

Deficient antiviral immune responses in childhood: Distinct roles of atopy and asthma

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Background: Impaired immune response to viral infections in atopic asthmatic patients has been recently reported and debated. Whether this condition is present in childhood and whether it is affected by atopy *per se* deserves further investigation.

Objective: We sought to investigate airway interferon production in response to rhinovirus infection in children who are asthmatic, atopic, or both and its correlation with the airway inflammatory profile.

Methods: Bronchial biopsy specimens and epithelial cells were obtained from 47 children (mean age, 5 ± 0.5 years) undergoing bronchoscopy. The study population included asthmatic children who were either atopic or nonatopic, atopic children without asthma, and children without atopy or asthma. Rhinovirus type 16 induction of IFN- λ and IFN- β mRNA and protein levels was assessed in bronchial epithelial cell cultures. The immunoinflammatory profile was evaluated by means of immunohistochemistry in bronchial biopsy specimens.

Results: Rhinovirus type 16-induced interferon production was significantly reduced in atopic asthmatic, nonatopic asthmatic, and atopic nonasthmatic children compared with that seen in nonatopic nonasthmatic children (all $P < .05$). Increased rhinovirus viral RNA levels paralleled this deficient interferon

induction. Additionally, IFN- λ and IFN- β induction correlated inversely with the airway T_H2 immunopathologic profile (eosinophilia and IL-4 positivity: $P < .05$ and $r = -0.38$ and $P < .05$ and $r = -0.58$, respectively) and with epithelial damage ($P < .05$ and $r = -0.55$). Furthermore, total serum IgE levels correlated negatively with rhinovirus-induced IFN- λ mRNA levels ($P < .05$ and $r = -0.41$) and positively with rhinovirus viral RNA levels ($P < .05$ and $r = 0.44$).

Conclusions: Deficient interferon responses to rhinovirus infection are present in childhood in asthmatic subjects irrespective of their atopic status and in atopic patients without asthma. These findings suggest that deficient immune responses to viral infections are not limited to patients with atopic asthma but are present in those with other T_H2-oriented conditions. (J Allergy Clin Immunol 2012;■■■:■■■-■■■.)

Key words: Asthma, viral infections, interferons, T_H2 inflammation, epithelial damage

Viral upper respiratory tract infections are, directly or indirectly, responsible for vast health care use worldwide. Viral infections are particularly important in asthmatic patients, in whom rhinovirus is the most frequent cause of exacerbations both in adults and children.^{1,2} Furthermore, data from longitudinal studies suggest that wheezing episodes associated with viral infections early in life are a major risk factor for the development of asthma later in life.³⁻⁵

Impaired immune response to viral infections has been proposed as a mechanism for increased susceptibility to infections in asthmatic patients.^{6,7} This concept is supported by studies reporting deficient virus-induced interferon production in bronchial epithelial cells, alveolar macrophages, and blood cells in adult asthmatic patients.⁸⁻¹⁰ However, the relevance of this concept has been questioned by other studies that did not confirm impaired antiviral responses in asthmatic patients.¹¹⁻¹³

The relationship between viral infections, innate immunity, and asthma is complex, particularly in childhood.¹⁴⁻¹⁷ Of note, none of the studies conducted thus far in children specifically examined these responses in the respiratory system. Whether an abnormal immune response is present in the airways of young children or whether it develops later as a consequence of long-term immune deregulation still needs to be investigated.

Another unresolved issue is the role of atopy. The majority of studies performed thus far on immune responses to viral infections examined patients with concomitant asthma and atopy. Interestingly, a recent longitudinal study showed that children who were sensitized to aeroallergens (even without respiratory symptoms) had a greater risk of having viral wheeze,¹⁸ suggesting that allergic sensitization *per se* could influence susceptibility to viral infections. Therefore dissecting the role of atopy from that of asthma in their effects on the immune responses to viral infections

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Abbreviations used

RV16: Rhinovirus type 16
vRNA: Viral RNA

is an important field of investigation that needs to be properly addressed. For these purposes, we investigated *ex vivo* rhinovirus-induced interferon production in bronchial epithelial cells from asthmatic children (both atopic or nonatopic), atopic children without symptoms of asthma, and control children. We also investigated the immunopathologic profile in airway biopsy specimens of these children and correlated these profiles with the *ex vivo* innate responses to rhinovirus.

METHODS**Study subjects**

The study population included 47 children who underwent bronchoscopy for appropriate clinical indications at the Department of Pediatrics of the University of Padova, Padua, Italy. Children were recruited from March 2007 to May 2008. Children were classified as atopic when they had increased total (determined by using paper radioimmunosorbent tests) and specific (determined by using RASTs) IgE levels. For further details, see the **Methods** section and **Table E1** in this article's Online Repository at www.jacionline.org.

According to previous studies evaluating similar populations,^{19,20} children were classified as having asthma if they had repeated episodes of wheezing, breathlessness, and coughing, particularly at night and in the early morning, that were present apart from colds. Symptoms had to be responsive to bronchodilators prescribed by the child's pediatrician. Data on the presence and reversibility of episodic symptoms were evaluated retrospectively at admission for bronchoscopy by a respiratory pediatrician after reviewing clinical records/prescriptions and making inquiries of the parents. The information was implemented by a questionnaire administered to parents. A detailed description is reported in the **Methods** section in this article's Online Repository. This approach, which is based mostly on clinical judgment and assessment of symptoms, is in accordance with Global Initiative for Asthma guidelines for the diagnosis of asthma in children aged 5 years and younger and allowed for consistent diagnostic criteria for all children, including those too young for spirometry.²¹ Spirometry was performed only in children who were able to cooperate with the test (≥ 6 years old).

Fiberoptic bronchoscopy was conducted in accordance with international guidelines.²² Written consent was obtained from children's parents after they were informed that bronchial brushings and biopsy specimens would be taken for research purposes. The study was performed according to the Declaration of Helsinki and was approved by the Ethics Committee of the Padova City Hospital.

Viral stocks

Rhinovirus type 16 (RV16; a major group rhinovirus) was obtained from the Health Protection Agency Culture Collections, Health Protection Agency Microbiology Services, Salisbury, United Kingdom, and used for all experimental conditions described. The virus was used at a multiplicity of infection of 5 for all experiments.²³

Primary bronchial epithelial cell cultures and cell processing

Human bronchial epithelial cells were harvested during bronchoscopy by means of brushing and were frozen as previously described.^{8,9} Total RNA levels and supernatants were obtained at the appropriate time points,^{8,9} as detailed in the **Methods** section in this article's Online Repository. In selected experiments cells were stimulated with inocula in which viral particles were

removed by means of ultrafiltration through membranes (Amikon, London, United Kingdom).²³

Quantitative TaqMan real-time RT-PCR

Quantitative real-time PCR was carried out with specific primers and probes for rhinovirus, IFN- β , IFN- λ (specific for IFN- λ_1), IL-8 (Qiagen), and 18S rRNA (a housekeeping gene), as detailed in the **Methods** section in this article's Online Repository. Interferon, IL-8 mRNA, and viral RNA (vRNA) expression were normalized to 18S rRNA levels, compared with standard curves, and expressed as copy numbers per microgram of RNA.

ELISA

ELISAs for detection of IFN- β , IFN- λ , and IL-8 levels in cell-culture supernatants were performed according to the manufacturers' instructions. Plates were read on a Labsystems plate reader and analyzed with SoftMax Pro software (Molecular Devices, Sunnyvale, Calif). Sensitivities, specificities, and sources for the individual ELISAs are detailed in the **Methods** section in this article's Online Repository.

Bronchial biopsy specimens

Bronchial biopsy specimens were processed for histologic and immunohistochemical analysis, as previously described.^{19,20,24} An analysis of epithelial damage was performed on sections stained with hematoxylin and eosin. Inflammatory cell counts (eosinophils, neutrophils, macrophages, and CD4⁺ T lymphocytes), as well as IL-4 expression, were quantified in the subepithelium by means of immunohistochemistry and expressed as the number of cells per square millimeter of examined tissue.

Statistical analysis

All cases were coded and the measurements were made without knowledge of clinical data to avoid observer bias. Comparisons among groups were evaluated with either ANOVA or the Kruskal-Wallis test, followed, when results were significant, by using Student *t* tests or Mann-Whitney *U* tests, as appropriate. Correlation coefficients were calculated by using the nonparametric Spearman rank method. *P* values of .05 or less were considered to indicate statistical significance.

RESULTS**Clinical findings**

The bronchoscopic procedures were well tolerated by all children, and no complications were encountered. During the procedure, bronchial brushings and biopsy specimens were collected from all subjects. Thirteen of the 47 primary cultures did not produce significant growth (4/12 from atopic asthmatic, 3 of 12 from nonatopic asthmatic, 3 of 11 from atopic nonasthmatic, and 3 of 12 from nonatopic nonasthmatic children). Therefore clinical and experimental data refer to the 34 of 47 children whose primary bronchial epithelial cells were successfully cultured (8 atopic asthmatic, 9 nonatopic asthmatic, 8 atopic nonasthmatic, and 9 nonatopic nonasthmatic children; **Table I**). No clinical differences were found between the patients included or not included in the analysis. None of the children in the nonasthmatic groups subsequently complained of wheezing episodes (mean follow-up after bronchoscopy, 49 months).

Age and sex were not significantly different among the 4 groups of children (additional analyses are shown in **Tables E2-E4** in this article's Online Repository at www.jacionline.org). Importantly, age at onset and symptom duration were not significantly different between atopic and nonatopic asthmatic children (**Table I**). Clinical

TABLE I. Clinical characteristics of the children in the study population

	Asthmatic atopic children (n = 8)	Asthmatic nonatopic children (n = 9)	Nonasthmatic atopic children (n = 8)	Nonatopic nonasthmatic children (n = 9)
No. and sex (male/female)	6/2	5/4	3/5	4/5
Age (y)	5 (2-13)	5 (3-7)	5 (2-12)	5 (2-7)
Age at onset of asthma symptoms (y)	2.9 ± 1.2	2.2 ± 0.4	—	—
Duration of asthma symptoms (y)	3.4 ± 1.2	3.1 ± 0.5	—	—
Monthly infective episodes in last year	1.3 ± 0.5	2.1 ± 0.4	1.5 ± 0.2	1.7 ± 0.3
Total IgE (U/mL)	749 ± 347*†	40 ± 10	376 ± 260*†	21 ± 8

Data are expressed as medians (ranges) or means ± SEMs.

* $P < .05$ compared with control children.

† $P < .05$ compared with asthmatic nonatopic children (t test).

indications for bronchoscopy did not differ significantly among the 4 groups, and these indications are summarized in the **Results** section in this article's Online Repository at www.jacionline.org. Groups were comparable in terms of the frequency of infective episodes in the previous year. Lung function values were obtained in all children able to cooperate with the test (3 atopic asthmatic, 2 nonatopic asthmatic, 2 atopic nonasthmatic, and 2 control children) and were normal in all children, except for 1 atopic asthmatic child (FEV₁ of 68% of predicted value). The number of hospitalizations or emergency department visits for asthma exacerbation was not different in atopic and nonatopic asthmatic children (see the **Results** section in this article's Online Repository).

No children were chronically treated with oral or high-dose inhaled corticosteroids. Eleven of 17 asthmatic children received inhaled salbutamol as needed, whereas the remaining 6 patients were taking low doses of inhaled corticosteroids (equivalent daily doses of beclomethasone ranging from 200-400 mg). This group of 6 patients represented equal numbers of atopic and nonatopic asthmatic children (n = 3 each). No differences were observed between asthmatic children treated with inhaled steroids and those who were not (see **Table E5** in this article's Online Repository at www.jacionline.org). Only 1 child (from the asthmatic nonatopic group) was treated with cysteinyl leukotriene receptor inhibitors. Finally, none of the children were treated with antibiotics at the time of bronchoscopy or in the 4 weeks before the study.

Ex vivo interferon induction and rhinovirus replication in bronchial epithelial cells

RV16 vRNA was undetectable in all primary cell cultures included in the study before the experimental infection. Similarly, IFN- λ and IFN- β mRNA levels were very low (close to the lowest limit of detection) in uninfected cells from all groups (see the **Results** section in this article's Online Repository).

Eight hours after RV16 infection, a significant induction in IFN- λ and IFN- β mRNA levels was observed in all subject groups (approximately 3-fold log₁₀ increase, $P < .001$). However, the induction of IFN- λ and IFN- β in both atopic and nonatopic asthmatic children was significantly less than that seen in nonatopic nonasthmatic children (**Fig 1, A and B**, and see **Fig E1** in this article's Online Repository at www.jacionline.org). Epithelial cells from atopic children without asthma showed a similar impairment in interferon induction after RV16 infection when compared with those from nonatopic nonasthmatic children (**Fig 1, A and B**, and see **Fig E1**).

Conversely, IL-8 mRNA and protein levels (used as a control) were similar in the 4 groups of subjects, demonstrating that epithelial cells were indeed responsive to viral stimuli. Finally,

inactivation by means of physical removal of the viral particles completely abrogated the rhinovirus-mediated induction of interferon mRNA (see **Figs E1 and E2** in this article's Online Repository at www.jacionline.org).

Twenty-one cell-culture supernatants were available to analyze the IFN- β and IFN- λ protein levels 48 hours after infection (see **Table E2**). IFN- β levels were similar among the groups in uninfected cell cultures (see **Fig E1**). RV16 infection resulted in significant increased IFN- β protein production in all groups (see **Fig E1**), but the levels were significantly lower in atopic asthmatic, nonatopic asthmatic, and atopic nonasthmatic children when compared with those in control children (**Fig 1, C**). IFN- λ protein levels were detectable in samples from 9 subjects only, too few for a meaningful analysis.

The reduction in IFN- λ and IFN- β mRNA levels was mirrored by a significant increase in RV16 vRNA levels (>1 log₁₀) in both atopic and nonatopic asthmatic children when compared with those seen in nonatopic nonasthmatic children (**Fig 1, D**). The levels of mRNA encoding for IFN- λ and IFN- β were correlated to each other (see **Fig E1**). Incremental changes in IFN- λ and IFN- β mRNA levels between 0 and 8 hours were inversely related to changes in vRNA levels between the same time points in the entire population (see **Figs E3 and E4** in this article's Online Repository at www.jacionline.org) and when groups were considered individually (data not shown).

Immunopathologic profiling in bronchial biopsy specimens

Because impaired interferon responses were similar in both atopic and nonatopic asthmatic children and in atopic children without asthma, we sought to determine the underlying pathogenic similarities among these conditions. The expression of the T_H2 cytokine IL-4 was significantly increased in bronchial biopsy specimens of atopic asthmatic children, nonatopic asthmatic children, and atopic children without asthma compared with that seen in nonatopic nonasthmatic children (**Fig 2, A**). Similarly, eosinophil levels were greater in the blood of atopic asthmatic children, nonatopic asthmatic children, and atopic children without asthma when compared with those seen in nonatopic nonasthmatic children (**Fig 2, B**). The number of eosinophils in airway biopsy specimens paralleled those in peripheral blood, but the overall comparison did not reach the level of statistical significance ($P = .08$; **Fig 2, C**). No significant differences were observed among the 4 groups of children in CD4⁺ T-lymphocyte, macrophage, and neutrophil counts (**Table II**). Additionally, prominent destruction of the bronchial epithelial

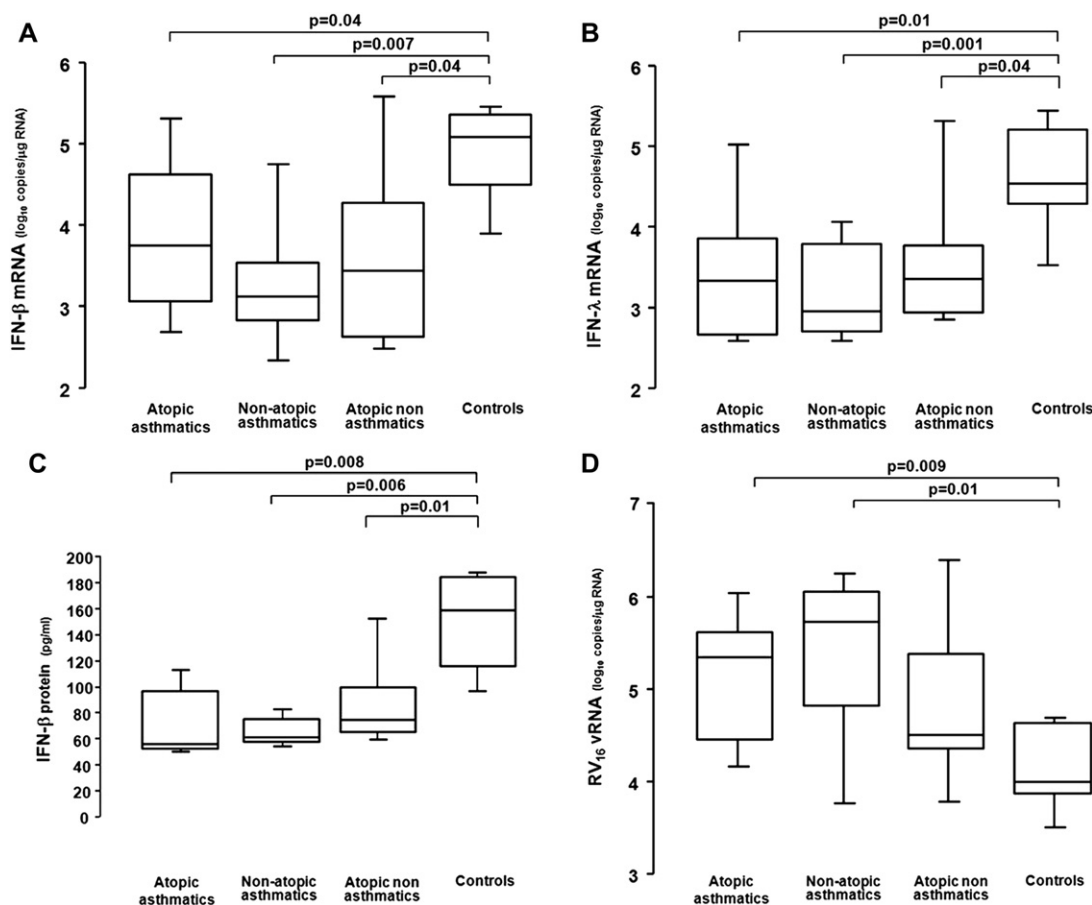


FIG 1. Interferon induction and rhinovirus vRNA levels. **A**, RV16-induced IFN-β mRNA levels at 8 hours ($P = .02$, Kruskal-Wallis test). **B**, RV16-induced IFN-λ mRNA levels at 8 hours ($P = .009$, Kruskal-Wallis test). **C**, RV16-induced IFN-β protein levels at 48 hours ($P = .02$, Kruskal-Wallis test). **D**, Rhinovirus vRNA levels at 8 hours ($P = .02$, Kruskal-Wallis test). *Bottom and top of the box plot: 25th and 75th percentiles; solid line, median; brackets, 10th and 90th percentiles.* Fig 1, A, B, and D: Atopic asthmatic children, $n = 8$; nonatopic asthmatic children, $n = 9$; atopic nonasthmatic children, $n = 8$; control children, $n = 9$. Fig 1, C: Atopic asthmatic children, $n = 6$; nonatopic asthmatic children, $n = 5$; atopic nonasthmatic children, $n = 5$; control children, $n = 5$.

layer was found in the 3 groups of children with asthma, atopy, or both (Fig 2, D). The photomicrographs in Fig 3 are representative examples of the pathologic changes (ie, eosinophilia, IL-4 expression, and epithelial damage) that were observed in airway biopsy specimens.

Correlations linking *ex vivo* immune responses to immunopathologic parameters

We then explored the interplay between the T_H2 microenvironment and the structural changes in the airways and the *ex vivo* immune responses and viral replication.

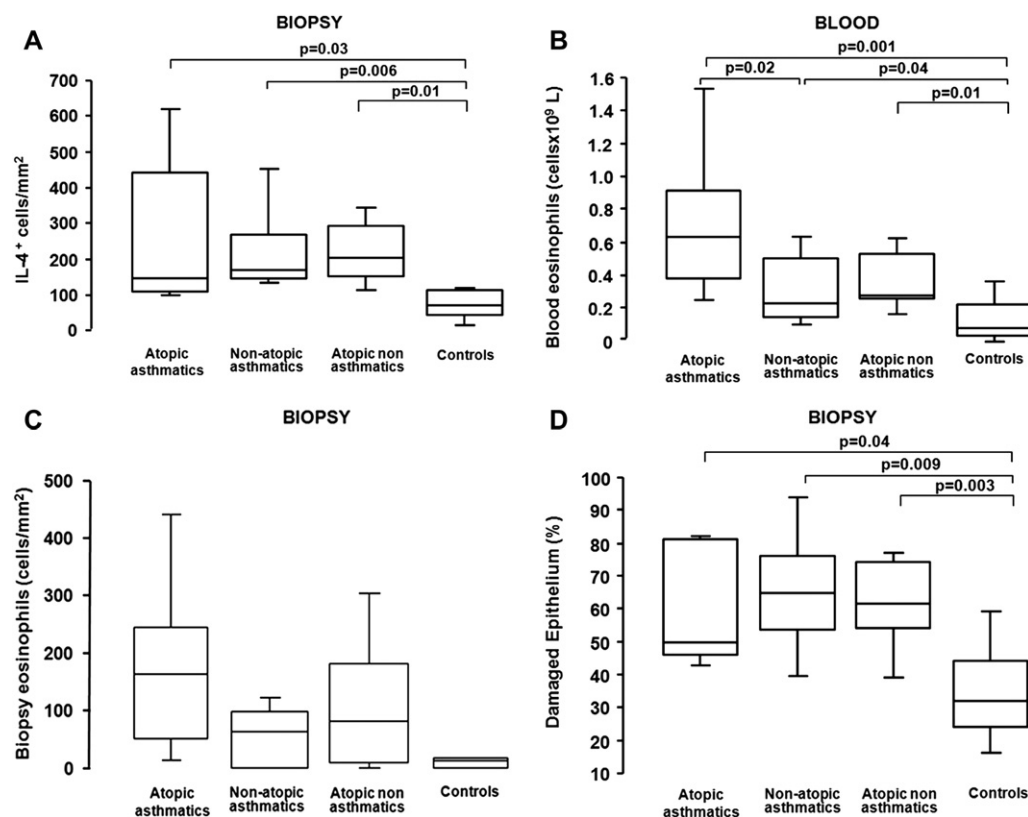
Ex vivo RV16-induced IFN-β mRNA levels at 8 hours were inversely correlated with airway eosinophil counts ($P < .05$ and $r = -0.38$). Consistently, when children were categorized into those with high or low levels of tissue eosinophilia (based on a previous report²⁵), we found that children with higher eosinophil levels exhibited weaker IFN-λ and IFN-β mRNA induction on RV16 infection (Fig 4, A and B). Children with high eosinophil counts also had a trend toward decreased IFN-β protein production and increased vRNA levels, which did not reach statistical significance (data not shown).

Furthermore, *ex vivo* RV16-induced IFN-β protein levels at 48 hours were inversely related to blood eosinophil counts ($P = .008$ and $r = -0.59$), the expression of IL-4 in airway biopsy specimens (Fig 4, C), and the degree of epithelial damage (Fig 4, D).

Finally, total serum IgE levels were negatively correlated with RV16-induced IFN-λ mRNA levels at 8 hours (Fig 5, A) and positively correlated with rhinovirus vRNA levels at the same time point (Fig 5, B; detailed analyses are presented in the Results section in this article's Online Repository).

DISCUSSION

In this study we investigated whether asthma, atopy, or both in children were associated with abnormal innate immune responses in terms of IFN-λ and IFN-β production after *ex vivo* RV16 infection. Our results showed that (1) children with atopic asthma, just as adults with this condition, had deficient epithelial interferon production in response to *ex vivo* viral infection; (2) asthmatic children show the same impaired response to rhinovirus, even in the absence of atopy; and (3) impaired interferon production occurs also in atopic children without symptoms of asthma.

**TABLE II.** Immunopathologic profile of the bronchial biopsy specimens

	Asthmatic atopic children (n = 6)	Asthmatic nonatopic children (n = 7)	Nonasthmatic atopic children (n = 6)	Nonatopic nonasthmatic children (n = 6)
CD4 ⁺ cells/mm ²	739 (476-958)	415 (137-1014)	581 (400-743)	373 (175-1108)
Macrophages/mm ²	493 (145-597)	113 (0-241)	184 (0-354)	56 (16-366)
Neutrophils/mm ²	514 (49-1023)	96 (0-244)	98 (0-742)	152 (98-219)

Data are expressed as medians (ranges). All P values are greater than .05, as determined by using the Kruskal-Wallis test.

Innate immune responses are the first defense against viral infections. Previous studies suggested that the immune response to viral infections is deficient in adult atopic asthmatic patients⁸⁻¹⁰ and that this deficiency correlates with the severity of virus-induced asthma exacerbations⁹ and asthma symptoms.¹² Furthermore, there is clinical evidence that asthmatic patients, once infected with either viruses^{1,26,27} or bacteria,²⁸⁻³⁰ have more severe clinical manifestations of the infective process. In the current study we found that primary epithelial cells of children with atopic asthma had a deficient production of interferon in response to rhinovirus, which was coupled to increased rhinovirus vRNA levels. This observation extends the previous findings in adults with atopic asthma.

Because we were interested in dissecting the role of atopy from that of asthma in the abnormal epithelial responses to viral infections, we extended our investigation to children with non-atopic asthma and to atopic children without asthma.

Interestingly, a similar impaired epithelial response to rhinovirus infection was found even in asthmatic children without allergic sensitization. At variance, a prior study examining the antiviral interferon responses in nonatopic children with asthma found that IFN- α induction in peripheral blood was not different from that seen in control subjects.¹⁵ This apparent discrepancy could be explained by the fact that we investigated airway epithelial cells (ie, the natural site at which respiratory tract infections occur). These data suggest the importance of local mechanisms over the systemic responses in the control of antiviral responses.

Perhaps surprisingly, we also found a similar impaired response to rhinovirus infection in atopic children who did not have symptoms of asthma, suggesting that atopy *per se* shares with asthma the abnormal epithelial responses that could increase susceptibility to infections. In support of our findings are the recent epidemiologic studies showing an increased risk of serious pneumococcal disease and *Streptococcus pyogenes* infections in

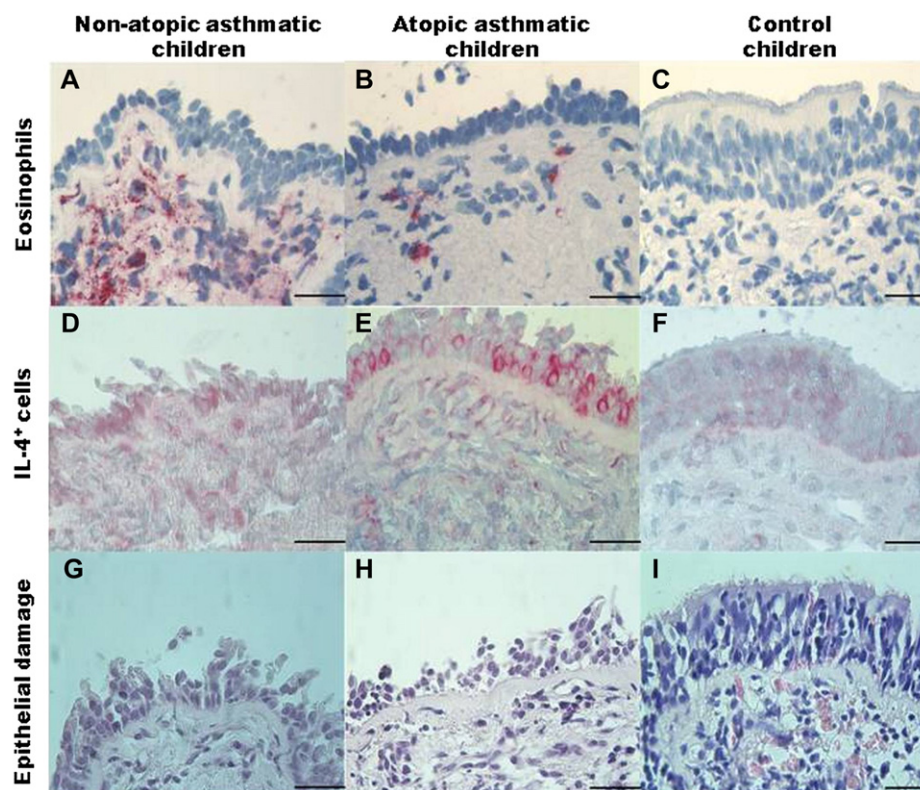


FIG 3. Representative microphotographs of pathologic changes in biopsy specimens related to the impaired innate responses observed *ex vivo*. **A-C**, Eosinophils as detected by means of immunostaining with the mAb anti-EG2 (eosinophils are stained in red). **D-F**, IL-4 positivity as detected by means of immunostaining with the mAb anti-IL-4 (positive cells are stained in red). **G-I**, Damage to the airway epithelium in sections stained with hematoxylin and eosin. Scale bars = 10 μ m.

patients with atopic conditions, such as atopic dermatitis and allergic rhinitis, even after adjustment for concomitant asthma.^{31,32}

A possible explanation for these observations could be the finding in our study that circulating IgE levels were inversely related to interferon production in bronchial epithelial cells, which is in agreement with the known negative modulation of IgE on innate immune responses.³³⁻³⁵ It is also possible that the interferon deficiency observed in atopic children without symptoms of asthma is related to the presence of T_H2 inflammation and epithelial damage in the airways, which are features typical of children with asthma and might *per se* affect innate immune responses. Despite a similar susceptibility to infections, the clinical consequences after a respiratory tract infection are different in asthmatic and atopic children without asthma, suggesting that factors other than deficient interferon production are needed for asthma exacerbations to occur.

We found that IFN- λ and IFN- β levels were significantly reduced in both children with atopy (and no asthma) and children with asthma (and no atopy). Of interest, there was no additive effect when considering that children with both atopy and asthma had similar reduced interferon levels. This observation indicates that each of these conditions (asthma and atopy) *per se* can independently influence antiviral responses with no synergic effect.

The availability of bronchial biopsy specimens in the children of our study has allowed us to investigate the possibility of a common link underlying the similar impaired response to viral infections in atopic and nonatopic asthmatic patients and in atopic subjects without symptoms of asthma. The airway pathology in

all these clinical conditions showed a T_H2 inflammatory response that was not present in the airways of control children. Furthermore, the increase in T_H2 inflammation, in terms of airway eosinophilia and IL-4 expression, was associated with decreased interferon induction in response to rhinovirus infection. These findings are in line with the known inhibitory effects of T_H2 cytokines on innate immune responses.^{36,37} Interestingly, another important pathologic abnormality that is common to both asthmatic and atopic children without asthma was the prominent damage to the bronchial epithelium that was related to enhanced IL-4 expression and deficient interferon production. The somewhat surprising observation of epithelial damage in atopic children without symptoms of asthma confirms previous studies in atopic adults, in whom the typical airway pathology (T_H2 inflammation, basement membrane thickening, and epithelial damage) was observed in the absence of the clinical and functional features of asthma.³⁸⁻⁴⁰ Moreover, there is increasing appreciation that, in addition to disturbance of immunologic pathways, a primary defect in the epithelial barrier could be a crucial pathogenetic event, even in patients with atopic conditions other than asthma.^{41,42}

The complex design and population recruitment in our study might present some limitations. Because children were undergoing bronchoscopy for appropriate clinical indications (as detailed in this article's Online Repository), they might not be considered comparable with the population as a whole. However, it was reassuring that these children had the same pathologic alterations that have been described in adults with asthma in whom these

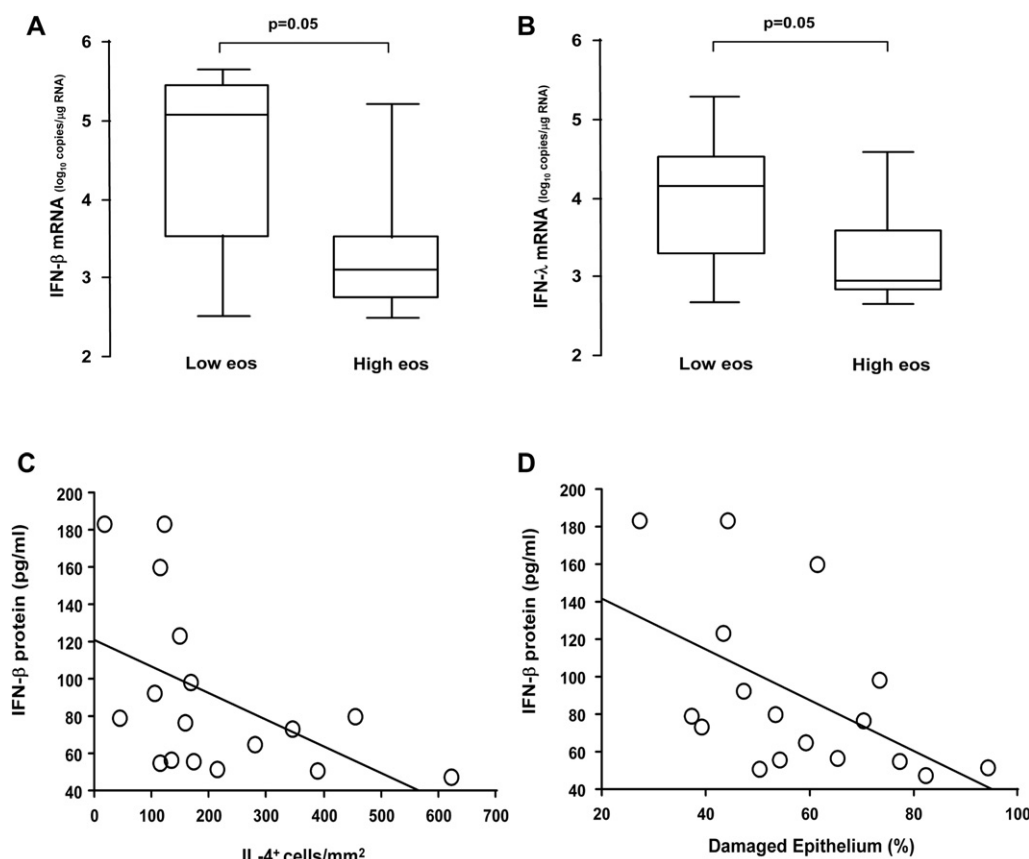


FIG 4. Association between immunopathologic profile and interferon production. **A** and **B**, RV16-induced IFN- β mRNA (Fig 4, A) and IFN- λ mRNA (Fig 4, B) levels at 8 hours stratified according to eosinophil (*eos*) levels in airway biopsy specimens. **C**, Correlation between RV16-dependent induction of IFN- β protein at 48 hours and IL-4 expression (Spearman rank correlation: $P = .02$, $r = -0.58$). **D**, Correlation between RV16-dependent induction of IFN- β protein at 48 hours and degree of epithelial damage (Spearman rank correlation: $P = .02$, $r = -0.55$).

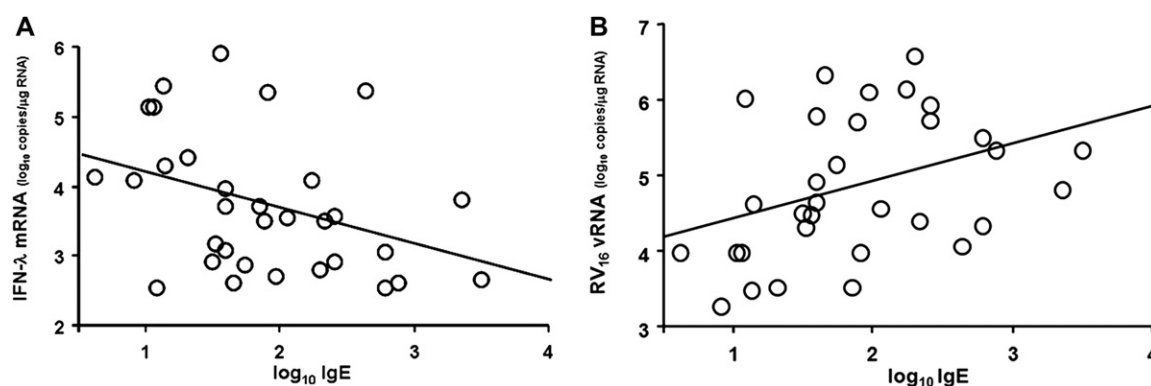


FIG 5. **A**, Correlation between total serum IgE levels and RV16-dependent induction of IFN- λ mRNA at 8 hours (Spearman rank correlation: $P < .05$, $r = -0.41$). **B**, Correlation between total serum IgE levels and RV16-dependent induction of vRNA levels at 8 hours (Spearman rank correlation: $P < .05$, $r = 0.44$).

selection biases did not apply. Furthermore, it should be noted that the indications for bronchoscopy were the same for all children, including the control subjects, who did not show impairment in interferon responses.

Another possible limitation is that because of the young age of our population, we had to rely largely on clinical assessment for the diagnosis of asthma. However, this approach fulfils Global

Initiative for Asthma guideline recommendations for the diagnosis of asthma in children 5 years and younger.²¹

Finally, the information on infective episodes was collected retrospectively at the time of bronchoscopy, which could have limited the accuracy of this information. Despite these potential limitations, we believe that the data observed are relevant and might have significant implications.

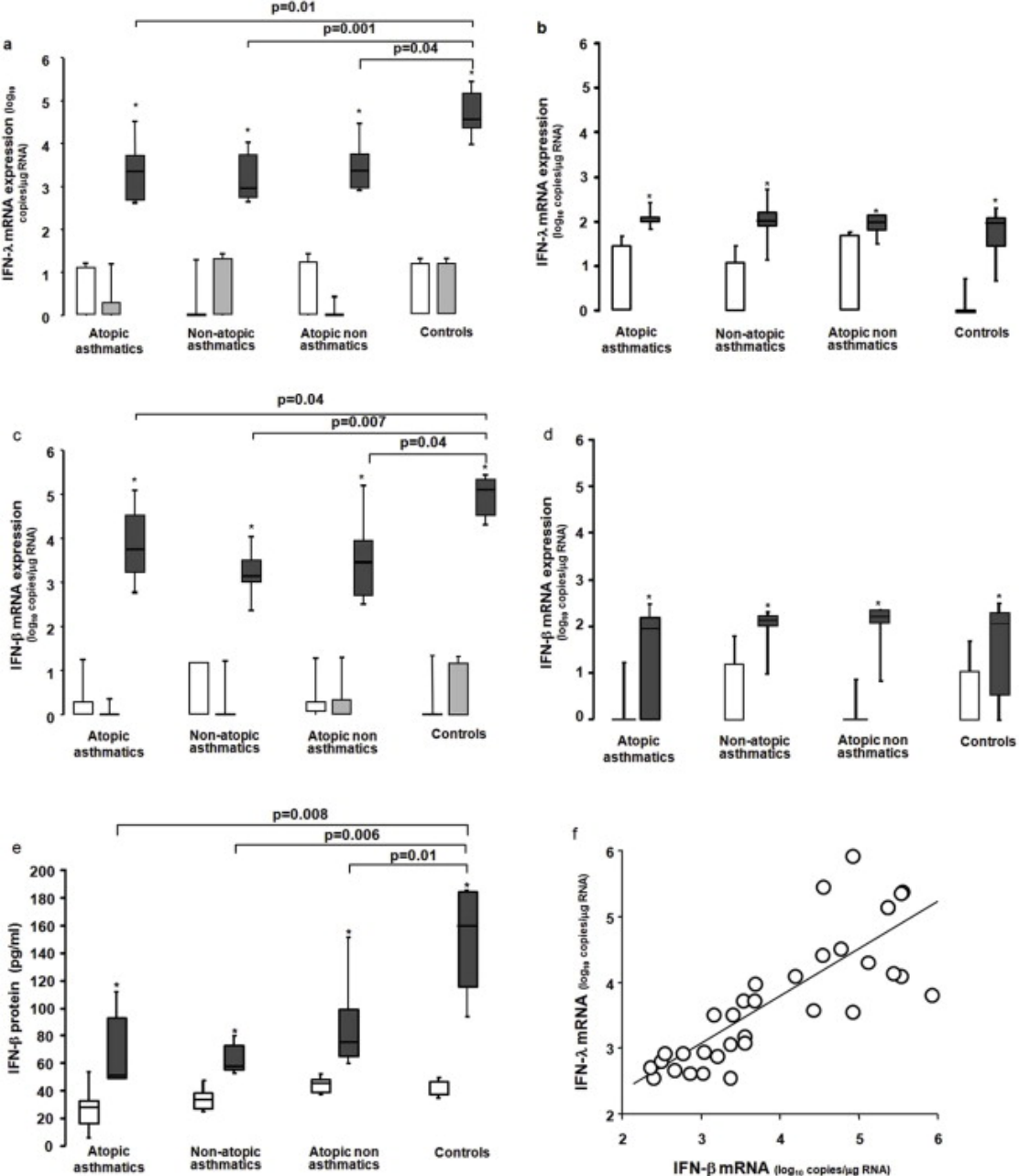
In conclusion, in our study we have shown that the deficient production of interferon by epithelial cells in response to rhinovirus, which was previously documented in adults with atopic asthma, can be seen not only in atopic and nonatopic asthmatic children but also in atopic children without asthma symptoms. The common link to the deficient immune response to rhinovirus in these conditions seems to be the airway T_H2 inflammatory profile and epithelial damage that correlate with the decreased interferon production. These data suggest that the impaired immune responses of the epithelium after viral infections are more likely related to the T_H2 inflammatory milieu in the airways rather than being an intrinsic characteristic of atopic asthma.

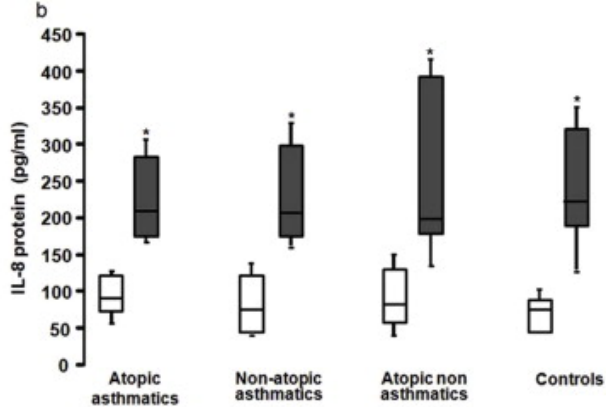
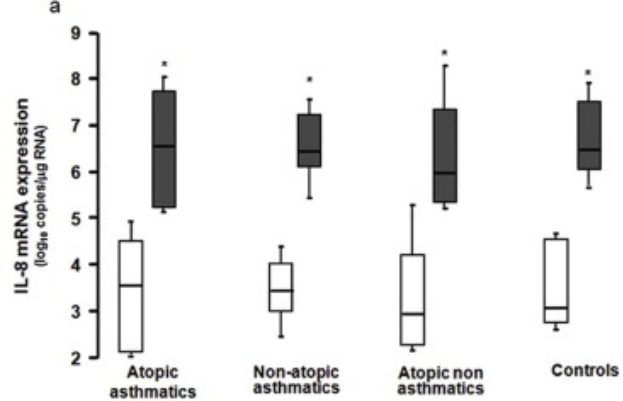
We thank Professor Manuel Cosio for insightful comments on the manuscript.

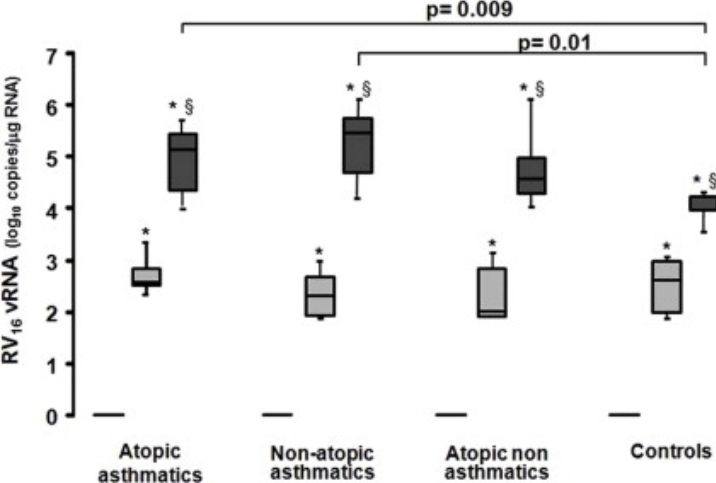
Clinical implications: Our observations of deficient immune responses to rhinovirus in asthmatic children irrespective of atopy and in atopic children without asthma contribute to identify early mechanisms of susceptibility to infections.

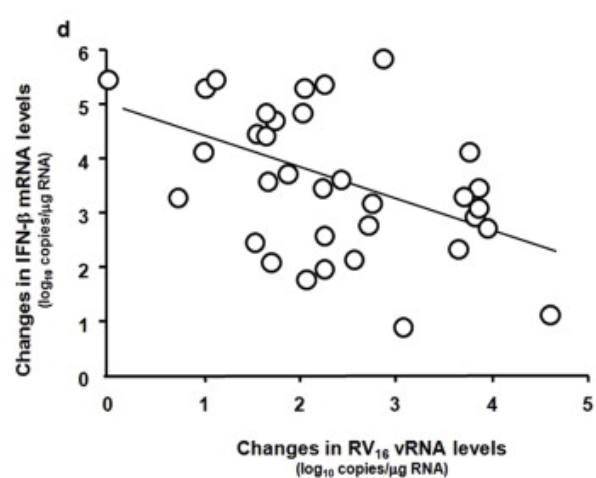
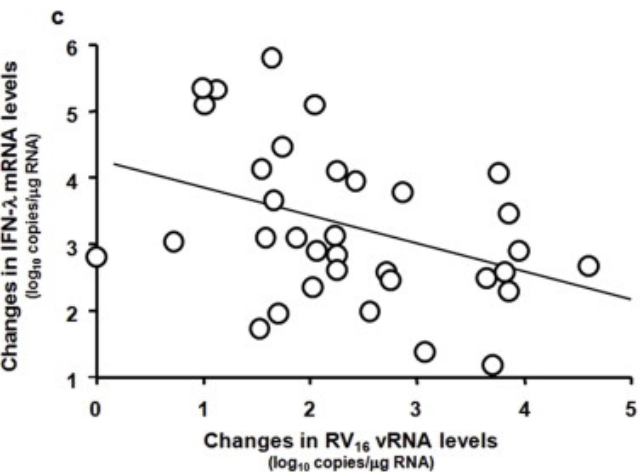
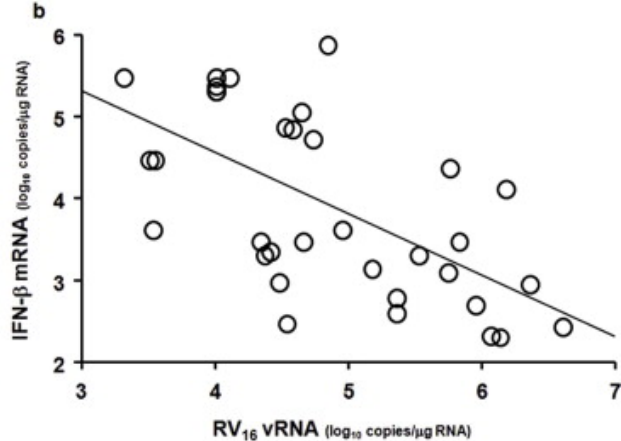
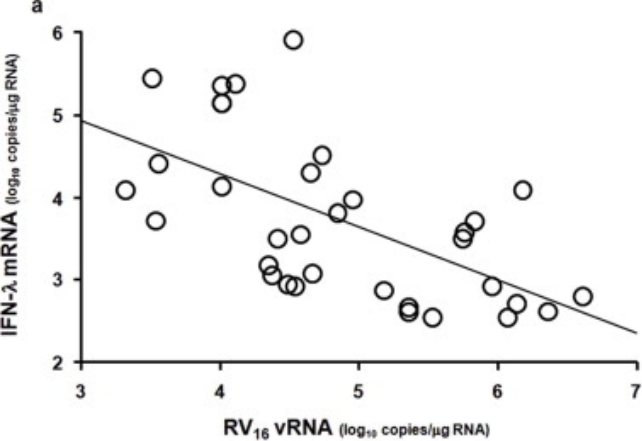
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Online Repository Materials

Deficient antiviral immune responses in childhood: distinct roles of atopy and asthma

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Methods

Subjects

Study population included 47 children who underwent fiberoptic bronchoscopy for appropriate clinical indications, at Department of Woman and Child Health, University of Padova – Italy. Bronchoscopy was conducted in accordance with safety criteria laid down in the guidelines for fiberoptic bronchoscopy in children ^{E1}.

All children underwent routine blood tests, including complete blood count, total (paper radioimmunosorbent test [PRIST]), and specific (radioallergosorbent test [RAST]) IgE to define the presence of atopy. The presence of atopy was defined by an increase in total (above age-related normal values) and specific IgE (>0.35 kU/L). In particular, specific IgE for the most common aeroallergens were investigated. These included: house dust mite (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*), moulds (*Alternaria alternata*), cat dander, trees (birch, olive, hazel, cypress), herbs (*Artemisiifolia*, *Artemisia absinthium*, *Parietaria judaica*), grass pollens (*Lolium perenne*, *Poa pratensis*; *Phleum pratense*, *Dactylis glomerata*, *Cynodon dactylon*), as well as ovalbumin, α and β lactoglobulin.

Children were defined as having asthma when they had repeated episodes of wheezing, breathlessness and cough, particularly at night and early morning, that were present even apart from colds. Moreover, in all asthmatic children, symptoms had to be responsive to bronchodilators prescribed by the child paediatrician ^{E2, E3}.

Data on presence and reversibility of episodic symptoms were evaluated retrospectively at admission for bronchoscopy by a respiratory pediatrician after reviewing clinical records/prescriptions and inquiring the parents. The respiratory pediatrician reviewed the personal child health record, in which symptoms are

recorded, in association with the therapy prescribed and the response to such therapy assessed by the family pediatrician. This information was implemented by a questionnaire administered to parents with a detailed investigation of symptoms pattern, relation to colds and presence of infective episodes. Parents were asked whether their children had respiratory infective episodes of any types including colds, pharyngitis, bronchitis, pneumonia, otitis or others. Moreover, they had to quantify their frequency in the last year on a monthly grading scale. Thus, in our study, the information was collected retrospectively at the time of bronchoscopy and it covered all the history from symptom onset, with a particular focus in the year preceding the bronchoscopy for the presence of infectious episodes. One out of three symptoms (wheezing, breathlessness and coughing) was considered to be sufficient. The reason for this choice was to have the same diagnostic criteria in all children, including those too young to perform spirometry. Spirometry was obtained in children able to cooperate with the test (in all children ≥ 6 yrs of age). This is in line with International Guidelines for asthma diagnosis and treatment indicating that in young children (≤ 5 yrs of age) the diagnosis of asthma has to be based largely on clinical judgment and on assessment of symptoms and physical findings ^{E4}.

Written consent was obtained from children's parents after they were informed that, during bronchoscopy, bronchial brushing and biopsies would be taken for research purposes. The study was performed according to the Declaration of Helsinki and was approved by the Ethics Committee of Padova City Hospital.

Virus stocks

Rhinovirus type 16 (RV16) (a major group rhinovirus) was obtained from the Health Protection Agency Culture Collections, Health Protection Agency Microbiology Services, Salisbury, UK, and used for all experimental conditions described. Viral stocks were prepared by infection of sensitive cell monolayers (Ohio HeLa) as described previously^{E5}. TCID50/ml values were determined and rhinovirus serotype were confirmed as RV16 by neutralization with serotype-specific antibodies (from American Type Culture Collection; ATCC, Rockville, MD, USA) before the experimental procedures as previously described^{E5}. Virus at multiplicity of infection (MOI) of 5 was used for all the experiments.

Primary bronchial epithelial cell cultures and cell processing

Human bronchial epithelial cells were harvested during bronchoscopy by brushing (>95% epithelial cells) and frozen in foetal calf serum (FCS) supplemented with 10% dimethyl sulfoxide (DMSO). The cultured cells were all cytokeratin positive and exhibited a basal cell phenotype, as evidenced by the expression of cytokeratin 13, irrespective of the type of donor of the original brushings. Primary bronchial epithelial cells harvested by bronchial brushing were seeded in a 25-cm² flask cells into hormonally supplemented bronchial epithelial growth medium (BEGM; Clonetics, San Diego, USA) containing 50U/ml penicillin and 50 mg/ml streptomycin^{E6, E7}. When the monolayer reached at least 80% confluence the cells were split and expanded in a 75-cm² flask. At confluence, cells were then split again, counted resuspended (1.8 x10⁵ cells/ml) and immediately seeded in 12-well plates (2 ml in each well) for the infection, avoiding further in-vitro passages before rhinovirus stimulation. At 80% confluency cells were starved in BEBM (no supplements) overnight before exposure

to RV16. Bronchial epithelial cells were exposed to rhinovirus at room temperature for 1 hour, and then washed with phosphate buffered saline (PBS) to eliminate unbound virus and incubated for different time point (0, 8 hr and 48 hr if available). Previous studies reported impaired RV16-induced IFN- λ and IFN- β mRNA^{E6, E7} at 8hrs, and impaired protein production at 48 hr after the infection^{E6, E7} in bronchial epithelial cells from adult asthmatic patients. For consistency with these studies we chose here the same time points, to be able to compare our data in children with previous analyses conducted in adults. At each time point (0, 8 and 48 hr) experiments were performed in duplicate. We prioritized 0 and 8 hr cultures and then, if enough cells were available, we also set up plates for the 48 hr time point.

Total RNA was extracted by RNeasy Mini Kit (Qiagen), according to manufacturer's instructions. In selected experiments, cells were stimulated with inocula from which virus particles were removed by ultrafiltration through membranes (Amikon, London, UK) to remove all molecules greater than 30 kDa (internal control), performed according to the manufacturer's instructions.

Quantitative Taqman® Real Time RT-PCR

Taqman® Real Time RT-PCR was used to assess rhinovirus vRNA levels. Real time PCR is the most sensitive tool to identify the presence of rhinovirus^{E8}. Previous studies documented a relationship between vRNA levels (by Real Time PCR) and the release of intact and viable virus (by conventional viral titration)^{E6,E9}.

Real Time PCR was also used to assess interferon (IFN)- β , - λ and interleukin (IL)-8 mRNA induction. 2 μ g of total RNA were used for cDNA synthesis (Omniscript RT kit,

Qiagen). Quantitative PCR was carried out using specific primers and probes for rhinovirus, (sense: 5'-GTG AAG AGC CSC RTG TGC T-3' 50nM, antisense 5'-GCT SCA GGG TTA AGG TTA GCC-3' 300nM, probe-5'-FAM-TGA GTC CTC CGG CCC CTG AAT G-TAMRA-3' 175nM), IFN- λ (sense: 5'- GGA CGC CTT GGA AGA GTC ACT -3' 300nM, antisense: 5'- AGA AGC CTC AGG TCC CAA TTC -3' 900nM, probe 5'-FAM AGT TGC AGC TCT CCT GTC TTC CCC G TAMRA-3' 175nM)^{E7}, IFN- β (sense: 5'- CGC CGC ATT GAC CAT CTA -3' 300nM, antisense: 5'- GAC ATT AGC CAG GAG GTT CTC A -3' 900nM, probe 5'-FAM TCA GAC AAG ATT CAT CTA GCA CTG GCT GGA TAMRA-3' 175nM), IL-8 (Qiagen) and 18S rRNA (housekeeping gene - sense: 5'- CGC CGC TAG AGG TGA AAT TCT -3', antisense: 5'- CAT TCT TGG CAA ATG CTT TCG-3' 300nM each,probe: 5'-FAM ACC GGC GCA AGA CGG ACC AGA TAMRA-3' 175nM)^{E7}. Reactions consisted of 2 μ l of cDNA (cDNA for 18S was diluted 1:100), 12.5 μ l 2x QuantiTect Probe PCR Master Mix (Qiagen). The set of primers and probes used for IFN- λ mRNA detection were specifically designed to detect IFN- λ 1. Reactions were performed on Rotor Gene 6000 (Corbet, Australia). IFN- λ , - β , IL-8 mRNA levels and viral RNA expression were normalised to 18S rRNA and compared to standard curves and expressed as copy numbers per mcg RNA.

ELISA

ELISAs for detection of IFN- β and IFN- λ and for IL-8 levels in cell culture supernatants were performed according to the manufacturers' instructions. Plates were read on a Labsystems plate reader and analyzed using SoftMax Pro software. Sensitivities and sources for the individual ELISAs were as follows: IFN- β (25 pg/ml;

Interferon Source, PBL Laboratories); IFN- λ (20 pg/mL; Ray Biotech Inc.) and IL-8 (31.2 pg/ml; R&D Systems). The ELISA kit for IFN- λ 1 used in the study shows ~90% cross reactivity with recombinant human IFN- λ 2 and around ~35% cross reactivity with recombinant human IFN- λ 3.

Bronchial Biopsy

As per ethical committee recommendations, we were allowed to obtain only one biopsy per child and this was obtained from the main carina. Bronchial biopsies were collected and processed for histological and immunohistochemical analysis as previously described^{E2, E3, E10}. Biopsies were considered suitable for examination when there was at least 1.0 mm of basement membrane length and 0.1 mm² of subepithelial area. Biopsies were fixed in 4% formaldehyde, dehydrated through alcohol series, embedded in paraffin wax and cut in 5 μ m thick sections. Analysis of epithelial damage was performed on sections stained with haematoxylin-eosin. The length of the incomplete epithelium, as well as the total length of epithelium (as assessed by the length of total basement membrane), were measured and results were expressed as % of the incomplete epithelium length over total basement membrane length. The epithelium was considered incomplete when the basement membrane was completely denuded or when it was covered by a single layer of basal cells with no intact ciliated cells or goblet cells. The number of inflammatory cells (eosinophils, neutrophils, macrophages, CD4-T-lymphocytes) were quantified in the subepithelium by immunoistochemistry and expressed as number of cells/mm² of examined tissue^{E2,E3}. Similarly the expression of IL-4 was quantified in the subepithelium and expressed as number of positive cells /mm² of examined tissue. In

a specific analysis, children were also categorized, based on the inflammatory profile of biopsies, in subjects with low or high levels of eosinophils in the airways using a previously reported cut-off value ^{E11}.

Statistical analysis

To avoid observer bias, the cases were coded, and the measurements made without knowledge of clinical data. Comparisons among groups were evaluated with either ANOVA or Kruskal-Wallis test followed, when significant, by Student's t-tests or Mann-Whitney U tests, as appropriate. Correlation coefficients were calculated using the non parametric Spearman's rank method. P values less than or equal to 0.05 was considered to indicate statistical significance. All tests were two tailed. Previous studies documented a significant difference in RV-induced IFN production in asthmatic patients compared to controls with 9-10 patients per group ^{E6, E7}. Moreover, as few as six to nine subjects are sufficient to show significant eosinophil recruitment in the airway biopsies of children with asthma or atopy ^{E2}. For these reasons it was feasible to select 8 to 9 patients per group in our complex study.

Results

Clinical findings

Atopic status was defined by an increase in total and specific IgE. Table E1 reports a detailed characterisation of the atopic children included in the study (grouped as follows: i) all children recruited to the study, ii) children with successful brushing cultures, iii) children with bronchial biopsies and iv) children with protein available for the analysis). No significant difference was found in the atopic status of these groups. Among patients recruited in the study, 75% of atopic asthmatic patients and 55% of atopic non-asthmatic patients ($P=0.30$) were sensitized to house-dust mite; 58% of atopic asthmatic patients and 45% of atopic non-asthmatic patients ($P=0.50$) were sensitized to moulds; 17% of atopic asthmatic patients and 18% of atopic non-asthmatic patients ($P=0.90$) were sensitized to cat dander; 17% of atopic asthmatic patients and 36% of atopic non-asthmatic patients ($P=0.28$) were sensitized to trees; 58% of atopic asthmatic patients and 63% of atopic non-asthmatic patients ($P=0.51$) were sensitized to grass pollens or herbs.

Rhinitis was present in 4 out of 8 asthmatic atopic children and in 2 out of 8 atopic non-asthmatic children. Atopic dermatitis was present in three children at recruitment (2 asthmatic atopic and 1 atopic non asthmatic). Four children (2 asthmatic atopic and 2 atopic non asthmatic) had suffered of atopic dermatitis during the first year of life but had no clinical manifestations thereafter. Symptoms of food allergy were reported in one atopic asthmatic child only.

Children were defined as having asthma when they had repeated episodes of wheezing, breathlessness and cough, particularly at night and early morning, that were present even apart from colds and were responsive to bronchodilators. One out

of three symptoms was considered to be sufficient, but among asthmatic children in our study (n=17), the great majority reported having either all three symptoms (n=6) or two out of three symptoms (n=6). Wheezing only was reported in 3 out of 17 subjects and cough alone in 2 subjects.

Lung function was assessed in all children who were able to cooperate with the test (i.e. those $\geq$ 6yrs) [n=9, of whom 3 Atopic Asthmatics, 2 Non-atopic asthmatics, 2 Atopics and 2 Controls, all within the normal range except from 1 atopic asthmatic who showed an FEV1 of 69%pred]. The pattern of symptoms in these children was similar to that in whom lung function was not tested.

Clinical indications for bronchoscopy did not differ significantly among the four groups and were as follows: recurrent pneumonia (n=3), chronic cough (n=2), difficult to treat asthma (n=1), obstructive sleep apnea syndrome (OSAS) (n=1), atelectasis (1) in children with atopic asthma; recurrent pneumonia (n=6), chronic cough (n=2), laryngomalacia (n=1) in children with non-atopic asthma; recurrent pneumonia (n=5), chronic cough (n=2), middle lobe syndrome (n=1) in atopic children without asthma; recurrent pneumonia (n=7), chronic cough (n=2) in control children. Patients with recurrent pneumonia underwent bronchoscopy when in stable conditions.

Finally, the number of children ever hospitalized for asthma exacerbation was similar in atopic and non-atopic asthmatic children. In particular, 2 out of 8 atopic asthmatics and 3 out of 9 non-atopic asthmatic children were previously hospitalized due to asthma exacerbations. In addition, the number of emergency room visits for asthma per patient in the previous year was not different between atopic and non-atopic asthmatic children [0 (0-1) vs 1 (0-3)].

Rhinovirus induced IFN- λ and - β mRNA induction.

Forty-seven bronchial brushings were initially seeded in 25-cm² flasks. Among these, an effective cell culture was established in 34 samples that provided the cells for the 0 and 8 hr time point. Twenty-one samples provided also enough cells to set up the 48 hr time point. No difference was found in the demographic characteristics between children included in the 8 hr and 48 hr time point analysis (Table E2).

IFN- λ and - β mRNA levels were virtually absent in uninfected cells both at 8 and 48 hr time point. RV16 resulted in a significant induction of IFN- λ and - β mRNA at 8 hr (n=34; figure E1 panel A and C) and at 48 hr (n=21; figure E1 panel B and D). A parallelism was found between RV-16 induced IFN- λ and - β mRNA induction at 8 hr after the infection (p<0.0001, r=0.81 - Figure E1 panel F). At 8 hr IFN- λ and - β mRNA induction was significantly impaired in asthmatic atopic, asthmatic non atopic and atopic non asthmatic children compared to non asthmatic non atopic children. Cell exposure to inocula from which virus particles were removed by ultrafiltration through membranes (f-RV) resulted in no induction of IFN mRNA (figure E1, panel A and C).

In line with previous studies^{E12}, IFN- λ and - β protein levels in 8 hr supernatants of cell cultures exposed to rhinovirus were undetectable (data not shown). IFN- β levels at 48 hr after RV16 infection were significantly higher in cell culture supernatants of non-atopic, non-asthmatic children (controls) compared to all other groups (Figure E1 panel E).

Rhinovirus induced IL-8 production.

The production of IL-8 was evaluated as a control. At 8hr after infection, neither RV16-induced IL-8 mRNA induction (figure E2 panel A) nor IL-8 protein production

(figure E2 panel B) significantly differed in the four groups of children.

Ex-vivo rhinovirus replication in bronchial epithelial cells.

RV16 vRNA was undetectable at all time points in all primary cell cultures exposed to medium alone (uninfected cells) included in the study. Levels of vRNA were no different at time 0 (after 1 hr exposure to rhinovirus) between groups and significantly increased in all groups at 8 hr after the infection (Figure E3). At 8 hr vRNA levels in cell cultures of atopic-asthmatic and non-atopic asthmatic children were significantly higher compared to non-atopic, non-asthmatic children (Figure E3). RV16-induced IFN- λ and IFN- β mRNA were strongly inversely related to vRNA levels at 8 hr after the infection (Spearman rank correlation: $P < 0.001$; $r = -0.62$ and $P < 0.001$; $r = -0.63$, respectively – Figure E4 panel A and B). Moreover, the changes in RV-16 vRNA levels between 0 and 8 hrs were negatively correlated with the changes in IFN- λ mRNA levels and IFN- β mRNA levels between the same time points ($P < 0.001$; $r = -0.62$ and $P < 0.001$; $r = -0.63$, respectively – Figure E4 panel C and D).

Effects of gender, age, and ICS treatment on biopsy findings and ex-vivo rhinovirus induced IFN mRNA induction

We examined the relationship between age and the main outcomes of this study (i.e. RV-16 induced IFN- λ and IFN- β mRNA levels, vRNA levels, expression of IL-4 and eosinophils counts) and we found that age was not significantly correlated with any of these parameters. In particular, all but 7 children included in our study were at preschool age ($n = 27$, 79%). When we excluded older children from the analysis, all the main findings were confirmed (Table E3).

We also performed a gender-stratified analysis and found no differences between males and females in any of those parameters (Table E4). Moreover, to evaluate whether inhaled corticosteroid treatment would modify the findings obtained from results bronchial biopsies (i.e., eosinophil counts and IL-4-producing cells) we performed a separate analysis of biopsies of asthmatic children using low-dose ICS (n=6) vs those not treated with ICS (n= 11). No difference was observed for either eosinophil counts or IL-4+ cells between these groups (table E5). In addition, a separate analyses failed to document any effect of ICS treatment on IFN- λ and IFN- β mRNA induction or on changes in vRNA levels following RV-16 infection (Table E5). This is in line with previous observation that in vitro steroid treatment does not affect rhinovirus induced interferon production in primary epithelial cells^{E6}.

Correlations linking ex-vivo immune responses to immunopathological parameters

When we looked at immunological connection between atopic status and immune responses to viral infections, we found that, in the entire study population, total serum IgE levels were negatively correlated with RV16-induced IFN- λ mRNA levels (Fig. 6a) and positively correlated with rhinovirus vRNA levels ($P < 0.05$; $r = 0.42$, Fig. 6b). Correlation between total IgE levels and IFN- β mRNA levels just failed to reach statistical significance ($p = 0.09$). Previous studies^{E9} and the current study (Figure E1 panel F) showed a parallelism between RV-16 induced IFN- λ and $-\beta$ mRNA induction in primary bronchial epithelial cells. Thus, the missed significance of the

300 correlation between IgE levels and IFN- β was likely due to statistical conditions
301 (variability, number of subjects) rather than a different biological behavior.

302

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- 340

341 FIGURE LEGENDS

342 Figure E1. Rhinovirus induced IFN- λ and - β in bronchial epithelial cells.

343 Levels of IFN- λ and - β mRNA at 8 hr (n=34 - Panel A and C) and at 48 hr (n=21 -
344 Panel B and D) after RV-16 infection in bronchial epithelial. Panel E) levels of IFN- β
345 protein at 48 hr after RV-16 infection in bronchial epithelial cells (n=21). Panel F)
346 Correlation between IFN- λ and - β mRNA levels at 8 hr following RV-16 infection
347 (n=34 - $p < 0.0001$, $r = 0.81$). White boxes, light grey boxes and black boxes represent
348 cells exposed to medium alone, inocula from which virus particles were removed by
349 ultrafiltration through membranes, or rhinovirus-16 respectively. * Significantly
350 different from cells exposed to medium alone and inocula with ultrafiltrated virus.

351

352 Figure E2 Rhinovirus induced IL-8 production in bronchial epithelial cells.

353 Panel A) Levels of IL-8 mRNA at 8 hr after RV-16 infection in bronchial epithelial cells
354 (n=34). Panel B) levels of IL-8 protein at 8 hr after RV-16 infection in bronchial
355 epithelial cells (n=34). White box and black boxes represent cells exposed to medium
356 alone, or rhinovirus-16 respectively. * Significantly different from cells exposed to
357 medium alone.

358

359 Figure E3: Levels of RV-16 vRNA in uninfected cells (White boxes), time 0 (light gray
360 boxes) and 8 hr (black boxes) after RV-16 infection in bronchial epithelial cells
361 (n=31). * Significantly different from cells exposed to medium alone; § Significantly
362 different from time 0.

363

364 Figure E4: Correlations between vRNA levels and RV-16 induced IFN- λ and IFN- β
365 mRNA levels in bronchial epithelial cells

366 Panel A and B) Correlation, at 8hr after the infection, between RV-16 vRNA levels
367 and RV-16 induced IFN- λ and IFN- β mRNA levels, respectively. Panel C and D)
368 Correlation between changes of RV-16 vRNA levels and changes of RV-16 induced
369 IFN- λ and IFN- β mRNA levels, respectively, at 8hr compared to time 0.
370

Table E1: Allergen sensitization in the atopic children (AA: atopics asthmatics , A: atopics without asthma)

Child	Samples available (brushing/biopsy/protein)	Positive specific IgE (RAST ⁺)
1 AA	brushing/protein	Dermatophagoides pteronyssinus, dermatophagoides farinae, β -lactoglobulin
2 AA	brushing/protein	Dermatophagoides pteronyssinus, dermatophagoides farinae, olive.
3 AA	brushing/biopsy/protein	Dermatophagoides pteronyssinus, dermatophagoides farina, lolium perenne, dactylis glomerata, phleum pratense, hazel.
4 AA	brushing/biopsy/protein	Alternaria alternata, ovalbumin.
5 AA	brushing/biopsy/protein	Dermatophagoides pteronyssinus, dermatophagoides farinae, alternaria alternata, cat dander, lolium perenne, dactylis glomerata.
6 AA	brushing/biopsy	Dermatophagoides pteronyssinus, dermatophagoides farinae, β -lactoglobulin
7AA	brushing/biopsy/protein	Dermatophagoides pteronyssinus, dermatophagoides farinae, lolium perenne, alternaria alternata, cat dander.
8 AA	brushing/biopsy	Dermatophagoides pteronyssinus, dermatophagoides farinae, artemisia absinthium, ovalbumin, β -lactoglobulin.
9 AA	brushing not grown	Alternaria alternata.
10 AA	brushing not grown	Dermatophagoides pteronyssinus, dermatophagoides farinae, alternaria alternata, ovalbumin, α -lactoglobulin, β -lactoglobulin.
11 AA	brushing not grown	Dermatophagoides pteronyssinus, dermatophagoides farinae, lolium perenne, poa pratensis, phleum pratense, alternaria alternata.
12 AA	brushing not grown	Alternaria alternata.
1 A	brushing/biopsy/protein	Dermatophagoides pteronyssinus, dermatophagoides farinae, alternaria alternata, β -lactoglobulin.
2 A	brushing/biopsy/protein	Alternaria alternata, cat dander, cynodon dactylon, artemisia absinthium, lolium perenne.
3 A	brushing	Alternaria alternata, cynodon dactylon, artemisia absinthium, cypress, ovalbumin, β -lactoglobulin, plantago lanceolata.
4 A	brushing/biopsy/protein	Dermatophagoides pteronyssinus, dermatophagoides farinae
5 A	brushing/biopsy	Dermatophagoides pteronyssinus, dermatophagoides farina, dactylis glomerata, cynodon dactylon, cat dander, artemisia absinthium, parietaria judaica, olive, hazel, cypress, lolium perenne, ovalbumin, β -lactoglobulin.
6 A	brushing/biopsy/protein	Artemisia absinthium, phleum pratense, lolium perenne, dactylis glomerata, cynodon dactylon, olive, wheat, alternaria alternata.
7 A	Brushing	Lolium perenne, dactylis glomerata, cynodon dactylon, artemisia absinthium, parietaria judaica.
8 A	brushing/biopsy/protein	Dermatophagoides pteronyssinus, ovalbumin, β -lactoglobulin
9 A	brushing not grown	Dermatophagoides pteronyssinus, dactylis glomerata, cynodon dactylon, lolium perenne
10 A	brushing not grown	Phleum pratense, olive, artemisia absinthium, cynodon dactylon, alternaria alternata
11 A	brushing not grown	Dermatophagoides pteronyssinus, dermatophagoides farinae, α -lactoglobulin

Table E2: Comparison of demographic characteristics between children included in the 8hr and 48 hr time point analysis

	Asthmatic atopic children		Asthmatic non-atopic children		Non-asthmatic atopic children		Non-atopic non-asthmatic children	
Time point	8 hr	48 hr	8 hr	48 hr	8 hr	48 hr	8 hr	48 hr
Number of patients	8	6	9	5	8	5	9	5
Gender (M/F)	6M/2F	4M/2F	5M/4F	2M/3F	3M/5F	3M/2F	4M/5F	3M/2F
Age (years)	5 (2-13)	5 (4-13)	5 (3-7)	5 (3-7)	5 (2-12)	5 (2-5)	5 (2-7)	5 (4-6)
Age at onset of asthma symptoms (years)	2.9±1.2	2.7±1.9	2.2±0.4	3.7±0.6	---		---	
Duration of asthma symptoms (years)	3.4±1.2	2.5±1.7	3.1±0.5	2.2±0.4	---		---	
Monthly infective episodes in last year	1.3±0.5	1.4±0.7	2.1±0.4	2.1±0.3	1.5±0.2	1.8±0.4	1.7±0.3	2±0.3
Total IgE (U/ml)	749±347*‡	767±345*‡	40±10	35±8	376±260*‡	315±145*‡	21±8	23±14

Data are expressed as median (range) or mean±SEM. *P<0.05 compared with control children, ‡ P<0.05 compared with non-atopic asthmatic children.

Table E3: Analysis of a subgroup of the entire study population: preschool children (i.e. children aged ≤ 5 yrs).

	Atopic Asthmatics (n=5)	Non-atopic Asthmatics (n=7)	Atopic non-asthmatics (n=6)	Controls (n=7)
IFN-α mRNA (log10 copies/ μ g RNA)	3.6 (2.8-3.9)*	3.2 (2.7-4.0)*	3.1 (2.8-3.7)*	4.8 (3.8-5.4)
IFN-γ mRNA (log10 copies/ μ g RNA)	4.1 (3.0-4.7)*	3.1 (2.7-4.2)*	3.1 (2.4-4.7)*	5.2 (4.2-5.4)
IFN-γ protein (fold induction/untreated)	2.4 (1.8-2.9)	1.5 (1.5-1.9)*	1.9 (1.4-2.9)*	4 (2.1-4.7)
RV16 vRNA (log10 copies/μg RNA)	5.3 (4.4-6)*	5.4 (4.1-6.1)*	4.6 (3.9-6.2)	3.9 (3.8-4.6)
Blood eosinophils (cells $10^9/L$)	0.5 (0.2-1.7)*	0.3 (0.09-0.7)*	0.3 (0.1-0.6)*	0.07 (0-0.4)
IL-4⁺ cells/mm²	113 (101-386)*	169 (56-451)*	204 (113-343)*	72 (14-119)

Values are expressed as median (range)

* significantly different from controls (p<0.05)

Table E4: RV16-induced IFN- λ mRNA, IFN- β mRNA, RV16 vRNA and immunopathological profile stratified by gender (atopic asthmatics, non-atopic asthmatics and atopic non-asthmatics)

	IFN- α mRNA (log ₁₀ copies/«gRNA)	IFN- β mRNA (log ₁₀ copies/«gRNA)	RV ₁₆ vRNA (log ₁₀ copies/«gRNA)	IL-4 (cells/mm ²)	Tissue Eosinophils (cells/mm ²)	Blood eosinophils (cells10 ⁹ /L)
Male (n=14)	3.5(2.5-5.9)	3.5(2.6-5.5)	5.3(3.5-6.3)	145(56-620)	63 (12-441)	0.3 (0.09-1.7)
Female (n=11)	2.9(2.5-4.1)	3.1(2.3-5.9)	4.8(3.3-6.6)	204(101-451)	81(0-304)	0.3 (0.1-0.7)

Table E5: RV16-induced IFN- λ mRNA, IFN- β mRNA, RV16 vRNA and immunopathological profile stratified by steroid use (atopic asthmatics and non-atopic asthmatics)

	IFN- α mRNA (log ₁₀ copies/«gRNA)	IFN- β mRNA (log ₁₀ copies/«gRNA)	RV ₁₆ vRNA (log ₁₀ copies/«gRNA)	IL-4 (cells/mm ²)	Tissue Eosinophils (cells/mm ²)	Blood eosinophils (cells 10 ⁹ /L)
No ICS (n=11)	2.9(2.5-5.4)	3.1(2.3-5.5)	5.3(3.3-6.3)	155(101-620)	90(0-441)	0.4(0.09-1.7)
ICS (n=6)	3.3(2.5-3.9)	3.3(2.6-3.6)	5.4(4.3-5.8)	157(56-386)	38(13-180)	0.4(0.2-0.8)