Imprinting of bronchial epithelial cells upon \textit{in vivo} rhinovirus infection in people with asthma

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Epithelial hyperresponsiveness is an intrinsic defect in bronchial epithelium from asthma patients, which increases upon rhinovirus exposure, but not in healthy individuals

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Abstract

\textbf{Background} Defective translocation of the translational repressor TIAR (T-cell internal antigen receptor) in bronchial epithelial cells (BECs) from asthma patients underlies epithelial hyperresponsiveness, reflected by an exaggerated production of a select panel of inflammatory cytokines such as CXCL-8, interleukin (IL)-6, granulocyte colony-stimulating factor, CXCL-10, upon exposure to tumour necrosis factor (TNF) and IL-17A. With this study we aimed to clarify whether epithelial hyperresponsiveness is a consistent finding, is changed upon \textit{in vivo} exposure to rhinovirus (RV)-A16 and applies to the bronchoconstrictor endothelin-1.

\textbf{Methods} BECs were obtained from asthma patients (n=18) and healthy individuals (n=11), 1 day before and 6 days post-RV-A16 exposure. BECs were cultured and stimulated with TNF and IL-17A and inflammatory mediators were analysed. The bronchoalveolar lavage fluid (BALF) was obtained in parallel with BECs to correlate differential cell counts and inflammatory mediators with epithelial hyperresponsiveness.

\textbf{Results} Epithelial hyperresponsiveness was confirmed in sequential samples and even increased in BECs from asthma patients after RV-A16 exposure, but not in BECs from healthy individuals. Endothelin-1 tended to increase in BECs from asthma patients collected after RV-A16 exposure, but not in BECs from healthy individuals. \textit{In vitro} CXCL-8 and endothelin-1 production correlated. \textit{In vivo} relevance for \textit{in vitro} CXCL-8 and endothelin-1 production was shown by correlations with forced expiratory volume in 1 s % predicted and CXCL-8 BALF levels.

\textbf{Conclusion} Epithelial hyperresponsiveness is an intrinsic defect in BECs from asthma patients, which increases upon viral exposure, but not in BECs from healthy individuals. This epithelial hyperresponsiveness also applies to the bronchoconstrictor endothelin-1, which could be involved in airway obstruction.

Introduction

Asthma is a heterogeneous inflammatory airway disease characterised by airway hyperresponsiveness and episodic worsening of the disease. Respiratory viral infections trigger this worsening of the disease, which is paralleled by enhanced airway inflammation \cite{1, 2}. Various cell types have been implicated in driving airway inflammation in asthma, ranging from cells implicated in allergy, innate immune cells and structural cells. Previously it was shown that bronchial epithelial cells (BECs) from asthma patients compared to those from healthy individuals display an exaggerated production of inflammatory proteins \cite{3-5}.

mRNAs encoding inflammatory proteins at large have a short half-life, which ensures their limited production, but also complicates the analyses of a possible aberrant regulation of inflammatory protein production. By prolonging the half-life of mRNA encoding these inflammatory proteins, upon exposure to...
interleukin (IL)-17A next to tumour necrosis factor (TNF) [6], we could visualise a synergistic/exaggerated production of inflammatory mediators by BECs from most asthma patients [7]. We referred to this synergistic/exaggerated production as epithelial hyperresponsiveness, which was due to a defective cytoplasmic translocation of a translational repressor, T-cell internal antigen receptor (TIAR). Consequently, TIAR cannot limit the production of inflammatory proteins. This defective translocation underlies an exaggerated and corticosteroid-unresponsive production of IL-8 (CXCL-8) and that of a select panel of other inflammatory mediators in mild, moderate and severe asthma patients. The clinical relevance of this epithelial hyperresponsiveness was shown by a strong correlation between in vivo neutrophilic inflammation and forced expiratory volume in 1 s (FEV1) reversibility and the in vitro CXCL-8 production by BECs from the same patients [7]. The current study was set up to clarify whether this epithelial hyperresponsiveness was consistent in repeated samples of BECs, and thus a genuine intrinsic defect. As rhinovirus (RV)-A16 infection results in neutrophilic inflammation [8], more so in asthma patients than healthy individuals [9], and correlates with clinical severity [10], we also aimed to determine whether an in vivo rhinovirus infection impacted this epithelial hyperresponsiveness.

So far, we have shown epithelial hyperresponsiveness for inflammatory mediators only. TIAR interacts through at least three RNA-recognition motifs with hundreds of mRNAs [11], which are present also in pre-proendothelin-1 mRNA [12] and that can give rise to endothelin (ET)-1 production. ET-1 is a potent bronchoconstrictor, which can be produced by BECs and which has been implicated in asthma and possibly airway hyperresponsiveness [13]. As rhinovirus infection results in a prolonged period of enhanced airway hyperresponsiveness we also set out to measure ET-1 production as a function of epithelial hyperresponsiveness.

Material and methods

Study design

Bronchial epithelial brushes were obtained 1 day before and 6 days after RV-A16 challenge in two trials, the RESOLVE (NTR1677) study with healthy individuals and mild asthma patients and the MATERIAL (NCT01520051) study with mild asthma patients; the setup (figure 1), inclusion and exclusion criteria and sampling time points of these were identical and have been described elaborately [7]. Patients with mild allergic asthma met the following criteria: a history of episodic chest symptoms, baseline FEV1 >80% predicted, airway responsiveness to methacholine (provocative concentration causing a 20% fall in FEV1 (PC20) <9.8 mg·mL⁻¹) and a positive skin prick test response to at least one of 10 common aeroallergens (grass mix, tree mix, house dust mite, cats, dogs, Alternaria alternata, Aspergillus fumigatus, Cladosporium herbarum, latex and rabbit). Healthy individuals from the RESOLVE study had a FEV1 >80% pred, PC20 >16 mg·mL⁻¹ and were skin prick test negative for the 10 common aeroallergens. All mild asthma patients and healthy controls were nonsmoking or had stopped smoking ≥12 months previously with ≤5 pack-years. Asthma patients were not allowed to use inhaled or systemic corticosteroids or treatment other than inhaled short-acting β₂-agonists within 2 weeks prior to the start of the study. None of the participants had a concomitant disease or a (chronic) inflammatory condition that would interfere with this study according to the judgement of a pulmonary physician. The characteristics of patients and healthy volunteers are provided in table 1. RV-A16 infection was confirmed by at least one of the indicators: increase in common cold score, increase in RV-A16 titre and RV-positive PCR in bronchoalveolar lavage. The PCR for rhinovirus was a pan-RV PCR.

BECs culturing and stimulation

BECs were obtained by bronchial brushes during bronchoscopy from asthma patients and healthy individuals and plated on six-well plates pre-coated with PureCol (Advanced Biomatrix, San Diego, CA, USA) in submerged cultures, which is referred to as passage 0 (P0). The cells were grown until confluence and then cultured on six-well plates (P1) in equal amounts and when confluent stimulated with recombinant human (rh)TNF (5 ng·mL⁻¹) (R&D Systems, Minneapolis, MN, USA) and rhIL-17A (100 ng·mL⁻¹) (R&D Systems) for 16 h for the assessment of released mediators in submerged cultures. Concentrations of TNF and IL-17A were optimised in earlier studies [14]. Baseline values (before RV-A16 exposure) for all cytokines, but not endothelin-1, from cultured BECs were obtained from a larger dataset established earlier [7]. The comparison presented here is based merely on the availability of paired samples of brushed and subsequently cultured epithelial cells, before and after RV-A16 exposure. Based on this availability, there were nine mild-asthma patients with hyperresponsive BECs and nine with normoresponsive BECs (i.e. with a response like that of BECs from healthy individuals). Hyperresponsive BECs are defined as CXCL-8 production of >10000 pg·mL⁻¹ upon TNF and IL-17A stimulation, and a synergistic effect (CXCL-8 production by TNF and IL-17A stimulation combined divided by CXCL-8 production with TNF...
alone and CXCL-8 production with IL-17A alone) >2.5. Additionally, we showed in the previous study that patients with hyperresponsive BECs did not respond to corticosteroids (CXCL-8 levels were not decreased with dexamethasone treatment post-TNF and -IL-17A stimulation), but we did not perform these experiments in BECs obtained post-RV16. Normoresponsive BECs are defined as CXCL-8 production of <10 000 pg·mL$^{-1}$ upon TNF and IL-17A stimulation. In addition, a synergistic effect <2.5 and decreased levels of CXCL-8 with dexamethasone treatment post-TNF and -IL-17A stimulation.

**Bronchoalveolar lavage fluid sampling**

Bronchoalveolar lavage fluid (BALF) was obtained sequentially from eight subsequent instillations with 20 mL of warm saline, according to standard procedures. Fractions 4–8 were pooled, after which cells were pelleted for subsequent cytospin preparations. Cytokines and endothelin were determined in the resulting supernatant. BALF recovery in healthy individuals (mean±SD 60.5±7.4% before and 65.1±6.4% after RV-A16) and asthmatics (mean±SD 69.7±7.4% before and 65.1±8.3% after RV-A16) were similar, enabling direct comparison. CXCL-8 from BALF was analysed using multiplex assays (BioRad). Differential cell counts were determined using Diff-Quick staining (RAL Diagnostics) in the BALF. When available, 400 cells were differentiated into eosinophils, neutrophils, macrophages/monocytes and lymphocytes in addition to columnar and squamous epithelial cells.

**Mediator analyses**

The *in vitro* experiments using BECs were performed in triplicate for each condition and equal volumes from triplicates were combined into one sample for cytokine measurements. CXCL-8, IL-6, granulocyte

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### TABLE 1 Characteristics of mild asthma patients and healthy individuals before and after rhinovirus (RV)-A16 challenge

|                      | Healthy individuals (Before RV-A16) | Healthy individuals (After RV-A16) | Mild asthmatics (Before RV-A16) | Mild asthmatics (After RV-A16) |
|----------------------|------------------------------------|-----------------------------------|---------------------------------|---------------------------------|
| **Subjects**         | 11                                 | 18                                | 18                              | 18                              |
| **Age (years)**      | 22.36 (19–31)                      | 21.77 (18–29)                     | 23.98 ±2.75                     | 23.53 ±2.55                     |
| **Sex ratio (male/female)** | 4/7                               | 7/11                              | 5/13                            | 8/12                            |
| **FEV$_1$ pre-bronchodilator (L)** | 3.93±0.41                         | 3.89±0.25                        | 4.11±0.79                       | 3.99±0.79                       |
| **FEV$_1$ pre-bronchodilator (% pred)** | 105.63±10.43                     | 104.9±8.79                       | 101.38±11.02                    | 98.44±13.67                     |
| **PC$_{20}$ (mg·mL$^{-1}$)** | >16                                | >16                               | 2.68 (0.13–7.35)                | 3.36 (0.38–9.51)                |
| **FeNO (ppb)**       | 16.52±8.15                         | 17.05±4.53                       | 58.02±31.72                     | 64.61±32                       |

Data are presented as n, mean (range) or mean±SD. Δ: change; FEV$_1$: forced expiratory volume in 1 s; PC$_{20}$: provocative concentration causing a 20% fall in FEV$_1$; FeNO$: exhaled nitric oxide fraction.
colony-stimulating factor (G-CSF), CXCL-10, CXCL-1, IL-1 receptor antagonist (IL-1RA), vascular endothelial growth factor (VEGF)-α, IL-1α, CXCL-12 and macrophage colony-stimulating factor (M-CSF) were measured by multiplex assays (BioRad) and read using a Bioplex system (BioRad). Endothelin-1 was analysed using ELISA (Duoset ELISA, DY1160; R&D Systems). Eosinophil cationic protein (ECP) and myeloperoxidase (MPO) were determined using ELISA, as described [15].

Statistical analysis
GraphPad Prism 8.4.2 was used for statistical analyses (t-tests, one-way and two-way ANOVA). p-values ≤ 0.05 were considered statistically significant. For associations, Pearson’s and Spearman’s correlation tests were performed with two-tailed analysis, depending on normal distribution.

Results
Enhanced hyperresponsive production of specific inflammatory mediators after RV-A16 challenge
As shown before [7] and here in particular, not all mild asthma patients have BECs displaying epithelial hyperresponsiveness (i.e. they are normoresponsive) to TNF and IL-17A (figure 2a–d). Nine patients had hyperresponsive BEC and remained hyperresponsive after infection with RV-A16. In addition, five out of nine patients with normoresponsive BECs turned hyperresponsive after RV-A16 infection, whereas this was not observed for BECs from healthy individuals (figure 2a–d). The epithelial hyperresponsiveness applies to a select panel of cytokines (CXCL-8, IL-6, G-CSF and CXCL-10), but not to others (CXCL-1, IL-1RA, VEGF-A, IL-1α, CXCL-12 and M-CSF) (supplementary figure E1A–F).

ET-1 production by BECs from asthma patients tended to increase after exposure to RV-A16, whereas there were no changes in ET-1 production by BECs from healthy individuals (figure 2e). As expected, because of similar TIAR-recognition motifs in CXCL-8 and pre-proendothelin-1 mRNA, ET-1 and CXCL-8 production upon TNF and IL-17A stimulation in BECs from both groups were significantly correlated both before and after RV-A16 exposure (figure 2f).

CXCL-8 from BECs in vitro correlated with BALF CXCL-8 and FEV1
Whereas CXCL-8 levels in BALF did not differ significantly between healthy controls and asthma patients before RV16, after RV-A16 challenge, CXCL-8 BALF levels increased in mild asthmatics, but not in healthy individuals (figure 3a). Next, we compared CXCL-8 production by BECs in vitro with CXCL-8 in BALF for each participant. Despite potential other sources of CXCL-8 and its consumption, we found a correlation for these after RV-A16 exposure, but not before RV-A16 (figure 3b).

RV-A16 infection induced a small but significant drop in FEV1 % predicted (figure 3c) in patients, but not in healthy individuals. We considered the drop too low to perform a relevant comparison with the change in ET-1 levels (by BECs collected after RV-A16 minus those before RV-A16 exposure). Instead, we compared ET-1 levels with FEV1 % predicted, which correlated significantly before, but not after RV-A16 exposure (figure 3d). However, the change in CXCL-8 levels (by BECs collected after RV-A16 minus those before RV-A16 exposure) correlated inversely with FEV1 % predicted measured after RV-A16 in patients and healthy individuals, but not before RV-A16 exposure (figure 3e).

RV-A16 reduced neutrophils in BALF from healthy individuals, but not for eosinophils
The change in BALF eosinophils (%) due to RV-A16 exposure did not differ significantly between patients and healthy individuals (figure 4a). Interestingly, the change in neutrophils (%) was significantly lower in healthy individuals compared to that in asthma patients (figure 4b). There were no changes in response to RV-A16 in BALF macrophages/monocytes, lymphocytes, columnar and squamous epithelial cells. Neither the percentage eosinophils nor that of neutrophils correlated with FEV1 % predicted before and after RV-A16 exposure (data not shown). Changes in BALF ECP (figure 4c) and MPO (figure 4d), indicating the activated eosinophils and neutrophils, respectively, did not differ significantly between asthma patients and healthy individuals. In addition, the ECP and MPO in the BALF did not correlate with FEV1 % predicted before and after RV-A16 exposure (data not shown).

Discussion
BECs from at least some mild asthma patients can display epithelial hyperresponsiveness [7], and this is a consistent finding. The intrinsic defect of epithelial hyperresponsiveness in asthma is maintained during weeks of cell culturing and is also observed in subsequent BEC samples obtained from the same patients, indicative of imprinting. Moreover, we showed that BECs collected after in vivo RV-A16 infection display increased epithelial hyperresponsiveness, even in BECs from some patients who showed no epithelial hyperresponsiveness prior to RV-A16 challenge. Finally, this increased epithelial hyperresponsiveness was
Healthy controls
Mild asthma patients

**CXCL-8 pg·mL⁻¹**

|        | Day –1 | Day 6 | Day –1 | Day 6 |
|--------|--------|-------|--------|-------|
| NS     | 0.9968 | 0.0069| 0.1903 | 0.0459|

**IL-6 pg·mL⁻¹**

|        | Day –1 | Day 6 | Day –1 | Day 6 |
|--------|--------|-------|--------|-------|
| NS     | 0.0846 | 0.0121| 0.8857 | 0.0503|

**G-CSF pg·mL⁻¹**

|        | Day –1 | Day 6 | Day –1 | Day 6 |
|--------|--------|-------|--------|-------|
| NS     | 0.0734 | 0.0053| 0.8857 | 0.0503|

**Endothelin (ET)-1 pg·mL⁻¹**

|        | Day –1 | Day 6 | Day –1 | Day 6 |
|--------|--------|-------|--------|-------|
| NS     | 0.8857 | 0.0503| 0.8857 | 0.0503|

**CXCL-8 before RV16 pg·mL⁻¹**

|        | Day –1 | Day 6 | Day –1 | Day 6 |
|--------|--------|-------|--------|-------|
| NS     | 0.02   | 0.02  | 0.02   | 0.02  |

**ET-1 before RV16 pg·mL⁻¹**

|        | Day –1 | Day 6 | Day –1 | Day 6 |
|--------|--------|-------|--------|-------|
| p=0.33 | R²=0.15|       |       |       |

**CXCL-8 after RV16 pg·mL⁻¹**

|        | Day –1 | Day 6 | Day –1 | Day 6 |
|--------|--------|-------|--------|-------|
| NS     | 0.02   | 0.02  | 0.02   | 0.02  |

**ET-1 after RV16 pg·mL⁻¹**

|        | Day –1 | Day 6 | Day –1 | Day 6 |
|--------|--------|-------|--------|-------|
| p=0.0017 | R²=0.31|       |       |       |

**FIGURE 2** Tumour necrosis factor (TNF) and interleukin (IL)-17A-induced mediator production by bronchial epithelial cells (BECs) from asthma patients and healthy individuals, before and after in vivo rhinovirus (RV)-A16 challenge. BECs collected after in vivo RV-A16 challenge from mild asthma patients (n=18), as compared to BECs collected before the viral challenge, produced upon 16 h of exposure to TNF and IL-17A significantly higher levels of a) CXCL-8, b) IL-6, c) granulocyte colony-stimulating factor (G-CSF), d) CXCL-10 and, trend-wise, e) endothelin (ET)-1. In contrast, BECs from healthy individuals (n=11) did not show an increase. For statistical analyses, two-tailed paired t-tests (before versus after RV16) and two-tailed unpaired t-tests (healthy controls versus asthma patients) were used. Bars and whiskers depict mean±SD. f) ET-1 production correlated with that of CXCL-8 after stimulation with TNF and IL-17A in BECs, both before (left panel) and after (right panel) RV-A16 challenge. Correlations were done with Pearson’s correlation coefficient. NS: nonsignificant. *: p<0.05; **: p<0.01.
a) 

![Graph showing CXCL-8 BALF levels before and after RV16 for Healthy controls and Mild asthma patients.](image)

b) 

![Graph showing correlation between CXCL-8 BALF before RV16 and ET-1 levels before RV16.](image)

c) 

![Graph showing FEV1% pred before and after RV16 for Healthy controls and Mild asthma patients.](image)

d) 

![Graph showing correlation between FEV1% pred before RV16 and ET-1 levels before RV16.](image)

e) 

![Graph showing correlation between FEV1% pred before RV16 and ΔCXCL-8 levels pg·mL⁻¹.](image)
shown only in BECs from asthma patients and not in those from healthy individuals. BALF CXCL-8 increased after RV-A16 at day 6, which correlated with increased CXCL-8 production in vitro by TNF- and IL-17A-stimulated BECs obtained after RV-A16 and with decreasing FEV\textsubscript{1} % predicted after RV-A16 challenge. TNF- and IL-17A-stimulated BECs obtained after RV16 produced higher ET-1 levels when compared to that of BECs obtained before RV16. ET-1 levels correlated with decreasing FEV\textsubscript{1} % predicted only before RV16.

As in our previous report, not all asthma patients have BECs that are hyperresponsive [7], although in this study a large proportion of the patients had BECs that were normoresponsive. Therefore, in this study, we did not find a significant difference in CXCL-8, IL-6, G-CSF, CXCL-10 and ET-1 at baseline (before RV16). Intriguingly, BECs from some of these patients developed epithelial hyperresponsiveness after viral infection, which lead us to speculate that there may be a predisposition for this defect in BECs at baseline.

BECs from asthma patients collected after RV-A16 infection released enhanced ET-1 trend-wise and this correlated with CXCL-8, in line with similar regulatory motifs in the mRNAs encoding CXCL-8 and pre-proendothelin-1, respectively. Despite the correlation between ET-1 and CXCL-8 at baseline, there was no enhanced ET-1 production by BECs collected at baseline from asthma patients. Possibly, the protease that clips pre-proendothelin-1 into endothelin-1 is not active in BECs at baseline. Although we found a weak but significant correlation between ET-1 and FEV\textsubscript{1} % predicted before RV-A16 exposure, there was no correlation post-RV-A16 exposure. This may be due to inflammation-induced oedema formation and

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**FIGURE 3** *In vitro* and *in vivo* CXCL-8 measurements, their correlation, and that with forced expiratory volume in 1 s (FEV\textsubscript{1}) % predicted and for endothelin (ET)-1. a) CXCL-8 bronchoalveolar lavage fluid (BALF) levels increased significantly after rhinovirus (RV)-A16 exposure in asthma patients (n=18), but not in healthy individuals (n=11). b) BALF CXCL-8 correlates with CXCL-8 released from bronchial epithelial cells (BECs) obtained after RV-A16 exposure when data for healthy and asthma participants are combined. c) RV-A16 infection in asthma patients (n=18) induced a significant drop in FEV\textsubscript{1} % predicted, but not for healthy individuals at day 4 after RV-A16 (n=11). d) ET-1 levels correlated significantly with FEV\textsubscript{1} % predicted before and not after exposure to RV-A16. e) The change (Δ) in CXCL-8 levels released *in vitro* by BECs obtained after RV-A16 correlated with FEV\textsubscript{1}, measured after RV-A16, but not before RV-A16 exposure. For statistical analyses, two-tailed paired t-tests (before versus after RV16) and two-tailed unpaired t-tests (healthy controls *versus* asthma patients) were used. Nonparametric Spearman correlation test was performed with two-tailed analysis. NS: nonsignificant. *: p<0.05; **: p<0.01.

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**FIGURE 4** The change in eosinophils, neutrophils, myeloperoxidase (MPO) and eosinophil cationic protein (ECP) at day 6 post-rhinovirus (RV)-A16 exposure. Comparison of deltas (Δ; percentage of cell numbers at day 6 after RV-A16 minus the baseline at day −1) in a) percentage eosinophils shows no significant difference, while b) the Δ of neutrophils in bronchoalveolar lavage fluid (BALF) of healthy individuals (n=11) was negative (lower during infection than at baseline) and significantly lower than the marginal increase (positive Δ) observed in mild asthmatics (n=18). The Δ of c) ECP and d) MPO levels in BALF were not statistically significantly different in mild asthma patients (n=18) and not in healthy individuals (n=10). The bars and whiskers depict means±SD. For statistical analyses, two-tailed nonparametric Mann-Whitney t-tests were used for figure 4a and c; unpaired t-tests were used for figure 4b and d. NS: nonsignificant. **: p<0.01.
mucus production in response to RV-A16 infection, which, besides bronchoconstriction, determines FEV₁. This is supported by the inverse correlation between the increase in pro-inflammatory CXCL-8 production and the FEV₁ after RV-A16 challenge.

There are several potential shortcomings of this study. Firstly, despite enhanced CXCL-8 production, there was no correlation with neutrophils and MPO. This may be due to various opposing effects. Neutrophil counts may not truly reflect neutrophilic inflammation, as activated neutrophils having released granular contents will not be detected as neutrophils in cell differentiations. In addition, neutrophilic inflammation may also be underestimated by neutrophil counts when neutrophil extracellular traps are formed [16, 17] whereby, in addition, not all granular MPO is being released. Secondly, as we did not assess viral particles in the cultures, we cannot exclude that there still was virus present, which may have contributed to the enhanced mediator productions. However, we consider this unlikely for several reasons. Infected cells tend to not adhere and thus expand in cultures and, in addition, we did not observe any cytopathic effects in our cultures. Furthermore, rhinoviruses are known to induce various soluble proteins such as IL-1RA [18] and IL-1α [19], which occurred in only a couple of BEC cultures (supplementary figure E1).

In all, we have shown that epithelial hyperresponsiveness is a consistent finding and this intrinsic defect of exaggerated cytokine/mediator production upon stimuli is retained and thereby imprinted in bronchial epithelial cells from asthma patients. Rhinovirus infection can induce and worsen epithelial hyperresponsiveness, and it is of interest to determine how long this enhanced hyperresponsiveness remains. Finally, we showed that epithelial hyperresponsiveness in BECs also affects production of the bronchoconstrictor ET-1, which may partly contribute to bronchoconstriction besides several other factors.

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Author contributions: A. Ravi conducted experiments, analysed data and prepared the manuscript. S. Chowdury and A. Dijkhuis conducted experiments. B.S. Dierdorp, T. Dekker and Y.S. Sabogal Piñeros conducted experiments. R. Kruize optimised and performed the endothelin-1 ELISA, and conducted experiments. C.J. Majoor performed bronchoscopies. P.J. Sterk contributed to study design and reviewed the manuscript. R. Lutter devised the study, contributed to the experimental set-up definition and prepared the manuscript. All authors approved the final version of the manuscript.

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