Allergy EUROPEAN JOURNAL OF ALLERGY AND CLINICAL IMMUNOLOGY EAACI

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

Journal:	*Allergy*
Manuscript ID:	ALL-2014-00633.R1
Wiley - Manuscript type:	Original Article: Experimental Allergy and Immunology
Date Submitted by the Author:	n/a
Complete List of Authors:	Contoli, Marco; University of Ferrara, Research Centre on Asthma and COPD Ito, Kazuhiro; Imperial College, Airway Disease, National Heath and Lung Institute Padovani, Anna; University of Ferrara, Research centre on Asthma and COPD Poletti, Donatella; University of Ferrara, ENT Unit, Department of Biomedical and Surgical Sciences Marku, Brunilda; University of Ferrara, Research centre on Asthma and COPD Edwards, Michael; Imperial College London, Department of Respiratory Medicine London Stanciu, Luminita; Imperial College London, Department of Respiratory Medicine Gnesini, Giulia; University of Ferrara, Research centre on Asthma and COPD Pastore, Antonio; University of Ferrara, ENT Unit, Department of Biomedical and Surgical Sciences Spanevello, Antonio; University of Insubria and Fondazione Maugeri, Respiratory Diseases Morelli, Paolo; Cros NT,, Center of Excellence for Clinical Trial Data Johnston, Sebastian; Imperial College London, Department of Respiratory Medicine Caramori, Gaetano; University of Ferrara, Research centre on Asthma and COPD Papi, Alberto; University of Ferrara, Research centre on Asthma and COPD
Keywords :	asthma, innate immunity, virus, basic mechanisms

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

Marco Contoli[1], Kazuhiro Ito[2], Anna Padovani[1], Donatella Poletti[3], [4], Brunilda Marku[1], Michael R. Edwards[4], Luminita A. Stanciu[4], Giulia Gnesini[1], Antonio Pastore[3], Antonio Spanevello[5], Paolo Morelli[6], Sebastian L. Johnston[4], Gaetano Caramori[1], Alberto Papi[1].

[1] Research Centre on Asthma and COPD, Department of Medical Sciences, University of Ferrara, Italy

[2] Airway Disease, National Heath and Lung Institute, Imperial College, London, UK.

[3] ENT Unit, Department of Biomedical and Surgical Sciences, University of Ferrara, Italy

[4] Airway Disease Infection Section, National Heart and Lung Institute, Imperial College and MRC and Asthma UK Centre in Allergic Mechanisms of Asthma, London, UK

[5] University of Insubria, Varese; Fondazione Maugeri, IRCCS, Tradate

[6] Cros NT, Verona, Italy.

Address correspondence to:

Marco Contoli, MD, PhD

Research Centre on Asthma and COPD, Department of Medical Sciences, University of Ferrara, Italy

Via Savonarola 9, 44121 Ferrara, Italy

Tel: +390532236908

Fax: +390532210297

Email: ctm@unife.it

1 Word count (text): 2554

2 Key words: asthma, innate immunity, virus, Th2 inflammation, interferon.

3

4

Abstract (word count: ~~246~~245)

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-λ1) induction following rhinovirus (RV)-16 infection. We also investigated the modulatory effects of Th2 cytokines on ~~signalling pathways involved in the rhinovirus-induced interferon production and inflammatory cascade including~~ Toll-like receptor (TLR)-3, interferon-responsive factor (IRF)-3, and nuclear factor (NF)-kB~~,~~ i.e. key molecules and transcription factors involved in the rhinovirus-induced interferon production and inflammatory cascade. Pharmacological and redox modulation ~~by the reducing agent N-acetylcysteine on~~of these pathways was also ~~tested~~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-kB 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~~ and production of IFNs~~.

Conclusions: IL-4 and IL-13, through inhibition of TLR3 expression and ~~oxidant-mediated inhibition of~~ signalling (IRF-3~~,~~), impair immune response to RV-16 infection. These data suggest that Th2 conditions increase susceptibility to infections and identify ~~a~~ pharmacological ~~approach~~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).~~(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).~~(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 ~~and~~b) not all asthmatic patients have ~~high~~a preponderant Th2 oriented inflammatory profile in the airways ~~(6). 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) and suggest that the Th2 inflammation influences the integrity of the innate immune responses and the outcomes of infections.~~

~~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). Thus, modulation of the intracellular redox state represents a potential pharmacological target for interfering with the intimate mechanisms that are related to viral infection and the immunological and inflammatory substrates and consequences of such infections.~~

(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 of 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-λ1) interferon production against rhinovirus infection in bronchial epithelial cells. We also ~~identified~~evaluated the ~~signalling~~

~~pathways involved in the inhibitory~~ effects of Th2 cytokines over key molecules (TLR-3) and transcription factors (IRF3 and NF-kB) involved in the innate immune responses to ~~viral infections~~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 (16). 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 (7,16,17).~~

~~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.~~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). ~~Atopic status was defined as the presence of at least one positive response to skin pricks tests for common local aeroallergens (the~~

The demographic characteristics ~~are reported~~of the patients enrolled in the ~~online supporting information).~~

~~The~~study and the experimental conditions of the cell cultures and ~~stimulations~~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 (~~18~~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-λ1), CXCL8 (Qiagen), TLR3 (Qiagen), and IL-4 (Qiagen) as detailed elsewhere (7,16,17). 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 copy numbers per microgram of RNA.~~

Quantitative real-time PCR was performed with specific primers and probes for rhinovirus, IFN-β, IFN-λ (specific for IFN-λ1), 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 $\log_{10}$ 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)~~(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 NFkB, IRF3 and STAT1 in 10-µg 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

~~The data are expressed as the means and the standard errors of the means of at least 4 experiments. Differences between the conditions at each time point were calculated with unpaired t tests. Multiple comparisons were first analysed with ANOVAs. When significant ($p< 0.05$) differences between groups/conditions were found, post hoc analyses were performed with paired or unpaired *t*-tests as appropriate. Values below .05 were considered significant. In cases of multiple comparisons, the reported p values refer to analyses that were adjusted with the Bonferroni correction.~~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 $<0{\cdot}05$ 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 ~~(19)~~(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 ~~(20).~~(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 uM) 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)-kB 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, ~~but~~although not always, characterized by enhanced Th2-type inflammation ~~(21).~~(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 inhibitory effects of Th2 inflammation on interferon production can favour increased susceptibility to infections in asthmatic patients.~~

~~The airway epithelial layer is the natural site at which respiratory viral infections occur. Recent investigations have evaluated the crosstalk between Th2 inflammation and antiviral interferon production at the bronchial epithelial level. Moriwaki and colleagues found that Th2 (IL-13) deficient mice exhibit greater enhancements of IFN λ expression following intratracheal instillation of poly-IC (which mimicked viral infection) than wild-type mice, whereas IFN λ expression following poly-IC is absent in the lungs of mice with allergen-induced asthma in which Th2 cytokines plays a central pathogenic role (8). Eosinophils are the effector cells of the Th2 inflammatory cascade. It has recently been shown that rhinovirus infection of bronchial epithelial cells cocultured with eosinophils results in the impairment of interferon β and λ production and increases rhinovirus replication (9). On the other side, previous studies have reported that interferon λ down-regulates the Th2 inflammatory response (22-25). Taken together, these data support the existence of a counter-regulatory relationship between Th2 inflammation and the innate immune response.~~

~~The mechanisms by which Th2 inhibits virus-induced interferon production at the airway epithelial level remain largely unexplored. Double-stranded RNA (dsRNA) produced during viral infection and replication is an important stimulus of the host innate immune response. This dsRNA is recognized and engaged by TLR3, which in turn activates transcription factors, such as nuclear factor (NF)-kB and IFN regulatory factor (IRF)-3/7, which lead to the production of proinflammatory cytokines, chemokines and antiviral molecules including interferons (26-28).~~

Previous studies have shown that oxidative stress negatively interferes with intracellular interferon signalling, including IRF3 activation (14,15), but can amplify the activation of the transcription factors involved in virus-induced interferon production and/or inflammation, such as NF-kB (29). It has also been reported that the exposure of epithelial cells to IL-4 and IL-13 cytokines leads to increased intracellular oxidative burst (30-32). In this study, we found that 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 (33). In accordance with previous observations (14), we also found that Th2 cytokines inhibited rhinovirus-induced IRF3 activation via a mechanism that can be modulated by antioxidants. 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. Interestingly, recent in vivo observations suggest that the expression (34) and activity (35) 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 than such impairments develop only in specific subgroups of patients, e.g., those with more severe asthma and those more prone to infections, in which Th2 inflammation is 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.

The outcome of the interferon-activated Janus kinase (JAK) signal transducer and activator of transcription (STAT) signalling pathway is the transcriptional induction of the expressions of

genes with antiviral properties. Specifically, STAT1 activation is a common mechanism of the antiviral actions of Type I (IFN-α and -β), Type II (IFN-γ) and Type III (IFN-λ) interferons (20). JAK-STAT signalling is activated following interferon-receptor engagement. It has also been previously shown that oxidative stress directly inhibits interferon-induced JAK-STAT activation and signalling (15). Therefore, the impairment of STAT1 that we documented in the presence of a Th2 inflammatory environment can be the result of impaired interferon release and consequent reduction in IFN-receptor stimulation but also of Th2 mediated oxidative stress being reversed by antioxidant (N-acetylcysteine) treatment.

NF-kB is a key element in the rhinovirus-induced inflammatory response and might also be involved in virus-induced interferon production. A recent study showed that rhinovirus-infected NF-kB p65-deficient mice exhibited reduced neutrophilic inflammation; however, interferon induction, antiviral responses and viral loads are unaffected (36). In our study, we found that IL-4 stimulation did not impair rhinovirus-induced NF-kB activation. Taken together, these experimental data confirm that NF-kB is required for pro-inflammatory responses, but its role in interferon induction by rhinoviruses is redundant, and the data also show that Th2 cytokines do not impair NF-kB activation potentially explaining why IL-4 and IL-13 had no effect on CXCL-8 in our model system.

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-kB is required for pro-inflammatory responses, but its role in interferon induction by rhinoviruses is not essential an may be redundant. The fact that Th2 cytokines do not impair NF-kB 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)~~(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 ~~confirm~~support the concept that ~~impaired immune responses to viral infection are not exclusive to~~the Th2 inflammation can dampen the anti-viral response and make the Th2 environment even without asthma ~~but are shared with other Th2 -driven diseases~~ (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 ~~(37). The inhibition of oxidative stress represents a possible pharmacological approach to potentiate the innate immune responses of these patients and to reduce their susceptibility to infections.~~(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 patent Blair ED, Killington RA, Rowlands DJ,

Clarke NJ, Johnston SL. Transgenic animal models of HRV with human ICAM-1 sequences. UK patent application No. 02 167 29.4, 18 July 2002 and International patent application No. PCT/EP2003/007939, 17 July 2003. licensed, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. Anti-virus therapy for respiratory diseases. UK patent application No. GB 0405634.7, 12 March 2004. licensed, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. Interferon-Beta for Anti-Virus Therapy for Respiratory Diseases. International Patent Application No. PCT/GB05/50031, 12 March 2004. licensed, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. The use of Interferon Lambda for the treatment and prevention of virally-induced exacerbation in asthma and chronic pulmonary obstructive disease. UK patent application No. 0518425.4, 9 September 2005. licensed, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. Anti-Virus Therapy for Respiratory Diseases. US Patent Application – 11/517,763, Patent No.7569216, National Phase of PCT/GB2005/050031, 04 August 2009. licensed, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. Interferon-beta for Anti-Virus Therapy for Respiratory Diseases. European Patent Number 1734987, 5 May 2010. licensed, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. Anti-Virus Therapy for Respiratory Diseases (IFNb therapy) Hong Kong Patent Number 1097181, 31 August 2010. licensed, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. Anti-Virus Therapy for Respiratory Diseases (IFNb therapy). Japanese Patent Number 4807526, 26 August 2011. licensed, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. Interferon-beta for Anti-Virus Therapy for Respiratory Diseases. New Hong Kong - Divisional Patent Application No. 11100187.0, 10 January 2011. Licensed, and a patent Burdin N, Almond J, Lecouturieir, V, Girerd-Chambaz Y, Guy, B, Bartlett N, Walton R, McLean G, Glanville N, Johnston SL. Induction of cross-reactive cellular response against rhinovirus antigens European Patent Number 13305152, 4 April 2013. pending. **Dr. Caramori** reports grants from AstraZeneca Italy, grants and personal fees from Boehringer Ingelheim, grants and other from GlaxoSmithKline Italy, grants from Menarini , grants from Almirall, outside the submitted work. **Dr. Papi** reports grants, personal fees, non-financial support and other from Chiesi, grants, personal fees, non-

financial support and other from Astrazeneca, grants, personal fees, non-financial support and other from GlaxoSmithKline, grants, personal fees, non-financial support and other from Boehringer Ingelheim, grants, personal fees, non-financial support and other from Merck Sharp & Dohme, personal fees and non-financial support from Menarini, personal fees and non-financial support from Novartis, personal fees and non-financial support from Zambon, grants, personal fees, non-financial support and other from Pfizer, grants, personal fees, non-financial support and other from Takeda, grants, personal fees, non-financial support and other from Mundipharma, outside the submitted work.

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)-kB (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 uM) 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.

References

1. Global Initiative for Asthma Global Strategy for Asthma Management and Prevention 2014. *Available from: wwwginasthmaorg*
2. Edwards MR, Bartlett NW, Hussell T, Openshaw P, Johnston SL. The microbiology of asthma. *Nature Reviews Microbiology* 2012;**10**:459–471.
3. Corne J, Marshall C, Smith S, Schreiber J, Sanderson G, Holgate S et al. Frequency, severity, and duration of rhinovirus infections in asthmatic and non-asthmatic individuals: a longitudinal cohort study. *Lancet* 2002;**359**:831–834.
4. Holt PG, Strickland DH. Interactions between innate and adaptive immunity in asthma pathogenesis: new perspectives from studies on acute exacerbations. *J Allergy Clin Immunol* 2010;**125**:963–972.
5. Hansel TT, Johnston SL, Openshaw PJ. ReviewMicrobes and mucosal immune responses in asthma. *The Lancet* 2013;**381**:861–873.
6. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 2012;**18**:716–725.
7. Baraldo S, Contoli M, Bazzan E, Turato G, Padovani A, Marku B et al. Deficient antiviral immune responses in childhood: distinct roles of atopy and asthma. *J Allergy Clin Immunol* 2012;**130**:1307–1314.
8. Moriwaki A, Matsumoto K, Matsunaga Y, Fukuyama S, Matsumoto T, Kan-O K et al. IL-13 suppresses double-stranded RNA-induced IFN-λ production in lung cells. *Biochemical and Biophysical Research Communications* 2011;**404**:922–927.
9. Mathur SK, Fichtinger PS, Kelly JT, Lee W-M, Gern JE, Jarjour NN. Interaction between allergy and innate immunity: model for eosinophil regulation of epithelial cell interferon expression. *Ann Allergy Asthma Immunol* 2013;**111**:25–31.
10. Beisswenger C, Kandler K, Hess C, Garn H, Felgentreff K, Wegmann M et al. Allergic airway inflammation inhibits pulmonary antibacterial host defense. *J Immunol*

2006;**177**:1833–1837.

11. Message SD, Laza-Stanca V, Mallia P, Parker HL, Zhu J, Kebadze T et al. Rhinovirus-induced lower respiratory illness is increased in asthma and related to virus load and Th1/2 cytokine and IL-10 production. *Proc Natl Acad Sci USA* 2008;**105**:13562–13567.

12. Papi A, Papadopoulos NG, Stanciu LA, Bellettato CM, Pinamonti S, Degitz K et al. Reducing agents inhibit rhinovirus-induced up-regulation of the rhinovirus receptor intercellular adhesion molecule-1 (ICAM-1) in respiratory epithelial cells. *The FASEB Journal* 2002;**16**:1934–1936.

13. Papi A, Contoli M, Gasparini P, Bristot L, Edwards MR, Chicca M et al. Role of xanthine oxidase activation and reduced glutathione depletion in rhinovirus induction of inflammation in respiratory epithelial cells. *J Biol Chem* 2008;**283**:28595–28606.

14. Kocic G, Sokolovic D, Jevtovic T, Veljkovic A, Kocic R, Nikolic G et al. Hyperglycemia, oxidative and nitrosative stress affect antiviral, inflammatory and apoptotic signaling of cultured thymocytes. *Redox Rep* 2010;**15**:179–184.

15. Di Bona D, Cippitelli M, Fionda C, Cammà C, Licata A, Santoni A et al. Oxidative stress inhibits IFN-alpha-induced antiviral gene expression by blocking the JAK-STAT pathway. *J Hepatol* 2006;**45**:271–279.

16. Zhao W, Qi J, Wang L, Zhang M, Wang P, Gao C. LY294002 inhibits TLR3/4-mediated IFN-b production via inhibition of IRF3 activation with a PI3K-independent mechanism. *FEBS Lett* 2012;**586**:705–710.

17. Lee KS, Lee HK, Hayflick JS, Yong C Lee, Kamal D Puri. Inhibition of phosphoinositide 3-kinase attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. *The FASEB Journal* 2006;**20**:455–465.

18. Cao W, Manicassamy S, Tang H, Kasturi SP, Pirani A, Murthy N et al. Toll-like receptor–mediated induction of type I interferon in plasmacytoid dendritic cells requires the rapamycin-sensitive PI(3)K-mTOR-p70S6K pathway. *Nat Immunol* 2008;**9**:1157–1164.

19. Fukao T, Koyasu S. PI3K and negative regulation of TLR signaling. *Trends in Immunology* 2003;**24**:358–363.

20. Kelly-Welch AE. Interleukin-4 and Interleukin-13 Signaling Connections Maps. *Science* 2003;**300**:1527–1528.

21. Contoli M, Message SD, Laza-Stanca V, Edwards MR, Wark PAB, Bartlett NW et al. Role of deficient type III interferon-lambda production in asthma exacerbations. *Nat Med* 2006;**12**:1023–1026.

~~17~~22. Wark PAB, Johnston SL, Bucchieri F, Powell R, Puddicombe S, Laza-Stanca V et al. Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. *J Exp Med* 2005;**201**:937–947.

~~18~~23. Papi A, Johnston S. Rhinovirus infection induces expression of its own receptor intercellular adhesion molecule 1 (ICAM-1) via increased NF-kappa B-mediated transcription. *J Biol Chem* 1999;**274**:9707–9720.

~~19~~24. Kennedy JL, Turner RB, Braciale T, Heymann PW, Borish L. Pathogenesis of rhinovirus infection. *Current Opinion in Virology* 2012;**2**:287–293.

~~20~~25. Rauch I, Müller M, Decker T. The regulation of inflammation by interferons and their STATs. *JAKSTAT* 2013;**2**:e23820.

~~21. Wenzel SE. Asthma: defining of the persistent adult phenotypes. *Lancet* 2006;**368**:804–813.~~

~~22. Srinivas S, Dai J, Eskdale J, Gallagher GE, Megjugorac NJ, Gallagher G. Interferon-lambda1 (interleukin-29) preferentially down-regulates interleukin-13 over other T helper type 2 cytokine responses in vitro. *Immunology* 2008;**125**:492–502.~~

~~23. Dai J, Megjugorac NJ, Gallagher GE, Yu RYL, Gallagher G. IFN-lambda1 (IL-29) inhibits GATA3 expression and suppresses Th2 responses in human naive and memory T cells. *Blood* 2009;**113**:5829–5838.~~

~~24. Koltsida O, Hausding M, Stavropoulos A, Koch S, Tzelepis G, Ubel C et al. IL-28A (IFN-λ2) modulates lung DC function to promote Th1 immune skewing and suppress allergic~~

~~airway disease. *EMBO Mol Med* 2011;**3**:348–361.~~

~~25. Edwards MR, Johnston SL. Interferon-lambda as a new approach for treatment of allergic asthma? *EMBO Mol Med* 2011;**3**:306–308.~~

~~26~~26. Slater L, Bartlett NW, Haas JJ, Zhu J, Message SD, Walton RP et al. Co-ordinated Role of TLR3, RIG-I and MDA5 in the Innate Response to Rhinovirus in Bronchial Epithelium. *PLoS Pathog* 2010;**6**:e1001178.

27. Hewson CA, Jardine A, Edwards MR, Laza-Stanca V, Johnston SL. Toll-like receptor 3 is induced by and mediates antiviral activity against rhinovirus infection of human bronchial epithelial cells. *J Virol* 2005;**79**:12273–12279.

~~27~~28. Wang Q, Nagarkar DR, Bowman ER, Schneider D, Gosangi B, Lei J et al. Role of double-stranded RNA pattern recognition receptors in rhinovirus-induced airway epithelial cell responses. *J Immunol* 2009;**183**:6989–6997.

~~29~~29. Bartlett NW, Slater L, Glanville N, Haas JJ, Caramori G, Casolari P et al. Defining critical roles for NF-κB p65 and type I interferon in innate immunity to rhinovirus. *EMBO Mol Med* Published Online First: 14 November 2012. doi:10.1002/emmm.201201650

30. Wang Q, Miller DJ, Bowman ER, Nagarkar DR, Schneider D, Zhao Y et al. MDA5 and TLR3 initiate pro-inflammatory signaling pathways leading to rhinovirus-induced airways inflammation and hyperresponsiveness. *PLoS Pathog* 2011;7:e1002070.

31. Wenzel SE. Asthma: defining of the persistent adult phenotypes. *Lancet* 2006;**368**:804–813.

32. Schnurr K, Borchert A, Kuhn H. Inverse regulation of lipid-peroxidizing and hydroperoxyl lipid-reducing enzymes by interleukins 4 and 13. *FASEB J* 1999;**13**:143–154.

33. Lee Y. IL-4-induced Oxidative Stress Upregulates VCAM-1 Gene Expression in Human Endothelial Cells. *Journal of Molecular and Cellular Cardiology* 2001;**33**:83–94.

34. Brinckmann R, Topp MS, Zalán I, Heydeck D, Ludwig P, Kuhn H et al. Regulation of 15-lipoxygenase expression in lung epithelial cells by interleukin-4. *Biochem J* 1996;**318**:305–312.

35. Koarai A, Sugiura H, Yanagisawa S, Ichikawa T, Minakata Y, Matsunaga K et al. Oxidative Stress Enhances Toll-Like Receptor 3 Response to Double-Stranded RNA in Airway Epithelial Cells. *Am J Respir Cell Mol Biol* 2010;**42**:651–660.

3036~~31.3233~~. Mueller T, Terada T, Rosenberg IM, Shibolet O, Podolsky DK. Th2 Cytokines Down-Regulate TLR Expression and Function in Human Intestinal Epithelial Cells. *The Journal of Immunology* 2006;**176**:5805–5814.

~~34~~37. Rochlitzer S, Hoymann H-G, Müller M, Braun A, U-BIOPRED consortium. No exacerbation but impaired anti-viral mechanisms in a rhinovirus-chronic allergic asthma mouse model. *Clin Sci* 2014;**126**:55–65.

38. Golebski K, Luiten S, van Egmond D, de Groot E, Röschmann KIL, Fokkens WJ et al. High Degree of Overlap between Responses to a Virus and to the House Dust Mite Allergen in Airway Epithelial Cells. *PLoS ONE* 2014;**9**:e87768.

39. Parsons KS, Hsu AC, Wark PAB. TLR3 and MDA5 signalling, although not expression, is impaired in asthmatic epithelial cells in response to rhinovirus infection. *Clin Exp Allergy* 2013;**44**:91–101.

~~35~~40. Sykes A, Edwards MR, Macintyre J, Del Rosario A, Gielen V, Haas J et al. TLR3, TLR4 and TLRs7-9 Induced Interferons Are Not Impaired in Airway and Blood Cells in Well Controlled Asthma. *PLoS ONE* 2013;**8**:e65921.

~~36~~41~~37~~. Rantala A, Jaakkola JJK, Jaakkola MS. Respiratory Infections in Adults with Atopic Disease and IgE Antibodies to Common Aeroallergens. *PLoS ONE* 2013;**8**:e68582.

42. Aksoy E, Vanden Berghe W, Detienne S, Amraoui Z, Fitzgerald KA, Haegeman G et al. Inhibition of phosphoinositide 3-kinase enhances TRIF-dependent NF-κB activation and IFN-β synthesis downstream of Toll-like receptor 3 and 4. *Eur J Immunol* 2005;**35**:2200–2209.

Figure 1

The data are now plotted as box and whiskers

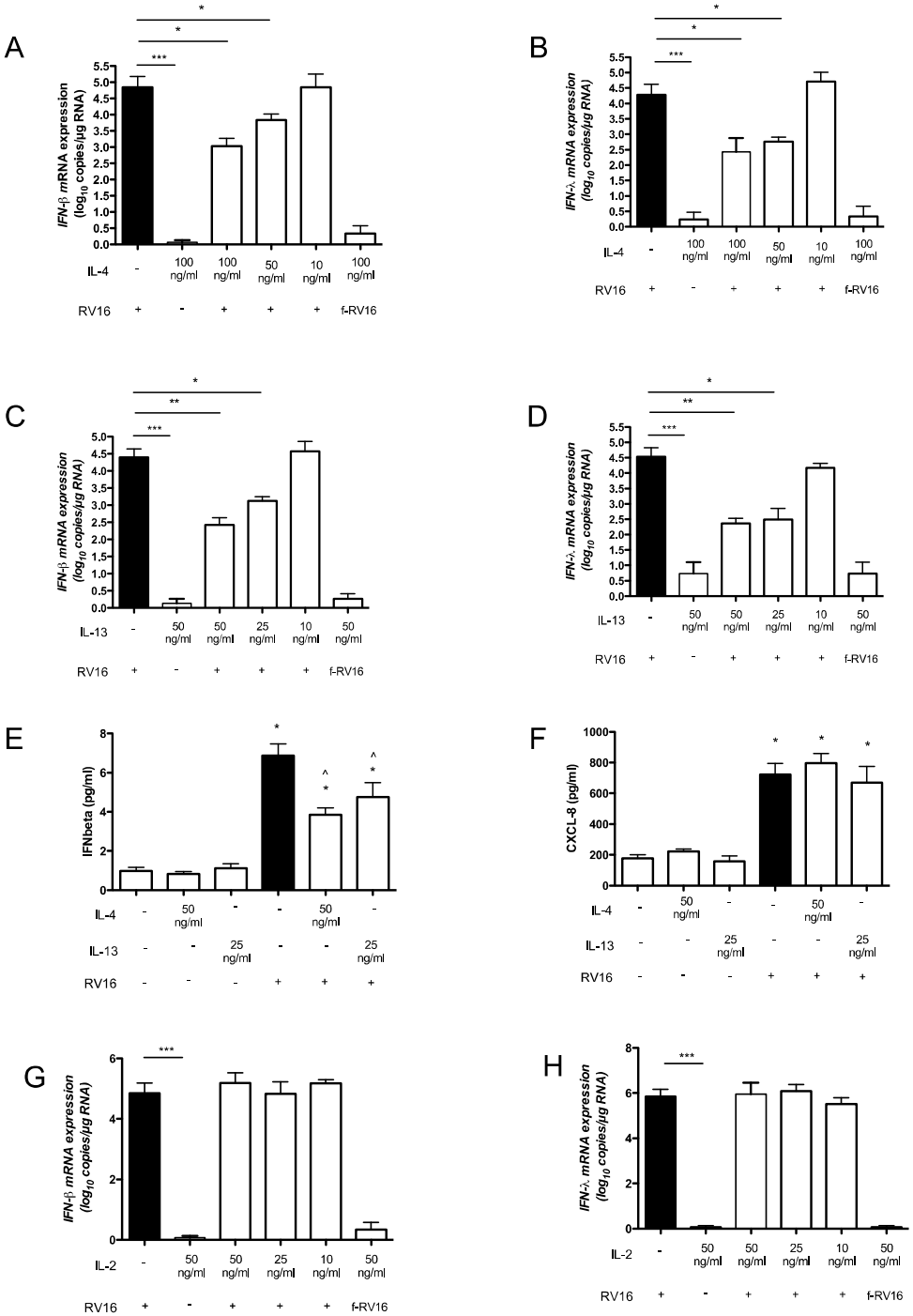


Figure 2

The data are now plotted as box and whiskers

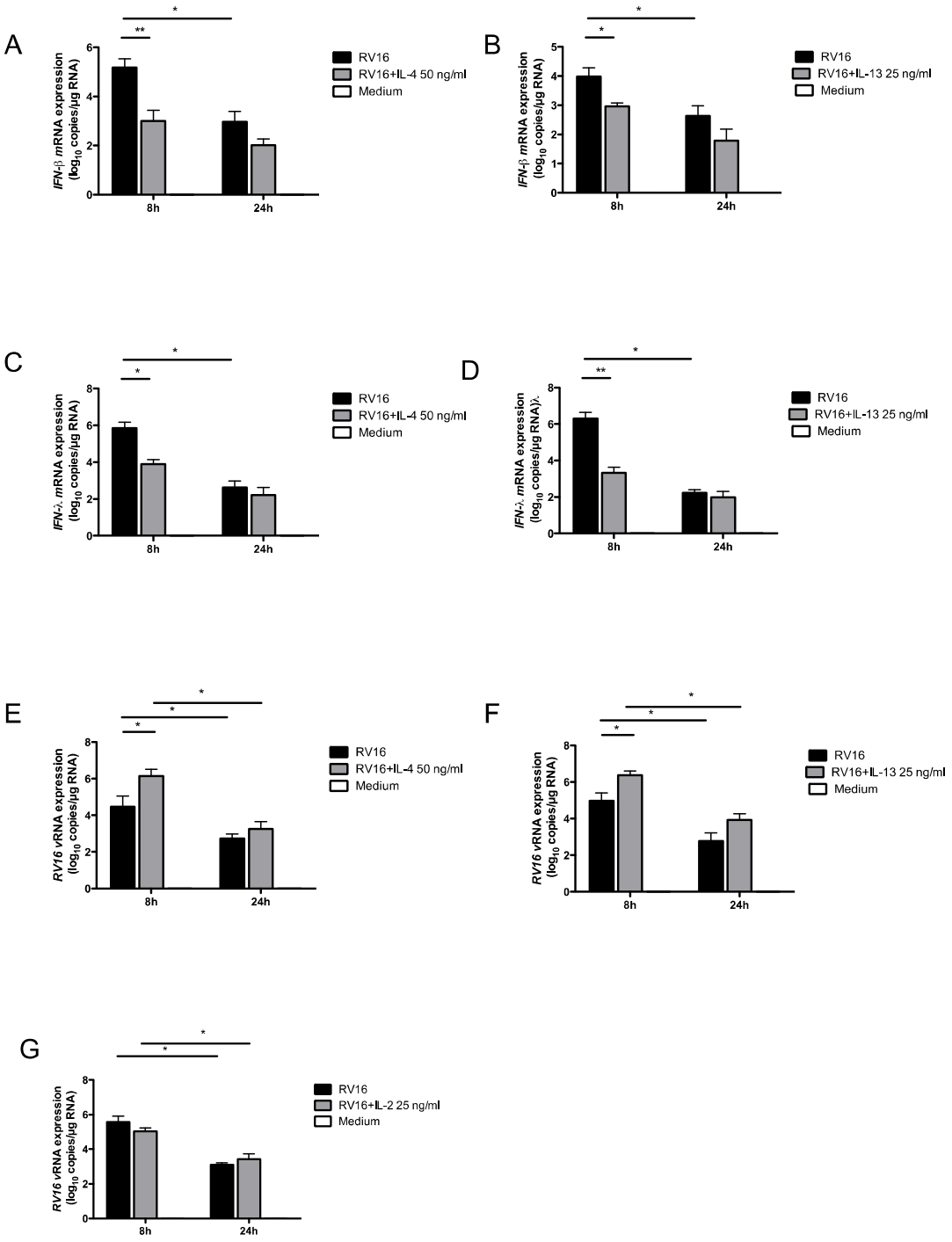
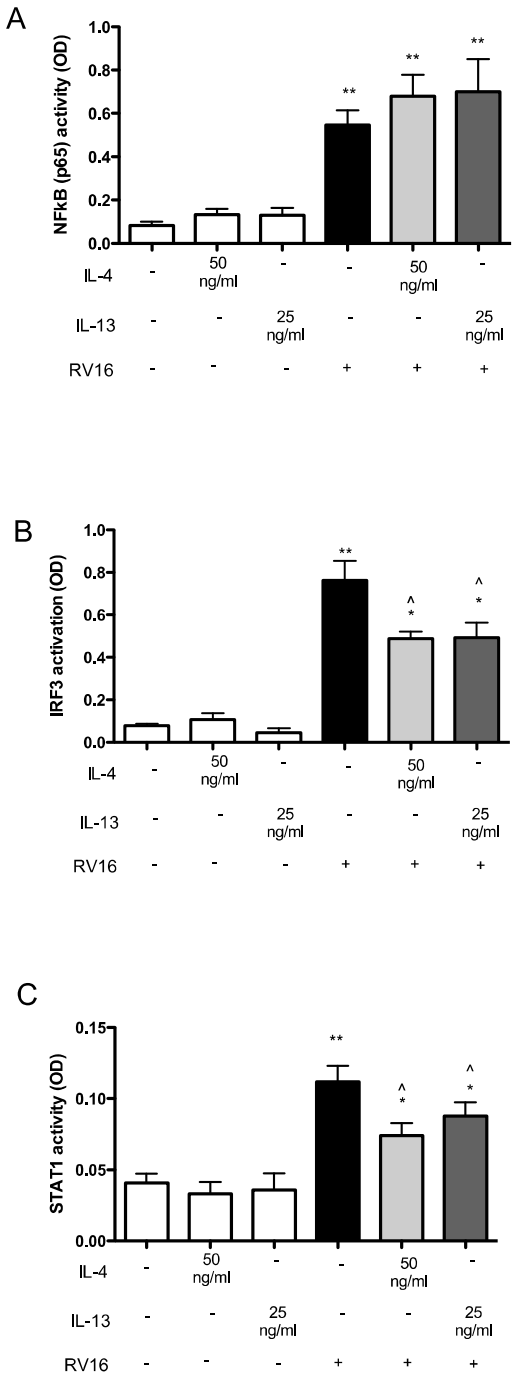


Figure 3 The data are now plotted as box and whiskers

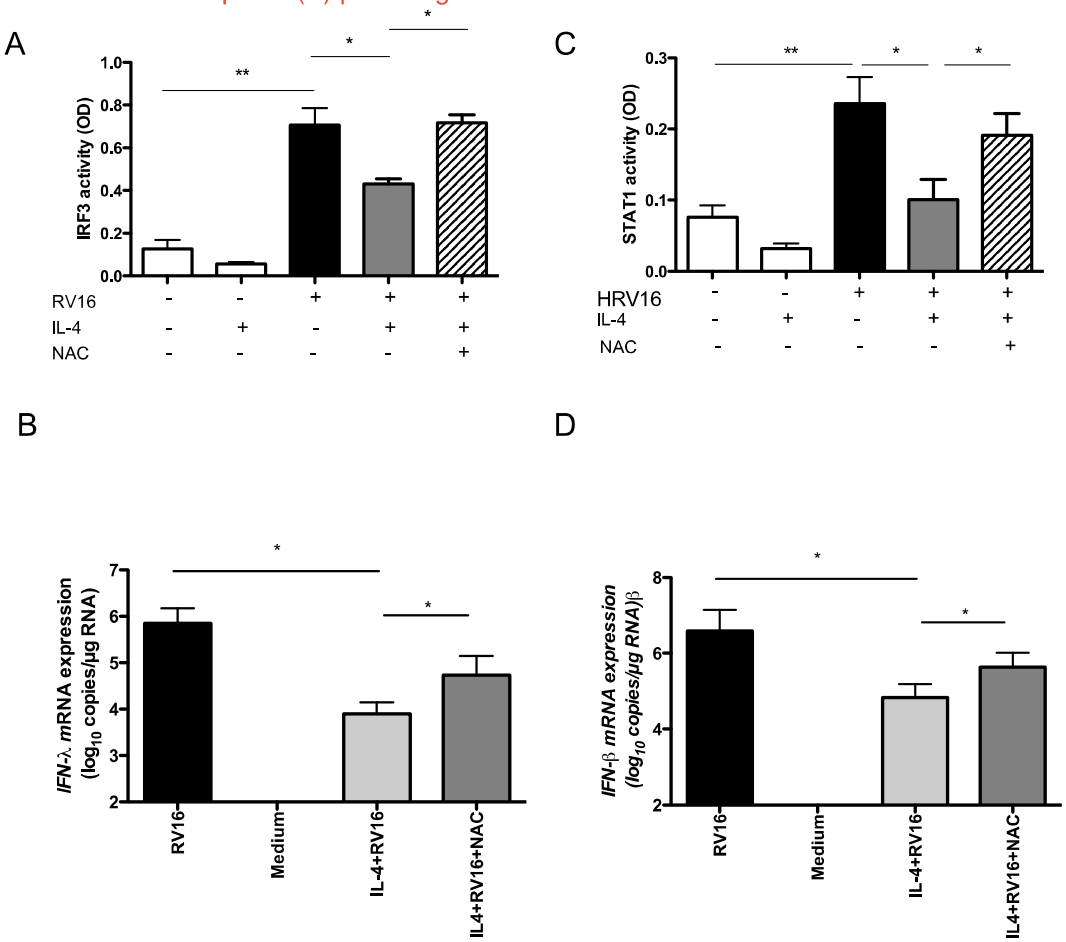


1

2

Figure 4

The data are now plotted as box and whiskers.
A new panel (E) providing new data has been added.

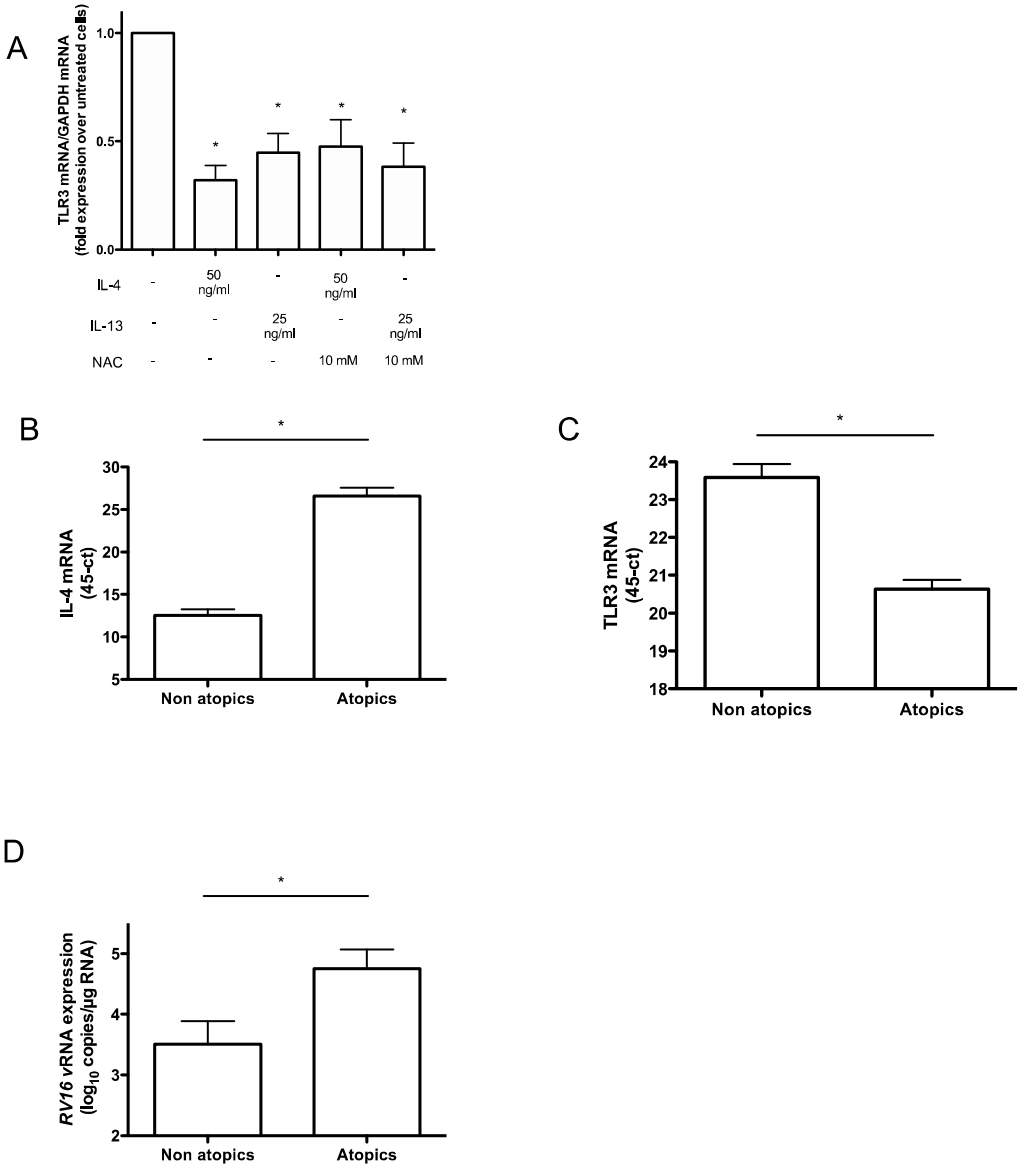


1

2

Figure 5

The data are now plotted as box and whiskers



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

Marco Contoli[1], Kazuhiro Ito[2], Anna Padovani[1], Donatella Poletti[3], Brunilda Marku[1], Michael R. Edwards[4], Luminita A. Stanciu[4], Giulia Gnesini[1], Antonio Pastore[3], Antonio Spanevello[5], Paolo Morelli[6], Sebastian L. Johnston[4], Gaetano Caramori[1], Alberto Papi[1].

[1] Research Centre on Asthma and COPD, Department of Medical Sciences, University of Ferrara, Italy

[2] Airway Disease, National Heath and Lung Institute, Imperial College, London, UK.

[3] ENT Unit, Department of Biomedical and Surgical Sciences, University of Ferrara, Italy

[4] Airway Disease Infection Section, National Heart and Lung Institute, Imperial College and MRC and Asthma UK Centre in Allergic Mechanisms of Asthma, London, UK

[5] University of Insubria, Varese; Fondazione Maugeri, IRCCS, Tradate

[6] Cros NT, Verona, Italy.

Address correspondence to:

Marco Contoli, MD, PhD

Research Centre on Asthma and COPD, Department of Medical Sciences, University of Ferrara, Italy

Via Savonarola 9, 44121 Ferrara, Italy

Tel: +390532236908

Fax: +390532210297

Email: ctm@unife.it

1 Word count (text): 2554

2 Key words: asthma, innate immunity, virus, Th2 inflammation, interferon.

3

4

Abstract (word count: 245)

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-λ1) 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)-kB, 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-kB 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 of 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-λ1) 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-kB) 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 $\log_{10}$ 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 NFkB, IRF3 and STAT1 in 10-µg 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 $<0{\cdot}05$ 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 uM) 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)-kB 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-

kB is required for pro-inflammatory responses, but its role in interferon induction by rhinoviruses is not essential an may be redundant. The fact that Th2 cytokines do not impair NF-kB 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 patent Blair ED, Killington RA, Rowlands DJ,

Clarke NJ, Johnston SL. Transgenic animal models of HRV with human ICAM-1 sequences. UK patent application No. 02 167 29.4, 18 July 2002 and International patent application No. PCT/EP2003/007939, 17 July 2003. licensed, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. Anti-virus therapy for respiratory diseases. UK patent application No. GB 0405634.7, 12 March 2004. licensed, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. Interferon-Beta for Anti-Virus Therapy for Respiratory Diseases. International Patent Application No. PCT/GB05/50031, 12 March 2004. licensed, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. The use of Interferon Lambda for the treatment and prevention of virally-induced exacerbation in asthma and chronic pulmonary obstructive disease. UK patent application No. 0518425.4, 9 September 2005. licensed, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. Anti-Virus Therapy for Respiratory Diseases. US Patent Application – 11/517,763, Patent No.7569216, National Phase of PCT/GB2005/050031, 04 August 2009. licensed, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. Interferon-beta for Anti-Virus Therapy for Respiratory Diseases. European Patent Number 1734987, 5 May 2010. licensed, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. Anti-Virus Therapy for Respiratory Diseases (IFNb therapy) Hong Kong Patent Number 1097181, 31 August 2010. licensed, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. Anti-Virus Therapy for Respiratory Diseases (IFNb therapy). Japanese Patent Number 4807526, 26 August 2011. licensed, a patent Wark PA, Johnston SL, Holgate ST, Davies DE. Interferon-beta for Anti-Virus Therapy for Respiratory Diseases. New Hong Kong - Divisional Patent Application No. 11100187.0, 10 January 2011. Licensed, and a patent Burdin N, Almond J, Lecouturieir, V, Girerd-Chambaz Y, Guy, B, Bartlett N, Walton R, McLean G, Glanville N, Johnston SL. Induction of cross-reactive cellular response against rhinovirus antigens European Patent Number 13305152, 4 April 2013. pending. **Dr. Caramori** reports grants from AstraZeneca Italy, grants and personal fees from Boehringer Ingelheim, grants and other from GlaxoSmithKline Italy, grants from Menarini , grants from Almirall, outside the submitted work. **Dr. Papi** reports grants, personal fees, non-financial support and other from Chiesi, grants, personal fees, non-

financial support and other from Astrazeneca, grants, personal fees, non-financial support and other from GlaxoSmithKline, grants, personal fees, non-financial support and other from Boehringer Ingelheim, grants, personal fees, non-financial support and other from Merck Sharp & Dohme, personal fees and non-financial support from Menarini, personal fees and non-financial support from Novartis, personal fees and non-financial support from Zambon, grants, personal fees, non-financial support and other from Pfizer, grants, personal fees, non-financial support and other from Takeda, grants, personal fees, non-financial support and other from Mundipharma, outside the submitted work.

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)-kB (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 uM) 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.

References

1. Global Initiative for Asthma Global Strategy for Asthma Management and Prevention 2014. *Available from: wwwginasthmaorg*
2. Edwards MR, Bartlett NW, Hussell T, Openshaw P, Johnston SL. The microbiology of asthma. *Nature Reviews Microbiology* 2012;**10**:459–471.
3. Corne J, Marshall C, Smith S, Schreiber J, Sanderson G, Holgate S et al. Frequency, severity, and duration of rhinovirus infections in asthmatic and non-asthmatic individuals: a longitudinal cohort study. *Lancet* 2002;**359**:831–834.
4. Holt PG, Strickland DH. Interactions between innate and adaptive immunity in asthma pathogenesis: new perspectives from studies on acute exacerbations. *J Allergy Clin Immunol* 2010;**125**:963–972.
5. Hansel TT, Johnston SL, Openshaw PJ. ReviewMicrobes and mucosal immune responses in asthma. *The Lancet* 2013;**381**:861–873.
6. Wenzel SE. Asthma phenotypes: the evolution fromclinical to molecular approaches. *Nat Med* 2012;**18**:716–725.
7. Baraldo S, Contoli M, Bazzan E, Turato G, Padovani A, Marku B et al. Deficient antiviral immune responses in childhood: distinct roles of atopy and asthma. *J Allergy Clin Immunol* 2012;**130**:1307–1314.
8. Moriwaki A, Matsumoto K, Matsunaga Y, Fukuyama S, Matsumoto T, Kan-O K et al. IL-13 suppresses double-stranded RNA-induced IFN-λ production in lung cells. *Biochemical and Biophysical Research Communications* 2011;**404**:922–927.
9. Mathur SK, Fichtinger PS, Kelly JT, Lee W-M, Gern JE, Jarjour NN. Interaction between allergy and innate immunity: model for eosinophil regulation of epithelial cell interferon expression. *Ann Allergy Asthma Immunol* 2013;**111**:25–31.
10. Beisswenger C, Kandler K, Hess C, Garn H, Felgentreff K, Wegmann M et al. Allergic airway inflammation inhibits pulmonary antibacterial host defense. *J Immunol*

2006;**177**:1833–1837.

11. Message SD, Laza-Stanca V, Mallia P, Parker HL, Zhu J, Kebadze T et al. Rhinovirus-induced lower respiratory illness is increased in asthma and related to virus load and Th1/2 cytokine and IL-10 production. *Proc Natl Acad Sci USA* 2008;**105**:13562–13567.

12. Papi A, Papadopoulos NG, Stanciu LA, Bellettato CM, Pinamonti S, Degitz K et al. Reducing agents inhibit rhinovirus-induced up-regulation of the rhinovirus receptor intercellular adhesion molecule-1 (ICAM-1) in respiratory epithelial cells. *The FASEB Journal* 2002;**16**:1934–1936.

13. Papi A, Contoli M, Gasparini P, Bristot L, Edwards MR, Chicca M et al. Role of xanthine oxidase activation and reduced glutathione depletion in rhinovirus induction of inflammation in respiratory epithelial cells. *J Biol Chem* 2008;**283**:28595–28606.

14. Kocic G, Sokolovic D, Jevtovic T, Veljkovic A, Kocic R, Nikolic G et al. Hyperglycemia, oxidative and nitrosative stress affect antiviral, inflammatory and apoptotic signaling of cultured thymocytes. *Redox Rep* 2010;**15**:179–184.

15. Di Bona D, Cippitelli M, Fionda C, Cammà C, Licata A, Santoni A et al. Oxidative stress inhibits IFN-alpha-induced antiviral gene expression by blocking the JAK-STAT pathway. *J Hepatol* 2006;**45**:271–279.

16. Zhao W, Qi J, Wang L, Zhang M, Wang P, Gao C. LY294002 inhibits TLR3/4-mediated IFN-b production via inhibition of IRF3 activation with a PI3K-independent mechanism. *FEBS Lett* 2012;**586**:705–710.

17. Lee KS, Lee HK, Hayflick JS, Yong C Lee, Kamal D Puri. Inhibition of phosphoinositide 3-kinase attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. *The FASEB Journal* 2006;**20**:455–465.

18. Cao W, Manicassamy S, Tang H, Kasturi SP, Pirani A, Murthy N et al. Toll-like receptor–mediated induction of type I interferon in plasmacytoid dendritic cells requires the rapamycin-sensitive PI(3)K-mTOR-p70S6K pathway. *Nat Immunol* 2008;**9**:1157–1164.

19. Fukao T, Koyasu S. PI3K and negative regulation of TLR signaling. *Trends in Immunology* 2003;**24**:358–363.

20. Kelly-Welch AE. Interleukin-4 and Interleukin-13 Signaling Connections Maps. *Science* 2003;**300**:1527–1528.

21. Contoli M, Message SD, Laza-Stanca V, Edwards MR, Wark PAB, Bartlett NW et al. Role of deficient type III interferon-lambda production in asthma exacerbations. *Nat Med* 2006;**12**:1023–1026.

22. Wark PAB, Johnston SL, Bucchieri F, Powell R, Puddicombe S, Laza-Stanca V et al. Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. *J Exp Med* 2005;**201**:937–947.

23. Papi A, Johnston S. Rhinovirus infection induces expression of its own receptor intercellular adhesion molecule 1 (ICAM-1) via increased NF-kappa B-mediated transcription. *J Biol Chem* 1999;**274**:9707–9720.

24. Kennedy JL, Turner RB, Braciale T, Heymann PW, Borish L. Pathogenesis of rhinovirus infection. *Current Opinion in Virology* 2012;**2**:287–293.

25. Rauch I, Müller M, Decker T. The regulation of inflammation by interferons and their STATs. *JAKSTAT* 2013;**2**:e23820.

26. Slater L, Bartlett NW, Haas JJ, Zhu J, Message SD, Walton RP et al. Co-ordinated Role of TLR3, RIG-I and MDA5 in the Innate Response to Rhinovirus in Bronchial Epithelium. *PLoS Pathog* 2010;**6**:e1001178.

27. Hewson CA, Jardine A, Edwards MR, Laza-Stanca V, Johnston SL. Toll-like receptor 3 is induced by and mediates antiviral activity against rhinovirus infection of human bronchial epithelial cells. *J Virol* 2005;**79**:12273–12279.

28. Wang Q, Nagarkar DR, Bowman ER, Schneider D, Gosangi B, Lei J et al. Role of double-stranded RNA pattern recognition receptors in rhinovirus-induced airway epithelial cell responses. *J Immunol* 2009;**183**:6989–6997.

29. Bartlett NW, Slater L, Glanville N, Haas JJ, Caramori G, Casolari P et al. Defining critical roles for NF-κB p65 and type I interferon in innate immunity to rhinovirus. *EMBO Mol Med* Published Online First: 14 November 2012. doi:10.1002/emmm.201201650

30. Wang Q, Miller DJ, Bowman ER, Nagarkar DR, Schneider D, Zhao Y et al. MDA5 and TLR3 initiate pro-inflammatory signaling pathways leading to rhinovirus-induced airways inflammation and hyperresponsiveness. *PLoS Pathog* 2011;**7**:e1002070.

31. Wenzel SE. Asthma: defining of the persistent adult phenotypes. *Lancet* 2006;**368**:804–813.

32. Schnurr K, Borchert A, Kuhn H. Inverse regulation of lipid-peroxidizing and hydroperoxyl lipid-reducing enzymes by interleukins 4 and 13. *FASEB J* 1999;**13**:143–154.

33. Lee Y. IL-4-induced Oxidative Stress Upregulates VCAM-1 Gene Expression in Human Endothelial Cells. *Journal of Molecular and Cellular Cardiology* 2001;**33**:83–94.

34. Brinckmann R, Topp MS, Zalán I, Heydeck D, Ludwig P, Kuhn H et al. Regulation of 15-lipoxygenase expression in lung epithelial cells by interleukin-4. *Biochem J* 1996;**318**:305–312.

35. Koarai A, Sugiura H, Yanagisawa S, Ichikawa T, Minakata Y, Matsunaga K et al. Oxidative Stress Enhances Toll-Like Receptor 3 Response to Double-Stranded RNA in Airway Epithelial Cells. *Am J Respir Cell Mol Biol* 2010;**42**:651–660.

36. Mueller T, Terada T, Rosenberg IM, Shibolet O, Podolsky DK. Th2 Cytokines Down-Regulate TLR Expression and Function in Human Intestinal Epithelial Cells. *The Journal of Immunology* 2006;**176**:5805–5814.

37. Rochlitzer S, Hoymann H-G, Müller M, Braun A, U-BIOPRED consortium. No exacerbation but impaired anti-viral mechanisms in a rhinovirus-chronic allergic asthma mouse model. *Clin Sci* 2014;**126**:55–65.

38. Golebski K, Luiten S, van Egmond D, de Groot E, Röschmann KIL, Fokkens WJ et al. High Degree of Overlap between Responses to a Virus and to the House Dust Mite Allergen in Airway Epithelial Cells. *PLoS ONE* 2014;**9**:e87768.

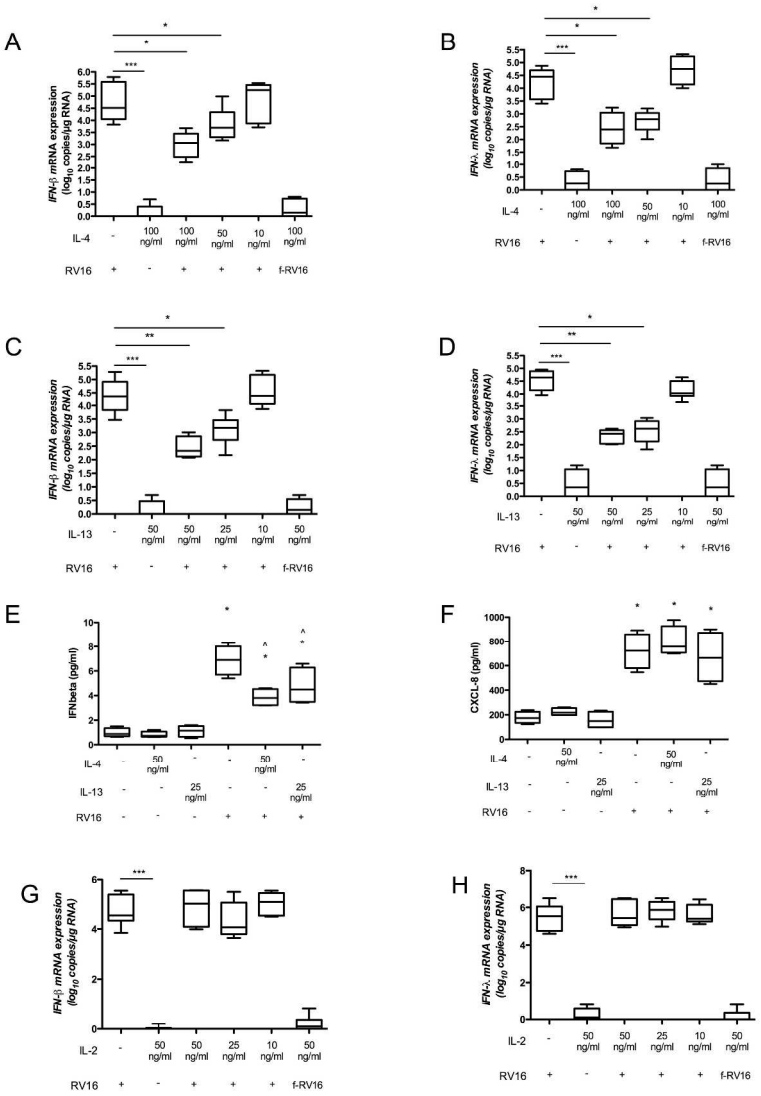
39. Parsons KS, Hsu AC, Wark PAB. TLR3 and MDA5 signalling, although not expression, is impaired in asthmatic epithelial cells in response to rhinovirus infection. *Clin Exp Allergy* 2013;**44**:91–101.

40. Sykes A, Edwards MR, Macintyre J, Del Rosario A, Gielen V, Haas J et al. TLR3, TLR4 and TLRs7-9 Induced Interferons Are Not Impaired in Airway and Blood Cells in Well Controlled Asthma. *PLoS ONE* 2013;**8**:e65921.

41. Rantala A, Jaakkola JJK, Jaakkola MS. Respiratory Infections in Adults with Atopic Disease and IgE Antibodies to Common Aeroallergens. *PLoS ONE* 2013;**8**:e68582.

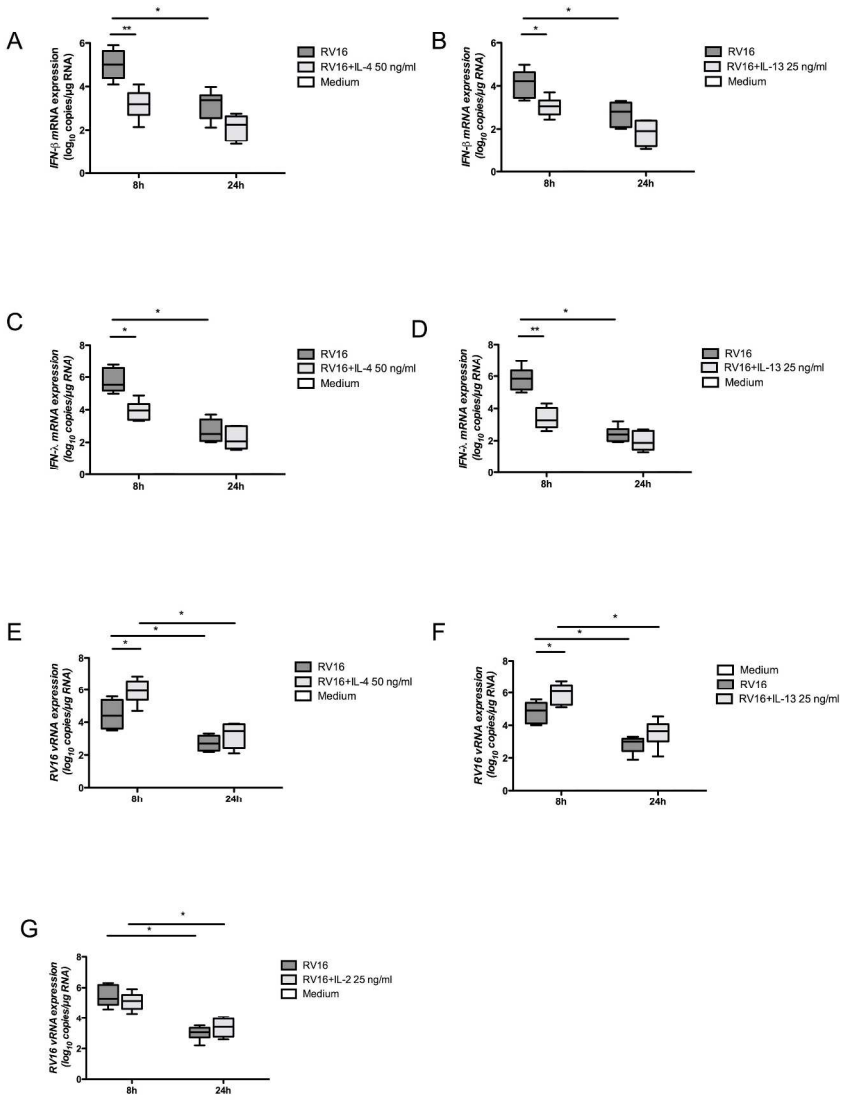
42. Aksoy E, Vanden Berghe W, Detienne S, Amraoui Z, Fitzgerald KA, Haegeman G et al. Inhibition of phosphoinositide 3-kinase enhances TRIF-dependent NF-κB activation and IFN-β synthesis downstream of Toll-like receptor 3 and 4. *Eur J Immunol* 2005;**35**:2200–2209.

Figure 1



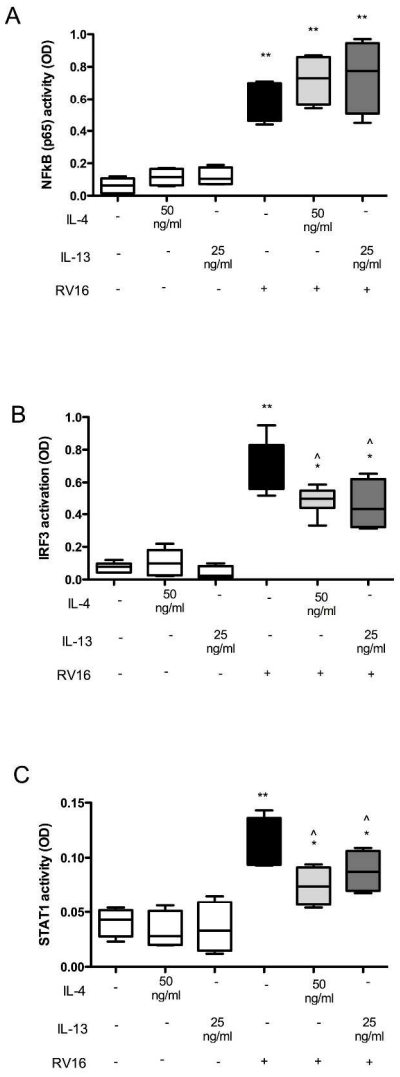
241x355mm (300 x 300 DPI)

Figure 2



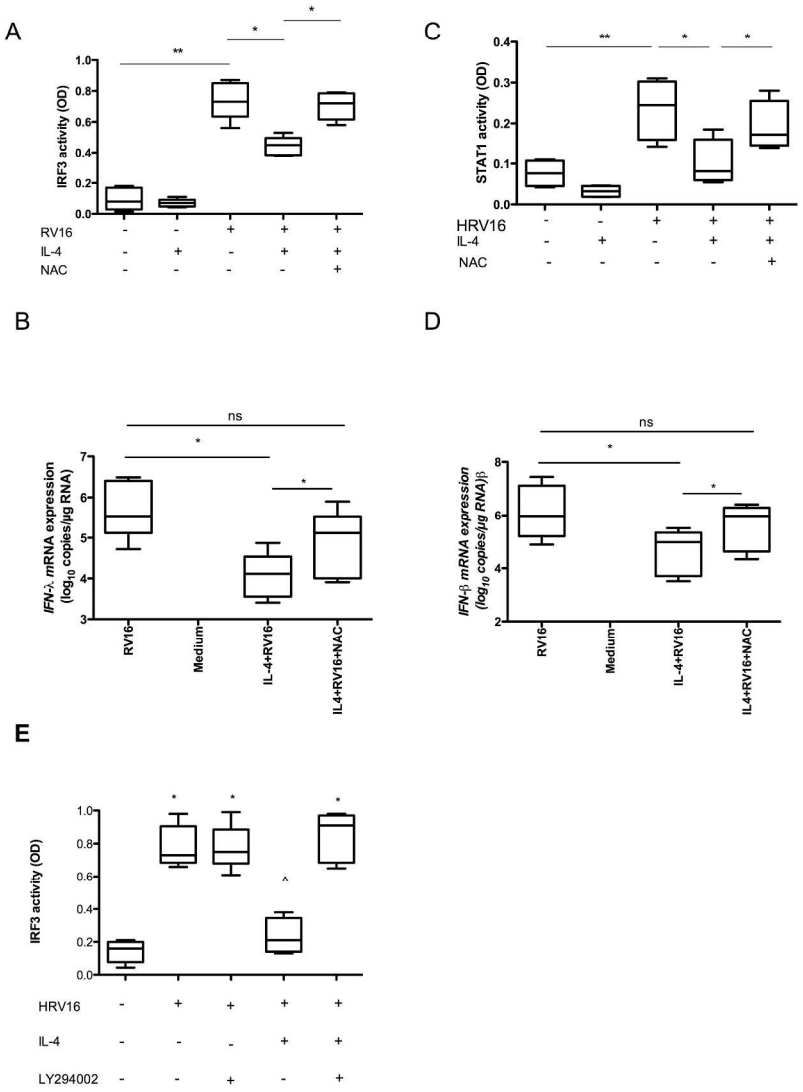
247x330mm (300 x 300 DPI)

Figure 3



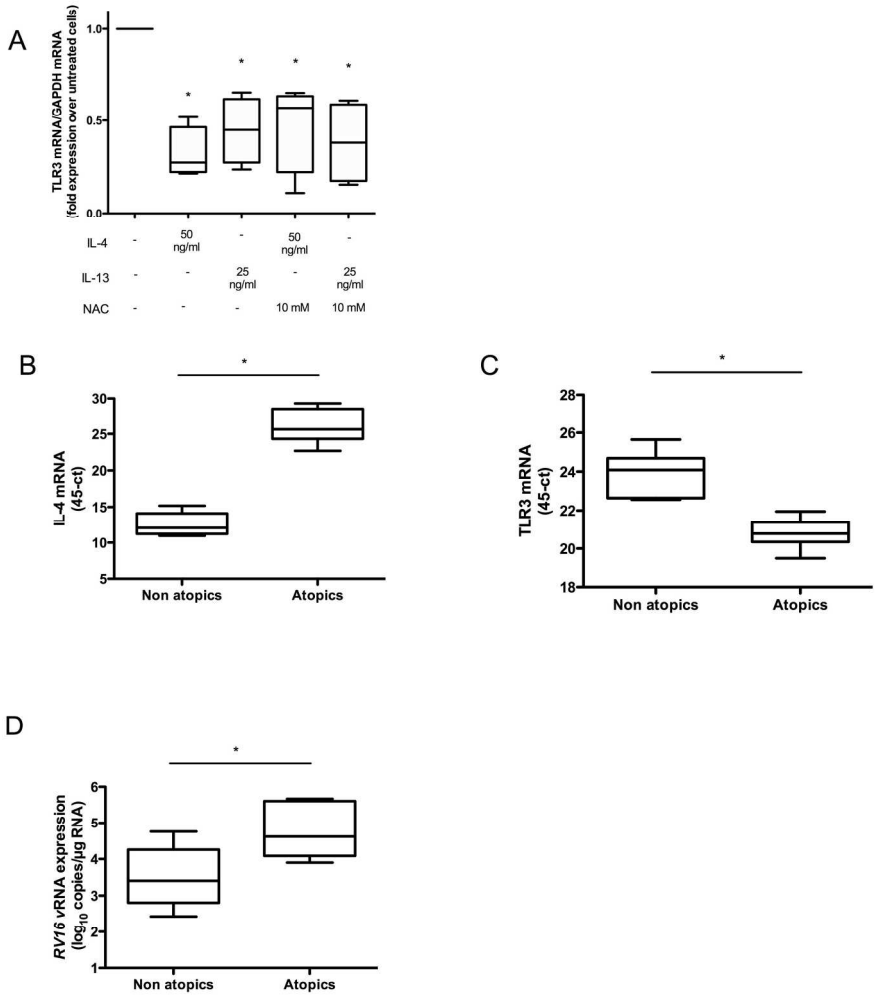
253x658mm (300 x 300 DPI)

Figure 4



244x328mm (300 x 300 DPI)

Figure 5



203x226mm (300 x 300 DPI)