Early View

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Imprinting of bronchial epithelial cells upon *in vivo* rhinovirus infection in asthma patients

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Author contributions: A.R. conducted experiments, analyzed data and prepared the manuscript. S.C. and A.D. conducted experiments. B.S.D., T.D. and Y.S.S.P conducted experiments. R.K. optimized and performed the endothelin-1 ELISA and conducted experiments. C.J.M. performed bronchoscopies. P.J.S. contributed to study design and reviewed the manuscript. R.L. devised the study, contributed to the experimental set-up definition and prepared the manuscript. All authors approved the final version of the manuscript.

Abstract

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 like CXCL-8, IL-6, G-CSF, CXCL-10, upon exposure to TNF and IL-17A. With this study we aimed to clarify whether epithelial hyperresponsiveness is a consistent finding, is changed upon *in vivo* exposure to rhinovirus-A16 (RV-A16) and, also applies to the bronchoconstrictor endothelin-1.

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 analyzed. The bronchoalveolar lavage fluid (BALF) was obtained in parallel with BECs to correlate differential cell counts and inflammatory mediators with epithelial hyperresponsiveness.

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. *In vitro* CXCL-8 and endothelin-1 production correlated. *In vivo* relevance for *in vitro* CXCL-8 and endothelin-1 production was shown by correlations with FEV₁ % predicted and CXCL-8 BALF levels.

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 characterized 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 [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 [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 IL-17A next to TNF [6], we could visualize 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 interleukin-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 FEV₁ reversibility and the *in vitro* CXCL-8 production by BECs from the same patients [7]. The current study was set up first to clarify whether this epithelial hyperresponsiveness was consistent in repeated samples of BECs and thus a genuine intrinsic defect. As rhinovirus-A16 (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 preproendothelin-1 mRNA [12] and that can give rise to endothelin-1 (ET-1) production. ET-1 is a potent bronchoconstrictor, which can be produced by BEC and that 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 which 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 (PC₂₀ <9.8 mg·mL⁻¹) and a positive skin prick test response to at least 1 of 10 common aeroallergens (grass mix, tree mix, house dust mite, cats, dogs, *Alternaria alternatum*, *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 non-smoking or had stopped smoking 12 months ago with ≤ 5 pack-years. Asthma patients were not allowed to use inhaled or systemic corticosteroids or treatment other than inhaled short-acting β_2 -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 judgment 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 titer 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 precoated 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 in BEBM medium (Lonza, Basel, Switzerland) supplemented with growth factors (Lonza) and Ciproxin (Sigma, Reading, UK) at 2 $\mu\text{g}/\text{mL}$. The cells were then passaged into 24-well plates (P1) in equal amounts and when confluent stimulated with recombinant human (rh)TNF (5 $\text{ng}\cdot\text{mL}^{-1}$) (R&D Systems, Minneapolis, MN, USA) and rhIL-17A (100 $\text{ng}\cdot\text{mL}^{-1}$) (R&D Systems) for 16 hours for the assessment of released mediators in submerged cultures. Concentrations of TNF and IL-17A were optimized in earlier studies [14]. Baseline values (before RV-A16 exposure) for all cytokines, but not endothelin-1, from cultured BECs were obtained from an earlier established larger data set [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 9 mild asthma patients with hyperresponsive and 9 with

normoresponsive (i.e. with a response alike BECs from healthy individuals) BECs. Hyperresponsive BECs are defined as CXCL-8 production of >10,000pg/ml upon TNF and IL-17A stimulation. In addition, 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) more than 2,5. We also showed in the previous study that patients with hyperresponsive BECs also 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 <10,000pg/ml upon TNF and IL-17A stimulation. In addition, a synergistic effect less than 2,5 and decreased levels of CXCL-8 with dexamethasone treatment post TNF and IL-17A stimulation.

BALF sampling:

BALF was obtained sequentially from 8 subsequent instillations with 20 ml of warm saline, according to standard procedures. Fractions 4 up to 8 were pooled, after which cells were pelleted for subsequent cytopsin preparations. Cytokines and endothelin were determined in the resulting supernatant. BALF recovery in healthy individuals ($60.5\% \pm 7.4\%$ before and $65.1\% \pm 6.4\%$ (mean $\pm$ SD) after RV-A16) and asthmatics ($69.7\% \pm 7.4\%$ before and $65.1\% \pm 8.3\%$ (mean $\pm$ SD) after RV-A16) were similar, enabling direct comparison. CXCL-8 from BALF was analyzed 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, lymphocytes in addition to columnar and squamous epithelial cells.

Mediator analyses:

The *in vitro* experiments using BECs were done in triplicates for each condition and equal volumes from triplicates were combined into one sample for cytokine measurements. CXCL-8, IL-6, G-CSF, CXCL-10, CXCL-1, IL-1RA, VEGF-a, IL-1a, CXCL-12 and M-CSF were measured by multiplex assays (BioRad) and read with a Bioplex (BioRad). Endothelin-1 was analyzed by ELISA (Duoset ELISA, DY1160; R&D systems). ECP and MPO were determined by 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, 5 out of 9 patients with normoresponsive BEC turned hyperresponsive after RV-A16 infection. In addition, 5 out of 9 patients with normoresponsive BEC turned hyperresponsive after RV-A16 infection. After RV-A16 challenge, however, BECs from more asthma patients displayed epithelial hyperresponsiveness, whereas this was not observed for BECs from healthy individuals (Figure 2A-D). The epithelial hyperresponsiveness applies to a

select panel of cytokines (C-X-C motif chemokine-8 (CXCL-8), interleukin-6 (IL-6), granulocyte-colony stimulating factor (G-CSF) and CXCL-10), but not to others (CXCL-1, interleukin-1-receptor antagonist (IL-1RA), vascular endothelial growth factor-A (VEGF-a), interleukin-1alpha (IL-1 α), CXCL-12 and macrophage colony-stimulating factor (M-CSF)) (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 prepro-endothelin-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 significantly differ 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 for each participant CXCL-8 production by BECs *in vitro* with CXCL-8 in bronchoalveolar lavage fluid (BALF). Despite potential other sources of CXCL-8 and its consumption, we found a correlation for these after RV-A16 exposure (Fig 2B, right panel), but not before RV-A16 (Figure 3B, left panel).

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 delta 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 delta 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, compared to that from asthma patients, but not for eosinophils

The change in BALF eosinophils (%) due to RV-A16 exposure did not significantly differ 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 eosinophilic cationic protein (ECP; Figure 4C) and myeloperoxidase (MPO; Figure 4D), indicating the activated eosinophils and neutrophils, respectively, did not differ significantly between asthma patients and healthy individuals. The ECP and MPO in the BALF also 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 that showed no epithelial hyperresponsiveness prior to RV-

A16 challenge. And finally, this increased epithelial hyperresponsiveness was 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 FEV₁% 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 FEV₁% predicted only before RV16.

Like in our previous report, not all asthma patients have BECs that are hyperresponsive [7], although in this study a large part 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 do make us speculate that there may be a predisposition for this defect in BECs at baseline.

BECs from asthma patients collected after RV-A16 infection released trend wise enhanced ET-1 and this correlated with CXCL-8, in line with similar regular motifs in the mRNAs encoding respectively CXCL-8 and preproendothelin-1. 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 preproendothelin-1 into endothelin-1 is not active in BECs at baseline. Although we found a weak but significant correlation between ET-1 and FEV₁ % predicted before RV-A16 exposure, there was no correlation post RV-A16 exposure. This may be due to inflammation-induced edema formation and mucus production in response to RV-A16 infection that, 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. First, despite enhanced CXCL-8 production, there was no correlation with neutrophils and MPO [17]. This may be due to various opposing effects. Neutrophil counts may not truly reflect neutrophilic inflammation as activated neutrophils having release 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 [18] 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. We do, however, 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. Also, rhinoviruses are known to induce various soluble proteins like IL-1RA [19] and IL-1 α [20] which occurred in only a couple of BEC cultures (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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Figure Legends:

Figure 1

Figure 1. Overview of study design. Screening was performed 14–56 days before RV-A16 challenge. Lung function tests, bronchoscopy and bronchoalveolar lavage fluid were collected at the indicated time points before and after RV-A16 challenge in mild asthma patients and healthy individuals.

Figure 2

Figure 2. TNF and IL-17A-induced mediator production by BECs from asthma patients and healthy individuals, before and after *in vivo* RV-A16 challenge. BECs collected after *in vivo* RV-A16 challenge from mild asthma patients ($n=18$; red symbols), as compared to BECs collected before the viral challenge, produced upon 16 hrs of exposure to TNF and IL-17A

significantly higher levels CXCL-8 **(A)**, IL-6 **(B)**, G-CSF **(C)**, CXCL-10 **(D)** and, trend wise, ET-1 **(E)**. In contrast, BECs from healthy individuals ($n=11$; black symbols) did not show an increase. For statistical analyses two-tailed, paired t-test (before vs after RV16), two-tailed unpaired t-test (healthy controls vs asthma patients) was used: ** $P<0.01$, * $P<0.05$ and ns; not significant. The bars and whiskers depict mean with standard deviation. 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 **(F)**. Correlations were done with Pearson's correlation coefficient: ** $P<0.01$, * $P<0.05$.

Figure 3

Figure 3

Figure 3. *In vitro* and *in vivo* CXCL-8 measurements and their correlation with FEV₁ % predicted and ET-1. CXCL-8 BALF levels increased significantly after RV-A16 exposure in asthma patients ($n=18$), but not in healthy individuals ($n=11$) **(A)**. BALF CXCL-8 correlates with CXCL-8 released from BECs obtained after RV-A16 exposure when data for healthy and asthma participants are combined **(B)**. RV-A16 infection in asthma patients ($n=18$) induced a significant drop in percentage FEV₁ predicted, but not for healthy individuals at day 4 after RV-A16 ($n=11$) **(C)**. ET-1 levels correlated significantly with FEV₁ % predicted before and not after exposure to RV-A16 **(D)** The change (delta) in CXCL-8 levels released *in vitro* by BECs obtained after RV-A16 correlated with FEV₁ measured after RV-A16, but not before RV-A16 exposure **(E)**. For statistical analyses two-tailed, paired t-test (before vs after RV16), two-tailed unpaired t-test (healthy controls vs asthma patients) was used: * $P<0.05$, ns; not significant. Non-parametric Spearman correlation test was performed with two-tailed analysis: *** $P<0.001$, * $P<0.05$, ns; not significant.

Figure 4

Figure 4. The change in eosinophils, neutrophils, MPO and ECP at day 6 post RV-A16 exposure. Comparison of deltas (percentage of cell numbers at day 6 after RV-A16 minus the baseline at day -1) in percentage eosinophils (A) shows no significant difference, while the deltas of neutrophils in BALF of healthy individuals ($n=11$) were negative (lower during infection than at baseline) and significantly lower than the marginal increase (positive delta) observed in mild asthmatics ($n=18$) (B). The deltas of ECP (C) and MPO (D) 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 mean with standard deviation. For statistical analyses two-tailed, non-parametric Mann-Whitney t-test was used for Figure 4A and 4C; unpaired t-test was used for Figure 4B and 4D: ** $P<0.01$, ns; not significant.

Supplementary figure legend:

Figure E1

Figure E1. TNF and IL-17A-induced mediator production by BECs from asthma patients and healthy individuals, before and after *in vivo* RV-A16 challenge, that did not change in response to RV-A16. CXCL-1 (A), IL-1RA (B), VEGF-a (C), IL-1 α (D), CXCL-12 (E) and M-CSF (F) did not differ in PBECs obtained before and after RV-A16 when stimulated with TNF and IL-17A stimulation for 16hrs in mild asthmatics ($n=11$) and healthy individuals ($n=18$). (black represents healthy individuals and red represents mild asthma patients). The bars and whiskers depict mean with standard deviation. For statistical analyses two-tailed, paired t-test was used ns; not significant.

Table:

	Healthy individuals		Mild asthmatics	
	Before RV-A16	After RV-A16	Before RV-A16	After RV-A16
Subjects (n)	11		18	
Age (years) *	22.36 (19-31)		21.77 (18-29)	
Sex ratio (M/F)	4 / 7		7 / 11	
Δ FEV ₁ pre-bronchodilator (L) †	3.93 (0.41)	3.89 (0.25)	4.11 (0.79)	3.99 (0.79)
Δ 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) *	>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)

*Mean (min-max)

† Mean (SD)

Table 1. Characteristics of mild asthma patients and healthy individuals before and after RV-A16 challenge.