

# Direct contact between dendritic cells and bronchial epithelial cells inhibits T cell recall responses towards mite and pollen allergen extracts *in vitro*

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## Summary

Airway epithelial cells (AECs) form a polarized barrier along the respiratory tract. They are the first point of contact with airborne antigens and are able to instruct resident immune cells to mount appropriate immune responses by either soluble or contact-dependent mechanisms. We hypothesize that a healthy, polarized epithelial cell layer inhibits inflammatory responses towards allergens to uphold homeostasis. Using an in-vitro co-culture model of the airway epithelium, where a polarized cell layer of bronchial epithelial cells can interact with dendritic cells (DCs), we have investigated recall T cell responses in allergic patients sensitized to house dust mite, grass and birch pollen. Using allergen extract-loaded DCs to stimulate autologous allergen-specific T cell lines, we show that AEC-imprinted DCs inhibit T cell proliferation significantly of Bet v 1-specific T cell lines as well as decrease interleukin (IL)-5 and IL-13 production, whereas inhibition of Phl p 5-specific T cells varied between different donors. Stimulating autologous CD4<sup>+</sup> T cells from allergic patients with AEC-imprinted DCs also inhibited proliferation significantly and decreased production of both T helper type 1 (Th1) and Th2 cytokines upon rechallenge. The inhibitory effects of AECs' contact with DCs were absent when allergen extract-loaded DCs had been exposed only to AECs supernatants, but present after direct contact with AECs. We conclude that direct contact between DCs and AECs inhibits T cell recall responses towards birch, grass and house dust mite allergens *in vitro*, suggesting that AECs-DC contact *in vivo* constitute a key element in mucosal homeostasis in relation to allergic sensitisation.

**Keywords:** allergy, dendritic cells, human, T cells, tolerance, mucosa

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## Introduction

The human lung is constantly exposed to viruses, bacteria, environmental factors and airborne allergens that potentially threaten our health [1]. The airway epithelial cells (AECs) cover the surfaces of the airways and are thus often the first point of contact with external antigens.

During homeostasis, epithelial conditioning may involve the induction or generation of specific dendritic cell (DC) subsets, resulting in T cell anergy or induction of regulatory T cells [referred to as Tr1 or inducible regulatory T cells (T<sub>regs</sub>)] that suppress undesirable hyper-responsiveness towards allergens [2]. DC subsets are divided commonly into two major subgroups: the

CD11c<sup>+</sup> myeloid DCs (mDCs; also referred to as 'conventional' DCs) and the CD11c<sup>−</sup> plasmacytoid DCs (pDCs), although this is an area of some debate [3]. There is convincing evidence that both mDCs and pDCs are involved in the pathogenesis of allergen-induced rhinitis, where mDCs are critical inducers of allergen-specific T helper type 2 (Th2) cells [4], whereas pDCs are suggested to be involved in tolerance induction towards inhaled allergens [5–7].

The mechanisms behind allergic reactions are well characterized, but the initial stages of allergen recognition by innate immune cells leading to the induction of a Th2 response and the synthesis of immunoglobulin (Ig)E are less clear. An imbalanced and overactive immune

response against allergens has long been thought to be the primary mechanism behind allergy. A more recent hypothesis points to the dysregulation of the epithelial barrier and its interactions with resident immune cells as the primary defect in the pathogenesis of allergic reactions [8,9].

Epithelial cells recognize pathogen-associated molecular patterns (PAMPs) through specialized pattern recognition receptors such as Toll-like receptors [10]. Upon recognition of antigens, AECs help to determine the functional properties of innate immune cells in the tissues by either soluble or contact-dependent mechanisms. AECs can recruit monocytes and fully differentiated DCs and condition them towards either regulatory or immunogenic responses [11–15]. In response to inhaled allergens, AECs secrete a variety of chemokines and cytokines, among them thymic stromal lymphopoietin (TSLP), interleukin (IL)-25, IL-33 and IL-1b, that play a role in the induction of inflammatory Th2 responses [12,16–19].

Transcriptomic analyses have shown that healthy individuals raise an epithelial response after intranasal allergen challenge, albeit following a different activation pattern compared to epithelium from allergic patients [20]. In epithelium from allergic patients the increase in transcribed proteins was connected with enhanced uptake properties, suggesting that there might be genetic disease-induced differences in how AECs react with allergens [21]. Several of the known allergens, such as those from house dust mite (HDM), are proteases and may thus be able to disrupt the epithelial barrier to gain access to the airways and facilitate allergen sensitization [22–24]. However, protease activity is not a general feature of all allergens and it still remains unclear exactly how important the protease activity of some allergens is for sensitization. Studies have linked Toll-like receptor (TLR)-4 on AECs to both proteinase-dependent and -independent allergic responses in mice [25,26]. A recent study found that TLR-4 was activated by proteases in the airways to initiate allergic airway disease immunity. These immune responses were induced by protease cleavage of the clotting protein fibrinogen, yielding fibrinogen cleavage products that acted as TLR-4 ligands on airway epithelial cells and macrophages, suggesting a potential link between allergic immune responses and fibrinogen and thereby indirectly to protease activity [27].

Other allergens, such as Phl p 1 from timothy grass, have been found to activate AECs by non-proteolytic mechanisms, while being able to be transported actively through the epithelium [28]. Birch pollen allergen is also transported actively through the respiratory epithelium, but only in allergic individuals [9].

Moreover, bronchial epithelial cells from asthmatics fail to polarize *in vitro*, unlike bronchial epithelial cells taken from healthy individuals, and airway epithelial cells in

asthmatic patients have a reduced expression of tight junction proteins [29–31], suggesting that compromised epithelial polarization also plays a role in allergic disorders. It has also been shown that inhibitory signals between AECs and DCs depend upon interactions with E-cadherin, a tight junction protein [32,33]. As loss of E-cadherin in cultured ECs has been shown to enhance TSLP production, it has been theorized that this leads to a loss of inhibitory signalling and facilitates allergic sensitization by activating DCs [34].

We have developed an *in-vitro* model to study how intact polarized AEC affect neighbouring cells and T cell responses. The model uses the 16HBE14o<sup>–</sup> bronchial epithelial cell line, which has been characterized to have a non-serous, non-ciliated phenotype and to form a confluent, polarized cell monolayer with the expression of both drug transport proteins and functional tight junctions [35]. With this model we have shown that AEC-imprinted monocyte-derived DCs (MDDCs) exhibit an altered phenotype with decreased levels of secreted inflammatory cytokines in response to activation by lipopolysaccharide (LPS) [36]. Furthermore, the AEC-imprinted DCs induced lower T cell proliferation in autologous Bet v 1-specific T cells, compared to non-imprinted DCs [36]. These results support the theory that an intact, healthy epithelial layer provides a microenvironment that supports tolerance to allergens. It is still unknown whether allergic individuals mount an exaggerated response towards allergens or/and fail to develop a tolerogenic response to maintain homeostasis. In addition, whether allergic reactions are triggered by inherent defects in the epithelium or certain Th2-inducing properties of allergens, or a combination of both, has yet to be clarified.

In the present study we have utilized our *in-vitro* model system to investigate how AEC-imprinting of DCs loaded with extract from three separate allergens, HDM, birch and timothy grass pollen, affects autologous T cell responses. To do this, allergen extract-loaded DCs, with or without AEC imprinting, were allowed to stimulate primary T cell responses as well as recall responses from pre-established birch and grass allergen-specific T cell lines.

## Material and methods

### Reagents, antibodies and cell lines

The antibodies used comprised: anti-CD11c [phycoerythrin (PE); BD Pharmingen, Albertslund, Denmark; cat. no. 555392 or peridinin chlorophyll (PerCP)-effluor 710; eBioscience, Frankfurt, Germany; cat. no. 460116], anti-CD80 (PE; BD Pharmingen; cat. no. 557227), anti-CD274 [fluorescein isothiocyanate (FITC); BD Pharmingen; cat. no. 558065], anti-human leucocyte antigen D-related

(HLA-DR) [FITC; BD Pharmingen; cat. no. 347400 or allophycocyanin (APC)-H7; BD Pharmingen; cat. no. 641393, IgG1 (FITC) BD Pharmingen; cat. no. 33814], IgG2a (APC; Nordic Biosite, Copenhagen, Denmark; cat. no. 400222), IgG1 (PE, BD cat. no. 349043), anti-CD40 (FITC; BD Pharmingen; cat. no. 555588), anti-CD23 (APC; eBioscience; cat. no. 17-0238-42), anti-ILT3 (APC; eBioscience; cat. no. 17-5139-42), anti-PD-L1 (FITC; BD Pharmingen; cat. no. 558065) and anti-CD83 (APC; BD Pharmingen; cat. no. 551073). The AEC line, 16HBE140-, was established by transformation of normal bronchial epithelial cells obtained from a 1-year-old male heart-lung transplant patient and was a kind gift from Professor Dieter C. Gruenert (California Pacific Medical Center Research Institute, University of California, San Francisco, CA, USA) [37]. Allergen extract from *Betula verrucosa*, *Phleum pratense* and *Dermatophagoides pteronyssinus* was prepared in-house [38]. Some extracts were labelled with FITC using an allergen : FITC molar ratio of 1 : 20 [38]. Endotoxin levels in allergen extracts were measured to be below 11 EU/mg.

### Culturing medium

The AEC line was cultured in two different types of medium. The minimum essential medium (MEM)-based culture medium used consisted of: MEM (Lonza, Basel, Switzerland; cat. no. BE12-125F) with the addition of 1% (V/V) L-glutamine (Lonza; cat. no. 17-605C), 1% (V/V) Na-Pyruvate (Lonza; cat. no. BE13-115E), 1% (V/V) NEAA (Lonza; cat. no. BE13-114E), penicillin (1000 U/ml)/streptomycin (1000 U/ml) (Invitrogen, Carlsbad, CA, USA; cat. no. 15140-122), 2.5% (V/V) HEPES (Lonza; cat. no. 17-737F), 4 ng/ml Gentamycin (Lonza; cat. no. BE02-012E) and 10% (V/V) heat-inactivated fetal calf serum (FCS) (Invitrogen; cat. no. 10108-165). The RPMI-based culture medium used to generate monocyte-derived dendritic cells consisted of RPMI (Lonza; cat. no. BE12-1155/U), 5% human AB-serum (Lonza; cat. no. 14-490E), 1% (V/V) Na-Pyruvate (Lonza; cat. no. BE13-115E), 1% (V/V) NEAA (Lonza; cat. no. BE13-114E), penicillin (1000 U/ml)/streptomycin (1000 U/ml) (Invitrogen; cat. no. 15140-122), 2.5% (V/V) and 4 ng/ml gentamycin (Lonza, cat. no. BE02-012E). Coating solution for filter inserts consisted of LHC basal medium (Invitrogen; cat. no. 12677), 1% W/V collagen, bovine type I (BD Bioscience; cat. no. 354231), 1% W/V fibronectin (BD Bioscience; cat. no. 354008) and 1% W/V bovine serum albumin (BSA).

### Co-culture model system

The co-culture model was used as described in previous work [36]. In brief, the AEC line was thawed and cultured in advance for 4–6 days. The culture inserts were made of polyethylene terephthalate with a pore size of

3 µm (BD Pharmingen; cat. no. 353093) and AECs were cultured on the top of the inverted filter inserts. The inverted inserts were placed into beakers with MEM-based culture media covering the insert to a level of 4 mm above the filter for 10–12 days at 37 °C with 5% CO<sub>2</sub>. At this point, filters were reverted and placed into wells with RPMI-based culture media, and MDDCs suspended in RPMI-based culture media were added to the basal side within the insert well.

### Transepithelial electrical resistance (TEER) measurements

To monitor and verify epithelial polarization the transepithelial electrical resistance TEER ( $\Delta R_{TEER}$ ) was measured daily, as described previously [36]. In brief, the mean resistance (in  $\Omega$ ) of three to five measurements per insert with cell layers ( $\overline{R_{Cell+insert}}$ ) was subtracted with the mean resistance of a cell culture insert without cells ( $\overline{R_{Insert}}$ ), and then multiplied with the insert surface area  $A_{Insert}$  (in cm<sup>2</sup>):

$$\Delta R_{TEER} = A_{Insert} \cdot (\overline{R_{Cell+insert}} - \overline{R_{Insert}}) \quad (1)$$

### Generation of monocyte-derived DCs

Blood samples were obtained from donors allergic to house dust mites, grass or birch in accordance with the guidelines of the local ethics committee. Respective allergen sensitization in donors was confirmed by specific IgE levels above 3.5 kU/l. CD14-positive cells were isolated using the EasySep<sup>®</sup> Human CD14 positive selection kit according to the manufacturer's protocol (Stemcell Technologies, Grenoble, France; cat. no. 18058). MDDCs were generated employing a previously described 7-day protocol with the addition of recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) (Berlex Leukine<sup>®</sup>; Genzyme, Cambridge, MA, USA) and recombinant human IL-4 (Peprotech, Hamburg, Germany; cat. no. 200-04) [39].

### Flow cytometric assessment of MDDC surface markers

After 24 h co-culturing, filter inserts were incubated for 25 min in phosphate-buffered saline (PBS) with 2 mM ethylenediamine tetraacetic acid (EDTA) at 4 °C and the loosely bound MDDCs were detached and collected by gently flushing the basal side of AECs on the filter inserts using PBS with EDTA. DCs were spun down, resuspended in RPMI-based media. Flow cytometry analyses were performed on a BD fluorescence activated cell sorter (FACS)-Calibur or BD FACSaria calibrated with fluorophore labelled beads, and compensation was adjusted for each experiment based on the applied antibodies. DCs were identified and gated by their expression of CD11c.

### Measurements of cytokine secretion

Cytokine content in cell supernatants was assessed by multiplex electrochemiluminescent immunoassays (MesoScale Discovery, Gaithersburg, MD, USA). MDDC-derived cytokines were assessed using a kit for measurement of eotaxin, monocyte chemoattractant protein-1 (MCP)-1, IL-1 $\beta$ , IL-6, tumour necrosis factor (TNF)- $\alpha$ , IL-10 and IL-12p70. TSLP was analysed on a single-spot kit. AEC-derived cytokines were assessed using a kit for measurement of eotaxin, eotaxin-3, IL-8, IP-10, MCP-1, MCP-4, MDC and macrophage inflammatory protein-1 $\beta$  (MIP-1 $\beta$ ) and T cell-derived cytokines were assessed using a kit for measurement of interferon (IFN)- $\gamma$ , IL-10, IL-12p70, IL-13, IL-2, IL-4 and IL-5. All cytokines in the respective kits were measured in a single well of a 96-well plate and all measurements were performed in duplicate or triplicate. All reagents were provided with the kit and analyses followed the manufacturer's guidelines. The electrochemiluminescence of the secondary ruthenium-conjugated antibody was read in a MesoScale Discovery Sector (MSD) Imager plate reader and analysed using the associated workbench software (MesoScale Discovery, Gaithersburg, MD, USA).

### T cell line generation

Pre-generated Bet v 1- or Phl p 5-specific T cell lines [40,41] were thawed, washed and cultured in RPMI with 5% human AB-serum (Lonza; cat. no 14-490E). Sixty-five IU/ml recombinant human (rh)IL-2 (Chiron, Emeryville, CA, USA; cat no 004184) was added on day 3 and 30 IU/ml of rhIL-2 was added on days 4 and 5. Cells were cultured for a total of 10 days before usage.

### DC T cell co-culture and analysis

Following AEC imprinting, DCs not used for FACS analysis were co-cultured together with pre-expanded autologous Bet v 1- or Phl p 5-specific T cells in duplicates at a ratio of 25 000 DCs to 100 000 T cells in 200  $\mu$ l/well in 96-well plates. Prior to co-culture, DCs were irradiated to prevent proliferation of any remaining AECs in culture. After 48 h of co-culture [ $^3$ H]-thymidine was added to cells at a concentration of 2.5  $\mu$ Ci/ml. After an additional 16–20 h, T cell proliferation was measured by thymidine incorporation.

### Statistics

Statistical analysis was performed by two-tailed *t*-test, with *P*-values below 0.05 considered significant, and labelled \*. *P*-values below 0.01 are labelled \*\* and *P*-values below 0.001 are labelled \*\*\*.

## Results

### Allergens' effect on AECs

To investigate how allergen exposure affects the epithelial microenvironment, birch, grass or HDM extracts were

added to the epithelium's apical side after epithelial polarization was established. Cell culture supernatants were collected 24 h later from the AECs basal side and analysed for cytokine production (Fig. 1). There was a general trend of decreased chemokine production by AECs after allergen extract administration. Significantly decreased levels of interferon gamma-induced protein 10 (IP-10) were secreted after birch and grass extract exposure (Fig. 1a), whereas MDC levels were decreased significantly after grass and HDM exposure (Fig. 1b). MIP-1 $\beta$  and MCP-1 also appeared to be down-regulated slightly after administration of all three allergens, respectively, but these changes were not significant (Fig. 1c,d). IL-10, IL-12, IL-6, IL-8, eotaxin and TNF- $\alpha$  levels were unchanged by allergen extract administration (results not shown).

### Allergens' effect on DC phenotype

The interaction between co-stimulatory receptors is an important signalling event during activation of naive and memory T cells, and we investigated how birch and grass pollen extracts affected the DC phenotype with or without the influence of the epithelium. The expression of membrane receptors on the surface of DCs was analysed by flow cytometry after 24 h of allergen administration, with or without DCs in direct contact with AECs (Fig. 2). The allergen extracts were either administered directly to the DCs on the epithelium's basal side or apically, thus allowing uptake across the epithelial layer.

We did not observe any phenotypical changes by direct administration of grass or birch pollen to DCs. The changes in DC phenotype were due to the epithelial contact and not to allergen administration, which is consistent with earlier findings [36]. CD80 and programmed death ligand 1 (PD-L1) were clearly up-regulated on DCs that had been in contact with the epithelium. An up-regulation of CD80 and PD-L1 was also observed on DCs that were cultured in AEC-conditioned supernatant; however, this change was not significant (data not shown). The membrane proteins CD23, ILT3 and CD209 were also investigated, but their expression was not affected by allergen extract exposure or epithelial contact (not shown).

### AEC-imprinted DC cytokine production in response to birch and grass extracts

Cytokines produced by DCs are important, and play a major role in T cell polarization. As the tested allergen extracts had no effect on any of the phenotypical markers investigated, we analysed DC culture supernatants for IL-10, IL-12p70, IL-6, TNF- $\alpha$ , eotaxin and MCP-1, 24 h after birch or grass pollen extract administration (Fig. 3). Administration of birch pollen extract induced increased secretion levels of eotaxin as well as

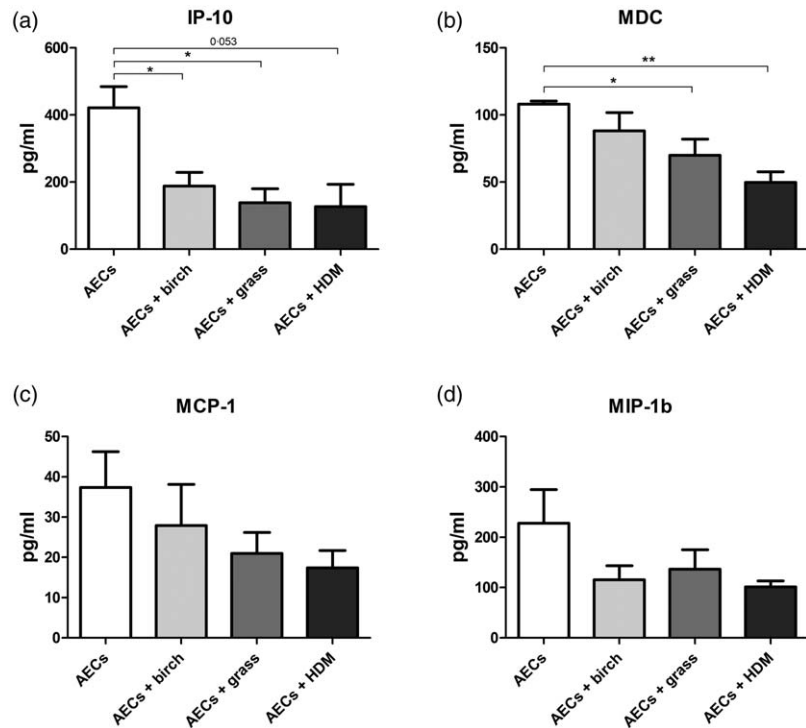


**Fig. 1.** Airway epithelial cell (AEC) cytokine secretion in response to allergen extract. After allowing the AECs to polarize, 100 µg/ml of birch, timothy grass or house dust mite (HDM) extracts were added to the AECs' apical side. Supernatants from AECs' basal side were collected after 24 h and analysed for levels of interferon gamma-induced protein 10 (IP-10) (a), myeloid dendritic cells (MDC) (b), monocyte chemoattractant protein 1 (MCP-1) (c) and macrophage inflammatory protein 1β (MIP-1β) (d). The results of three independent experiments are shown and error bars represent standard error of the mean (s.e.m.).

of IL-10 by AEC-imprinted DC (Fig. 3a). Eotaxin secretion by AEC-imprinted DCs was also increased after administration of grass extract, suggesting that this was most probably a general result of the AEC imprinting. However, grass extract failed to induce elevated levels of IL-10 (Fig. 3b). Extracts from grass and birch allergen had no effect on cytokine levels of MCP-1, IL-12, TNF-α and IL-6 in supernatants in any samples (not shown).

#### Allergen-specific T cell line proliferation and cytokine production

After DCs sample allergens in the periphery they migrate to the draining lymph node, where they encounter and activate naive or memory T cells in sensitized individuals. To investigate the activation of memory T cells in our model, AEC-imprinted DCs were exposed to either birch or grass extract, and subsequently co-cultured with autologous T cell lines specific for either Bet v 1 or Phl p 5. After 3 days of co-culture T cell proliferation was measured by thymidine incorporation (Fig. 4). Co-culturing the Bet v 1-specific T cells together with AEC-imprinted DCs resulted in a significantly decreased T cell proliferation compared with T cells stimulated with non-imprinted DCs (Fig. 4a). The same effect was observed whether the allergen extract was administered to the AECs' apical side or directly to DCs in contact with the AECs' basal side. The down-regulation was not observed with Phl p 5-specific T cells from grass allergic donors (Fig. 4b). Furthermore, the decreased prolifera-

tion was not observed with DCs cultured in AEC-conditioned supernatant, which did not have any effect on T cell proliferation in either T cell line (results not shown).

The efficacy of T cell responses depends heavily upon the production of T cell-specific cytokines. After 48 h of DC-T cell co-culture, supernatants were collected and analysed for a panel of Th1/Th2-specific cytokines (Fig. 5). AEC-imprinted DCs induced lower levels of Th2 cytokines IL-5 and IL-13 from Bet v 1-specific T cells compared to co-cultures with non-imprinted DCs, but only changes in IL-5 secretion were significant (Fig. 5a). Down-regulation of IL-5 and IL-13 was not seen with the Phl p 5-specific T cells (Fig. 5b). This correlated well with the decreased proliferation of the Bet v 1-specific T cells, showing that the AEC-imprinted DCs affected not only the proliferation rate of the T cells but also their cytokine production. The other investigated cytokines showed similar down-regulatory trends, although the changes between the two investigated set-ups were not significant (results not shown).

#### Primary T cell proliferation and cytokine production

Working with fully differentiated Th2 cells in the form of Th2 cell lines restricts the study of how polarization schemes can be modulated. In order to study the effect of AEC imprinting on freshly isolated CD4<sup>+</sup> T cells from donors sensitized to birch, grass or house dust mites, T cells were isolated and co-cultured with autologous AEC-

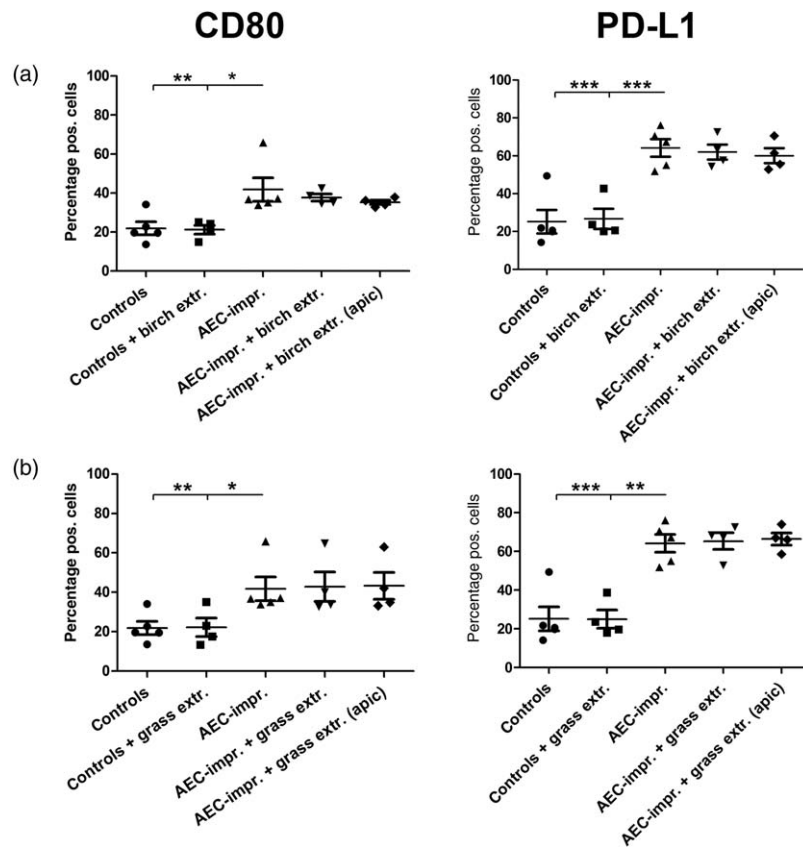


**Fig. 2.** Birch and timothy grass pollen effect on dendritic cell (DC) phenotype. Airway epithelial cell (AEC)-imprinted DCs were exposed to birch (a) or grass pollen (b) extract either directly, with extract added to the AECs' basal side, or indirectly with allergen extract added to the apical side of the AECs, allowing for DC uptake across the epithelial layer. After 24 h DCs were collected and analysed by flow cytometry for expression of CD80 and programmed death ligand 1 (PD-L1). DCs were discriminated by gating on CD11c<sup>+</sup> cells and distinct populations and frequency of positive cells was calculated based on the total number of CD11c<sup>+</sup> cells. The figure illustrates five independent experiments and error bars represent standard error of the mean (s.e.m.).

imprinted DCs after allergen extract administration (Fig. 6).

After restimulation, T cells co-cultured previously with AEC-imprinted DCs did not proliferate to the same degree as T cells co-cultured with non-AEC imprinted DCs. This trend was observed with all three allergen extracts investigated and all donors followed this trend, although the degree of inhibition varied between donors. The decreased proliferation was, however, only significant with the primary T cells co-cultured with AEC-imprinted DCs with grass pollen (Fig. 6b).

To investigate the T cell responses fully, supernatants were harvested 48 h after restimulation and screened for T cell-specific cytokines (Fig. 7). Restimulating *de-novo* T cells from patients allergic to grass, co-cultured previously with AEC-imprinted DCs, produced decreased levels of IL-13 and IL-5 (Fig. 7a). *De-novo* T cells from HDM allergics also showed a trend of decreased cytokine production; however, only changes in IL-10 secretion were significant (Fig. 7c). No change in cytokine secretion was observed after restimulation of T cells from birch allergics (Fig. 7b).

## Discussion

There is emerging evidence that the pathogenesis of allergy and asthma depends heavily upon the interactions

between the exposed epithelial surface and its neighbouring DC populations [42]. The local microenvironment in which DCs sample allergens has been suggested to play a major role in DC maturation and profiling of immune responses [10,43,44]. DCs are recruited continuously to the airways from the circulation, either as fully differentiated DCs or as monocyte precursors that mature into DCs. AECs set the microenvironment in which the DCs sample the allergens, and subsequently migrate to local draining lymph nodes to stimulate T cells.

The objective of this study was to investigate the effect of DCs, taking up allergens in direct association with intact, healthy airway epithelium on T cell activation. Using a previously characterized *in-vitro* model system of the bronchi [36], we have shown that DCs in contact with intact and polarized AECs exhibit a more tolerogenic phenotype after LPS stimulation [36], and we hypothesized that DCs in contact with an intact epithelium would induce tolerance and help to control hyperimmunogenic responses towards allergens. In this study we investigated the effect of AEC imprinting of DCs in the presence of extracts from the most common allergens, birch, grass and HDM, using specific T cell responses and primary T cell responses as end-points.

Although our *in-vitro* model does not simulate an environment completely similar to the airways of allergic individuals, it enables settings of a healthy environment,

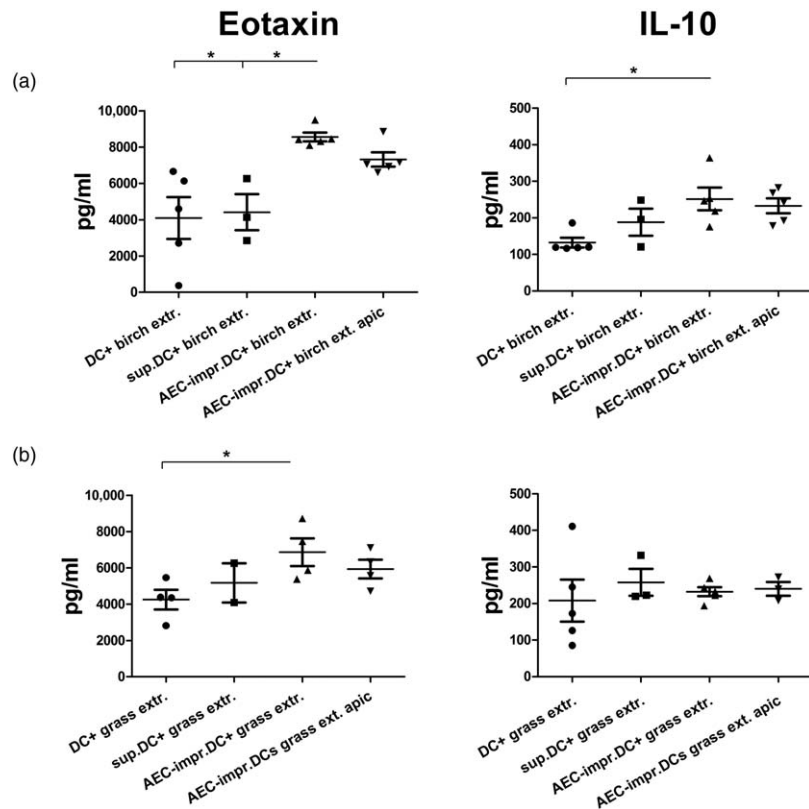


**Fig. 3.** Dendritic cell (DC) cytokine production induced by birch (a) or timothy grass (b) pollen. DCs were cultured with either airway epithelial cell (AEC) supernatant (sup.DC) or in direct contact with AECs (AEC-impr.DC). The figure represents results from five individual experiments with direct contact with AECs and three individual experiments with AEC supernatants with each allergen extract. Error bars represent standard error of the mean (s.e.m.).

making it possible to investigate the regulatory interactions between epithelium and DCs present during homeostasis. The dysregulation and dysfunction of the airway epithelium seen in asthmatic patients have been hypothesized to be responsible for skewed signalling from AEC, promoting inflammatory responses instead of upholding homeostasis [9,45,46].

Exposing the AEC line, 16HBE14o<sup>-</sup>, to birch pollen, grass pollen or HDM extracts showed a general trend of down-regulation of the production of the chemokines IP-10, MDC, MIP-1 $\beta$  and MCP-1, which are all associated with the recruitment of monocytes, DCs and T cells [47–50], implicating that these allergens do not induce any inflammatory signalling from AEC at steady state conditions.

This is in contrast to studies on another human bronchial epithelial cell line, BEAS-2B, where induced expression of MCP-1, regulated upon activation T cell-derived and secreted (RANTES) and IP-10 was reported after administration of Der p 1, which is a major allergen of HDM [51]. One of the main differences between the BEAS-2B and the 16HBE14o<sup>-</sup> cell lines is the former's inability to form tight junctions and polarize, suggesting that a polarized epithelium alters signalling properties. However, such a link between differences in AEC signalling AND lack of polarization has to be investigated further in future studies.

We did not observe any changes in membrane receptor expression on DCs after allergen extract exposure. We found that CD80 and PD-L1 were both up-regulated on DCs regardless of extract addition after AEC imprinting. A study by Rate *et al.* has shown that AEC-imprinted DCs up-regulate their expression of CD80 using the same 16HBE14o<sup>-</sup> cell line, which is consistent with our observations [14]. In contrast to the co-stimulatory properties of CD80, PD-L1 interacts with PD-1 on T cells, and acts as an inhibitor of T cell proliferation [52,53]. One group has reported up-regulation of CD80 and CD86 after Bet v 1 administration on DCs [54] and that this up-regulation was observed in both allergic and non-allergic individuals, although it was significant only on the DCs from allergics. We cannot exclude that this discrepancy may be caused by the use of purified allergen (Bet v 1) *versus* the allergen extract used in our experiments or differences between recombinant and natural allergens. Expression of CD86 and OX40L was also investigated; however, we did not find any change in CD86 due to AEC or allergen exposure and we did not find any OX40L expression on our DCs (results not shown). OX40L has been suggested to play a crucial role in the initiation of inflammatory Th2 responses and has been associated closely with TSLP activation of DCs [16]. However, we have not detected any TSLPR expression on our monocyte-derived DCs. This corresponds well with

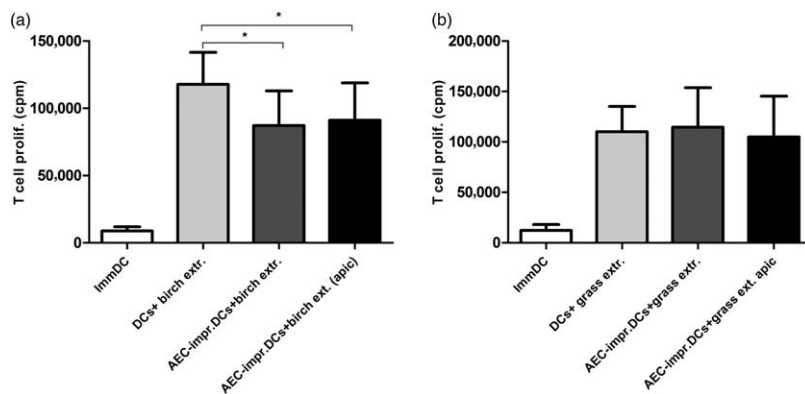


**Fig. 4.** Proliferation of allergen-specific autologous T cells. Seventy-two h after co-culturing airway epithelial cell (AEC)-imprinted dendritic cells (DCs) with either an autologous Bet v1-specific T cell line (a) or Phl p 5-specific T cell line (b) proliferation was investigated by thymidine incorporation. The figure shows mean results of eight individual donors for birch-specific T cell lines and four individual donors for grass-specific T cell lines. Error bars represent standard error of the mean (s.e.m.).

studies showing that myeloid DCs are the main responders to TSLP, and not monocyte-derived DCs [55].

The study by Rate *et al.* also showed that AEC-conditioned DC increased expression of Th1-associated chemokines with little change in Th2-associated chemokines [56]. Although the DC phenotype was mainly unaffected after exposure to extract from either allergen, we observed an induced production of eotaxin and IL-10 in AEC-imprinted DCs, which is in contrast with their findings. However, as discussed below, they found similar effects of AEC-imprinted DCs on T cells [48]. The increased expression of IL-10 was observed only after birch pollen administration and not after grass pollen administration. This was observed only after direct

contact between DCs and AECs, indicating that the differences in cytokine secretion are due most probably to the interactions between allergen, AECs and DCs. As supernatant from AECs and DCs cannot be separated after co-culture, we cannot exclude that the increased cytokine production originated from the AECs. However, AECs did not produce any eotaxin or IL-10 in response to allergen alone or from AEC–DC interaction alone.

Increased IL-10 production in APCs in response to allergen has been observed in *ex-vivo* studies, where administration of Phl p 5 to samples of oral epithelium induced higher levels of IL-10 from oral Langerhans cells [57], but the effect of epithelial cell contact was not investigated in that study. The increased IL-10 production after

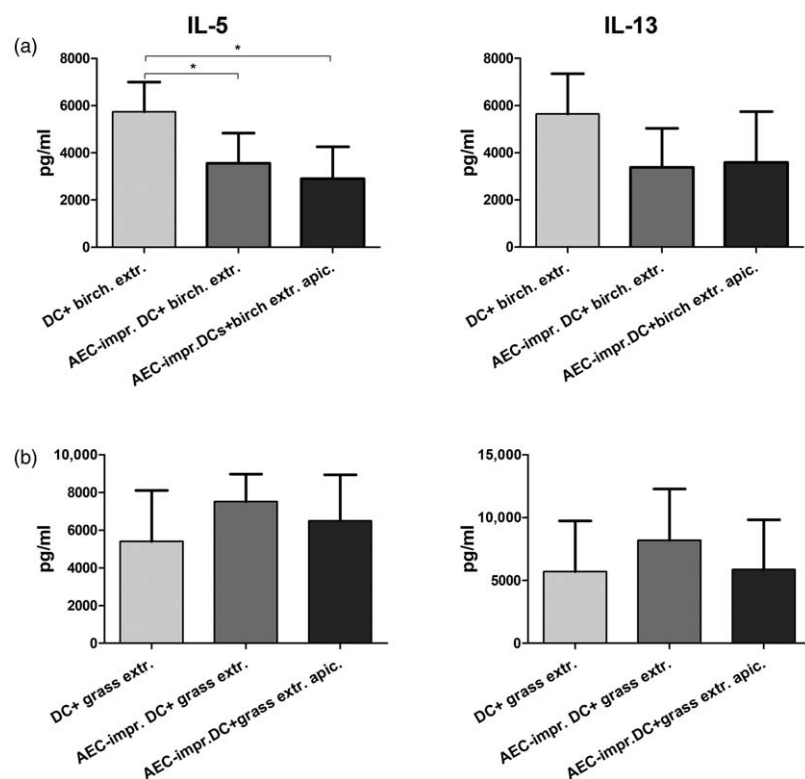


**Fig. 5.** Allergen-specific T cell cytokine production after dendritic cell (DC) co-culture. Forty-eight h after co-culturing airway epithelial cell (AEC)-imprinted DCs with either an autologous Bet v1-specific T cell line (a) or Phl p 5-specific T cell line (b) supernatants were harvested and analysed for T cell cytokines. The figure shows mean results of eight individual donors for birch-specific T cell lines and four individual donors for grass-specific T cell lines. Error bars represent standard error of the mean (s.e.m.).

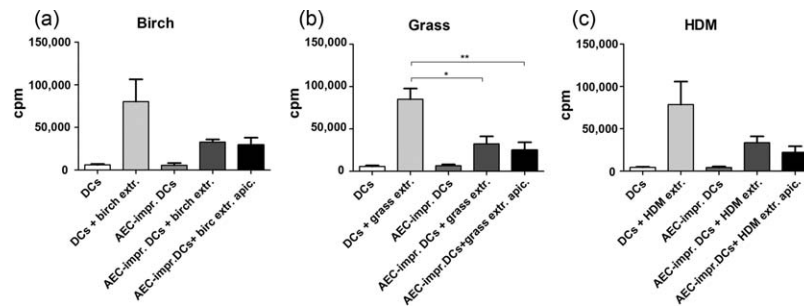


**Fig. 6.** Proliferation of restimulated primary T cells. Primary T cells were expanded with extract-pulsed airway epithelial cell (AEC)-imprinted dendritic cells (DCs) and restimulated with autologous allergen extract-loaded peripheral blood mononuclear cells (PBMCs). T cell proliferation from birch (a), timothy grass (b) and house dust mite (HDM) (c) donors was analysed after 72 h by thymidine incorporation. The figure shows mean results of four individual timothy grass- and HDM-allergic donors, respectively, and three individual birch-allergic donors. Error bars represent standard error of the mean (s.e.m.).

birch administration could potentially be responsible for the inhibited proliferation and cytokine secretion observed with the birch-specific T cell lines, but not with the grass allergen-specific T cell lines. However, addition of anti-IL-10 receptor to co-cultures in preliminary experiments did not reinstate T cell proliferation, although this has yet to be investigated in detail (data not shown). Furthermore, the effect of allergen extracts on the DC phenotype was similar, regardless of how the extracts were administered, suggesting that it is actually the interactions between the epithelium and DCs that are important for the imprinting, and not the amount of extract available or whether it is taken up through the epithelium. Moreover, the airway epithelium is capable of producing several cytokines in response to allergens that can influence DCs, such as TSLP, IL-33 and IL-25, and this may be addressed in future experiments to characterize fully the interplay between AEC and DC. However, inhibition by the AEC-imprinted DCs on the birch-specific T cell lines was observed only with DCs that had been in direct contact with AEC and not with DCs exposed only to AEC supernatants, indicating that AEC cytokines mentioned above are not the direct cause of the imprinting.

Any steady state inhibitory signals transmitted from intact, polarized epithelium to the neighbouring DCs would be expected to inhibit T cell proliferation from both Bet v 1- and Phl p 5-specific T cell lines. Eight birch allergic donors and four grass allergic donors were investigated and analysing individual donors separately revealed that a decreased T cell proliferation was present in six of the eight tested birch allergic donors, whereas AEC-imprinted DCs induced decreased proliferation in two of four grass allergic donors. Thus, the observed differences between these two allergen models in the present study could be due to testing an insufficient number of grass-allergic donors.

It could be questionable whether working with activation of pre-established T cell lines is an optimal read-out

for evaluating the tolerogenic properties of AEC-imprinted DCs. As these T cells have been cultured for several generations, they are highly reactive towards their selected epitopes and may not be highly sensitive to inhibitory signals from the DC. In order to investigate further if AEC-imprinting of DCs could moderate the responses of less committed T cells, we investigated the effects of AEC imprinting with T cells purified directly from whole blood from allergic donors. The CD4<sup>+</sup> T cells were first co-cultured for 10 days with AEC-imprinted DCs, after which they were restimulated with allergen extract-loaded PBMCs and analysed for proliferation and cytokine production.

As with the Bet v 1-specific T cell lines, the primary T cell cultures from birch, grass and HDM-allergic patients expanded with AEC-imprinted DCs exhibited a decreased proliferation upon restimulation, although the inhibition was significant only with the T cells from grass-allergic patients. Moreover, the proliferation data are strengthened by the measured T cell cytokine secretion. For grass and HDM, T cell cytokine secretion follows the proliferation profiles, where IL-10, IL-13 and IL-5 secretion were reduced strongly for primary T cell cultures expanded with AEC-imprinted DCs. However, this was not observed with birch extract, where AEC-imprinting mainly induced a strong but non-significant reduction of IL-10 production. This is in contrast to experiments with the Bet v 1-specific T cell lines, where both a decreased proliferation and cytokine response were observed after AEC-imprinted DC co-culture. It is puzzling that restimulation of primary T cells from birch allergic donors failed to show significantly lower proliferation and cytokine responses upon restimulation in contrast to the findings with Bet v 1-specific T cell lines. In general, however, AECs seem to possess the capacity to imprint DC to down-regulate both proliferation and cytokine production of both established T cell lines and primary T cell cultures. It should be noted that this trend of lowered

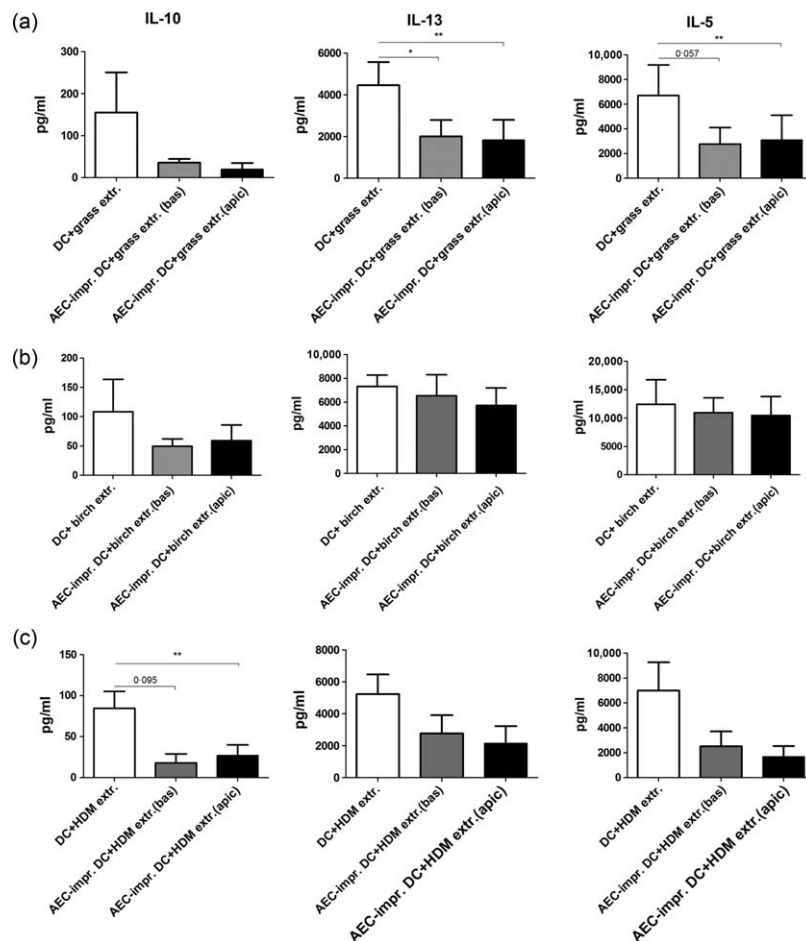


**Fig. 7.** Cytokine production from restimulated primary T cells. Forty-eight h after restimulating primary T cells from timothy grass (a), birch (b) and house dust mite (HDM) (c) allergic donors, supernatants were collected and analysed for a panel of T cell-specific cytokines. Cytokine analyses after peripheral blood mononuclear cell (PBMC) restimulation of primary T cell lines co-cultured with autologous dendritic cells (DCs) are represented as the difference in cytokine levels between restimulation with allergen extract and restimulation without allergen extract. The figure shows mean results of four individual timothy grass- and HDM-allergic donors, respectively, and three individual birch-allergic donors. Error bars represent standard error of the mean (s.e.m.).

cytokine secretion and T cell proliferation is observable even though a limited number of repeat experiments were performed. To further strengthen and validate these findings these experiments would need to be repeated on a larger scale, and should be investigated further in future studies.

The down-regulating effects on T cell responses, specifically Th2 responses, after AEC-imprinting have also been seen with HDM in a study by Rate and colleagues which generated AEC-imprinted DCs by adding monocytes in a mixed culture with semi-confluent AECs [14]. This would suggest that the regulatory effects of AEC-imprinted DCs do not necessarily require a fully polarized epithelium in all cases. Several studies with both primary AECs and cell lines have shown that AEC-imprinted APCs, whether in direct contact with AECs or exposed only to AEC supernatant, can induce regulatory T cells and inhibit inflammatory Th2 proliferation [12,14,57]. The present study demonstrates further that AECs are able to directly dampen antigen-specific T cell responses through interactions with DCs. We did not observe any skewing towards non-Th2 responses of the *de-novo* Th cells through co-culture with AEC-imprinted DCs, as no induction of IL-10 or IFN- $\gamma$  was seen (data not shown).

This would suggest that AECs do not induce an alternative activation of regulatory T cells or Th1 cells and that, in our model, T cell responses are either suppressed entirely or follow regulatory pathways which do not involve IL-10. This could possibly involve transforming growth factor (TGF)- $\beta$ -producing Th3 cells that have been shown to induce tolerance and suppress Th1, Th17 and Th2 responses in the gut mucosa [58], and TGF- $\beta$  production is to be investigated in future experiments. As T cell tolerance can be a result of anergy, apoptosis or secretion of regulatory effector cytokines it is also important to characterize fully the mechanism of T cell tolerance in future investigations.

In conclusion, the current study suggests strongly that allergen-specific immune responses are modulated by AEC. Future studies investigating the effect of unpolarized AEC or with compromised polarization in the current model are needed to demonstrate fully the role of polarized epithelium and the direct involvement of molecules important for polarization in promoting inhibitory immune responses. Such comparative studies are made easier with the emerging use of *in-vitro* co-culture models, which may help to understand and unravel the complex interplay that is present between epithelium and immune cells in airway

disorders. With more understanding of these mechanisms it will become easier to identify key pathways responsible for upholding homeostasis and may also reveal possible molecular target for future treatments.

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### Author contributions

D. P., S. H. and P. A. W. conceived and designed the experiments. D. P. performed the experiments and D. P. and P. A. W. analysed the data. D. P., S. H. and P. A. W. wrote the paper.

### Disclosure

D.P., P.A.W. and V.R.W. are employees of ALK, which develops and manufactures allergy vaccines.

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