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Take home message: Airway epithelial regulation of T cell activity may be impaired during virus-induced exacerbations in asthma.

Running title: Epithelial regulation of T cell activity

Key words: Airway epithelial cells; Allergic inflammation; Asthma immunology; T cell-modulation

To the Editor

In allergen-sensitized asthmatic individuals, allergen-specific type-2 T-helper (Th2) cells proliferate and secrete type-2 cytokines (e.g. interleukin (IL)-4, -5 and -13), driving the airway inflammatory response that gives rise to the clinical symptoms of asthma. Both early-life sensitization to aeroallergens and lower respiratory viral infections are important environmental risk factors for developing asthma. Respiratory viral infections are also the most common trigger for asthma exacerbations. Of interest, many asthma susceptibility genes are expressed in the airway epithelium[1], which forms the first continuous line of defense against inhaled environmental insults, including viruses and aeroallergens. Impaired immune regulation and failure to maintain tolerance to allergens is thought to contribute to allergic sensitization. Asthma epithelium may be deficient in its innate immune defense against virus infections, resulting in increased viral replication upon rhinovirus infection compared to non-asthma-derived epithelial cultures[2]. Furthermore, there is evidence for loss of the mucosal immune barrier in asthma, with disruption of epithelial integrity[1,3]. This may not only lead to increased permeability, but also result in the release of pro-inflammatory mediators, specifically of cytokines that drive type-2 responses[3,4]. We recently observed that the ability of allergens to disrupt epithelial barrier function is related to the development of type-2-mediated inflammation in asthma[5,6]. Furthermore, we demonstrated that healthy murine lung epithelium is a potent inhibitor of T cell proliferation and that this inhibition is lost upon viral infection[7]. It is unknown if this immune regulatory effect is displayed by human epithelium and is dysregulated in asthma. We hypothesize that changes in this regulatory effect translate into aberrant regulation of T cell responses in asthma. We studied the epithelial regulation of T cell proliferation and cytokine responses upon epithelial stimulation with a viral mimic, using co-culture of human T cells and primary bronchial epithelial cells (PBECs) from healthy controls and asthma patients.

Normal PBECs obtained from Lonza (Walkersville, MD) were used, except for the comparison between healthy and asthmatic epithelium, when PBECs derived by bronchial brushings from 5 healthy individuals and 5 patients with mild-moderate asthma, all non-smokers, were used (see fig. 1E for further characteristics). The study was approved by the Medical Ethics Committee of the UMCG. All subjects gave their written informed consent. PBECs were cultured in hormonally-supplemented bronchial epithelium growth medium (BEGM, Lonza) as described before[8-10]. PBECs were seeded in 12-well plates, grown to ~90% confluence, placed in BEGM/1% FCS overnight and pre-treated with/without 12.5µg/ml poly-(I:C) for 8 hours and washed before placing in co-culture with T cells. $CD4^+$ T cells were isolated from 10 ml peripheral blood of healthy non-allergic non-smoking volunteers by Ficoll-Hypaque (Lymphoprep; Nycomed, Oslo, Norway) by density-gradient centrifugation followed by MACS sorting using $CD4^+$ T cell Isolation Kit II (Miltenyi Biotec, San Diego, CA). T cells were seeded in duplicates at a concentration of $1.25*10^5$/ml in 12 well plates with/without PBECs (2:1 PBECs:T cells) or in the upper well of a transwell system (0.4µM pores, Corning Costar) placed above PBECs. T cells were stimulated for 96 hours in the presence/absence of PBECs in BEGM/1% FCS or in conditioned-medium from 24 hours PBEC culture. T cell proliferation was induced by α-CD3/α-CD28 immobilized to beads (Dynabeads, Invitrogen, Carlsbad, CA) and analyzed by labeling with 10 µM carboxy fluoroscein succinimidyl ester (CFSE, Life Technologies, Grand Island, NY) just prior to stimulation. CFSE dilution measurements were performed using flow cytometry (Calibur, BD) in viable $CD4^+$/7-amino-actinomycin-$D^{negative}$ T cells. The division index was calculated at (100-Y)/Y, where $Y(\%) = x_0 + x_1/2 + x_2/4 + x_3/8 + x_4/16$, x_0 = % of T cells that have not divided and x_{1-4} = T cells within progressive CFSE division gates. Levels of interferon (IFN)-γ, IL-4, IL-5, IL-10 and IL-13 were analyzed in cell-free

supernatants using a multiplex ELISA kit (Millipore, Billerica, MD) and Luminex technology (Luminex Corporation, Austin, TX).

We first studied the regulatory effects healthy human PBECs on T cell proliferative responses and observed an almost complete prevention of α-CD3/α-CD28-induced T cell proliferation, with suppression of all T cells (fig. 1A, B), without significant effects on T cell viability (not shown). We previously observed in mice that T cell proliferation is equally well suppressed by lung epithelial cells, whether they were cultured submerged or at the air-liquid interface (data not shown). Here, we show that the suppressive effect was still present when PBECs and T cells were separated in a transwell system, suggesting the effect does not require direct cell-cell contact (fig. 1B). In addition, the suppressive effect could be transferred by conditioned-medium derived from 24-hours culture of the epithelial cells (fig. 1C), indicating the involvement of one or several soluble mediators. Assessing cytokine production, there was no significant effect on the type-1 cytokine IFN-γ, while healthy PBECs strongly inhibited the α-CD3/α-CD28-induced secretion of the type-2 cytokines IL-4, IL-5, IL-10 and IL-13 (fig. 1D). PBECs cultured alone did not secrete detectable levels of any of these cytokines.

To study whether airway epithelium in asthma is deficient in its immunosuppressive capacity, we compared the effect of PBECs from asthma patients and healthy controls (fig. 1E). Both asthma-derived and control-derived PBECS formed confluent cell monolayer, without morphological differences as observed by microscopy, and both markedly inhibited T cell proliferation (fig. 1F). However, asthma-derived PBECs were significantly less potent in doing so (fig. 1F). Furthermore, PBECs from both asthmatics and healthy controls had a similar capacity to inhibit type-2 cytokine production, as shown for IL-13 (fig. 1G), without significant differences between the subject groups.

We previously showed that regulatory effect of healthy murine epithelial cells was lost upon viral infection[7]. Respiratory RNA viruses act on Toll-like receptor (TLR)3, potentially mediating airway inflammation in virus-induced asthma exacerbations[11,12]. Therefore, we used the TLR3 agonist poly-(I:C) as a viral mimic. PBECs from controls and asthmatics were exposed to poly-(I:C) and then washed, leaving the cells viable and fully confluent, before T cells were added. Pre-treatment with poly-(I:C) strongly reduced the suppressive effect of both control and asthma-derived PBECs on T cell proliferation (fig. 1H, 1I) and attenuated the inhibitory effect of control PBECs on type-2 cytokine secretion, as shown for IL-13 (fig. 1J).

Together, our data suggest that asthma and viral infection compromise the immunosuppressive capacity of airway epithelium, which may be most impaired during virus-induced exacerbations of asthma, resulting in severe inflammation and a loss of asthma control. Such a major compromise of epithelial immune suppressive capacity was not found and not expected in the epithelium from stable asthma patients studied here. Viral infection of murine pulmonary epithelial cells strongly reduces their T cell suppressive capacity[7] and our current data indicate that viral infection of human airway epithelial cells will have a similar effect. Even without using live virus, the epithelial immunosuppressive effect on T cells was strongly attenuated upon exposure to the viral mimic and TLR3 agonist poly-(I:C). We speculate that such inhibition of epithelial immune regulation could enhance type-2 cytokine secretion during viral infection in early life, an important risk factor for asthma development, and during virus-induced asthma exacerbations. Indeed, viral infection is accompanied by an increase in type-2 cytokine secretion in mouse models[13] and in asthmatic airways, where type-2 cytokine levels relate to exacerbation severity[14].

In conclusion, we show that human bronchial epithelium exerts potent inhibitory effects on T cell proliferation and type-2 cytokine secretion. These effects are attenuated following exposure to a viral mimic, and asthma epithelium displays reduced inhibition of T cell proliferation. Identification of the responsible mechanisms and mediator(s) involved in epithelial immune regulation may provide new targets for novel disease modifying therapeutic and preventive strategies in asthma.

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Figure legend

Figure 1. Primary bronchial epithelial cells (PBECs) inhibit α-CD3/α-CD28-induced T cell proliferation and associated type-2 cytokine production, an effect that is attenuated by viral stimulation and in asthma. **A)** Representative CFSE dilution measurement. **B)** Proliferation of T cells cultured in direct contact with PBECs (Lonza) compared to T cells and PBECs separated in a transwell system (*n=5*). **C)** Proliferation of T cells cultured in conditioned medium (CM) of 24 hour-PBEC (Lonza) cultures (*n=5*). **D)** IFN-γ, IL-4, IL-5, IL-10 and IL-13 levels measured in cell-free supernatants by multiplex ELISA after T cell stimulation with α-CD3/α-CD28-coupled beads for 4 days in the absence/presence of PBECs ((Lonza, *n=9-10*). **E)** Characteristics of the subjects from whom bronchial brushings were taken. Stable mild-moderate asthma patients were included based on the presence of allergy (assessed by skin test or Phadiatop) and bronchial hyperresponsiveness (assessed by PC_{20} AMP<16mg/ml, PC_{20} methacholine<8mg/ml or PC_{20} histamine<8mg/ml), and the absence of other lung diseases and of any corticosteroid, long-acting β-agonist, and long-acting anticholinergic use for at least 4 weeks preceding the study. Medians (range) or number (%) are presented. FEV_1% pred =forced expiratory volume during the first second as percentage of predicted. **F)** Proliferation of T cells cultured with/without PBECs from controls or asthma patients. **G)** IL-13 levels in cell-free supernatants of the T cells co-cultured with/without PBECs from controls or asthma patients (*n=5*). **H)** Proliferation of T cells cultured alone, with PBECs (Lonza), or with PBECs (Lonza) that were pre-treated with 12.5μg/ml poly-(I:C) (*n=5*). **I)** Proliferation of T cells cultured with PBECs from asthma patients that were pre-treated with/without 12.5μg/ml poly-(I:C) (*n=3*). **J)** IL-13 levels in cell-free supernatants of the co-cultures described in H. Means ± SEM are shown. *=p<0.05, **=p<0.01, and ***=p<0.001

as analyzed by 1-way ANOVA with Bonferroni's multiple comparison test in all graphs except for panel D and I, where an unpaired and paired Student's t-test was used, respectively.