

Th2-cytokines impair innate immune responses to rhinovirus in respiratory epithelial cells

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Abstract

Background: Asthma and other Th2 inflammatory conditions have been associated with increased susceptibility to viral infections. The mechanisms by which Th2 cytokines can influence immune responses to infections are largely unknown.

Methods: We measured the effects of Th2 cytokines (IL-4 and IL-13) on bronchial epithelial cell innate immune antiviral responses by assessing interferon (IFN- β and IFN- λ 1) induction following rhinovirus (RV)-16 infection. We also investigated the modulatory effects of Th2 cytokines on Toll-like receptor (TLR)-3, interferon-responsive factor (IRF)-3, and nuclear factor (NF)- κ B, i.e. key molecules and transcription factors involved in the rhinovirus-induced interferon production and inflammatory cascade. Pharmacological and redox modulation of these pathways was also assessed.

Results: Th2 cytokines impaired RV16-induced interferon production, increased rhinovirus replication and impaired TLR3 expression in bronchial epithelial cells. These results were replicated in vivo: we found increased IL-4 mRNA levels in nasal epithelial cells from nasal brushing of atopic rhinitis patients and a parallel reduction of TLR3 expression and increased RV16 replication compared to non-atopic subjects. Mechanistically, Th2 cytokines impaired RV-16 induced activation of IRF3, but had no effects on RV16-induced NF- κ B activation in bronchial epithelial cell cultures. N-acetylcysteine and phosphoinositide 3-kinase (PI3k) inhibitor restored the inhibitory effects of Th2 cytokines over RV-16 induced activation of IRF3.

Conclusions: IL-4 and IL-13, through inhibition of TLR3 expression and signalling (IRF-3), impair immune response to RV-16 infection. These data suggest that Th2 conditions increase susceptibility to infections and identify pharmacological approaches with potential to restore impaired immune response in these conditions.

Introduction

Asthma is a chronic disorder of the airways that is typically inflammatory in nature and affects millions of children and adults (1).

Viral infections of the respiratory tract early in life are associated with an increased risk of developing asthma later in life and are also the most frequent causes of asthma exacerbations in both children and adults (2). Rhinoviruses are the respiratory virus type that is most frequently detected during asthma exacerbations (3). The innate immune response is at the forefront of the defence against respiratory infections.

Impaired innate immune responses have been reported to be a possible mechanism of increased susceptibility to infections among asthmatic patients (2,4,5). The molecular mechanisms underlying such a deficiency are still largely unknown.

The cytokines produced by the Th2 subset of lymphocytes, such as IL-4 and IL-13, have been proven to be important drivers of allergic airway inflammation in asthma, although a) the mechanisms underlying their roles in the complex pathogenesis are still widely debated b) not all asthmatic patients have a preponderant Th2 oriented inflammatory profile in the airways (6) nor allergen driven clinical manifestations. We recently documented that in vivo Th2 inflammation is associated with impaired ex vivo immune responses to rhinovirus infection in bronchial epithelial cells from asthmatic children and also in cells from atopic children without any established clinical manifestations of asthma (7). These data accord with previous in vitro (8-10) and in vivo data (11) suggest that the Th2 inflammation influences the integrity of the innate immune responses and the outcomes of infections. Thus, intercepting the mechanisms of Th2 interference upon innate immune response may improve the ability to counteract the susceptibility to infections in these clinical conditions.

Oxidative stress has previously been found to influence the molecular pathways that are commonly involved in rhinovirus-induced intracellular signalling and proinflammatory activities (12,13). Furthermore, it has been shown that oxidative stress can impair innate immune responses by

interfering with the intracellular interferon signalling (14,15). In addition, phosphoinositide 3-kinases (PI3K) is a large family of intracellular signalling kinases involved in the both innate immune response to infections and Th2 inflammation (16-20). Thus, modulation of the intracellular redox state and of PI3k immunoregulatory activities represent potential pharmacological targets of interference with the substrates of immunological and inflammatory responses to viral infections.

Here, we evaluated the effects of Th2 cytokines (IL-4 and IL-13) on innate immune responses as assessed by type I (IFN- β) and type III (IFN- λ 1) interferon production against rhinovirus infection in bronchial epithelial cells. We also evaluated the effects of Th2 cytokines over key molecules (TLR-3) and transcription factors (IRF3 and NF- κ B) involved in the innate immune responses to rhinovirus and tested potential methods of pharmacological modulation.

Methods

Cell cultures and in vitro rhinovirus infections

The immortalized human bronchial epithelial cell line BEAS-2B was obtained from the American Type Culture Collection (Rockville, Md, USA) and cultured as previously described (21). Primary human bronchial epithelial cells (HBECs) were obtained from bronchial brushings of non-atopic, non-asthmatic and non-smoking subjects (who underwent bronchoscopy for clinical reasons – Supplementary Table 1) and cultured as previously described (7,21,22).

Primary epithelial cells of the nasal mucosae of five atopic rhinitis and six non-atopic healthy non-smoker volunteers were harvested by nasal scraping and freshly cultured in BEG medium (Supplementary Table 2). When confluent, total RNA was extract according to manufacturer's instructions (Qiagen).

The demographic characteristics of the patients enrolled in the study and the experimental conditions of the cell cultures and stimulations are detailed in the online supporting information and in Supplementary Table 1 and 2.

The study was approved by the local ethics committee of the University Hospital of Ferrara, and informed consent was obtained from each participant in accordance with the principles outlined in the Declaration of Helsinki.

Viral stocks

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

Quantitative TaqMan real-time RT-PCR

Quantitative real-time PCR was performed with specific primers and probes for rhinovirus, IFN- β , IFN- λ (specific for IFN- λ_1), CXCL8 (Qiagen), TLR3 (Qiagen), and IL-4 (Qiagen) as detailed elsewhere (7,21,22). Quantitative real-time PCR results were normalized to 18S rRNA or GAPDH expression (housekeeping genes). Interferon mRNA, CXCL8 mRNA, and viral RNA (vRNA) expression were normalized to 18S rRNA levels, compared with standard curves, and expressed as log₁₀ copy numbers per microgram of RNA. An arbitrary level equal to 1 copy of IFN-beta, -lambda mRNA or vRNA was assigned if undetectable.

Interferon and CXCL8 protein assays

The enzyme-linked immunosorbent assays (ELISA) used for the detections of IFN- β , IFN- λ , and CXCL8 levels in cell-culture supernatants were performed according to the manufacturers' instructions. The sensitivities, specificities, and sources for the individual ELISAs have been previously detailed (7).

Measurement of transcription factor activation

Nuclear extracts were prepared from the BEAS2B cells using a nuclear protein extraction kit (Active Motif, Rixensart, Belgium). Activated NF κ B, IRF3 and STAT1 in 10- μ g nuclear extracts were detected by evaluating their bindings to oligonucleotides targeting each binding site using

ELISA-based assays (TransAM Transcription Factor Assay kits for NFkB, IRF3 and the STAT family; Active Motif, La Hulpe, Belgium) following the manufacturer's recommendations.

Statistical analyses

Comparisons between conditions were performed by means of paired or unpaired non parametric tests (Wilcoxon test or Mann-Whitney test respectively). After the evaluation of the effects of rhinovirus infection (e.g. on interferon induction and/or transcription factors activation), in case of multiple tests, paired comparisons were performed using a Hierarchical method to control for multiplicity based on concentration, timing or modulatory intervention. A P-value of <0.05 was considered significant. All analyses were performed with GraphPad Prism 5.0 for Windows (GraphPad Software, Inc., La Jolla, California).

Results

Effects of Th2 cytokines on rhinovirus-induced interferon production and rhinovirus replication

To evaluate the effects of the Th2 status of the environment on rhinovirus-induced interferon (IFN) production, human bronchial epithelial cells (HBECs) and BEAS-2B cells were pre-treated with IL-4 and IL-13 prior to infection. Twenty-four-hour pre-treatment with IL-4 and IL-13 produced significant dose-dependent inhibition of rhinovirus-induced IFN- β mRNA expression and IFN- β protein release at 8 hrs after infection in both the BEAS-2B cell line (Supplementary Figure 1) and in HBECs (Figure 1A, 1C and 1E). Rhinovirus mediated induction of IFN- λ mRNA expression was also diminished 8 hrs after infection in both the BEAS-2B (Supplementary Figure 1) and HBECs (Figure 1B and 1D). IFN- λ protein was undetectable in the cell culture supernatants of any of the experimental conditions assessed here. Neither IL-4 nor IL-13 had any modulatory effects on the rhinovirus-induced pro-inflammatory cytokine production, such as CXCL8 (Figure 1F). IL-2 (used as control; Figure 1G and 1H) had no effect on rhinovirus-induced IFN induction. The inhibitory effect of Th2 cytokine pre-treatment on IFN production was observed at 8 but not 24 hrs after the infection (Figure 2A-D).

The impaired IFN expression following rhinovirus infection in the cell cultures that were pre-treated with Th2 cytokines was paralleled by increased rhinovirus replication in both the BEAS-2B (data not shown) and HBECs (Figure 2E, 2F). Levels of IFN- β and IFN- λ mRNA and of rhinovirus vRNA were virtually undetectable (close to the lowest limit of detection) in uninfected cells and in cells stimulated only with Th2 cytokines (Figure 1 and 2).

Effects of Th2 cytokines on rhinovirus-induced activation of transcription factors involved in interferon production and signalling.

To investigate the mechanisms involved in the inhibitory effects of the Th2 cytokines on rhinovirus-induced IFN production, we evaluated the effects of IL-4 and IL-13 on rhinovirus-induced activation of the transcription factors involved in interferon production, such as nuclear factor-kB (NF-kB) and IFN regulatory factor (IRF)-3 (24), and STAT1, which is a transcription factor that is activated by interferon-receptor engagement, and this activation leads to the transcription of IFN-stimulated genes (25). NF-kB, IRF-3 and STAT1 were activated 2 hrs after rhinovirus infection (Figure 3A-C). In the BEAS-2B epithelial cells, 12-hrs pre-treatment with IL-4 (50 ng/ml) and IL-13 (25 ng/ml) significantly inhibited rhinovirus-induced IRF3 activation and subsequent STAT1 activation (Figure 3B and 3C) but also resulted in a slight increase of NF-kB activation that was not statistically significant (Figure 3A).

Effects of antioxidants on Th2 cytokine-induced impairment of the innate immune response to rhinovirus.

Pre-treatment with exogenous NAC (10 mM) 30 min before rhinovirus infection abolished the inhibitory effect of the 24-hr pre-treatment with Th2 cytokines on rhinovirus-induced IRF-3 (Figure 4A) and STAT1 activations (Figure 4B) and restored the rhinovirus-induced expressions of IFN- λ (Figure 4C) and IFN- β mRNA (Figure 4D).

Effects of Phosphoinositide 3-kinases (PI3K) on Th2 cytokine-induced impairment of the innate immune response to rhinovirus.

We found that pre-treatment with the phosphoinositide 3-kinases (PI3k) inhibitor LY294002 (3.3 μ M) before rhinovirus infection restored the inhibitory effect of Th-2 cytokines on rhinovirus induced IRF-3 activation, suggesting that the inhibition of phosphoinositide 3-kinases counteract the Th-2 induced impairment of the innate immune response to rhinovirus (Figure 4E).

Effects of Th2 cytokines and oxidants on TLR3 expression.

TLR-3 it is one of the key molecule activated by double stranded RNA produced during rhinovirus infection which in turn activates transcription factors such as nuclear factor (NF)- κ B and IFN regulatory factor (IRF)-3/7 leading to the production of proinflammatory cytokines, chemokines and antiviral molecules including interferons (26-30). Twenty-four-hour IL-4 pre-treatment significantly inhibited the baseline expression of TLR3 in the respiratory epithelial cells (Figure 5A). This inhibition was not affected by concomitant cell exposure to the antioxidant N-acetyl cysteine (NAC, 10 mM; figure 5A).

The expression of TLR3 mRNA was evaluated in the nasal epithelial cells from atopic rhinitis patients. Compared to the non-atopic healthy subjects, the expression of IL-4 mRNA was significantly higher in the primary nasal cells obtained from patients with atopic rhinitis, which confirms the Th2-related nature of the inflammatory process present in the upper airways of these subjects (Figure 5B). Conversely, TLR3 mRNA expression was significantly lower in the primary nasal cells obtained from patients with atopic rhinitis compared to those of healthy subjects (Figure 5C). Consistent with the latter finding, increased viral replication was observed in the primary cultures of the nasal epithelial cells from the atopic subjects compared to those of the controls (Figure 5D).

Discussion

Asthma is a chronic inflammatory disorder of the airways that is typically, although not always, characterized by enhanced Th2-type inflammation (31). In this study, we documented that Th2 cytokines (IL-4 and IL-13) impaired components of innate immunity, such as the production of types I (IFN- β) and III IFN (IFN- λ), in response to rhinovirus infection.

The mechanisms by which a Th2 immunologic pattern inhibits virus-induced interferon production at the airway epithelial level, i.e. at the primary site of respiratory viral infections, remain largely unexplored. Here we evaluated the effects of Th2 cytokines over TLR-3 molecule and STAT1, IRF3 and NF-kB transcription factors, i.e. some of the key steps involved in rhinovirus induced interferon production (26-30).

In line with previous observations (14), we found that Th2 cytokines inhibited rhinovirus-induced IRF3 activation. We also found impaired activation of STAT1 following rhinovirus infection in the presence of Th2 cytokines. It has been previously reported that a) the exposure of epithelial cells to IL-4 and IL-13 cytokines leads to increased intracellular oxidative burst (32-34) and b) oxidative stress negatively interferes with intracellular interferon signalling, including IRF3 activation (14,15) and interferon-induced JAK-STAT activation and signalling (15). Interestingly, here we show that the antioxidant NAC restores rhinovirus-mediated activation of IRF3 and STAT1 and the downstream IFN- β and IFN- λ mRNA induction. These data suggest that the inhibition of oxidative stress represents a possible pharmacological approach to potentiate the innate immune responses to rhinovirus in a Th2 inflammatory milieu.

Oxidative stress can also amplify the activation of the NF-kB transcription factors involved in virus-induced interferon production and/or inflammation (35). A recent study showed that rhinovirus-infected NF-kB p65-deficient mice exhibited reduced neutrophilic inflammation, while interferon induction, antiviral responses and viral loads were unaffected (29). In our study we found that IL-4 stimulation did not impair rhinovirus-induced NF-kB activation, while it affected rhinovirus induced

interferon induction. Taken together, these experimental data confirm that NF- κ B is required for pro-inflammatory responses, but its role in interferon induction by rhinoviruses is not essential and may be redundant. The fact that Th2 cytokines do not impair NF- κ B activation is consistent with IL-4 and IL-13 having no effect on CXCL-8 induction in our model.

The stimulation of the bronchial epithelial cell line BEAS-2B with Th2 inflammatory cytokines (IL-4 and IL-13) led to reduced baseline expression of TLR3 via a mechanism that was not mediated by oxidants. Similar results have previously been shown in human intestinal epithelial cells (36). Thus, the impaired IRF3 activation documented here might be the consequence of the Th2 mediated impairment of TLR3 expression (which leads to impaired IRF3 activation) and also the consequence of a direct Th2-induced oxidant-mediated effect. Interestingly, we showed for the first time in the present study reduced *in vivo* levels of TLR3 associated with enhanced Th2 inflammation in the nasal epithelial cells of atopic rhinitis patients compared to normal controls. In line with these concepts, it has been recently shown that house dust mite (HDM)-sensitised mice have impaired rhinovirus-induced interferon production that is paralleled by a strong Th2-skewed inflammatory airway response (37). In addition, the pre-exposure of airway epithelial cells to HDM before rhinovirus infection deregulates TLR3-mediated production of cytokines and inflammatory mediators (38). Therefore HDM could suppress the IFN system either directly or via a Th-2 mediated pathway, the latter requiring further investigation given that IL-4/IL-13 signalling is quite distinct from HDM signalling. Interestingly, recent *in vivo* observations suggest that the expression (39) and activity (40) of TLR3 are not impaired in the bronchial epithelial cells of mild asthmatic subjects compared to healthy subjects. These data might suggest that mechanisms other than those associated with the TLR pathways are involved in the impairment of interferon production in the bronchial epithelial cells of asthmatic subjects. It is possible that such impairments favour increased susceptibility to infections in specific subgroups of patients, e.g., those with more severe asthma and/or those patients with a predominant Th2 airway inflammation where such impairment is expected to be more pronounced. It is also conceivable that impaired TLR3 expression in the upper respiratory tract i.e. nasal mucosa

could be a mechanism that is responsible for increased susceptibility that is primarily compartmentalized to upper respiratory infections in Th2-oriented diseases such as atopic rhinitis.

Finally, we recently found that asthmatic children, irrespective of atopic status, and atopic children, irrespective of the presence of asthma, exhibit impaired immune responses at the bronchial epithelial level in terms of type I (IFN- β) and type III (IFN- λ) production after rhinovirus infection. Enhanced Th2-mediated airway inflammation is the common biological substrate between these children (7). In this study, we documented that the development of a Th2 environment negatively affects the molecular mechanisms that govern the innate immune responses to viral infections. Indeed, TLR3 expression was found to be impaired in the epithelial cells of the nasal mucosa of atopic patients. In accordance with this concept, we observed increased viral replication associated with increased IL-4 expression in the nasal mucosa of atopic rhinitis patients compared to normal subjects. These data support the concept that the Th2 inflammation can dampen the anti-viral response and make the Th2 environment even without asthma (e.g. allergic rhinitis) a more susceptible condition to viral infections. This concept is consistent with recent observations that subjects with atopic diseases (including asthma, rhinitis and dermatitis) experience more frequent upper and lower respiratory tract infections than do non-atopic controls (41). In this scenario any pharmacological approach able to down-regulate Th2 inflammation has the potential to recover a defective innate immune responses. Previous studies showed that: a) IL-4 intracellular signalling leads to the activation of PI3k (20) and b) inhibition of PI3k resulted in attenuated Th2 inflammatory response in a mouse model of allergic asthma (17) and enhanced TLR signalling (19,42). Interestingly, in our study we found that the inhibition of PI3k restored the inhibitory effect of 24 hr pre-treatment with Th-2 cytokines on rhinovirus induced IRF-3 activation, suggesting that the inhibition of phosphoinositide 3-kinases counteract the Th-2 induced impairment of the innate immune response to rhinovirus.

In conclusion we showed that Th2 inflammation can dampen the anti-viral response and increase susceptibility viral infections of Th2 driven immunological conditions. In our experimental setting, pre-treatment with either antioxidants or inhibitors of PI3ks prevented the Th2-induced impairment of innate immune response to rhinovirus infection. Further studies are needed to elucidate

more extensively at system biology level the complex interactions in the signalling pathways that links Th2 inflammation to impaired immune response to infections.

Statement of contribution

Marco Contoli and Kazuhiro Ito conceived, designed and supervised all the study and experimental procedures; they directly contributed in the laboratory work and wrote the first draft of the manuscript. **Alberto Papi** co-designed and co-supervised all the study and experimental procedures and acts as guarantor for the studies. **Donatella Poletti (DP)** performed the nasal brushings. **Antonio Pastore** supervised the work of DP and advised on the scientific aspects of the study. **Anna Padovani, Brunilda Marku and Giulia Gnesini** performed the laboratory work and were in charge of the biological sample management and processing. **Antonio Spanevello, Gaetano Caramori, Michael R Edwards, Luminita Stanciu and Sebastian L. Johnston** advised on the scientific aspects of the study and contributed in the manuscript finalization. **Paolo Morelli** supervised the statistical analysis. All authors have approved the final version for publication.

Disclosure statement

Dr. Contoli reports grants from Chiesi, personal fees from Chiesi, personal fees from AstraZeneca, personal fees from Boehringer Ingelheim, personal fees from Chiesi, personal fees from Astrazeneca, personal fees from Novartis, personal fees from Menarini, personal fees from Mundipharma, personal fees from Almirall, personal fees from Zambon, outside the submitted work. **Dr. Ito** reports other from Pulmocide Ltd, outside the submitted work. **Dr. Padovani** has nothing to disclose. **Dr. Poletti** has nothing to disclose. **Dr. Marku** has nothing to disclose. **Dr. Edwards** has nothing to disclose. **Dr. Stanciu** has nothing to disclose. **Dr. Gnesini** has nothing to disclose. **Dr. Pastore** has nothing to disclose. **Dr. Spanevello** has nothing to disclose. **Dr. Morelli** has nothing to disclose. **Dr. Johnston** reports grants and personal fees from Centocor, grants and personal fees from Sanofi Pasteur, grants and personal fees from GSK, grants and personal fees from Chiesi, grants and personal fees from Boehringer Ingelheim, personal fees from Grünenthal, grants and personal fees from Novartis, grants, personal fees and other from Synairgen , outside the submitted work; In addition, Dr. Johnston has a

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Figure legends

Figure 1. Dose-response effects of 24-hr pre-treatment with Th2 cytokines (IL-4: panel A and B – IL-13: panel C and D) on rhinovirus-induced interferon mRNA induction (IFN- β : panel A and C and IFN- λ : panel B and D) in human bronchial epithelial cells (HBEC) of non-atopic, non-asthmatic subjects. Panel E: Effects of 24-hr pre-treatment with Th2 cytokines (IL-4 and IL-13) on rhinovirus (RV) 16-induced IFN- β production in HBECs (*p<0.01 vs. untreated and uninfected cells and IL-4- or IL-13-treated cells; ^p<0.05 vs. RV16 infected cells). Panel F: Effects of Th2 cytokines (IL-4: 50 ng/ml and IL-13: 25 ng/ml) on rhinovirus-induced CXCL-8 production in HBECs (*p<0.01 vs. untreated and uninfected cells and IL-4- or IL-13-treated cells). Effects of IL-2 on rhinovirus-induced IFN- β mRNA (Panel G) and IFN- λ mRNA (Panel H) in HBECs (***p<0.001; **p<0.01; *p<0.05; RV16: rhinovirus 16; f-RV16: cells inoculated with virus stock from which the virus had been removed by molecular weight filtration) (all panels n=6).

Figure 2. Time course of the effects of 24-hr pre-treatment with Th2 cytokines (IL-4 and IL-13) on rhinovirus-induced IFN- β and IFN- λ mRNA induction and rhinovirus replication in human bronchial epithelial cells (HBECs). (**p<0.01; *p<0.05) (all panels n=6).

Figure 3. Effects of 24-hr pre-treatment with Th2 cytokines (IL-4: 50 ng/ml and IL-13: 25 ng/ml) on rhinovirus-induced nuclear factor (NF)- κ B (Panel A), interferon responsive factor 3 (IRF3; Panel B) and STAT1 (Panel C) activation in BEAS-2b cell (**p<0.01 and *p<0.05 vs. untreated and uninfected cells and IL-4- or IL-13-treated cells; ^p<0.05 vs. rhinovirus (RV) 16-infected cells) (all panels n=4).

Figure 4. Effects of 24-hr pre-treatment with IL-4 (50 ng/ml) on rhinovirus (RV) 16-induced interferon responsive factor 3 (IRF3; Panel A) and STAT1 (Panel B) activation in the presence or absence of cell exposure to N-acetylcysteine (NAC, 10 mM) 30 minutes prior to infection (**p<0.01; p<0.05). Effects of N-acetylcysteine (NAC, 10 mM) 30 minutes prior to infection on the inhibitory effects of 24-hr pre-treatment with IL-4 (50 ng/ml) on rhinovirus (RV) 16-induced IFN- λ mRNA in BEAS-2B cells (Panel C) and IFN- β mRNA (Panel D) (ns: not significant; *p<0.05). (Panel E) Effects of phosphoinositide 3-kinases (PI3k) inhibitor LY294002 (3.3 μ M) before rhinovirus infection on the inhibitory effect of 24-hr pre-treatment with IL-4 (50 ng/ml) on rhinovirus (RV) 16-induced IRF-3 activation (*p<0.05 vs. untreated and uninfected cells; ^p<0.05 vs. rhinovirus (RV) 16-infected cells and vs cells infected with RV16 and exposed to LY294002) (all panels n=5).

Figure 5. Panel A) Effects of 24-hr pre-treatment with Th2 cytokines on TLR3 mRNA expression in BEAS-2B cells in the presence or absence of cell exposure to N-acetylcysteine (n=5). The expression of TLR3 was evaluated by real-time RT-PCR, the results were normalized to GAPDH, and the expression levels are represented as the fold-changes in expression vs. medium-treated cells (*p<0.05 vs. medium-treated cells). Panel B) Baseline IL-4 mRNA levels in the primary cell cultures of epithelial cells of the nasal mucosae of non-atopic (n=6) and atopic (n=5) subjects. Panel C) Baseline TLR3 mRNA levels in the primary cell cultures of epithelial cells of the nasal mucosae of non-atopic (n=6) and atopic subjects (n=5). D) Rhinovirus (RV) 16 vRNA levels 8 hr after the infection of primary nasal mucosa epithelial cells cultures of non-atopic (n=6) and atopic (n=5) subjects.

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Figure 1

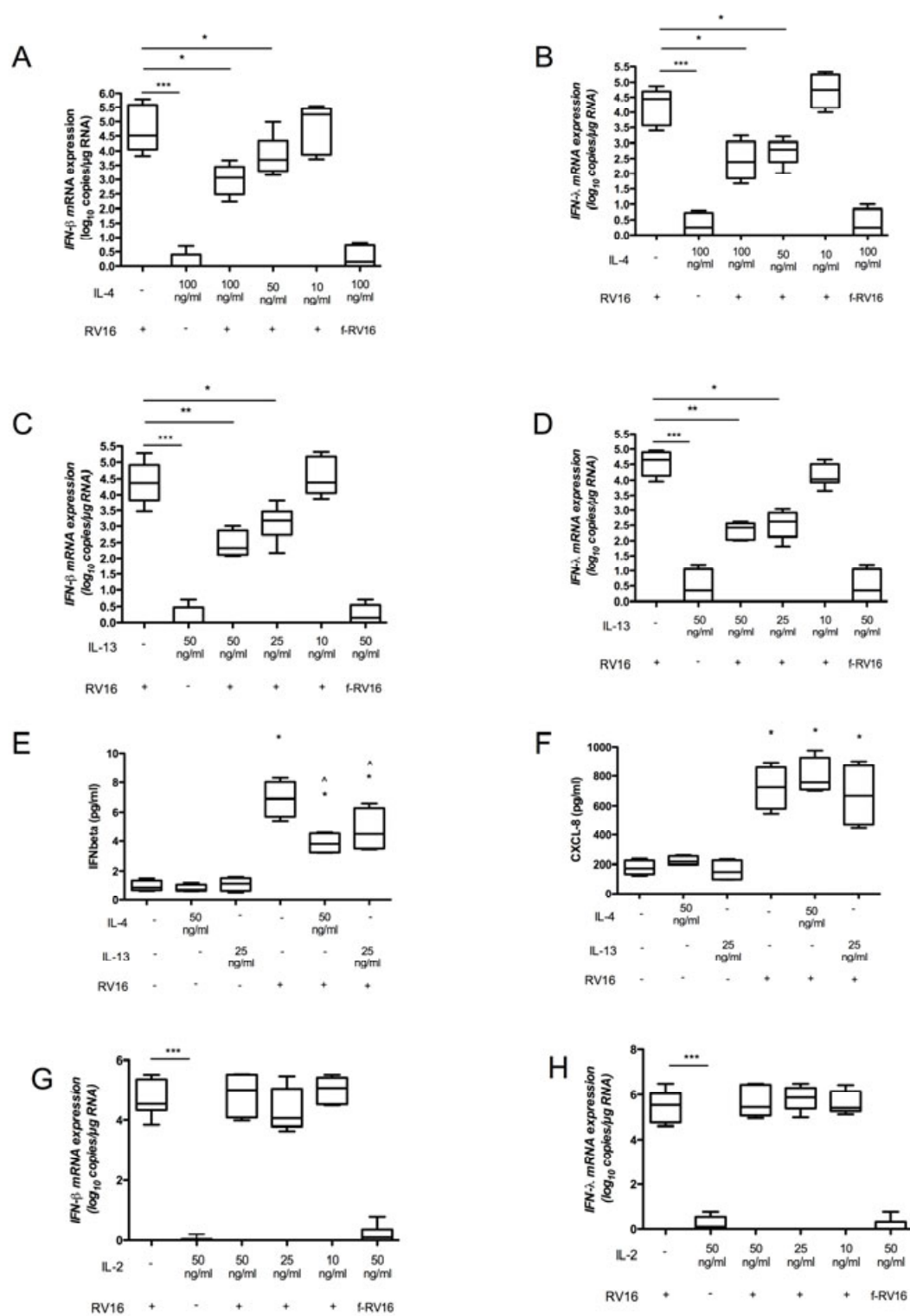


Figure 2

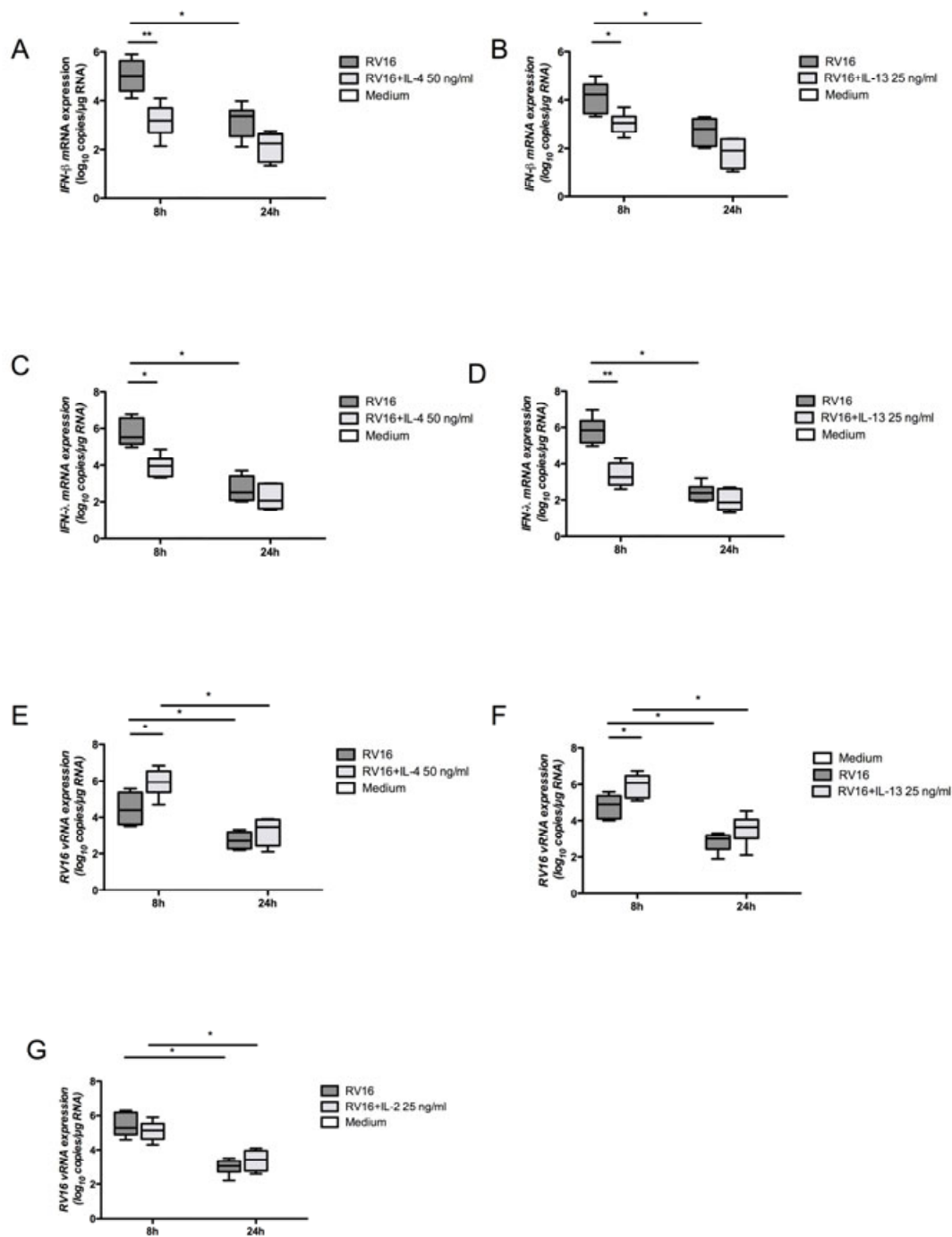


Figure 3

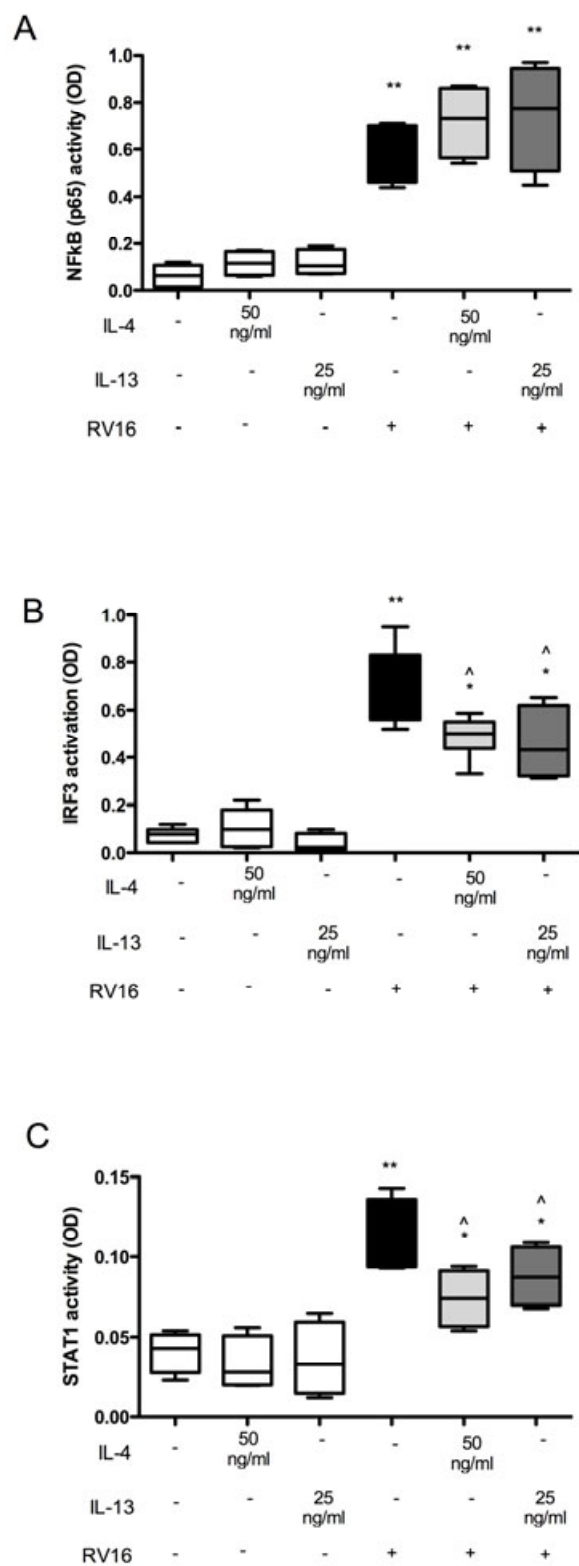


Figure 4

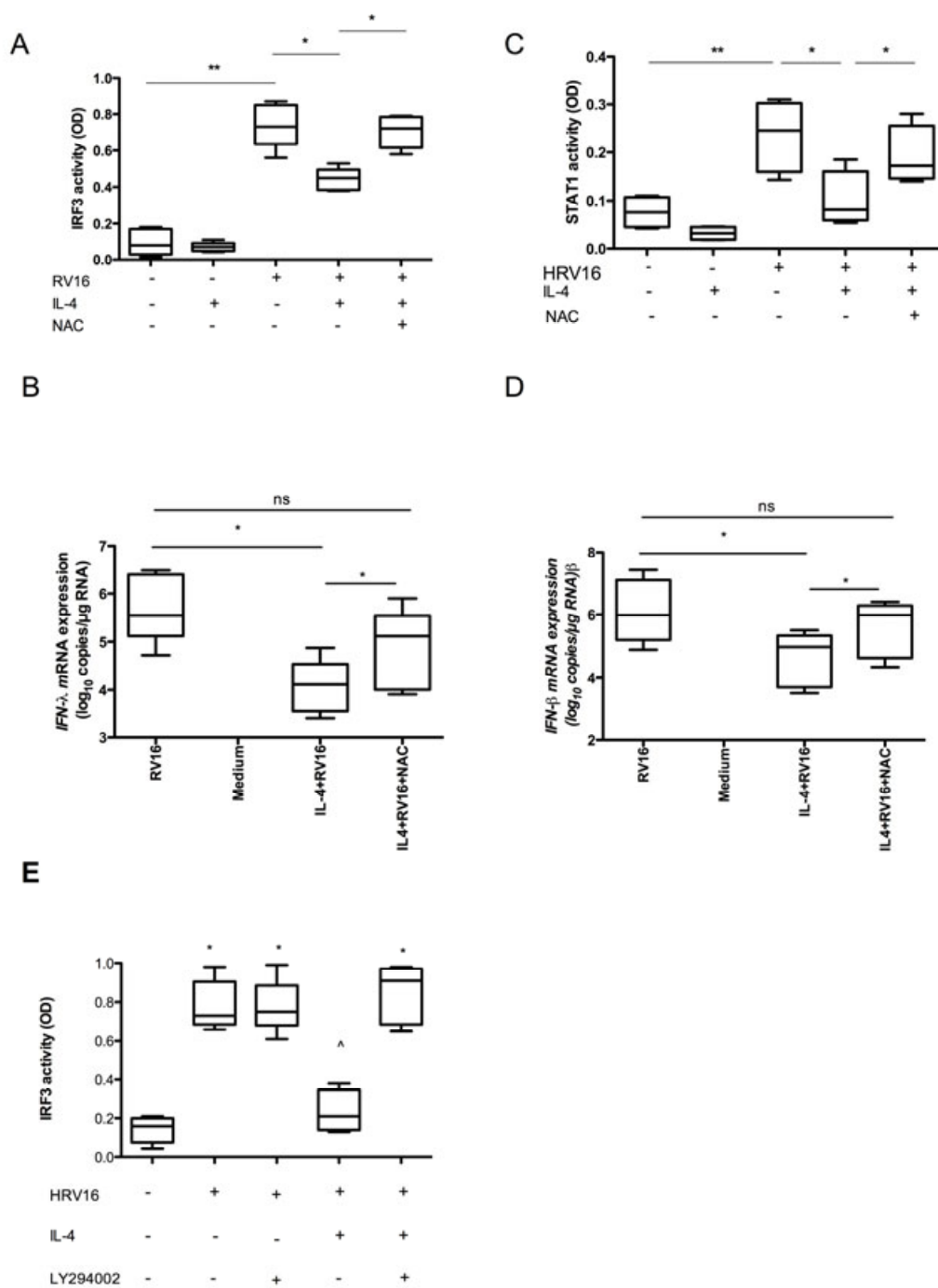
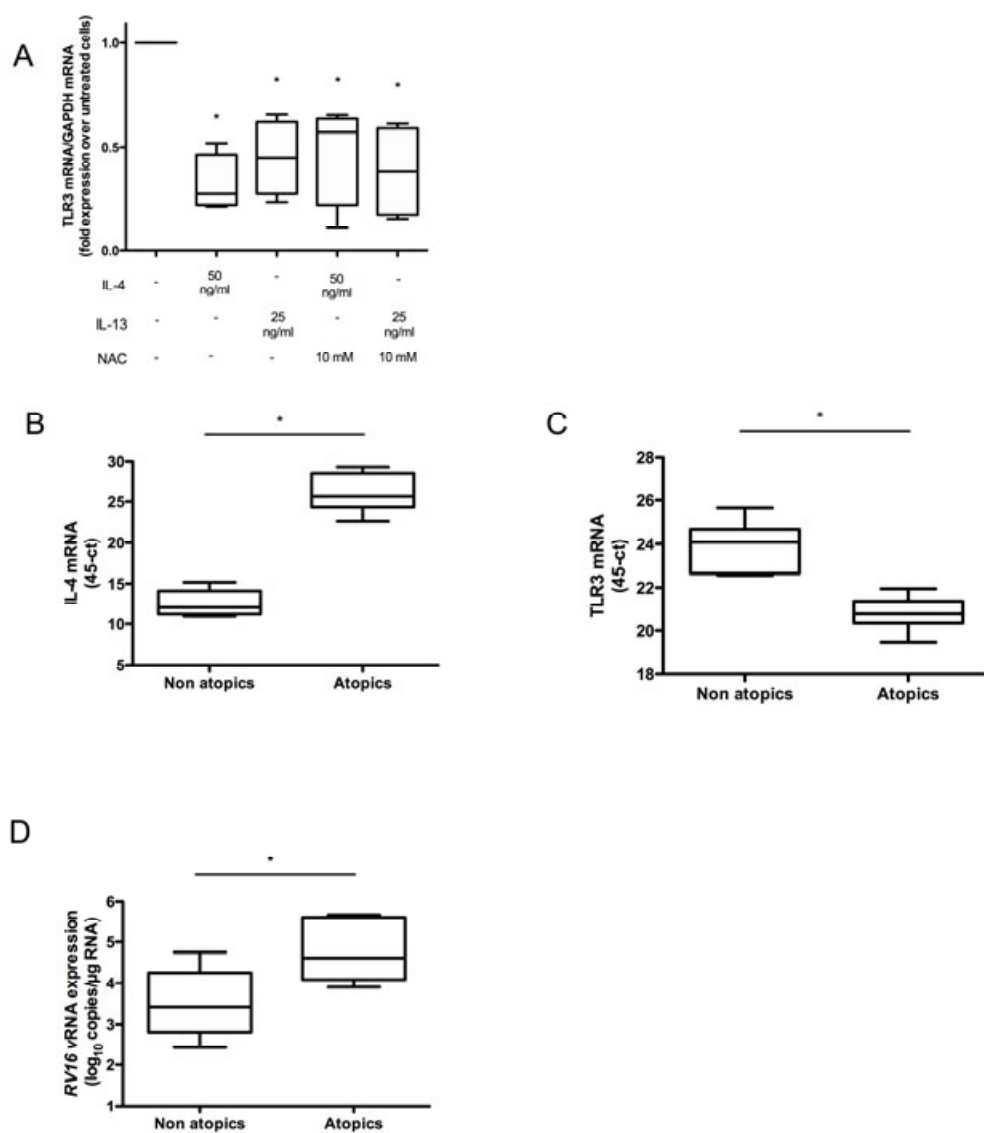


Figure 5



Online supporting information

**Th2-cytokines induce the impairment of innate immune responses to rhinovirus in
respiratory epithelial cells**

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1 Methods

2 **Study population**

3 Primary human bronchial epithelial cells (HBECS) were obtained from bronchial brushings
4 of non-atopic, non-asthmatic and non-smoking subjects who underwent bronchoscopy for clinical
5 reasons (the subject factors for these patients and indication for bronchoscopy are detailed in
6 Supplementary Table 1). Nasal symptoms were not investigated in these patients.

7 Nasal epithelial cells were obtained by nasal brushings from atopic and non-atopic patients
8 with persistent rhinitis (demographic characteristics of these patients are reported in supplementary
9 Table 2). Atopic status was defined as the presence of at least one positive response to skin pricks
10 tests for common local aeroallergens. The diagnosis of rhinitis was based on the presence of 1 or
11 more of the following nasal symptoms: congestion, rhinorrhoea (anterior and posterior), sneezing,
12 and itching (1). All patients suffered of persistent rhinitis defined as the presence of symptoms for
13 at least 4 days/week for more than 4 consecutive weeks (1).

14 The nasal and the bronchial epithelial cells were not obtained from the same individuals.

15 None of the patients included in the study (who underwent bronchoscopy or nasal brushing)
16 suffered of symptoms typical of asthma (such as such as wheezing, shortness of breath, chest
17 tightness and cough that vary over time in their occurrence, frequency and intensity) making the
18 diagnosis of concomitant asthma unlikely according to international guideline (2).

19

20 **Cell cultures and in vitro rhinovirus infections**

21 BEAS-2B cells, an immortalized human bronchial epithelial cell line, were obtained from
22 the American Type Culture Collection (Rockville, Md, USA) and cultured as previously described
23 (3-5). Primary human bronchial epithelial cells (HBECS) were obtained from bronchial brushings of
24 healthy (non-atopic, non-asthmatic and non-smoking) subjects (undergoing bronchoscopy for
25 clinical reasons) and cultured, as previously described (3-5).

26 Before the infection, the cells were exposed for 24 hrs to media (in which foetal calf serum

1 and/or hormone supplementation were removed) in the presence or absence of different
2 concentrations of IL-4, IL13 and IL-2. The rhinovirus infections were performed in 6-well plates
3 with the epithelial cell layer at approximately 80% confluence. After 1 hr infection at room
4 temperature with gently shaking, the medium with the unbound virus was removed and fresh
5 medium added to the cultures. Rhinovirus induced IFN- β and IFN- λ 1 production was evaluated at 8
6 and 24 hrs after the infection (3-6). Rhinovirus induced activation of transcription factors was
7 evaluated at 1 hr after rhinovirus infection (7). To assess the role of oxidative stress in the activation
8 of the transcription factors involved in rhinovirus induced interferon production cells were exposed
9 to N-acetyl cysteine [10 mM] for 30 min before the infection (8,9). To assess the role of
10 phosphoinositide 3-kinases (PI3k) on Th2-induced modulation of innate immune response to
11 rhinovirus infection cells were exposed to IL-4 (50 ng/ml) for 24 hr in the presence of the broad
12 spectrum phosphoinositide 3-kinases (PI3k) inhibitor LY294002 (3.3 μ M). In selected experiments
13 cells were stimulated with inocula in which viral particles were removed by means of ultrafiltration
14 through membranes (Amikon, London, United Kingdom).

15 Primary epithelial cells of nasal mucosa of 5 atopic rhinitis and 6 non-atopic healthy
16 volunteers were harvested by nasal scraping and freshly cultured in BEG medium. When confluent
17 total RNA was extract according to manufacture's instruction (Qiagen). Atopic status was defined
18 as the presence of at least one positive skin prick test to the common local aeroallergens.

19 The study was approved by the local ethics committee of the University Hospital of Ferrara,
20 and informed consent was obtained from each participant in accordance with the principles outlined
21 in the Declaration of Helsinki.

22 23 **References**

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1 Supplementary Table 1: clinical characteristics of the patients who underwent bronchoscopy

2

Patient ID (Initials)	Age	Gender	History of asthma	Atopy	Smoking habit	Indication for bronchoscopy
IV	31	Female	No	No	Never smoked	Emophtoe with negative high resolution CT scan
ZA	27	Male	No	No	Never smoked	Emophtoe with negative high resolution CT scan
FF	56	Male	No	No	Never smoked	Emophtoe with negative high resolution CT scan
BE	69	Female	No	No	Never smoked	Emophtoe with negative high resolution CT scan
MV	63	Female	No	No	Never smoked	Emophtoe with negative high resolution CT scan
OBG	30	Male	No	No	Never smoked	Solitary pulmonary nodule (15 mm) of the middle lobe

3

4

5

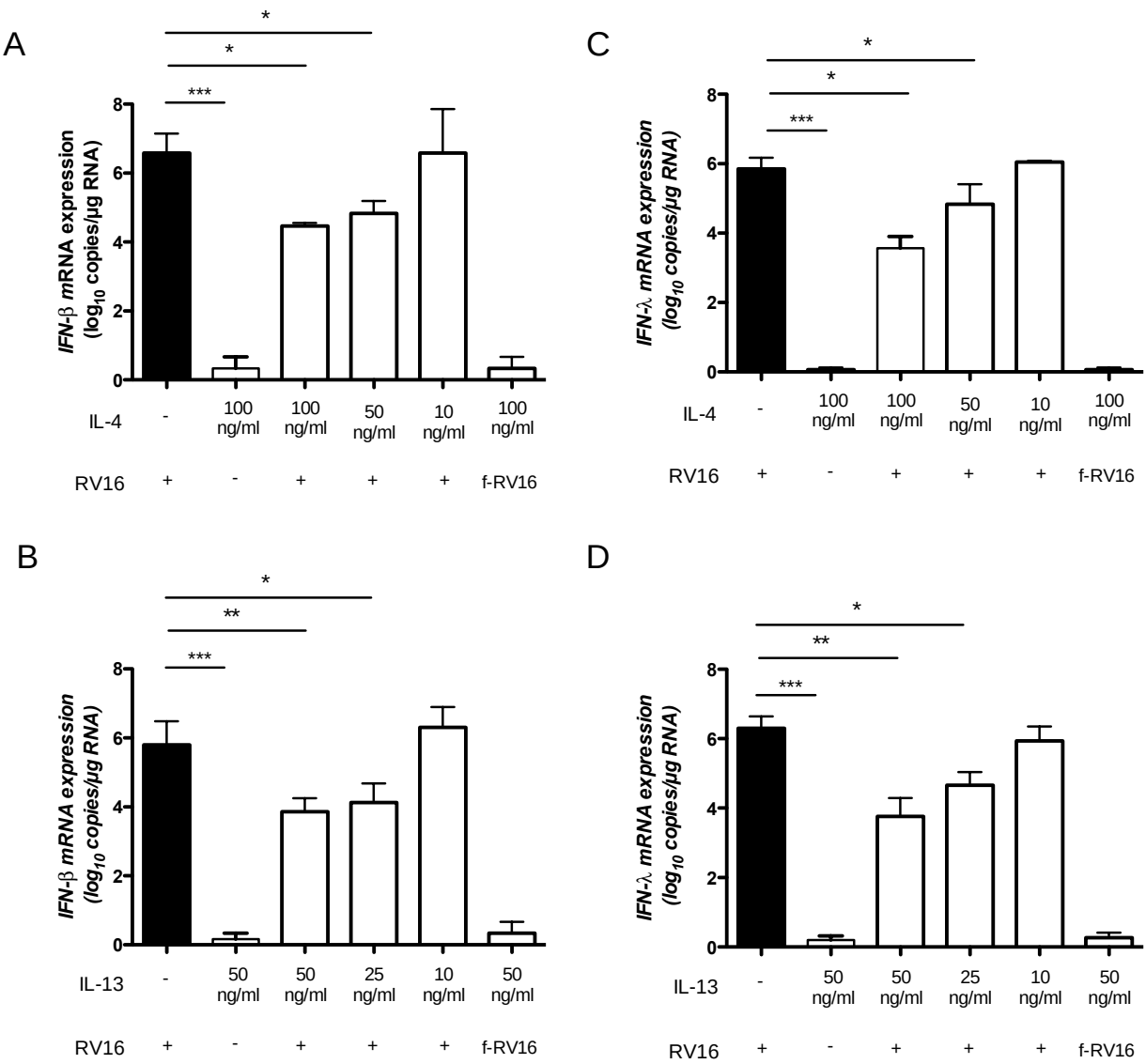
1 Supplementary Table 2: characteristics of the rhinitis subjects who underwent nasal brushing

Patient ID	Age	Gender	Positivity at Skin prick testing
Atopic patients			Mites, Grasses,
1A	39	male	Cat
2A	32	female	Mites
3A	43	male	Grasses, Alternaria
4A	24	male	Mites, Cat
5A	27	female	Grasses, Cat
Non-atopic patients			
1NA	52	male	None
2NA	24	male	None
3NA	29	male	None
4NA	39	female	None
5NA	25	female	None
6NA	30	male	None

2

3

1 Supplementary figure 1



2
3
4 Figure s1: Dose-response effects of 24-hr pre-treatment with Th-2 cytokines (IL-4: panel A and C –
5 IL-13: panel B and D) on rhinovirus-induced interferon mRNA induction (IFN-β: panel A and B
6 and IFN-λ: panel C and D) in bronchial epithelial cell line BEAS-2b (***p<0.001; **p<0.01;
7 *p<0.05)