

The novel use of the human nasal epithelial cell line RPMI 2650 as an *in vitro* model to study the influence of allergens and cytokines on transforming growth factor- β gene expression and protein release

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Summary

Background The epithelial accumulation of mast cells is a feature of allergic rhinitis and this has been linked to the expression of the known mast cell chemoattractant transforming growth factor- β (TGF- β) at this site. Little is known concerning the regulation of TGF- β gene expression or protein release by nasal epithelial cells. To address this we have utilized the RPMI 2650 human nasal epithelial cell line, which has some features that closely resemble normal nasal epithelium and has been reported to secrete a TGF- β -like molecule.

Objectives To investigate the regulation of TGF- β gene expression and protein secretion in RPMI 2650 nasal epithelial cells following exposure to allergens (house dust mite (HDM) and grass pollen) and mast cell associated T-helper type 2 (Th2) cytokines (IL-4, IL-13, and TNF- α).

Methods Light and scanning electron microscopy was used to evaluate the morphology of RPMI 2650 cells in culture, enzyme-linked immunosorbent assay was used to investigate their TGF- β secretory capacity and the identification of the TGF- β isotype(s) involved, flow cytometry was used to demonstrate the presence of TGF- β receptors on the RPMI 2650 cells, and the quantitative real-time TaqMan PCR was used to measure TGF- β gene expression.

Results TGF- β_2 was identified as the main isotype secreted by the RPMI 2650 cells. HDM allergens and TNF- α increased both TGF- β gene expression and protein release from these cells, whereas grass pollen, IL-4, and IL-13 were without effect.

Conclusions The RPMI 2650 nasal epithelial cell line represents a valid *in vitro* model to evaluate the regulation of TGF- β biology. In this system HDM allergens have stimulatory activity that is fundamentally different from that of grass pollen allergens, and the Th2 cytokines IL-4 and IL-13 are without effect. The ability of TNF- α to up-regulate both TGF- β gene expression and protein release indicates that mast cell–epithelial interactions concerning TGF- β are bi-directional and this may be fundamental to epithelial immunoregulation. The availability of a model system, such as the RPMI 2650 cells, will enable the early evaluation of future novel and targeted interventions directed toward the aberrant responses of upper airway structural cells.

Keywords gene expression, grass pollen, house dust mite, IL-4, IL-13, nasal epithelial cell line, protein release, RPMI 2650, transforming growth factor- β , TNF- α .

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Introduction

Mast cells play an important pathophysiological role in the clinical expression of allergic rhinitis (AR) [1], and their numbers are increased within the airway epithelium in persistent disease [2]. While some research into the area of nasal luminal mast cell accumulation has been undertaken [3, 4], many questions as to the relevant biological events underlying the epithelial localization and accumulation of

mast cells in AR remain unanswered. Although it is recognized that there are uncommitted progenitors within the nasal mucosal tissue, in the form of CD34⁺, IgE⁺ and tryptase[−] cells [5], which are considered to mature *in situ* under the influence of growth factors such as the stem cell factor, the local accumulation within the epithelium is likely to be a reflection of the epithelial expression of mast cell chemotactic factors that attract and immobilize the cell at that site within the tissue. We have previously provided evidence of increased epithelial immunoreactivity for the known mast cell chemoattractant, transforming growth factor- β (TGF- β) in AR, and evidence that nasal mast cells express receptors for TGF- β [6]. In this study [6], the TGF- β isoforms, TGF- β_1 , TGF- β_2 , and TGF- β_3 , all had increased

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epithelial expression in AR and positive correlations were evident between the extent of immunoreactivity for each of TGF- β_1 and TGF- β_2 and epithelial mast cell numbers. Others have previously reported that gut mast cells express TGF- β RI and TGF- β RII and that gut mast cells chemotact to all three isoforms of TGF- β [7].

No studies in rhinitis have investigated the specific factors that regulate TGF- β synthesis and release by nasal epithelial cells. Although the relationship between TGF- β expression and mast cells within the epithelium may reflect the relevance of TGF- β to mast cell immobilization at this site, it could also reflect the relevance of mast cell-derived products, such as IL-4, IL-13, and TNF- α , to epithelial cell activation. To explore this we have utilized a nasal epithelial cell line, RPMI 2650 (ATCC CCL-30) as an *in vitro* nasal model for the investigation of the effects of allergen exposure (house dust mite (HDM) extracts and grass pollen) and cytokine exposure (IL-4, IL-13 and TNF- α) on TGF- β protein secretion and gene expression, using ELISA and real-time TaqMan RT-PCR, respectively. The human nasal epithelial cell line RPMI 2650 used in these experiments originates from an anaplastic squamous cell carcinoma of the nasal septum [8], and has been predominantly utilized in studies of argininosuccinate synthetase [9], with only a few studies aimed at the characterization of the cell line itself. RPMI 2650 epithelial cells have been shown to closely resemble normal human nasal epithelium with respect to their karyotype [10], cytokeratin polypeptide expression patterns [11, 12], and the presence of mucoid material on the cell surface [8]. In other respects, however, RPMI 2650 cells display differences from normal nasal epithelium, in that they lack cilia and show little differentiation, and as such do not form a pseudo-stratified epithelial layer comprising different types of cells. The RPMI 2650 cell line has been developed for use as an *in vitro* cell model to study nasal mucosal permeability and metabolism [9], although there are limitations for its use in this respect [13]. These cells have also been shown to secrete TGF- α and TGF- β -like molecules [14] and although the precise isotype of TGF- β produced has not as yet been determined, it was felt that they could potentially provide a model to investigate regulatory events underlying TGF- β synthesis and release. As part of these studies, we have further characterized the RPMI 2650 cell line by evaluating the TGF- β isotype(s) released on activation and have explored, by flow cytometry, the presence of TGF- β receptors on the RPMI 2650 cell surface.

Materials and methods

Cell line culture and maintenance

RPMI 2650 human nasal septal squamous cell carcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The tumour cell line (passage 26–46) was grown in uncoated vented tissue culture flasks (TC flask 75 cm², Triple Red Ltd, Thame, Oxfordshire, UK) and incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. Cells were maintained in Vitacell (ATCC's-modified Eagle's medium) supplemented with 10% fetal calf serum (Gibco, Paisley, UK), 2 mM L-glutamine (Gibco), and penicillin 100 U/mL (Gibco), and streptomycin 100 µg/mL (Gibco). The culture medium was changed every 48 h.

Once the cells were almost confluent (around 7 days), they were detached from the culture flasks by washing twice with HBSS (Gibco) (calcium and magnesium free) and then incubating for 7 min with a 0.25% trypsin-EDTA solution (Gibco). This resulted in lifting of the cells off the plastic culture surface. The cells were then detached into 15 mL Falcon tube (Beckton Dickinson, Franklin Lakes, NJ, USA). The cell suspension was centrifuged at 570 g for 10 min at 20 °C to form a cell pellet. The cell pellet was resuspended in culture medium and passed through a 0.6 mm needle to ensure adequate dispersion of the cells. Cells were counted using a Neubauer haemocytometer (Fisher Scientific, Loughborough, UK) and viability established by the Trypan blue dye exclusion method. The cells were then either re-seeded into culture flasks or transferred to culture plates or wells in preparation for experiments. For propagation of the cell line, the cells were seeded into 75 cm² flasks, at approximately 0.5 × 10⁶ cells/mL. The cell line was used within 20 passages from the original frozen stock in order to minimize the phenotypic drift associated with long-term culture. For the challenge experiments, Primaria 24-well culture plates (Falcon, Beckton Dickinson) were used. The cells were seeded at 0.5 × 10⁶ cells in 0.5 mL of culture medium. A cell count was performed at 90% confluence, which normally occurred around 4 days post-seeding. This averaged 1.5 × 10⁶ cells/well and was reproducible throughout the study.

Flow cytometry

This was carried out using the commercially available Fluorokine kit (R&D Systems, Abingdon, UK) to determine the percentage of cells bearing the receptor for TGF- β_1 , using a human TGF- β_1 biotin conjugate, according to the manufacturer's instructions. As a negative staining control, an identical sample of cells was stained with 10 µL of biotinylated negative control reagent. There were four treatment categories: (1) non-stimulated cells; (2) non-stimulated cells that had been pre-incubated overnight with human monoclonal anti-TGF- β_2 (CAT-152) (1 mg/mL) (Cambridge Antibody Technology, Cambridge, UK); (3) cells that had been stimulated with HDM extract (5000 U/mL for 24 h); and (4) HDM (5000 U/mL)-stimulated cells that had been pre-incubated overnight with anti-TGF- β_2 antibody (1 mg/mL) (CAT-152).

For specificity testing, one tube containing cells was pre-treated with purified mouse IgG1 (Sigma, Poole, Dorset, UK) (10 µL of 1 mg/mL/10⁶ cells) for 15 min at room temperature in order to block Fc-mediated interactions. In a separate tube, 20 µL of anti-human TGF- β_1 blocking antibody was mixed with 100 µL of TGF- β_1 biotin and incubated for 15 min at room temperature. Then 1 × 10⁵ Fc-blocked cells in a volume of 500 µL were added to the tube containing anti-TGF- β_1 blocking antibody and fluorokine mixture. The reaction was then allowed to continue as described in the protocol. Analysis was performed using the fluorescent-activated cell scanning (FACScan) system (Beckton Dickinson, CA, USA), with the CellQuest software (Beckton Dickinson, San Jose, CA, USA) supplied. Fluorescence was measured and displayed on a single-parameter histogram using a log scale. The minimum number of cell events collected for analysis was set at 10 000.

Allergen and cytokine preparation

HDM extract (Allergopharma, Reinbek, Germany) was constituted in serum-free medium and used at the following concentrations: 625, 1250, 2500, and 5000 U/mL. Enzymatic activation was performed using 1 mM L-cysteine (Sigma). Control solutions consisted of serum-free medium and serum-free medium containing L-cysteine.

Aquagen SQ mixed grass solution (ALK Abello, Copenhagen, Denmark) was constituted in serum-free medium and used at the following concentrations: 100, 1000, 10 000, and 100 000 SQ-U/mL.

IL-4, IL-13, and TNF- α (all from PeproTech, London, UK) were used at the following concentrations, 1, 3, 10, and 30 ng/mL, achieved by performing serial dilutions of the stock solution with serum-free medium.

Allergen and cytokine challenge

When the cells in the T75 cm² flasks were 90% confluent, they were transferred to 24-well Primaria (Falcon) tissue culture plates (Beckton Dickinson) in preparation for the challenge experiments. A cell density of 1×10^6 cells/mL was used, and of this 500 μ L aliquots were transferred onto the 14-well plates, giving a final cell number of 5×10^5 cells/well. After 72 h, when the cells had reached about 80% confluence, they were washed twice with 500 μ L of unsupplemented MEM. The culture medium was then replaced with 500 μ L of serum-free medium, consisting of MEM supplemented with L-glutamine, penicillin, and streptomycin as previously described, but with the addition of 1% bovine serum albumin (Sigma), and the serum-free supplement ITS (Sigma), which contains 1% of insulin, transferrin, and sodium selenite. The cells were incubated for 24 h in the serum-free conditions to render the cells quiescent, thus producing growth arrest which would synchronize cell proliferation upon subsequent stimulation. After 24 h in serum-free medium, the cells were washed once with 500 μ L of unsupplemented MEM, and the cells were stimulated with either HDM extract (625, 1250, 2500, and 500 U/mL), grass pollen (100, 1000, 10 000, and 100 000 SQ-U/mL), IL-14, IL-13, or TNF- α (1, 3, 10, and 30 ng/mL) in volumes of 500 μ L. Incubations were performed at four-time-points, 2, 5, 8, and 24 h post-challenge, following which the supernatants and cell lysates were harvested.

Supernatant collection and cell lysis

Following the challenge with allergens or cytokines, and at a specific time-point, the culture supernatants were carefully aspirated, collected, and centrifuged at 759g for 10 min at 4 °C to remove any cells. The cell-free media were then divided into two (250 μ L) aliquots and stored at -20 °C for later measurements using an ELISA method.

Following supernatant collection, the cells were lysed with a solution containing Guanidium isothiocyanate (4 M) (Sigma) and 2-mercaptoethanol, which denatures proteins including RNases, using a volume of 200 μ L/well. The culture plate was then incubated for 10 min on a rocker platform at room temperature, following which the lysates were collected and stored at -80 °C in preparation for RNA extraction.

Quantification of transforming growth factor- β protein using an enzyme-linked immunosorbent assay

Release of TGF- β_1 and TGF- β_2 into culture supernatants of RPMI 2650 cells was measured using corresponding ELISA kits, according to the manufacturer's instructions (R&D Systems). As the ELISA only measured levels of the active protein, activation of the supernatants was undertaken in order to measure the total (latent+active) levels of both proteins.

Quantification of transforming growth factor- β_2 gene using quantitative real-time TaqMan polymerase chain reaction

For gene expression studies, the following doses, based on our preliminary studies, were studied: HDM (2500 U/mL), grass pollen (100 000 SQ-U/mL), IL-4, IL-13, and TNF- α (10 ng/mL), all analysed at 2, 5, 8, and 24 h. For analysis of TGF- β_2 expression, total RNA was extracted from cell lysates using the RNeasy column extraction method (Qiagen, Crawley, UK) according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed using the Reverse Transcription System (Promega, Southampton, UK) according to the manufacturer's instructions. TGF- β_2 primer (Qiagen) and probe (Eurogentec, Seraing, Belgium) sequences labelled with a 5'-reporter dye FAM (6-carboxy-fluorescein) and a 3'-quencher dye TAMRA (6-carboxy-N,N,N',N'-tetramethyl-rhodamine) were as follows: forward primer, 5'-GACCAACCGGCGGAAGA-3'; reverse primer, 5'-CAG-CAATTATCCTGCACATTTCTAA-3'; and probe, FAM-5'-CAATAGGCCGCATCCAAAGCACGT-3'-TAMRA.

Primers directed against the house-keeping genes Ubiquitin C (UBC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as the endogenous controls (Eurogentec). For each sample, measured in duplicate, the PCR reaction contained 25 ng of cDNA template, 3.2 pM fluorogenic probe, 15 pM of forward and reverse primers, and 7.50 μ L qPCR mix (Eurogentec), in a final volume of 12.5 μ L. Separate normalizing control samples were run, which contained 1 μ L of UBC and GAPDH primer and probe mixes (Eurogentec). RT-negative samples were used to demonstrate that the signals obtained were RT dependent and not because of genomic contamination, although this was irrelevant for the TGF- β_2 primer set since it spans an intron and therefore does not detect genomic DNA. The PCR protocol was as follows: 95 °C, 10 min, followed by 40 cycles of denaturation 95 °C, 15 s, and annealing/extension 60 °C, 1 min. Thermocycling and real-time detection of PCR products were performed on an iCyclerIQ sequence detection system (Bio-Rad, Hercules, CA, USA) and following completion of the PCR reaction, the thresholds for fluorescence emission baseline were set automatically at 10 times the background levels on the FAM and Yakima yellow dye layers. Expression levels in RPMI 2650 cells for the target gene TGF- β_2 were calculated using the $\Delta\Delta C_T$ method and expressed relative to the geometric mean of the house-keeping genes, GAPDH and UBC. Samples were measured in duplicate and in eight independent experiments.

Statistical analysis

Statistical analyses were performed using SPSS for Windows (SPSS Inc, Chicago IL, USA). The data was presumed not to

be normally distributed, and therefore comparisons between groups were made using the non-parametric Mann–Whitney *U*-test. The level of significance was accepted as $P < 0.05$.

Results

Light and scanning electron microscopy

Light and scanning electron microscopic features of the RPMI 2650 cells are shown in Fig. 1. Scanning electron microscopic evaluation of the RPMI 2650 cells revealed that these cells formed several overlapping layers. The cells were clustered, with the clusters connected by smaller or larger cell bridges, and composed of round and slightly flattened cells with prominent cell processes. The surfaces of the round cells were folded with projections suggestive of microvilli. In contrast, the flatter cells had a smoother surface with only a few microvilli. There was no evidence of cilia, and gaps of varying sizes could be observed between individual cells and cell clusters.

Flow cytometry The flow cytometric data are summarized in Table 1, while the analysis is shown in Fig. 2. The data show a low level of TGF- β receptor expression on non-stimulated RPMI 2650 cells. Following overnight incubation of non-stimulated RPMI 2650 cells with anti-TGF- β_2 antibody, the level of TGF- β receptor expression increased approximately three fold. HDM stimulation had no significant effect on the levels of TGF- β receptor expression, while

the effect of overnight pre-incubation with anti-TGF- β_2 antibody again increased the levels of TGF- β receptor expression in these HDM-stimulated cells. Specificity testing revealed a very low level of TGF- β receptor expression, suggesting that the assay is highly specific.

Transforming growth factor- β isotype profile

The levels of TGF- β_1 in the culture supernatants from stimulated and non-stimulated RPMI 2650 cells were all below the detection limits of the ELISA assay. However, we were able to detect and measure the levels of TGF- β_2 in non-stimulated RPMI 2650 cells. TGF- β_2 thus appeared to be the main isotype of secreted TGF- β in these cells. No measures were made of TGF- β_3 .

Transforming growth factor- β_2 protein and gene expression levels in RPMI 2650 cells following exposure to house dust mite extract and grass pollen

There was evidence of a significant time-dependent increase in baseline TGF- β_2 production by non-stimulated cells ($P < 0.001$), consistent with generation of TGF- β by RPMI 2650 cells. In addition, there was a significant concentration-dependent effect of HDM extract stimulation on TGF- β_2 protein concentrations in the recovered supernatant, with significant elevations at 2, 5, 8, but not 24 h post-exposure (Fig. 3). This effect on TGF- β_2 release by HDM allergen

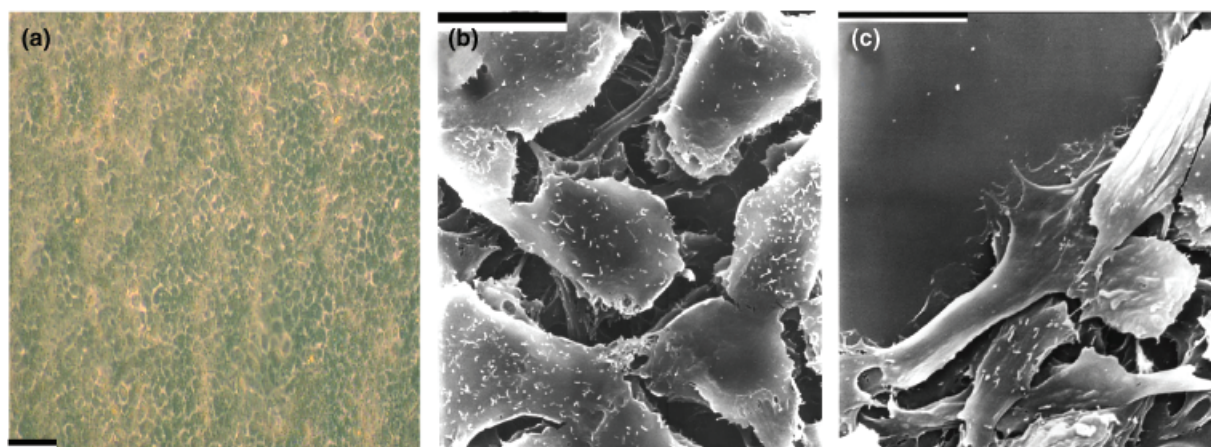


Fig. 1. (a) Light and (b, c) scanning electron microscopic features of RPMI 2650 cell layers. (a) scale bar = 50 μ m, (b) Magnification $\times 2.5K$; scale bar = 12 μ m; (c) Magnification $\times 3.0K$; scale bar = 10 μ m.

Table 1. Flow cytometric data for TGF- β receptor expression on RPMI 2650 cells expressed as median fluorescence values and percentage gated cells (values expressed represent means of $n = 2$)

Treatment group	Negative staining control		Biotinylated rh TGF- β_1	
	Median fluorescence	% gated cells	Median fluorescence	% gated cells
Non-stimulated cells	101.72	0.95	331.5	2.31
Non-stimulated cells pre-incubated overnight with anti-TGF- β_2 antibody	236.1	0.8	934.9	4.62
HDM-stimulated cells	76.76	0.99	280.07	1.56
HDM-stimulated cells pre-incubated overnight with anti-TGF- β_2 antibody	234.43	0.69	482.61	8.21
Specificity testing	46.14	1.03		

TGF- β_2 , transforming growth factor- β_2 ; HDM, house dust mite.

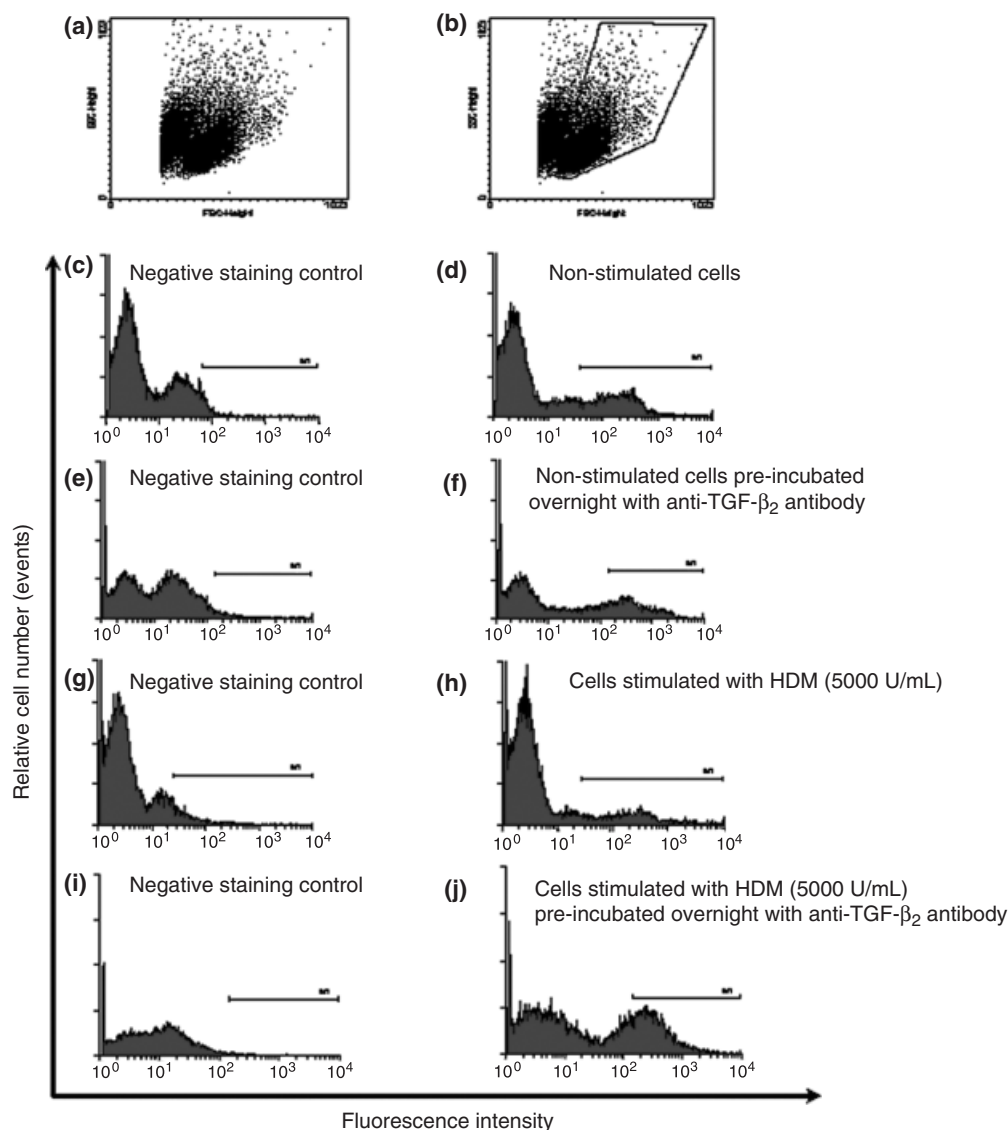


Fig. 2. Flow cytometric analysis of transforming growth factor- β (TGF- β) receptor expression on RPMI 2650 cells ($n = 2$). Cells from confluent cultures were stained with human TGF- β_1 biotin conjugate directly labelled with avidin-FITC reagent. Flow cytometric results show: (a) ungated and (b) gated RPMI 2650 cell populations, and (c)–(j) fluorescence intensity histograms on a log scale for 10 000 cells from each sample. A protein (soybean trypsin inhibitor) that has been biotinylated to the same degree as the TGF- β_1 cytokine was used as the negative control agent. The bar, M1, indicates the gated cell population from which the data for percentage of fluorescence shown in Table 1 were derived.

extract was evident at 625 U/mL, the lowest dose tested. Following treatment with HDM extract (2500 U/mL), analysis of TGF- β_2 gene transcription showed that the treatment caused a significant increase in TGF- β_2 mRNA at 5 and 8 h post-stimulation (Fig. 3).

In contrast, no significant concentration- or time-dependent effect of grass pollen stimulation on TGF- β_2 protein release into the supernatant was evident (Fig. 4). Similarly, no significant changes in TGF- β_2 mRNA levels resulted after stimulation with 10 000 SQ-U/mL of grass pollen allergen (Fig. 4).

Transforming growth factor- β_2 protein and gene expression levels in RPMI 2650 cells following exposure to interleukin-4, -13, and tumour necrosis factor- α

IL-4 and IL-13 stimulation did not have any significant effects on TGF- β_2 protein secretion or gene expression (Fig. 5).

TNF- α stimulation resulted in a significant increase in TGF- β_2 secretion at the 10 ng/mL doses at 24 h with the gene expression significantly increased following stimulation with TNF- α (10 ng/mL) at 2, 8, and 24 h (Fig. 5).

Discussion

This study has utilized the RPMI 2650 epithelial cell line to evaluate the regulation of generation and release of TGF- β by nasal epithelial cells. These cells have previously been identified as releasing a TGF- β -like molecule [14], although the specific isotypes have not been characterized. We have demonstrated that this cell line expresses the gene for TGF- β_2 and that this growth factor can be generated and released by RPMI 2650 cells. Whilst we identified gene expression for TGF- β_1 in these cells, measures of protein levels of this growth factor in supernatants were all below the level of

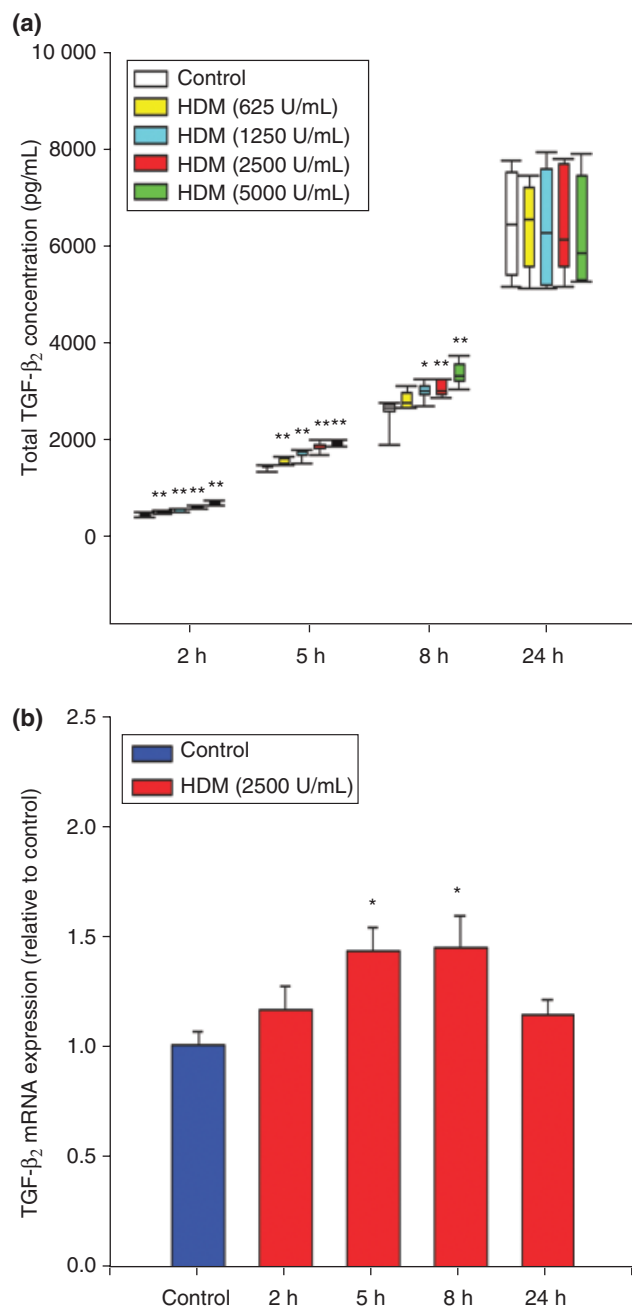


Fig. 3. (a) Transforming growth factor-β₂ (TGF-β₂) protein production by RPMI 2650 cell cultures ($n = 8$) after exposure in medium alone or with house dust mite (HDM) extract allergen. The box plots show the median and interquartile range, and the bars show the 10th and 90th percentiles. * $P < 0.05$; ** $P < 0.005$ vs. control, untreated cells. (b) Effect of HDM stimulation on TGF-β₂ gene expression in RPMI 2650 cells. Data are mean $\pm$ SEM ($n = 8$) and are normalized relative to TGF-β₂ gene expression in untreated (control) cells. * $P < 0.05$ vs. untreated control cells.

detection of the corresponding ELISA assay. This suggested that either the synthesis of TGF-β₁ was too low to allow detection, or that these anaplastic cells failed to transport the protein to allow its localized release. TGF-β₂ thus appeared to be the predominant isotype generated and released by these cells. TGF-β₂ is also the predominant TGF-β isotype expressed by nasal epithelial cells *in vivo* [6]. Using flow cytometry, we have also demonstrated that the RPMI 2650 cells express TGF-β receptors.

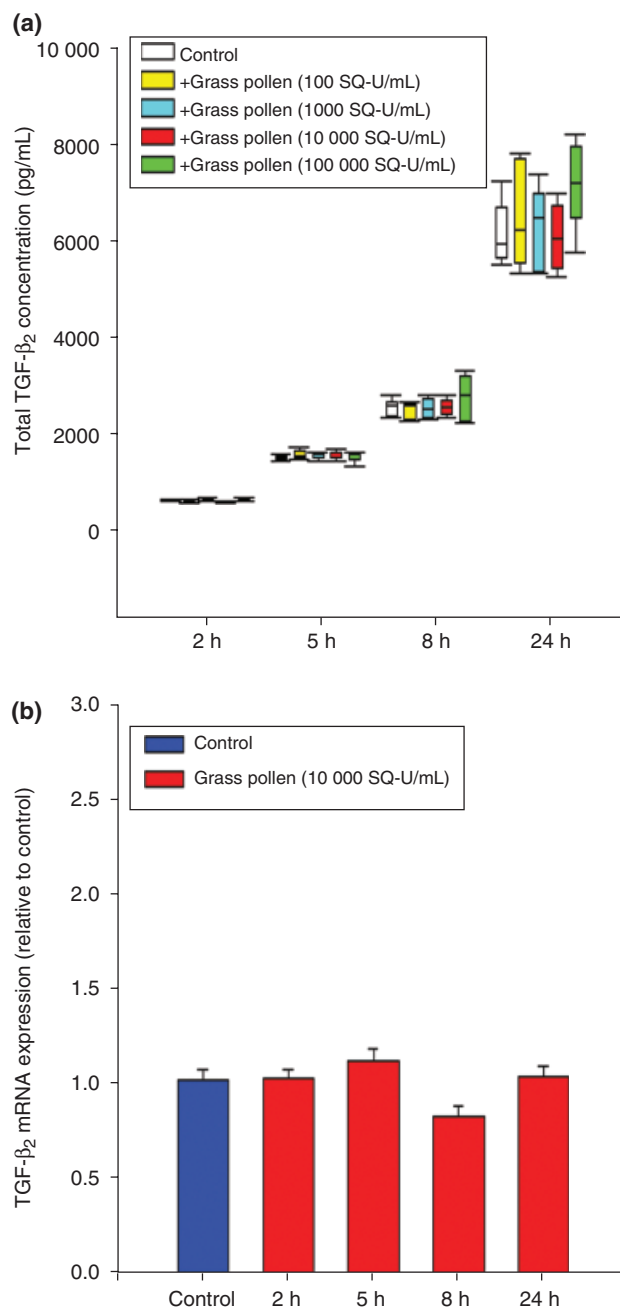


Fig. 4. (a) Transforming growth factor-β₂ (TGF-β₂) protein production by RPMI 2650 cell cultures ($n = 8$) after exposure in medium alone or with grass pollen allergen. The box plots show the median and interquartile range, and the bars show the 10th and 90th percentiles. * $P < 0.05$ vs. control, untreated cells. (b) Effect of grass pollen stimulation on TGF-β₂ gene expression in RPMI 2650 cells. Data are mean $\pm$ SEM ($n = 8$) and are normalized relative to TGF-β₂ gene expression in untreated (control) cells.

With the RPMI 2650 cell line we have shown a time-dependent increase in TGF-β₂ supernatant concentrations. The addition of HDM allergen extracts resulted in a concentration-dependent increase in this release. This was evident at 2, 5, and 8 h but not at 24 h. The results showed a significant increase in TGF-β₂ release at HDM concentrations as low as 625 U/mL. The equivalent dose-response profile for grass pollen challenge was not evident, even when the grass pollen allergen stimulus was increased up to

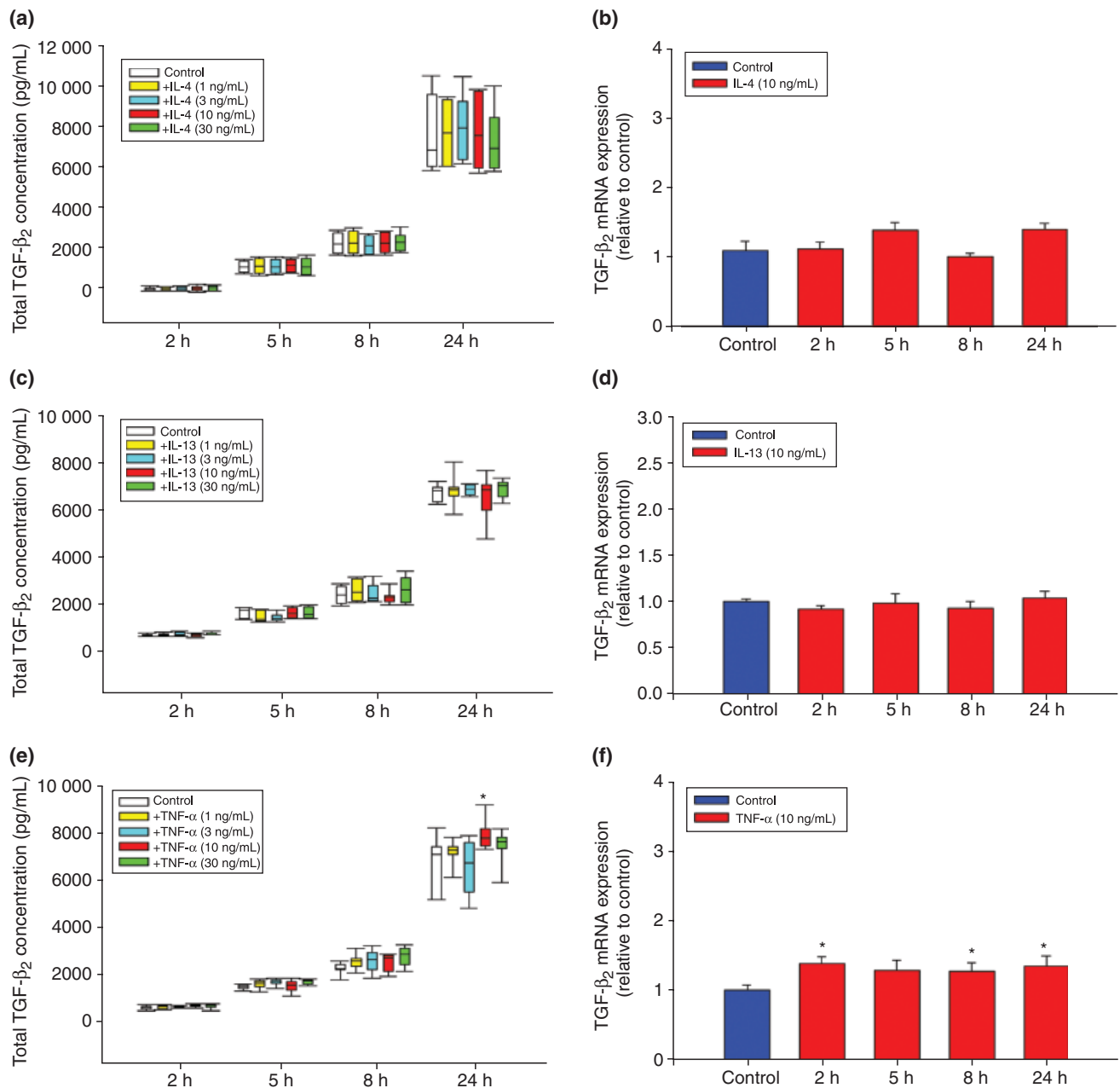


Fig. 5. (a, c, e) Transforming growth factor- β 2 (TGF- β 2) protein production by RPMI 2650 cell cultures ($n = 8$) after exposure in medium alone or with IL-4, IL-13, and TNF- α . The box plots show the median and interquartile range, and the bars show the 10th and 90th percentiles. (b, d, f) Effect of IL-4, IL-13, and TNF- α treatment on TGF- β 2 gene expression in RPMI 2650 cells. Data are mean $\pm$ SEM ($n = 8$) and are normalized relative to TGF- β 2 gene expression in untreated (control) cells.

100 000 SQ-U/mL. Previous *in vivo* studies have identified that nasal allergen challenge with grass pollen allergens at concentrations of 100–10 000 SQ-U produces a dose-dependent increase in nasal symptoms and plasma protein exudation [15]. With the 500 μ L challenge volume *in vitro*, the highest dose of grass pollen allergen used, at 50 000 SQ-U, is substantially greater than that required *in vivo* to induce cell activation and symptoms. These findings thus identify a fundamental difference between HDM allergens and grass pollen allergens in their ability to stimulate nasal epithelial cell activation. This difference was also evident when analysis was made of the TGF- β 2 gene expression responses to

challenge by real-time quantitative TaqMan RT-PCR analysis. The basis for these different responses to HDM and grass pollen requires further elucidation, and may be a manifestation of innate differences in the cell donor's allergic responses to these allergens. Furthermore, there may be differences between these allergens in their protease activity, although controversy exists regarding this [16–18].

Mast cells have been shown to contain preformed cytokines within their intracellular granules, including IL-4, IL-13, and TNF- α [19, 20]. These cytokines have relevance to epithelial cell activation. TNF- α is recognized to be a prominent cytokine involved in epithelial cell activation and *in vitro*

studies have reported that IL-4 and IL-13 have direct effects on epithelial function. IL-4 has been shown to enhance mucin expression by bronchial epithelial cells [21]. TNF- α , as well as IL-4 and IL-13, have been shown to enhance granulocyte macrophage-colony stimulating factor and IL-8 release in asthmatic bronchial epithelial or alveolar cell lines [22–24]. The effects of IL-4, IL-13, and TNF- α on the release of TGF- β_2 from bronchial epithelial cells have been investigated with conflicting findings. One study found evidence of synergistic inhibitive effects of TNF- α and IL-4, acting alone or in concert, on TGF- β production by the 16HBE human bronchial epithelial cell line [25]. In contrast, another study, using primary bronchial epithelial cell cultures from bronchial brushings, demonstrated the ability of both IL-4 and IL-13 to stimulate release of TGF- β_2 from bronchial epithelial cells [26]. We have been unable to detect any effect of IL-4 or IL-13 stimulation on TGF- β_2 gene expression or on protein release from RPMI 2650 cells. A range of concentrations from 1 to 30 ng/mL were studied. By contrast, the same range of concentrations of TNF- α did produce a significant stimulatory response. There was a significant stimulatory effect of TNF- α on TGF- β release into the culture supernatant with the 10 ng/mL at the 24 h time-point. In the studies evaluating the effects of stimulation on gene expression, the selected 10 ng/mL concentration of TNF- α generated a significant increase in TGF- β_2 gene expression at 2, 8, and 24 h. Thus, these cells were able to respond to cytokine stimulation and fundamental differences existed between the ability of TNF- α , on the one hand, and IL-4 and IL-13, on the other, to activate the RPMI 2650 epithelial cells. Of interest, the TNF- α , although stimulating TGF- β_2 gene expression between 2 and 24 h post-exposure, only stimulated protein release at 24 h in contrast to the studies with HDM allergen, which had less of a sustained effect on gene expression but increased TGF- β culture supernatant concentrations between 2 and 8 h post-exposure. This greater effect of HDM allergen may relate not only to *de novo* synthesis but also to cleavage of preformed TGF- β from its storage binding sites through proteolytic activity. In support of this, the study of the kinetics of TGF- β release revealed protein release prior to any effect on gene up-regulation. By contrast, the binding of released TGF- β to receptors and binding sites on the epithelial cell surface may have limited the ease of detection of TGF- β release following TNF- α stimulation. The identification that TNF- α can stimulate TGF- β gene expression and protein release from nasal epithelial cells adds another dimension to our previous findings of a correlation between TGF- β epithelial immunoreactivity *in vivo* and intraepithelial mast cell numbers in AR. We have previously demonstrated that nasal allergen challenge is associated with the local release of TNF- α [27], and the correlation could thus be, in part, accounted for by TNF- α release from mast cells regulating epithelial TGF- β expression. It is probable that this is not the only explanation as the identification of TGF- β_2 receptors on mast cells and the known potent mast cell chemotactic activity of TGF- β suggest that the mast cell–epithelial interaction is bi-directional. In view of the known immunoregulatory functions of TGF- β , it is possible that this mast cell–epithelial cell interaction could serve to limit the influence of subsequently recruited cells, such as T lymphocytes, thereby

limiting their potentiating role in exaggerating the inflammatory response.

These studies have all been conducted on the nasal epithelial cell line, RPMI 2650, which is less well characterized than a number of bronchial epithelial cell lines. These cells are not fully formed epithelial cells, with our scanning electron microscopic assessment of the RPMI 2650 cells confirming the findings of a previous study [12], namely that the cells grew in multiple layers and that they did not possess any cilia. Despite these findings, this cell line has been shown to closely resemble normal human nasal epithelium with respect to its karyotype [10], cytokeratin polypeptide expression patterns [11, 12], and the presence of mucoid material on the cell surface [8]. Both monolayer [28] and multilayer [9] growth patterns have been observed with the RPMI 2650 cell line. The trans-epithelial electrical resistance of the RPMI 2650 cell sheets cultured on porous membranes has been reported to range from 106 to 122.8 Ωcm^2 [9], which is similar to that reported for freshly excised human nasal epithelium (69.7–80.3 Ωcm^2) [29]. These values are typical of leaky-type polarized epithelia, although RPMI 2650 cells have been observed to form tight junctions [9]. We used flow cytometry to evaluate the ability of these cells to express TGF- β receptors. This suggested a low level of expression on non-stimulated RPMI 2650 cells. HDM stimulation resulted in a small but insignificant increase in the level of TGF- β receptor expression. However, the addition of anti-TGF- β_2 human antibody to either the non- or HDM-stimulated cells resulted in an appreciable increase in the levels of TGF- β receptor expression. This is consistent with the TGF- β_2 ELISA results. The ELISA data revealed that there was a significant baseline production of TGF- β_2 by RPMI 2650 cells. Therefore, under normal circumstances, one would expect the TGF- β receptor expression on the cell surfaces to be almost saturated, thus resulting in an apparently low level of TGF- β receptor expression when using the cytokine flow cytometry, as the technique detects the biotinylated TGF- β_1 bound to available TGF- β receptors. However, the addition of anti-TGF- β_2 antibody to non- and HDM-stimulated cells, and its subsequent binding to exogenous TGF- β_2 , should therefore make less of it available for receptor binding, and in the process free more receptors for the biotinylated TGF- β_1 cytokine, with a resultant increase in the apparent receptor number on flow cytometric analysis.

In conclusion, we have demonstrated that the RPMI 2650 nasal epithelial cell line generates TGF- β_2 and expresses TGF- β receptors and is thus relevant to the study of the regulation of this growth factor. HDM allergens related to *Dermatophagoides pteronyssinus* stimulate both release and gene expression for TGF- β_2 , whereas grass pollen allergens do not. No effect of the T-helper type 2 cytokines, IL-4 or IL-13, was discernable in this model, whereas TNF- α promoted TGF- β_2 gene expression and to a lesser extent protein release. As mast cells accumulate within the epithelium in AR generating and releasing TNF- α in the process, it is likely that mast cell–epithelial cell interaction contributes to the epithelial expression of TGF- β and that this interaction may serve an important immunomodulatory role. This represents the first documented use of the RPMI 2650 cell line as an *in vitro* model of human nasal epithelial cells in the study of interactions between upper airway epithelial cells and

regulatory factors such as cytokines and allergens. While the results of this study will require further validation, the use of the RPMI 2650 cell line in allergy studies represents an attractive prospect potentially offering significant benefits in accessibility, time, and economy when compared with primary cell cultures.

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