to induce EMT and promotes invasion of cancer (*19*). Recent reports have suggested that inflammation plays an important role in tumour progression. Inflammatory cells in the tumour micro-environment produce various inflammatory cytokines, which are involved in the EMT of cancer cells. Previous reports have shown that various pro-inflammatory cytokines, including tumour necrosis factor-α (TNF-α), are produced from activated macrophages (*20*), and that these cytokines augment TGF-β-induced EMT in A549 cells (*21*), whereas TNF-α by itself does not induce EMT in A549 cells (*22*). However, the roles of macrophage-derived inflammatory cytokines in TGF-β-induced EMT of lung cancer cells and the molecular mechanisms underlying this process are not fully understood.

In the present study, we examined the effect of factors derived from a mouse macrophage cell line RAW 264.7 on TGF-β-induced EMT in A549 cells. We found that RAW 264.7-derived factors enhance some phenotypes of TGF-β-induced EMT in A549 cells, including upregulation of fibronectin and N-cadherin. We also showed that the effects of conditioned medium of RAW 264.7 cells on TGF-β-induced EMT is inhibited by a neutralizing antibody against TNF-α, suggesting that the secretion of TNF-α from RAW 264.7 cells is critical for TGF-β-induced EMT. We further found that interleukin (IL)-1β is produced by RAW 264.7 cells and augments TGF-β-induced EMT in A549 cells. Interestingly, the effect is partially mediated by nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) signalling which is suppressed by a specific inhibitor dehydroxy methyl epoxyquinomicin (DHMEQ). Effects of other pharmacological inhibitors on enhancement of TGF-β-induced EMT by TNF-α and IL-1β were also evaluated. Our findings suggest that TNF-α and IL-1β endogenously secreted from RAW 264.7 cells enhance TGF-β-induced EMT in A549 cells at least in part through NFκB signalling.

Materials and Methods

Cell culture and reagents

RAW 264.7, a macrophage-like cell line established from an ascites of tumour induced in mouse by intraperitoneal injection of Abelson leukaemia virus, was a kind gift from Dr Tadashi Muroi (NIH Sciences). A549 and RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO/Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin G and 100 μg/ml of streptomycin. NMuMG cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml of penicillin G, 100 μg/ml of streptomycin and 10 μg/ml of insulin. Cells were grown in a 5% CO_2 atmosphere at 37°C. Recombinant human TGF-β1, TNF-α and IL-1β were obtained from R&D Systems (Minneapolis, MN, USA). Recombinant human IL-6 was purchased from Peprotech (Rocky Hill, NJ, USA). Lipopolysaccharide (LPS) was from SIGMA (St Louis, MO, USA) and GM6001, a pan-matrix metalloproteinase (MMP) inhibitor, was from Calbiochem (San Diego, CA, USA). DHMEQ has been described previously (*23*, *24*). U0126 was from Promega (Fitchburg, WI, USA). SP600125 and SB203580 were from Calbiochem.

Preparation of conditioned medium of RAW 264.7 cells

RAW 264.7 cells (4.5×10^6) were cultured in 10 ml growth medium in a 100-mm dish with or without LPS for 24 h. A549 cells were pre-cultured in a 6-well plate for 24 h with or without TGF-β. Culture medium was replaced with 2 ml/well of the obtained conditioned medium containing TGF-β where indicated.

Quantitative reverse transcription polymerase chain reaction

Total RNAs were extracted using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) as described previously (*25*). First-strand cDNAs were synthesized using the SuperScript III First-Strand Synthesis System (Life Technologies). Quantitative real-time PCR (qRT–PCR) analysis was performed as described previously (*25*). Amplification data were quantified using the standard curve method. All samples were run in duplicate, and the results were averaged and normalized to expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences are available as Supplementary Table I.

Immunoblotting

A549 cells were washed with PBS and lysed with Radioimmunoprecipitation assay buffer [20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS] or cell lysis buffer containing 0.5% Nonidet P-40, 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulphonyl fluoride and 10 mg/ml aprotinin (*26*, *27*). SDS polyacrylamide gel-electrophoresis (SDS–PAGE) and immunodetection were performed as described previously (*28*). Antibodies used for immunoblotting included anti-fibronectin (SIGMA), anti-N-cadherin (BD Biosciences, San Jose, CA, USA), anti-E-cadherin (BD), anti-α-tubulin (SIGMA) and anti-phospho-Smad2 (Cell Signaling Technology, Danvers, MA, USA).

Determination of morphological changes

Morphological changes of the cells were quantitatively determined by calculating cell circularity as described (*29*) by Image J software. Circularity value decreases by morphological change from a pebble-like shape to an elongated shape.

ELISA

Amounts of TNF-α secreted by RAW 264.7 cells were determined in 24 h conditioned media using the Quantikine Mouse TNF-α Immunoassay (R&D Systems), according to the manufacturer's instructions.

Cell invasion assay

Cell invasion assay was performed as described previously with some modifications (*30*). Briefly, cells were pre-treated with or without 5 ng/ml TGF-β and 20 ng/ml TNF-α and seeded in the upper chambers of type-IV collagen-coated (Nitta Gelatin, Osaka, Japan) 12-well culture inserts. After 8 h, cells that had migrated through the collagen-coated-inserts were visualized using crystal violet staining.

Luciferase reporter assays

Luciferase reporter assays were performed as described previously (*31*), by using 9xCAGA-luc (*32*) and NFκB-luc (*33*) as a reporter and TK-Rluc as an internal control.

Neutralizing antibody

Goat anti-mouse TNF-α neutralizing antibody and goat control IgG were obtained from R&D systems. A549 cells were pre-treated with or without 5 ng/ml TGF-β for 24 h, and culture medium was replaced with conditioned medium of RAW 264.7 cells containing either neutralizing antibody or control IgG (10 μg/ml).

Statistical analysis

Student's *t*-test was used to calculate the significance of differences between the two samples. The Tukey–Kramer test of the R statistical analysis programme was used for multiple data comparisons (*34*). A $P<0.05$ was considered to indicate statistical significance.

Results

Secreted factors from RAW 264.7 cells enhance TGF-β-induced EMT of A549 cells

As previously reported (14), TGF-β induces EMT of human lung adenocarcinoma A549 cells that is characterized by morphological changes from a pebble-like shape to an elongated shape (Fig. 1A). These morphological changes were quantitatively determined by the circularity of the cells (*29*) (Fig. 1B). We examined whether secreted factors from mouse RAW 264.7 macrophage cells affect the TGF-β-induced EMT of A549 cells. As shown in Fig. 1A, morphological changes induced by TGF-β were further enhanced by co-culture with RAW 264.7 cells (Fig. 1A and B). To study these effects at a molecular level, qRT–PCR analyses to examine the expression of hallmark EMT genes were performed using human-specific primers. TGF-β-induced expression of the mesenchymal markers fibronectin and N-cadherin were enhanced by the co-culture of the cells (Fig. 1C). Conversely, expression of the epithelial marker E-cadherin was strongly downregulated by TGF-β, but co-culture of the cells with RAW 264.7 did not elicit an additional effect.

We next determined whether the enhanced EMT was caused by secreted factors from RAW 264.7 cells. Since RAW 264.7 macrophage cells become activated upon treatment with *Escherichia coli*-derived LPS, we examined the effect of conditioned medium of RAW 264.7 cells treated with or without LPS on A549 cells. TGF-β-induced expression of fibronectin and N-cadherin mRNAs was enhanced by the addition of the conditioned medium, which was more significant when the conditioned medium of LPS-treated RAW 264.7 cells was used (Fig. 2A). The effect of the conditioned medium was not observed for repression of E-cadherin by TGF-β, which is in agreement with the

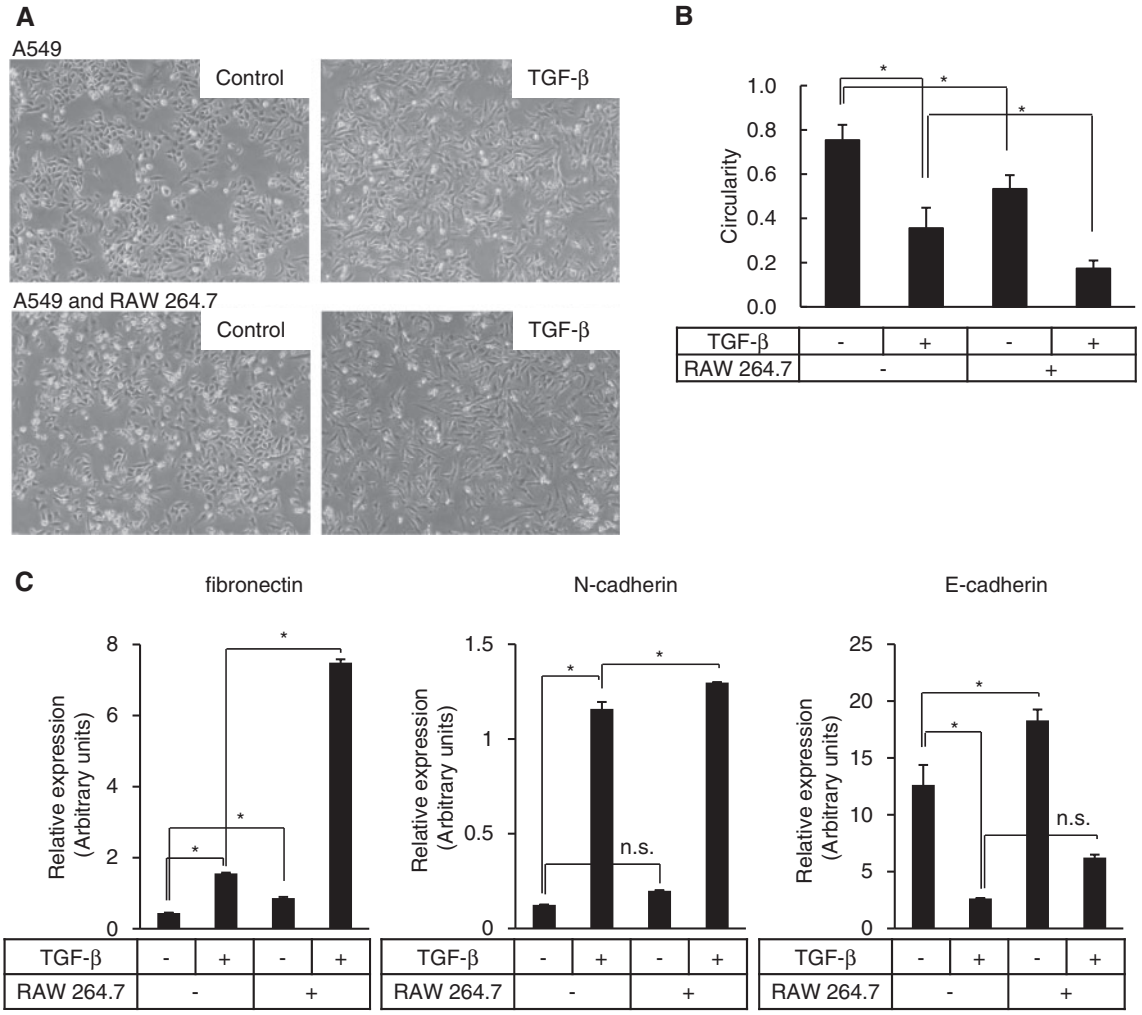

Fig. 1 Effect of co-culture with RAW 264.7 cells on TGF-β-induced EMT of A549 cells. (A) Human lung adenocarcinoma A549 cells were pre-cultured with or without 5 ng/ml TGF-β for 24 h and plated at a density of 5.0×10^4 cells/well in a 6-well plate. The same number of mouse macrophage RAW 264.7 cells were plated and incubated with or without TGF-β for 4 days followed by phase contrast microscopic imaging. (B) Cell circularity was calculated using ImageJ software. In total, 10 cells from each treatment in (A) were measured and the results were averaged. (C) qRT–PCR analysis of EMT marker expression by human-specific primers. A549 cells were co-cultured with RAW 264.7 cells for 3 days with or without TGF-β. *$P<0.05$; Error bars, SDs; n.s., not significant.

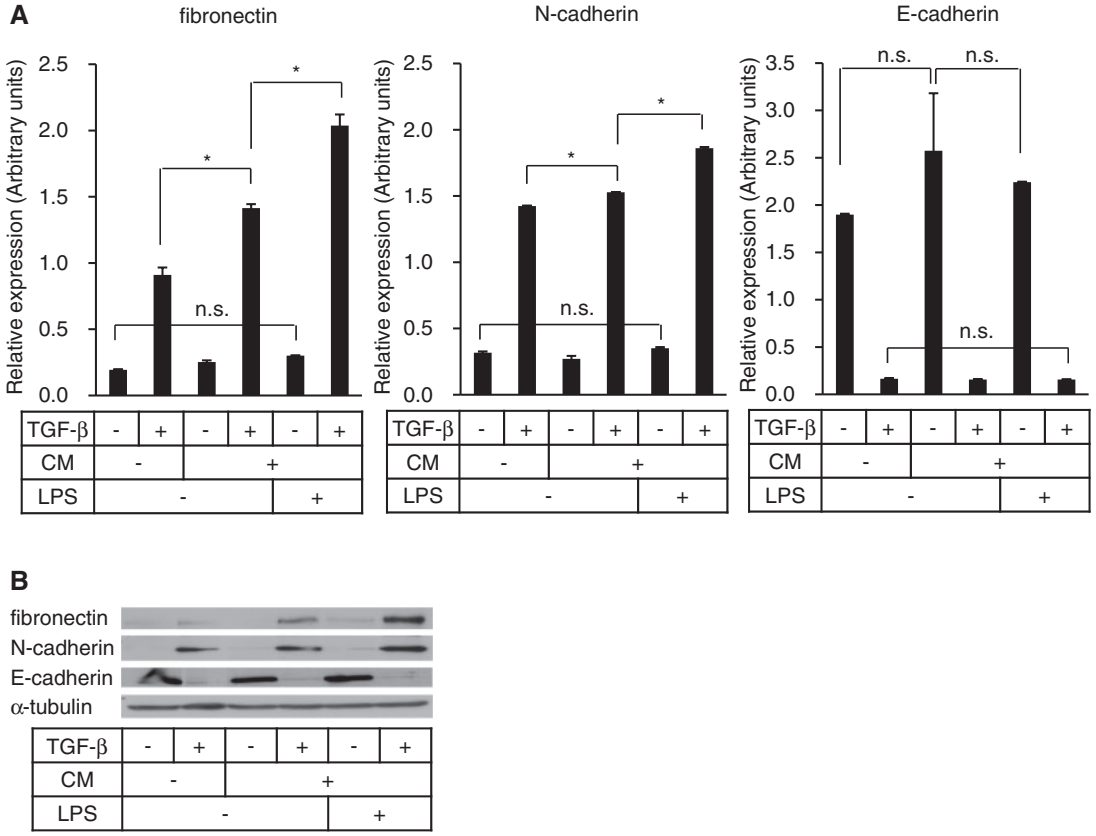

Fig. 2 Effect of conditioned medium of RAW 264.7 cells on TGF-β-induced EMT of A549 cells. (A) A549 cells were cultured with conditioned medium of LPS-activated (0.1 μg/ml) RAW 264.7 cells and stimulated with 5 ng/ml TGF-β. Expression of EMT markers was quantified using qRT–PCR. (B) Expression of fibronectin, N-cadherin and E-cadherin proteins in TGF-β-stimulated A549 cells cultured with conditioned medium of RAW 264.7 cells. A549 cells were cultured as in (A). α-tubulin expression is shown as the loading control. $*P<0.05$; CM, conditioned medium; error bars, SDs; n.s., not significant.

results of co-culture assays (Fig. 1C). Of note, treatment of A549 cells with LPS alone did not affect TGF-β-induced EMT phenotypes (Supplementary Fig. 1). The effects of conditioned medium on the expression of fibronectin, N-cadherin and E-cadherin determined using qRT–PCR analysis were confirmed at the protein expression level by immunoblot analysis (Fig. 2B). Secreted factor(s) from activated RAW 264.7 cells therefore enhance EMT of A549 cells stimulated with TGF-β.

TNF-α is secreted from RAW 264.7 cells and enhances TGF-β-induced EMT of A549 cells

We next attempted to identify RAW 264.7-derived factors that enhance the TGF-β-induced EMT in A549 cells. We speculated that production of such factors is increased following treatment of RAW 264.7 cells with LPS. Since TNF-α is reported to be secreted from activated macrophages, we examined the effects of LPS on TNF-α expression in RAW 264.7 cells. We confirmed that TNF-α was expressed in RAW 264.7 cells, which were upregulated following LPS treatment (Fig. 3A). ELISA analysis showed that >600 pg/ml of TNF-α was present in the conditioned medium prepared from the RAW 264.7 cells and that TNF-α concentrations were increased following LPS treatment (Fig. 3B).

When recombinant TNF-α was added to A549 cells, cellular morphology changed both in the absence and presence of TGF-β (Fig. 3C and D). Induction of fibronectin and N-cadherin by TGF-β was also enhanced by TNF-α addition (Fig. 3E). In contrast, E-cadherin expression was strongly suppressed by TGF-β treatment alone and additional effects of TNF-α were not observed. The effects of TNF-α on cellular morphology and mesenchymal marker expression are similar to those observed in the co-culture experiments and those using conditioned medium of RAW 264.7 cells. Notably, E-cadherin expression was not repressed following both co-culture with RAW 264.7 cells and the use of conditioned medium without TGF-β (Fig. 1C and Fig. 2A and B). In contrast, addition of recombinant TNF-α partially inhibited E-cadherin expression without TGF-β stimulation, suggesting that secreted TNF-α was insufficient for effectively regulating E-cadherin expression.

TNF-α enhances TGF-β-induced motility of A549 cells

On the basis of the effect of TNF-α on TGF-β-induced EMT of A549 cells, we next examined its role in cell invasiveness that accompanies with EMT. Cell invasion assay showed that TNF-α enhanced the TGF-β-induced invasion of A549 cells (Fig. 4A). During EMT, MMPs play important roles in stimulating cell invasion. Expression of MMP-9 and MMP-2 was significantly enhanced by TNF-α and TGF-β, but not by TNF-α alone (Fig. 4B). The effect of TNF-α on

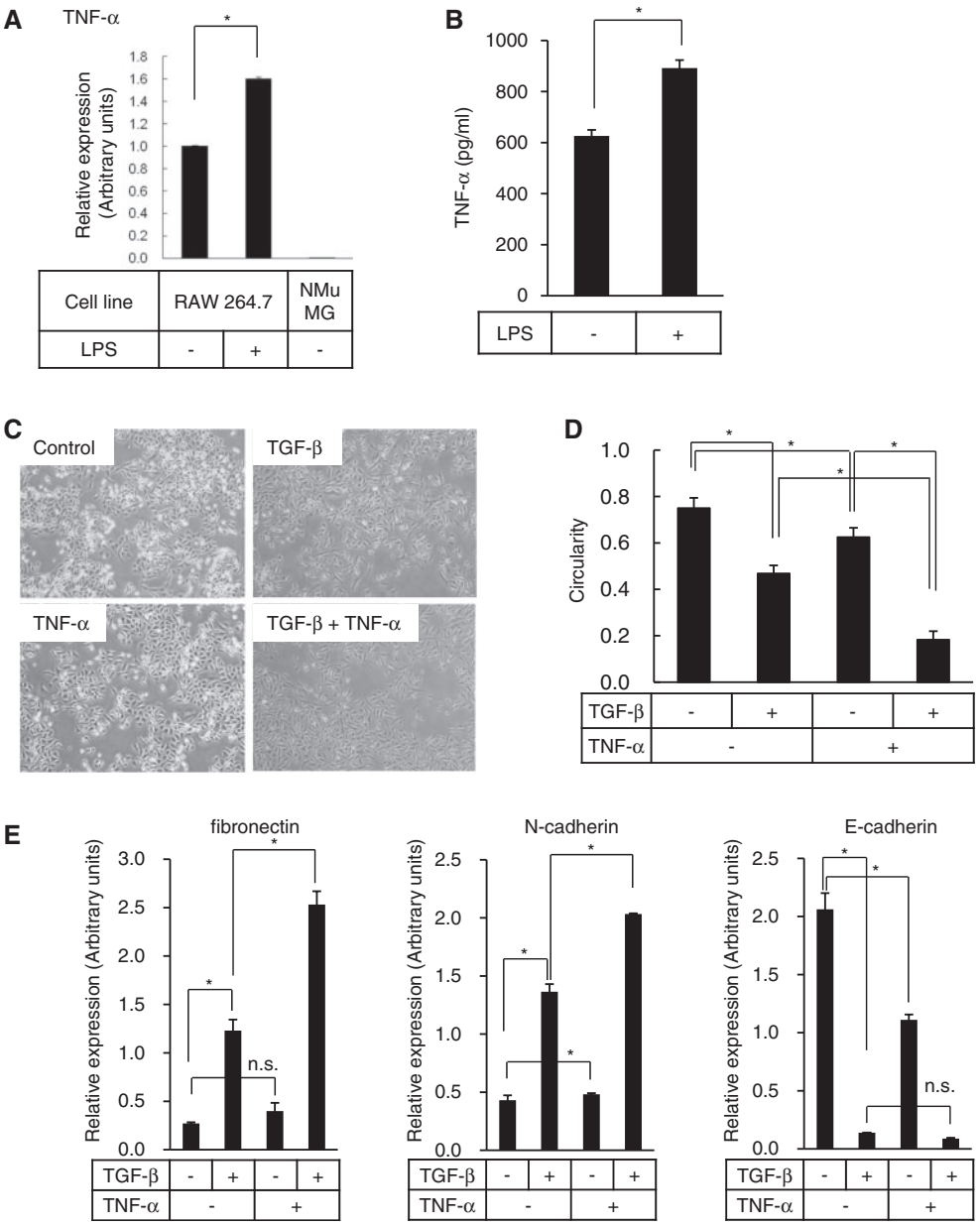

Fig. 3 Enhancement of TGF-β-induced EMT phenotype by TNF-α. (A) Expression of TNF-α mRNA in RAW 264.7 cells. Cells were treated with 0.1 μg/ml LPS, and TNF-α expression was quantified using qRT–PCR. Normal murine mammary gland NMuMG cells served as a negative control. (B) Quantification of secreted TNF-α protein from RAW 264.7 cells stimulated with LPS (0.1 μg/ml). TNF-α protein in the conditioned medium of RAW 264.7 cells was measured using ELISA. (C) Phase-contrast microscopic images of A549 cells treated with 5 ng/ml of TGF-β and 20 ng/ml of TNF-α. Cells were treated with the cytokines for 24 h. (D) Circularity of the cells in (C) was measured as in Fig. 1B. (E) qRT–PCR analysis of EMT marker expression in A549 cells. Cells were treated with 5 ng/ml of TGF-β and 20 ng/ml of TNF-α as indicated for 24 h. $^*P < 0.05$; Error bars, SDs; n.s., not significant.

cell invasiveness was dependent in part on MMPs, as shown by the effect of the pan-MMP inhibitor GM6001 (Fig. 4C).

Blocking TNF-α partially abrogates the effects of RAW 264.7-derived factors on the TGF-β-induced EMT in A549 cells

We next evaluated to what extent the effect of conditioned medium of RAW 264.7 cells is attributable to TNF-α by adding anti-mouse TNF-α neutralizing antibody to the conditioned medium of RAW 264.7 cells. When A549 cells were incubated with conditioned medium treated with anti-TNF-α neutralizing antibody, TGF-β-induced expression of fibronectin and N-cadherin was partially suppressed, whereas their basal expression was not significantly affected (Fig. 5). On the basis of these findings, we concluded that TNF-α is secreted from RAW 264.7 cells and enhances the EMT phenotype of A549 cells induced by TGF-β.

IL-1β is produced by RAW 264.7 cells and enhances TGF-β-induced EMT of A549 cells

The observation that anti-TNF-α neutralizing antibody was capable of partially inhibiting the effects of RAW 264.7-derived conditioned medium prompted us

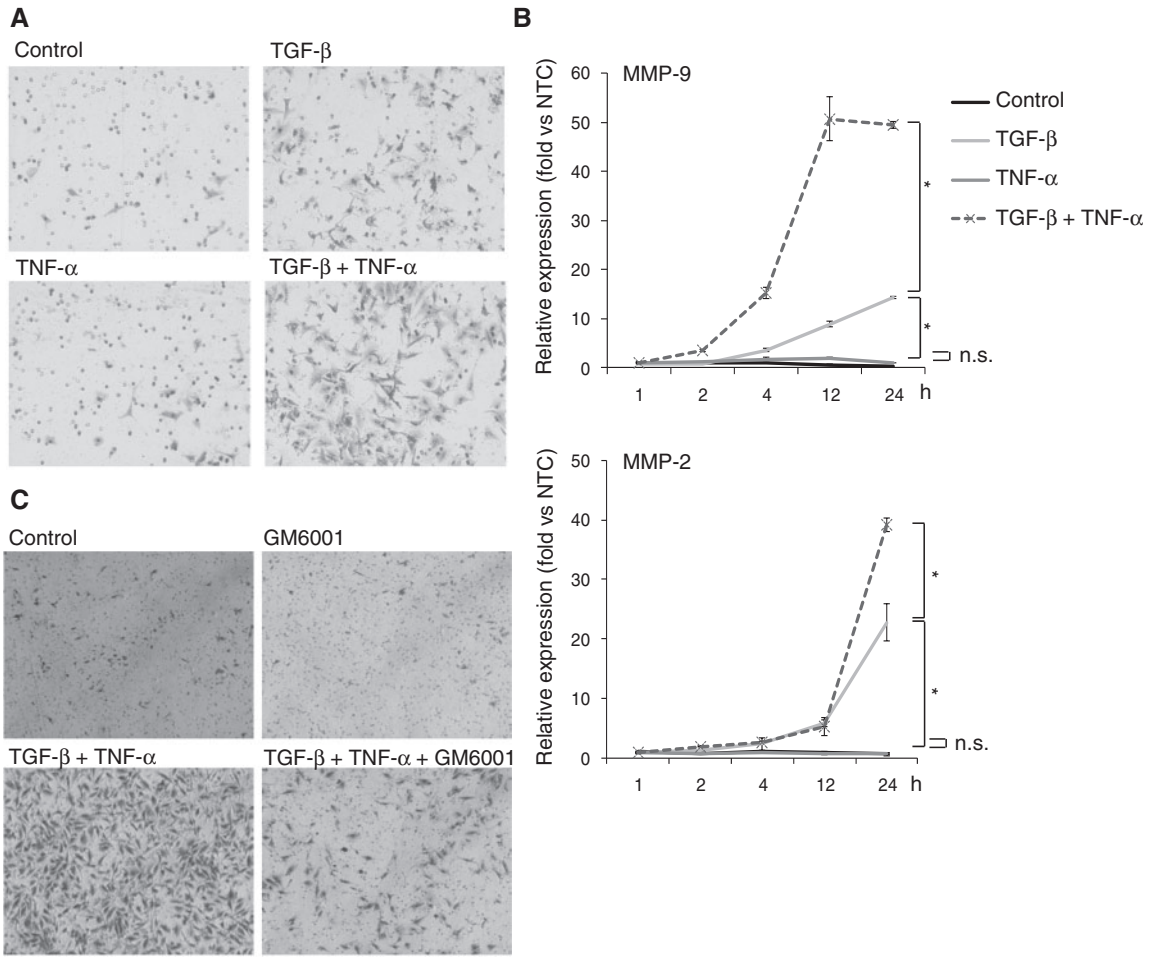

Fig. 4 Effect of TNF-α on the migration A549 cells. (A) Chamber cell invasion assay was performed using A549 cells stimulated with 5 ng/ml TGF-β and 20 ng/ml TNF-α. (B) Expression levels of MMP-2 and MMP-9 in A549 cells treated with TGF-β and TNF-α were analysed using qRT–PCR. (C) Effect of a pan-MMP inhibitor GM6001 on migration of A549 cells. Cells were seeded on Transwells as in (A), and cultured with 10 μM GM6001 in addition to TGF-β and TNF-α for 8 h. NTC, no treatment control at 1 h; $*P<0.05$; Error bars, SDs; n.s., not significant.

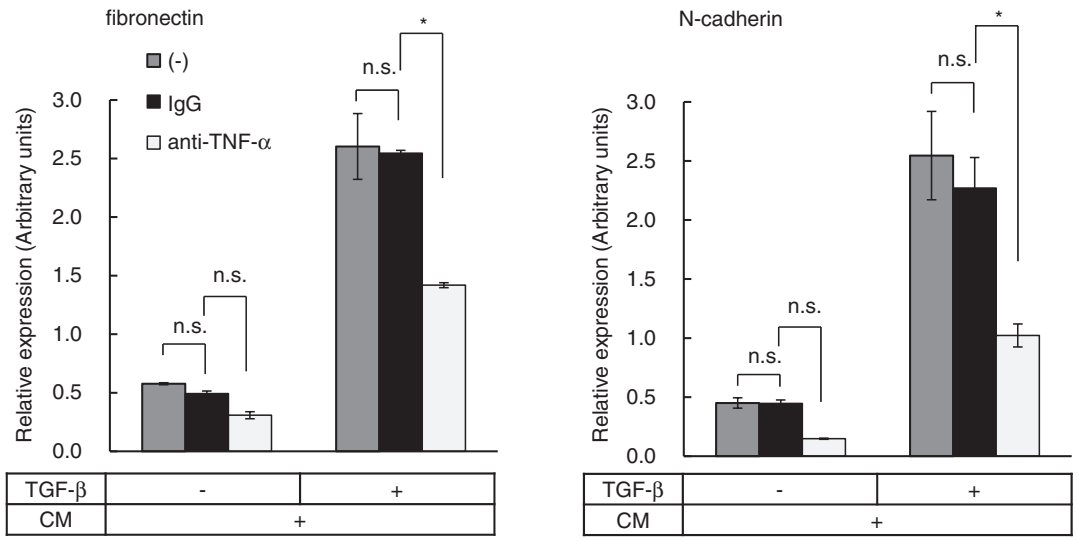

Fig. 5 Effect of TNF-α neutralizing antibody on the expression of mesenchymal markers. A549 cells were cultured in the conditioned medium of RAW 264.7 cells with anti-mouse TNF-α or control IgG. Total RNA was extracted and expression levels of fibronectin and N-cadherin were measured using qRT–PCR. $*P<0.05$; CM, conditioned medium; Error bars, SDs; n.s., not significant.

to search for other secreted factors in the conditioned medium of RAW 264.7 cells that are able to enhance EMT of A549 cells. As shown in Fig. 6, the expression of IL-1β and IL-6, inflammatory cytokines produced by activated macrophages, was also detected in RAW 264.7 cells and upregulated by LPS (Fig. 6A). Similar to TNF-α, IL-1β significantly enhanced the expression of TGF-β-induced fibronectin and N-cadherin (Fig. 6B). In contrast, we did not observe such an effect following IL-6 treatment. Additionally, no cooperative effect of TNF-α and IL-1β was observed for TGF-β-induced expression of EMT markers (Fig. 6C). These results suggest that RAW 264.7 cells secrete multiple proinflammatory cytokines, including TNF-α and IL-1β, to enhance TGF-β-induced EMT in A549 cells.

Effect of NFκB inhibitor DHMEQ on EMT of A549 cells

We further attempted to evaluate the molecular mechanisms underlying enhanced TGF-β-induced EMT by TNF-α. A mixture of inflammatory cytokines has been reported to increase the expression of TGFBR1 encoding TβRI in A549 cells, leading to enhanced Smad2 phosphorylation (*35*). We also observed the upregulation of TGFBR1 mRNA by TNF-α, IL-1β and TGF-β (Supplementary Fig. 2A). However, phosphorylation of Smad2 did not change under our experimental conditions (Supplementary Fig. 2B). Furthermore, we found that TNF-α did not enhance the transcriptional activity of the 9xCAGA-luc reporter, which consists of tandemly repeating Smad binding elements (Fig. 7A), suggesting that TNF-α failed to activate TGF-β signals in the present experimental conditions. Activation of TNF-α-induced NF-κB signals was confirmed by the NF-κB-luc reporter, which was not activated by TGF-β. We also quantified the expression levels of several EMT-related transcriptional regulators by qRT–PCR. We found that expression levels of δEF1 and SIP1 were highest when the cells were stimulated with TNF-α, IL-1β and TGF-β (Supplementary Fig. 2C). In contrast, co-stimulation with TNF-α and IL-1β did not enhance TGF-β-induced expression of other transcriptional regulators. Therefore, δEF1 and SIP1 might function as downstream components of the TGF-β-induced EMT enhanced by TNF-α and IL-1β.

Finally, we studied the involvement of signalling pathways downstream of TNF-α and IL-1β in the enhancement of TGF-β-induced EMT. We examined the effect of the NFκB inhibitor DHMEQ on the enhancement of EMT by TNF-α and IL-1β. As a positive control, induction of the intercellular adhesion molecule 1 (ICAM-1) by TNF-α and IL-1β was efficiently inhibited by DHMEQ addition (Fig. 7B). Fibronectin expression was partially inhibited by DHMEQ, whereas that of N-cadherin was not affected (Fig. 7C). We also performed qRT–PCR analysis of A549 cells transfected with RelA small interfering RNA (siRNA). We observed that the expression levels of fibronectin in A549 cells transfected with three different siRNAs for RelA were lower than those in the cells transfected with control siRNA, which was in agreement with the result using DHMEQ (Supplementary Fig. 3). We then used several kinase inhibitors to examine whether other signalling pathways downstream of TNF-α and IL-1β enhance TGF-β-induced EMT. We found that U0126, an mitogen-activated extracellular signal regulated kinase kinase (MEK) inhibitor, weakly inhibited the induction of fibronectin expression (Fig. 8). We also found that SB203580, a p38 mitogen-activated protein kinase inhibitor, inhibited the enhancement of TGF-β-induced N-cadherin expression by TNF-α and IL-1β, though it upregulated the expression of N-cadherin, as well as that of fibronectin induced by TGF-β alone. Thus, NFκB, ERK and p38 pathways appear to play different roles as downstream components for both TNF-α and IL-1β.

Discussion

Previous studies focused on the roles of TGF-β and inflammatory cytokines on EMT during lung fibrosis and used A549 cells as a cell line of alveolar epithelial origin (*22*). Kasai *et al*. reported that TGF-β, but not TNF-α or IL-1β, induces EMT of A549 cells. Subsequent studies, however, revealed that TGF-β-induced EMT is augmented by either TNF-α or IL-1β (*36*, *37*), or a mixture of inflammatory cytokines which include TNF-α and IL-1β (*35*). Enhanced TGF-β-induced EMT by TNF-α was observed not only in A549 cells, but also in normal bronchial epithelial cells, suggesting that enhanced EMT by TNF-α may be important in other pathological processes of lung diseases (*21*, *38*, *39*). Recently, Borthwick *et al*. (*21*) reported enhanced TGF-β-induced EMT by co-culture of A549 cells with THP-1 human macrophage cells. However, they did not provide direct evidence that THP-1 cell-derived TNF-α is involved in the enhancement of TGF-β-induced EMT and did not study the molecular mechanisms involved. We utilized a neutralizing antibody against TNF-α, and revealed that endogenous TNF-α derived from RAW 264.7 cells plays an important role in the enhancement of TGF-β-induced EMT of A549 cells. We observed enhancement of TGF-β-induced EMT of A549 cells by stimulation of RAW 264.7 cells with LPS. It has been reported that LPS directly affects epithelial cells via its receptor TLR4. However, LPS neither activated NFκB pathway nor enhanced TGF-β-induced EMT of A549 cells in our analysis (Supplementary Figs. 1 and 4). Absence or reduced CD14 and TLR4 possibly explains such an impairment of LPS response of A549 cells in our condition (*40*, *41*). Partial inhibition of the effects of RAW 264.7-derived conditioned medium by the TNF-α antibody also suggested the importance of other secreted factors. Multiple inflammatory cytokines, including IL-1β, IL-8 and IL-6, are produced from activated macrophages. Whether endogenous IL-1β secreted from RAW 264.7 enhances TGF-β-induced EMT, should be evaluated in future studies. It has been reported that IL-8 does not exhibit this enhancing effect (*21*), and we did not observe enhancement of EMT by IL-6. We have not ruled out the possibility that enhanced EMT resulted from crosstalk between A549 cells and RAW 264.7 cells. The effects of cancer cells on macrophages have been extensively studied. For example, cancer cells produce the chemoattractant MCP-1. Versican, an extracellular matrix

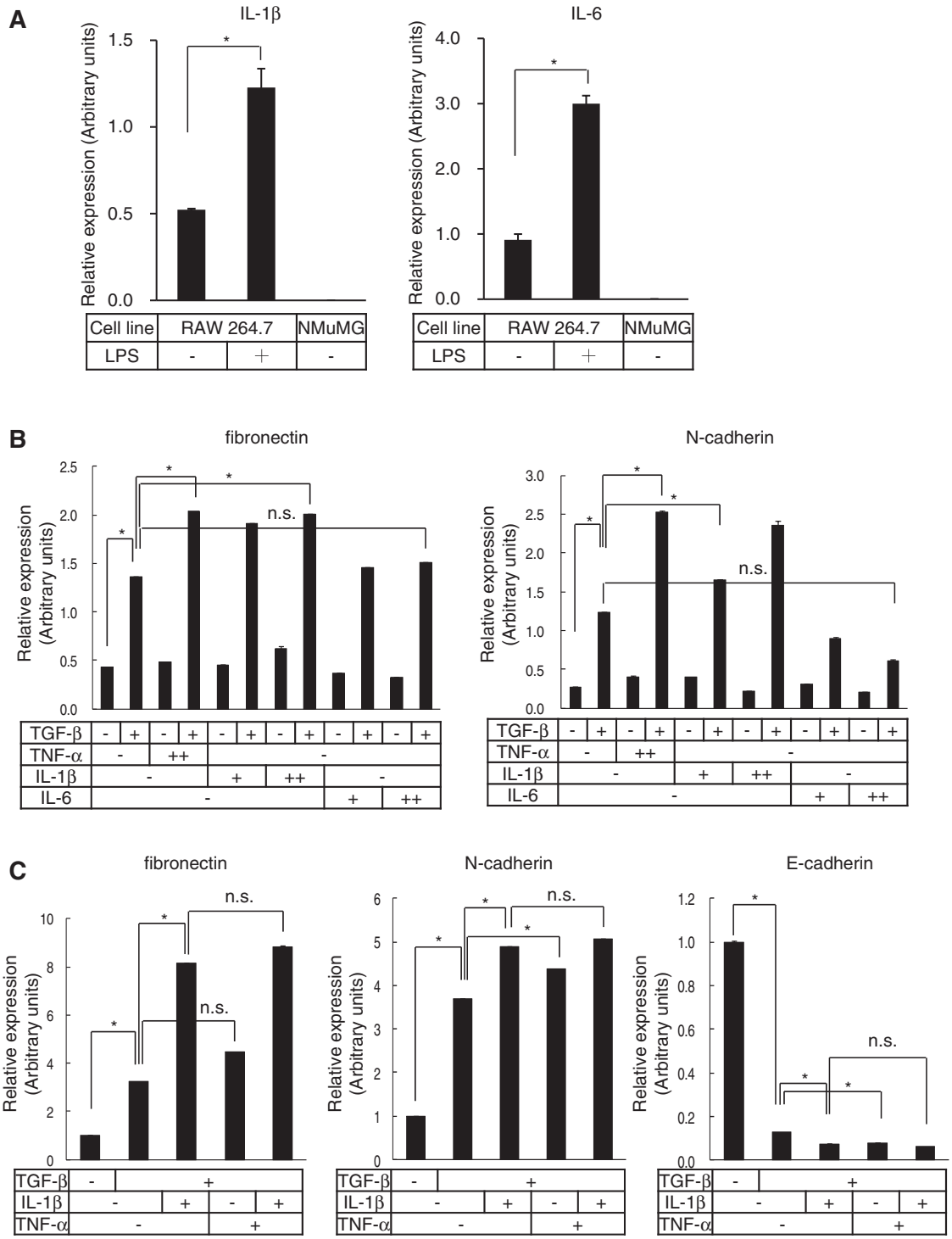

Fig. 6 Effect of IL-1β and IL-6 on TGF-β-induced expression of EMT markers. (A) qRT–PCR analysis of expression of IL-1β and IL-6 in RAW 264.7 cells treated with 0.1 μg/ml LPS was performed. NMuMG cells served as a negative control. The samples used in Fig. 3A were used for the analysis. (B) Effect of IL-1β and IL-6 on the expression of fibronectin and N-cadherin was determined using qRT–PCR in A549 cells. Cells were treated with 5 ng/ml TGF-β, 20 ng/ml TNF-α, 1 ng/ml (+) or 20 ng/ml (++) of IL-1β and IL-6 for 24 h. (C) A549 cells were treated with 1 ng/ml IL-1β, 20 ng/ml TNF-α, or both in the presence of TGF-β. $*P<0.05$; Error bars, SDs; n.s., not significant.

proteoglycan, is also secreted from cancer cells and is reported to activate macrophages and induce cancer cell invasion and metastasis (*42*). Whether A549 cells affect RAW 264.7 cells to secrete the factors that regulate EMT of A549 cells requires further investigation.

The mechanisms underlying the enhancement of TGF-β-induced EMT by TNF-α in A549 cells are poorly understood. Liu reported upregulation of TGFBR1 by inflammatory cytokines (*35*). However, upregulation of TGFBR1 mRNA was primarily induced by TGF-β in our analysis, and the effect of

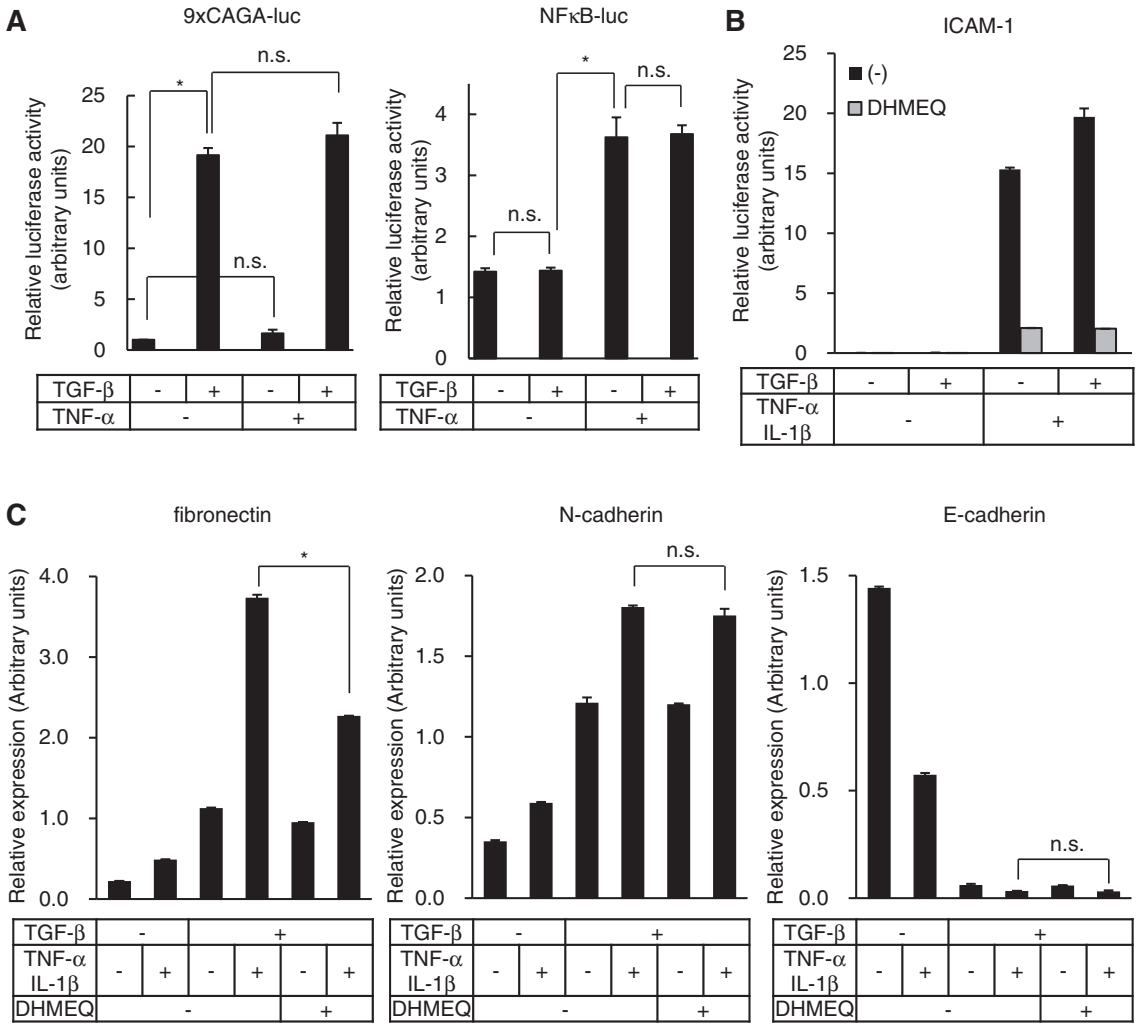

Fig. 7 Effect of DHMEQ on the enhancement of EMT marker expression by TNF-α and IL-1β. (A) A 9xCAGA-luc luciferase construct consisting of tandemly repeated Smad binding elements and NF-κB-luc luciferase reporter construct were transfected in A549 cells and stimulated with 5 ng/ml of TGF-β and 20 ng/ml of TNF-α for 24 h (9xCAGA-luc) or 6 h (NFκB-luc). (B) Inhibition of TNF-α and IL-1β-induced ICAM-1 expression by DHMEQ. A549 cells were pre-treated with 10 μg/ml of DHMEQ or DMSO as a control for 3.5 h and stimulated by TNF-α and IL-1β for 4 h. ICAM-1 expression was measured by qRT–PCR. (C) A549 cells were treated as in (B), and stimulated with TGF-β, TNF-α and IL-1β for 24 h. Expression of EMT markers was analysed by qRT–PCR. $*P<0.05$; Error bars, SDs; n.s., not significant.

TNF-α was observed only in the presence of TGF-β. Nevertheless, TGF-β-induced phosphorylation of Smad2, and Smad-induced 9xCAGA luciferase reporter activity did not change following the addition of TNF-α. We also observed that TGF-β does not enhance TNF-α-induced transcriptional activity of NFκB-luc, and that enhanced expression of target genes by TNF-α was selective to fibronectin and N-cadherin. Although many reports have revealed crosstalk between TNF-α and TGF-β signalling pathways in a variety of cells (*43*, *44*), the present analyses suggest that the cooperation of these cytokines appears to be exerted at the transcription level of each target gene depending on the context of their *cis*-regulatory elements, indicating differences in the transcriptional responses of target genes (*34*, *45*, *46*). Thus, neutralizing antibody against TNF-α inhibited the expression of both fibronectin and N-cadherin. In contrast, only the expression of fibronectin was inhibited by DHMEQ and U0126, whereas that of N-cadherin was inhibited by SB203580. Combinatorial effects of the several inhibitors on EMT phenotypes including expression of fibronectin and N-cadherin in the context of co-stimulation by TGF-β and inflammatory cytokines need to be evaluated in the future analyses.

The importance of EMT in cancer pathophysiology is not limited to cancer cell invasion and metastasis. Asiedu et al. reported generation of breast cancer stem cells by TNF-α and TGF-β (*47*). Based on our analyses suggesting the importance of TNF-α on TGF-β-induced EMT in A549 cells at a level of endogenous secretion from RAW 264.7 cells, complex mechanisms of enhancement by TNF-α require further examination to develop methods for controlling tumour cell invasion and cancer stem cells.

Supplementary Data

Supplementary Data are available at *JB* online.

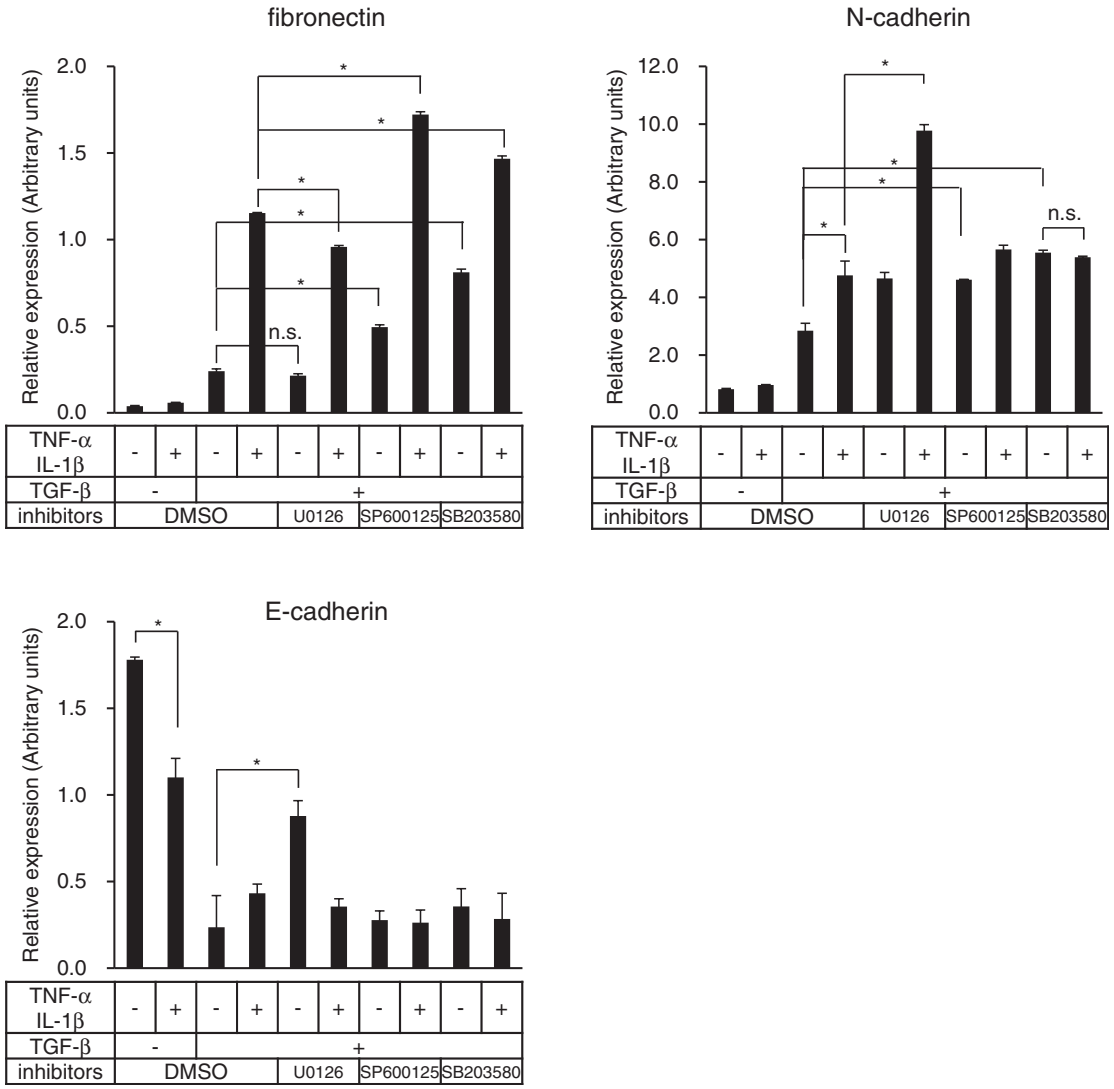

Fig. 8 Effect of kinase inhibitors on enhancement of TGF-β-induced EMT by TNF-α and IL-1β. A549 cells were pre-treated with either 10 μM U0126 (MEK inhibitor), 1 μM SP600125 (JNK inhibitor) or 1 μM SB203580 (p38 inhibitor) for 3.5 h. Cells were then stimulated with 5 ng/ml TGF-β, 20 ng/ml TNF-α and 1 ng/ml IL-1β for 24 h. Expression of EMT markers was determined by qRT–PCR after 24-h stimulation and normalized by GAPDH. $*P<0.05$; Error bars, SDs; n.s., not significant.

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Conflict of interest

None declared.

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