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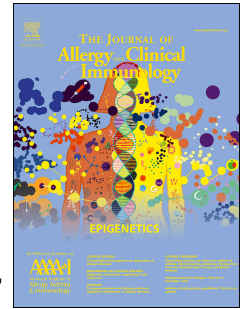
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Interferon-induced epithelial response to rhinovirus-16 in asthma relates to inflammation and FEV₁

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Capsule summary: Bronchial epithelial cells from mild asthma patients in response to *in vivo* rhinovirus-16 challenge, display a marked interferon-induced response 6 days post-infection, which correlates with eosinophilic inflammation and virus-induced decline of lung function.

Key words: transcriptomics, bronchial epithelial cells, virus-induced asthma symptoms

To the editor:

Asthma is a chronic inflammatory airway disease characterized by periodic worsening of asthma symptoms (loss of asthma control or, when more severe, exacerbation), which is often triggered by respiratory viral infections, particularly by rhinoviruses (RV) (1). The leading explanations for virus-induced worsening of asthma are an inadequate anti-viral response (2) and/or an exaggerated T_H2 response (3). Impaired production of innate interferons (2) by primary bronchial epithelial cells (PBECs) *in vitro* and that of type-1 interferons by bronchoalveolar lavage cells from asthmatics (4) have been reported, although this was not confirmed by others (5, 6). Importantly, *in vivo* data that can clarify the underlying mechanism is still lacking. We determined whether the transcriptomic profiles of PBECs from mild asthma patients obtained by bronchial brush before and 6 days after RV16 challenge, at the peak of respiratory symptoms (7), is an interferon-driven or T_H2 -driven (3) response. Furthermore, we assessed whether the response genes correlated with airway function and inflammation.

Bronchial epithelial brushes were obtained 1 day before and 6 days after RV16 challenge from the RESOLVE (NTR1677-(7) study and the MATERIAL (NCT01520051) study, the setup and time points of which were identical (Figure 1a). Patients with allergic asthma were not on inhaled or systemic corticosteroids. Detailed description of patients (table E1), inclusion and exclusion criteria, study procedures, RNA isolation and sequencing are provided in the online supplement.

A total of 174 genes (p value-adjusted <0.05) were differentially expressed between days -1 and 6 of which 131 were enhanced and 43 attenuated. Gene set enrichment analysis (GSEA) with gene ontology (GO) of differentially expressed genes, showed highly significant overlap with type I interferon response and defence response gene sets. Analysis of upregulated genes with interferome v2.01 showed that 15 interferon response genes can be induced by all interferons, 57 by both type I and II interferons, and 4 specific by type I interferon only (Figure E1a). Interestingly, T_H2 response genes (POSTN, SERPINB2, CLCA1) were not increased in PBECs 6 days after RV16 exposure (Figure E1b). The heat map shows 21 interferon (IFN) response genes (selected by GSEA) that were on average three-fold upregulated in PBECs after RV16 challenge (Figure 1b). The Z scores of these interferon response genes, increased for most patients after RV16 exposure (Figure 1c). Ingenuity pathway analysis (IPA) performed using all differently expressed genes to identify altered pathways showed both type I and II interferon response signaling pathways were upregulated (Figure 1d). The viral loads in BAL cells at day 6 after RV16 challenge (Figure E2a), but not those from nasopharyngeal swabs, correlated positively with the increased IFN response genes in PBECs (Figure E2b). Similar to our previous study (7) on low dose 10 TCID₅₀ RV16 challenge, asthma symptoms (ACQ) were increased 6 days after RV16 challenge (Figures E2c and 1e), which correlated with the Z score of IFN response genes (Figure 1f). Expression of IFI6, IFITM1 and IFITM3 after RV16 challenge, but no other IFN response

genes, displayed a significant inverse correlation with drop in FEV₁ (Figure 1g). IFN response genes did not correlate with PC₂₀ (Figure E2d).

BALF ECP (Figure 2a), but not percentage eosinophils (Figure 2b), significantly increased after RV16 challenge. There was a strong correlation between percentage eosinophils with IFN response genes after RV16 challenge in mild asthmatics (Figure 2c). ECP levels in BALF also strongly correlated with IFN response genes after and even before RV16 challenge (Figure 2d). Eosinophils in blood did not increase at day 6 after RV16 (Figure E3a), but interestingly correlated with IFN response genes only after RV16 (Figure E3b). FeNO levels in exhaled breath did not increase after RV16 (Figure E3c), but a significant positive correlation was seen with IFN response genes only after RV16 challenge (Figure E3d). The BALF neutrophil percentage and MPO did not increase after RV16 challenge (Figures E3e, E3g) and no correlation was observed between neutrophils and MPO with IFN response genes, neither before nor after RV16 challenge (Figures E3f, E3h). The innate cytokines CXCL-10, CXCL-8 and IL-6 were significantly increased in BALF after *in vivo* RV16 challenge (Figure 2e). Given that CXCL-10 can be induced by interferon, not surprisingly the Z scores of IFN response genes positively correlated with the change in CXCL-10 levels in BALF after RV16 challenge (Figure 2f), but not with CXCL-8 and IL-6.

Our findings clearly indicate that there is no intrinsic defect in the interferon response *in vivo*. There was a positive correlation between IFN response genes and viral load in BAL cells, indicating that the intensity of the interferon-induced response was dependent on the viral load. Furthermore, the IFN response genes, but not the viral load, correlated with FeNO, lung function parameters and asthma symptom scores after RV16 challenge. Similarly, eosinophilic inflammation after RV16 challenge, as reflected by eosinophil percentage and ECP, strongly correlated with interferon-induced responses. As ECP also correlated with interferon-induced responses before RV16 challenge, this is suggestive of a similar

underlying process before and after RV16 that is amplified by viral exposure. These above-mentioned correlations are also specific as IFN response genes did not correlate with markers of neutrophilic inflammation and the PC₂₀. Together this indicates that the interferon response may underlie the drop in FEV₁, asthma symptoms, FeNO and eosinophilic responses in asthma patients after RV16 exposure. These findings also substantiate interferon-induced cytokine CXCL-10 as an indicator of virus-induced exacerbations (8). In contrast to earlier reports on viral challenges in asthma we found no IL-13-driven response in PBECs at day 6 (9), nor an increase in BALF neutrophils. It is not likely that these differences were due to a difference in atopic status for the present asthma patients as skin prick testing, IgE levels and blood eosinophils are similar to those reported in other studies with RV16 challenges (Table E1). We cannot exclude that the differences are due to the later time point of sampling. Similarly, we cannot exclude an attenuated early interferon response because of sampling at day 6. In fact, we found that IL-33, which was overexpressed in a murine model of virus-induced asthma exacerbations and in PBECs from asthma patients 2 days after *in vivo* RV16 challenge as compared to appropriate controls (Ravanetti, L. et al., JACI in press), inhibited epithelial innate interferon production. Thus, IL-33 may underlie an initial deficient innate antiviral response, which may lead to a late marked interferon-induced gene response.

The supplemental file provides data from healthy controls that participated in the RESOLVE study. Even though based on few samples, interestingly at day 6 there is no interferon-induced response in PBECs from healthy controls exposed to RV16 *in vivo*. As virus was detected in BALF cells equally in healthy controls and patients, we consider it likely that the interferon response in healthy individuals had passed already. Currently, we are sampling 2 days after RV16 challenge in healthy individuals and asthma patients to clarify this.

In conclusion, we found a prominent interferon-induced response in PBECs from mild asthma patients during virus-induced loss of asthma control, at day 6. Strikingly, the increased

interferon-induced response induced by RV16 relates to the drop in FEV₁, increased ACQ scores, and enhanced airway inflammation (CXCL-10, ECP).

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Author's contribution: A.R. conducted experiments, analyzed the data and prepared the manuscript. J.K. supervised the transcriptome analyses. A.D. conducted experiments. S.M.B. and Y.S.S.P. carried out the RESOLVE and MATERIAL studies. P.I.B. and C.J.M. performed the clinical interventions. P.J.S. devised the study and prepared 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.

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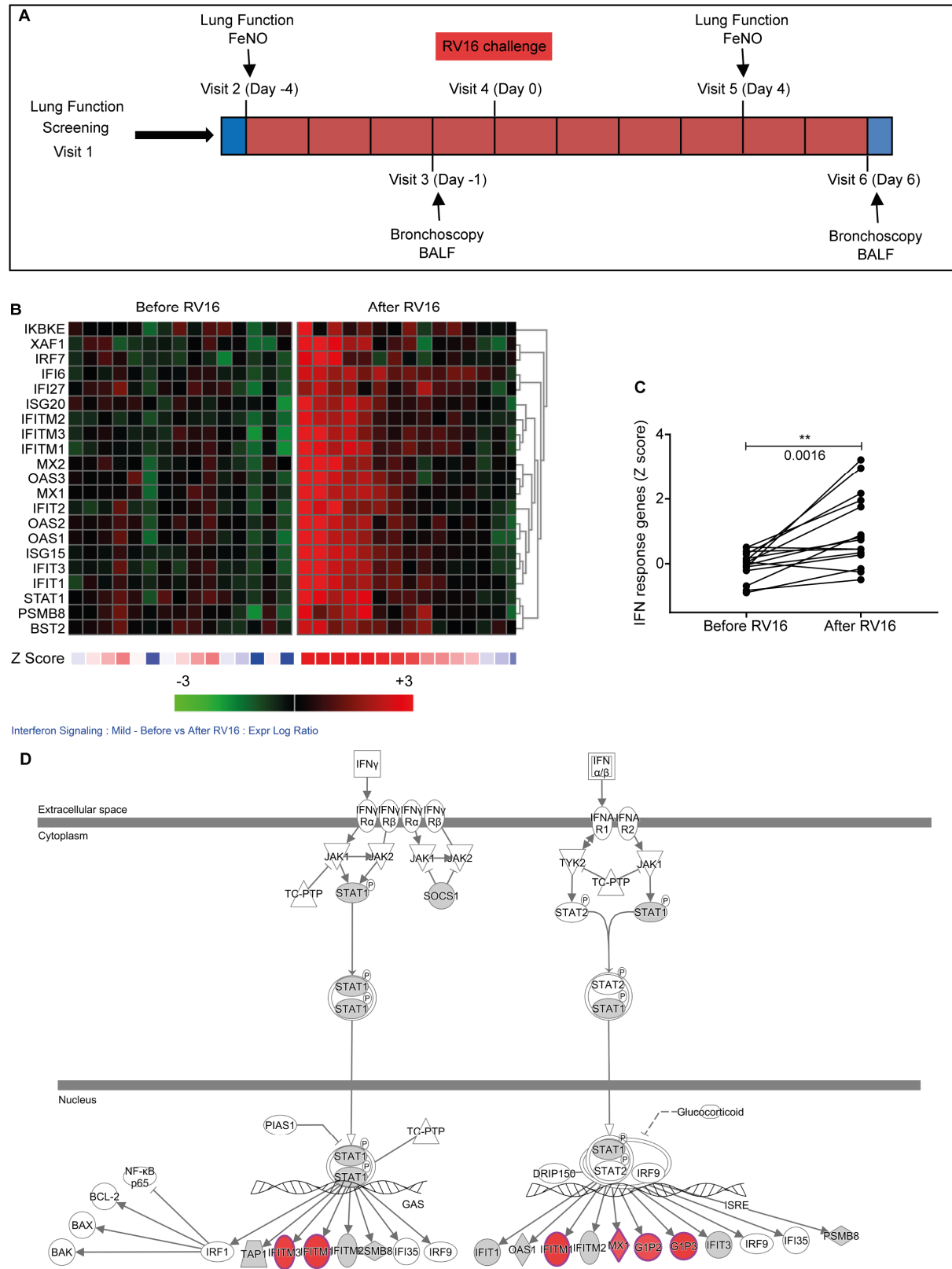
Figure Legends:

Figure 1. Enhanced type I interferon response in PBECs from mild asthmatics after *in vivo* RV16 challenge correlates with lung function. (A) Study overview showing RV16 challenge, in relation to sampling and clinical assessment. (B) Heat maps of 21 interferon response genes in PBECs from mild asthmatics ($n=15$) before and 6 days post-RV16. The intensity of average gene expression is represented as Z scores below the heat maps (blue for low and red for high gene expression). (C) As B, Z scores of interferon response genes plotted per patient; $**P<0.01$, paired t-tests. (D) Ingenuity pathway analysis for differentially expressed genes in asthma PBECs. Red and grey nodes represent upregulated genes, white

nodes represent genes that were not differentially expressed, but are in the pathway. **(E)** ACQ scores increased in mild asthma patients ($n=15$) at day 6 after RV16 challenge compared to baseline; $**P<0.01$, paired t-tests. **(F)** IFN response genes after RV16 challenge positively correlated with ACQ scores at day 6 after RV16. **(G)** The changes (Δ) in IFI6, IFITM1, IFITM3 in PBECs after RV16 infection correlated with change (Δ) in FEV₁% after RV16 challenge compared to before RV16 ($n=15$). All correlations were done by Pearson's correlation coefficient. $*P<0.05$.

Figure 2. IFN response genes in PBECs after RV16 challenge correlate with eosinophilic inflammation and CXCL-10 in BALF. BALF ECP **(A)** and percentage of BALF eosinophils **(B)** before and after RV16 challenge ($n=15$); $**P<0.01$, paired t-tests. IFN response genes correlated with percentage eosinophils **(C)** and ECP **(D)** after RV16 challenge ($n=15$). **(E)** BALF innate cytokines CXCL-10 ($n=15$), CXCL-8 ($n=14$) and IL-6 ($n=14$) before and after RV16 challenge; $*P<0.05$, $**P<0.01$ paired t-tests **(F)**. Z scores for IFN response genes correlated with change in BALF CXCL-10 after RV16 challenge ($n=15$) **(G)**. All correlations were done by Pearson's correlation coefficient; $**P<0.01$, $***P<0.001$.

Figure 1



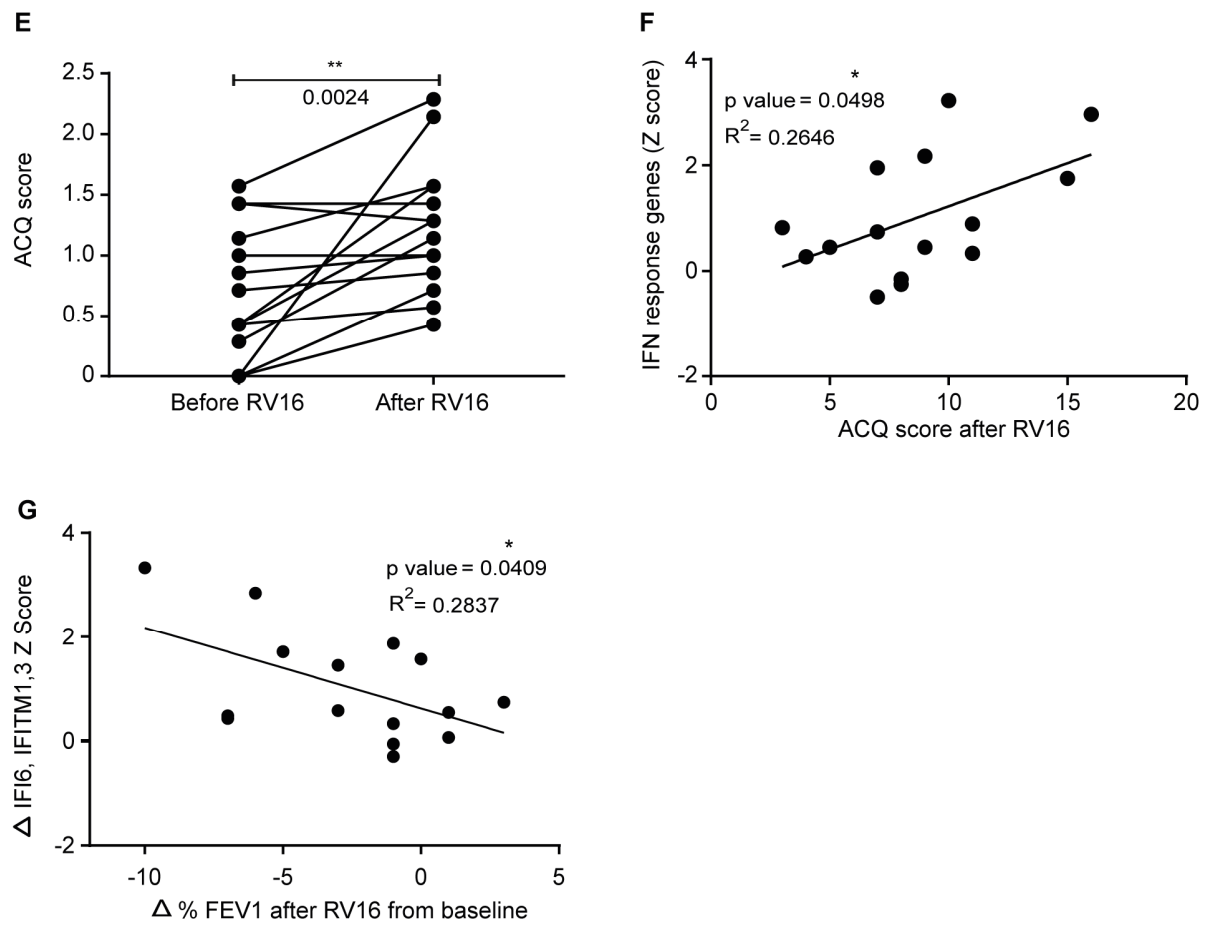
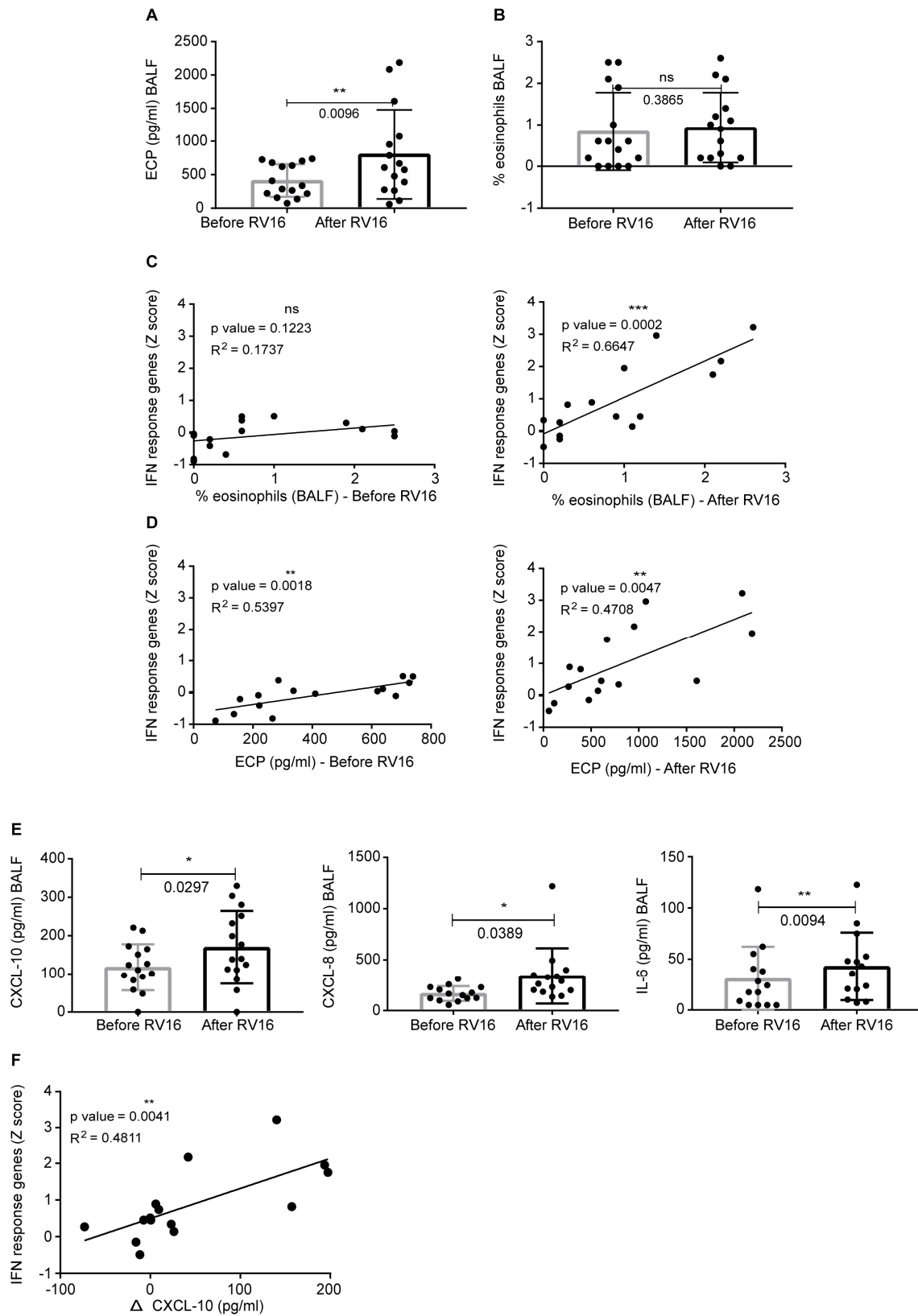


Figure 2



Supplementary text

Introduction

Parallel to the study with asthma patients, eleven healthy controls from the RESOLVE study (NCT1677) were subjected to RV16. The characteristics of the healthy controls are provided in table E2. Unfortunately, we could determine the transcriptomic profile in PBECs from four healthy individuals before and 6 days after RV16 challenge only. This was due to inappropriate handling of the samples and not to low quality of the RNA samples obtained from the controls. The characteristics of four healthy controls taken for transcriptomics are provided in table E3.

Material and Methods

Study procedures

The prebronchodilator FEV₁ was measured by standard spirometry according to the recommendations by the European Respiratory Society (1). The drop in FEV₁ was determined by the change in FEV₁ % predicted after RV16 challenge, compared to FEV₁ % predicted before RV16, at baseline. Methacholine bronchoprovocation tests by the standard tidal volume method with a maximum dose of 16 mg/ml were done to measure the PC₂₀ (2). Bronchoalveolar lavage (BAL) fluid and bronchial brushes were collected by standard bronchoscopic procedure (3). BALF recovery (73% ± 6.6%; mean ± SD) was similar in patients and healthy controls, allowing for direct comparison. The 73% recovery is in line with 68% recovery in our routine diagnostics that we obtain for 8 subsequent washes with 20 ml of saline. FeNO was measured by a chemiluminescence analyser (4). CXCL-10, CXCL-8, IL-6 was determined by Luminex according to the manufacturer's protocol (Bio-Rad Laboratories, Veenendaal, The Netherlands). Eosinophil cationic protein (ECP) and myeloperoxidase (MPO) were measured by ELISA as described previously (5). Rhinovirus

was detected by quantitative PCR in the nasal swabs and BAL cells collected 6 days after RV16 infection (6).

Subjects

The study protocols were reviewed and approved by the ethical review committee and were in accordance with the declaration of Helsinki. All patients and controls provided written informed consent. The studies were conducted in one center only, at the Department of Respiratory Medicine of the Academic Medical Center, Amsterdam, The Netherlands. 10 mild asthma patients and all healthy controls were from the RESOLVE study and 5 mild asthma patients were from the MATERIAL study. PBECs from asthma patients in the MATERIAL study were obtained only from patients in the placebo group. Patients with mild allergic asthma met the following criteria: a history of episodic chest symptoms, baseline forced expiratory volume in 1 second (FEV_1) $>80\%$ predicted, airway responsiveness to methacholine (provocative concentration causing 20% fall in FEV_1 , $PC_{20} < 9.8$ mg/mL), skin prick test positive for at least one of 10 common aeroallergens. We cannot exclude a bias in the male, female ratio in mild asthma patients.

The study design for healthy controls is similar to that of the mild asthma patients. Healthy individuals had a $FEV_1 >80\%$ predicted, $PC_{20} >16$ mg/mL, skin prick test negative for 12 common aeroallergens. To determine the atopic status of these patients, skin prick tests were done with 10 locally relevant aeroallergens: grass mix, tree mix, house dust mite, cats, dogs, alternaria alternatum, aspergillus fumigatus, cladosporium herbarum, latex, rabbit. (ALK, Hørsholm Denmark). All volunteers were aged between 18 and 40 years, were non-smoking or had stopped smoking 12 months ago with ≤ 5 pack years, were negative for serum neutralizing antibodies against RV16 (1 in 4 or lower) and did not have concomitant disease or a (chronic) inflammatory condition that would interfere with this study according to the

judgment of a pulmonary physician. Volunteers with an underlying viral airway infection on day 1, as determined by PCR on nasal swabs, were withdrawn from the study. The criteria for successful infection are at least one of these: RV16 seroconversion, positive Common Cold Score or detection of RV in nasal swab or lavage.

RNA isolation and sequencing

The brushes which consisted predominantly (>95%) of PBECs, were pooled (2 brushes for each sample) and centrifuged at 1240 rpm (Rotanta 460S) for 10 minutes at 4°C. The pellet was dissolved in 1 ml of TRIzol™ and stored at -80°C until RNA was isolated. After all samples were obtained, they were thawed at room temperature and, after 200 µl of chloroform was added, shaken vigorously for 30 seconds. The samples were kept at RT for 10 mins and centrifuged at 16,000g for 15 mins at 4°C. The aqueous phase was cleaned up and concentrated with protocol 5.3 using the Nucleospin® RNA XS extraction kit (Macherey-Nagel). No carrier RNA was added as it would interfere with RNA sequencing. The quality and concentration of the samples were assessed by fragment analyser (Advanced Analytical Technologies, Inc.). RNA from four brushes from healthy controls was of sufficient quality for transcriptome analysis. A dataset consisting of 38 samples were sequenced on Illumina NextSeq 500 sequencer.

cDNA preparation and RNA sequencing

The NEBNext Ultra Directional RNA library prep kit for Illumina was used to process the samples. The samples preparation was performed according to the protocol “NEBNext Ultra Directional RNA Library Prep Kit for Illumina” (NEB #7420S/L). Briefly, oligo-dT magnetic beads were used to isolate mRNA from total RNA. cDNA synthesis was performed after fragmentation of this mRNA. This was used for ligation with sequencing adapters followed by PCR amplification of the resulting product. The quality and yield after sample preparation

was measured with the fragment analyser. The size of the resulting products was consistent with the expected size distribution between 300-500 base pairs. To evaluate the quality of the library preparation and kits used, the raw data was sampled and mapped to annotated genomic references. Mapping positions were classified as intragenic, exonic, intergenic, intronic and rRNA. Clustering and DNA sequencing was performed according to the manufacture's protocol using the Illumina NextSeq 500. A concentration of 1.6pM was used as input. The reads were trimmed for adapter sequences using Trimmomatic v0.30 before the alignment. Presumed adapter sequences were removed from the read when the bases matched a sequence in the adapter sequence set (TruSeq adapters) with 2 or less mismatches and an alignment score of at least 12. The reference of 'Homo_sapiens.GRCh37.75' was used to align the reads. The mapping to the reference sequence was done using a short reader aligner based on Burrows-Wheeler transform. The default of mismatch rate of 2% (3 mismatches in a read of 150 bases) was used.

Sequence data analysis

The frequency of the reads mapped on the transcript was determined as counts, which is used as an input for downstream analysis. Additionally, RPKM and FPKM (reads/fragments per kilobase of exon per million reads mapped) values were calculated. The read counts were loaded into the DESeq2 package, a statistical package within the R/BioConductor platform to determine the differentially expressed genes (paired). Image analysis, base calling and quality check was performed with Illumina data analysis pipeline RTA v2.4.11 and Bcl2fastq v2.17. Z scores used for correlations are calculated in log₂ gene expression values, where the average (of the cohort) is subtracted from every sample value and divided by the standard deviation (of the cohort). The datasets can be accessed in GEO with <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE106388>

Supplementary Results

IFN response genes were not enhanced in healthy controls at day 6 after RV16 challenge

Contrary to what was found for PBECs from mild asthma patients, interferon response genes showed no increase in PBECs from healthy controls after RV16 challenge at day 6 (Figure E4a and E4b).

Supplementary figure legends:

Figure E1. Type of IFN response in PBECs from mild asthmatics after RV16 and that of the IL-13-driven T_H2 response genes in mild asthmatics and healthy controls. (A) The

Venn diagram represents the type of interferons that can induce the interferon response genes in PBECs after RV16. (B) The Z score of T_H2 response genes (CLCA1, POSTN, SERPINB2) plotted individually did not significantly differ before and after RV16 challenge in mild asthmatics ($n=15$) and healthy controls ($n=4$).

Figure E2. Viral copies in BAL correlate with IFN response genes but not with PC_{20} . (A)

Viral copies measured in BAL cells from healthy ($n=11$) and mild asthma patients ($n=15$) did not differ 6 days after RV16 challenge. (B) Viral load in BAL cells correlated positively with Z scores of IFN response genes, but no correlation was observed with viral load in the nasal swab ($n=15$); Pearson's correlation analysis: $**P<0.01$. (C) ACQ scores measured at day 1 before RV16 and 6, 13 days after RV16 ($n=15$). (D) There was no correlation observed between $2\log PC_{20}$, reflecting airway hyperresponsiveness, and interferon response genes before and after RV16 challenge (Pearson's correlation analysis).

Figure E3. Blood eosinophils, FeNO, BALF neutrophils and MPO before and after RV16 and their correlation with IFN response genes. (A) Blood eosinophils did not

increase at day 6 after RV16 in mild asthmatics ($n=15$). (B) IFN response genes correlated

with blood eosinophils only after RV16 in mild asthma patients **(C)** FeNO levels were increased in mild asthma patients at baseline but did not significantly differ before and after RV16 challenge in both mild asthmatics ($n=15$) and healthy controls ($n=12$); $**P<0.01$, $***P<0.001$, unpaired t-tests. **(D)** IFN response genes in PBECs also correlated with FeNO more significantly after RV16 challenge in mild asthmatics ($n=15$); Pearson's correlation analysis: $**P<0.01$. **(E)** The percentage of neutrophils in BALF did not change and **(F)** did not correlate with interferon response genes before and after RV16 challenge in mild asthmatics ($n=15$). **(G)** The MPO levels in BALF were not altered significantly before and after RV16 challenge ($n=15$). **(H)** Interferon response genes did not correlate with MPO levels before and after RV16 challenge ($n=15$).

Figure E4. PBECs from healthy controls show no enhanced IFN response genes after RV16 at day 6. (A) Heat maps of interferon responses genes in PBECs from healthy controls before and 6 days after *in vivo* RV16 exposure ($n=4$). **(B)** No differences were observed in Z scores of interferon response genes plotted per healthy control ($n=4$).

Supplementary tables

Table E1. Characteristics of mild asthma patients before and 6 days after RV16 challenge.

	Mild asthma patients	
	Before RV16	After RV16
Subjects (n)	15	
Age (years) *	22.82 (18-38)	
Sex ratio (M:F)	0.54	
tIgE (kU/L) †	140.77 (126.96)	
Number of positive skin prick tests †	4.52 (2.06)	
Δ FEV₁ pre-bronchodilator (L) †	3.80 (0.85)	3.73 (0.91)
Blood eosinophils (10⁹/L) †	0.23 (0.23)	0.23 (0.21)
Δ FEV₁ pre-bronchodilator (% pred) †	97.80 (6.37)	95.51 (8.06)
PC₂₀ (mg/ml) *	2.14 (0.09 - 7.35)	2.25 (0.13 - 8.00)
FeNO (ppb) †	58.03 (37.14)	63.23 (34.54)

*Mean (min-max) † Mean (SD)

Table E2. Characteristics of healthy controls before and 6 days after RV16 challenge.

	Healthy controls	
	Before RV16	After RV16
Subjects (n)	16	
Age (years) *	22.53 (19-31)	
Sex ratio (M:F)	0.25	
Δ FEV₁ pre-bronchodilator (L) †	4.13 (0.69)	4.03 (0.55)
Δ FEV₁ pre-bronchodilator (% pred) †	107.69 (10.37)	106.36 (10.13)
PC₂₀ (mg/ml) *	>16	
FeNO (ppb) †	20.8 (11.09)	21.09 (13.01)

*Mean (min-max) † Mean (SD)

Table E3. Characteristics of healthy controls before and 6 days after RV16 challenge, taken for transcriptomics

	Healthy controls	
	Before RV16	After RV16
Subjects (n)	4	
Age (years) *	21.75 (20-25)	
Sex ratio (M:F)	0.25	
Δ FEV₁ pre-bronchodilator (L) †	3.85 (0.30)	3.80 (0.30)
Δ FEV₁ pre-bronchodilator (% pred) †	106.25 (10.30)	105.25 (10.23)
PC₂₀ (mg/ml) *	>16	
FeNO (ppb) †	13.20 (4.30)	14.50 (3.74)

*Mean (min-max) † Mean (SD)

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