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MECCANISMI INFIAMMATORI ED IMMUNOLOGICI DELLE RIACUTIZZAZIONI DI ASMA E BRONCOPNEUMOPATIA CRONICA OSTRUTTIVA (BPCO) INDOTTE DA INFEZIONE VIRALE

Inflammatory and immunological mechanisms of virus induced asthma and chronic obstructive pulmonary disease (COPD) exacerbations

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INTRODUCTION

Asthma and chronic obstructive pulmonary disease (COPD) are the two most prevalent chronic airway diseases. Much of the morbidity, mortality and health care costs of the diseases are associated with exacerbations. Exacerbations are acute episodes that punctuated the natural history of the disease characterized by increased symptoms and airflow obstruction determining quality of life impairment and with possible effect on long term history of the disease. Thus, the prevention and the treatment of exacerbations are major tasks in the management of both asthma and COPD. However currently available treatment are only partially effective in reducing the incidence of such events.

Over the last decade evidence has emerged implicating virus respiratory tract infections as a major cause of exacerbations both in asthma and in COPD. In particular, rhinovirus (i.e. the etiologic cause of the common cold) is the most frequently identify virus during exacerbations of both asthma and COPD.

The comprehension of the inflammatory and immunological mechanisms that pave the way for exacerbation following respiratory tract infection in asthmatic and COPD patients will give the opportunity to highlight novel potential targets for pharmacological intervention. The recent development of *in vivo* experimental models of rhinovirus induced asthma and COPD exacerbations represents the unique tool to evaluate pathogenetic mechanisms and novel pharmacological pathways for treating and preventing COPD and asthma exacerbations.

The aim of the present thesis is to review the data available on inflammatory and immunological mechanisms of virus induced exacerbations of asthma and COPD and to present the results of the studies conducted by the Candidate to enlighten the emerging field of virus induced asthma and COPD exacerbations.

ROLE OF VIRUS INFECTION IN ASTHMA EXACERBATIONS

Asthma and exacerbations: definition

Asthma is a chronic inflammatory disease of the airways characterized by recurrent acute or subacute episodes of progressively worsening shortness of breath, cough, wheezing, and chest tightness or some combination of these symptoms (1). These events are named exacerbations. In addition exacerbations are characterized by worsening in lung function below the patient's usual values. Acute exacerbations are one of the most important (if not the most important) events in the natural history of asthma. They result in excess medication use, emergency department visits, hospitalisation, and even death. Additionally, asthma exacerbations reduce quality of life and lead to time off work and school with the associated emotional and financial stress this entails. From a societal perspective, exacerbations are the main driver of asthma-related costs, accounting for almost 50% of total costs (2).

Epidemiology of asthma exacerbation

In line with the increasing prevalence of asthma there has been an increasing burden of disease associated with acute exacerbations. Much of the morbidity and virtually all of the deaths associated with asthma are due to acute exacerbations and even among patients receiving optimal asthma therapy 20% will experience a severe exacerbation (3). In the USA there are an estimated 2 million visits to Emergency Departments and 500,000 hospital admissions per year due to asthma (1). In the UK over 17 million working days are lost every year due to asthma, with the total annual cost to the UK economy estimated at over £1 billion. The annual average treatment costs per patient are > 3.5 times higher for asthmatics who experience an exacerbation (4).

For many years general opinion has been that respiratory virus infections were the cause of the common cold, and were able to produce serious pulmonary complications only in infants, the elderly and immunocompromised subjects. It is now widely recognised that respiratory viral infections of the airways are also the most important causes of asthma exacerbations both in children and adults (5). The health costs of asthma exacerbations are enormous in terms of days lost from work or school, general practitioner consultations, hospital admissions and mortality. Despite optimised therapy for stable asthma with inhaled glucocorticoids and long-acting β_2 agonists asthma exacerbations continue to occur suggesting that the pathogenesis of asthma exacerbations and of stable asthma are likely to be different (6). The cellular and molecular mechanisms underlying these events have been studied and partially elucidated in the last few years but more studies are needed to fully understand the mechanisms of asthma exacerbations so that new more effective therapies can be developed.

Role of respiratory viruses infection in asthma exacerbations: clinical evidence

i) Prevalence of respiratory viruses infection in asthma exacerbations.

Initial studies designed to establish the relationship between virus infection and asthma exacerbations utilised virus isolation in cell culture from upper airway samples or detection of increasing titres of virus-specific serum antibodies to recognise respiratory virus infections. Since the techniques were relatively insensitive the frequency of viral detection was low. The development of molecular virology techniques, in particular of reverse transcription-polymerase chain reaction (RT-PCR) assays for the identification of respiratory viruses in biological samples, has provided a more sensitive tool to evaluate the role of respiratory viruses in the natural history of asthma. Clinical studies performed using RT-PCR suggest that the proportion of virus-induced asthma exacerbations is likely be around 80 to 85% in school-aged children (7) and 60-75% in

adults of the total number of exacerbations, rhinovirus being the virus type most frequently identified (8).

ii) Severity of virus-induced asthma exacerbations.

Respiratory viral infections not only contribute substantially to asthma morbidity in mild-moderate asthma, but they also appear to be associated with more severe exacerbations. In fact, upper respiratory viral infections are strongly associated in time with hospital admissions for asthma both in adults and in children (9-11). A recent study showed that asthma exacerbations in which a virus was detected were associated with a more compromised lower forced expiratory volume in one second (FEV_1), higher hospital admission rate and longer hospital stay. In this study, sputum lactate dehydrogenase (LDH) level, a marker of cell lysis and virus-induced bronchial epithelium damage, was significantly higher in the patients with viral infection and was the major predictor of length of hospital stay suggesting evidence for causation and not just association of viral infection in severe asthma exacerbations (8). These data also suggest that the degree of virus-induced epithelial damage to the lower airways is the major determinant of the severity of the exacerbation.

Winter peaks in asthma mortality have been observed in adults over 45 years of age, suggesting that respiratory virus infections, which are more frequent in winter, may also precipitate asthma deaths in these age groups (12-14).

iii) Synergism between viral infection and atopy

There are both clinical and experimental evidence indicating that respiratory viruses can act synergistically with other factors such as allergic sensitisation to exacerbate asthma.

The risk of wheezing in children is increased by the concomitant presence of virus infection and exposure to an allergen the child is sensitised to. Indeed, a large majority of wheezing episodes during childhood can be linked to infection with rhinovirus, and they are most likely to occur in those who have rhinovirus infection together with evidence of atopy or sputum eosinophilia (15). In a study of adult asthmatics with asthma exacerbations, the risk of hospital admission was strongly associated with the combination of allergic sensitisation, exposure to allergens the patient was sensitised to and concurrent viral infection (16).

Also human experimental infection indicates a synergistic effect between virus infection and allergen exposure. Indeed, several studies have demonstrated that rhinovirus enhances allergen-induced inflammatory responses in the lower airways, including histamine release, eosinophil recruitment and the induction of late-phase airway response (17, 18).

In rhinitic patients, experimental rhinovirus infection performed after nasal allergen challenge increase significantly the levels of both eosinophil peroxidase (EPO) and myeloperoxidase (MPO), in nasal lavage fluid suggesting a synergistic effect between allergen and rhinovirus in the activation of eosinophils and neutrophil granulocytes (19). The same authors in a previous study, showed that in patients with seasonal allergic rhinitis, experimental infection performed 4 days before allergen nasal challenge caused a significant increase of interleukin (IL)-8, eosinophil cationic protein (ECP) and α2-macroglobulin levels in the nasal lavage fluid (20). Finally, atopic adult with mild asthma and elevated levels of total serum immunoglobulin E (IgE) show greater lower respiratory tract symptoms during experimental rhinovirus infection compared with asthmatic patients with lower levels of IgE (21).

These synergistic effects have also been described in animal models. After respiratory syncytial virus (RSV) infection in mice sensitised to a specific allergen there is an increase in

airway hyperresponsiveness and of the number of Th2 lymphocytes in the animals previously exposed to the allergen compared with infected mice but not allergen challenged (22).

However, not all the clinical studies have reached the same conclusions: for instance a study performed on adult atopic asthmatic failed to show any additive or synergistic effect between allergen challenge and virus infection (23) and one study has even shown a protective effect of allergen exposure on virus infection (24).

Several causes could be responsible for the differences observed in different clinical and experimental settings. One possible explanation relates to the anti-inflammatory and antiviral immune response that could be evoked in some conditions by chronic allergen exposure. Indeed, chronic allergen exposure could induce the production in the airways of anti-inflammatory cytokines, such as IL-10, and/or antiviral mediators, such as interferon-γ (IFN)-γ, with consequent down regulation of airway inflammation and/or of the viral infectivity. Another possible mechanism is associated to the increase in the levels of exhaled nitric oxide (NO) occurring during asthmatic exacerbations (25). As discussed in details below, NO may play an important role in host defences against viruses. Also, differences in the timing and doses of allergens involved in different clinical/experimental setting could account for the differences observed in the outcomes of these studies.

Further studies are required to elucidate the complexity of the interactions between allergen exposure and respiratory virus infection on the modulation of airway inflammation during asthmatic exacerbations (26).

Role of respiratory viruses infection in asthma exacerbations: experimental models

Much of our current knowledge regarding virus-asthma interactions comes from experimental models. Obtaining samples from the lower airways for studying the mechanisms of asthma exacerbations is difficult for both logistical and ethical reasons. Experimental rhinovirus infection in human volunteers has long been used to study the pathogenesis of the common cold and this technique has also been used in subjects with mild asthma. The results of these studies will be reviewed here (Figure 1).

i) Experimental infection: clinical and functional consequences

Experimental rhinovirus infection of asthmatic patients can cause both cold and asthma symptoms (21, 27, 28). Several studies suggest that experimental rhinovirus infection can increase airway responsiveness. Increased airway responsiveness usually begins early after RV viral infection both in asthmatic and in nonasthmatic atopic subjects (29, 30). Rhinovirus-induced increases in airway responsiveness are greater in subjects with lower baseline pulmonary function (31) and in asthmatics with more severe cold symptoms (32). By detailed monitoring it has been possible to detect reductions in both peak expiratory flow (PEF) (32) and FEV_1 in a fraction of atopic asthmatic patients in the acute phase of experimental rhinovirus infection (28). However, it should be underlined that not every asthmatic patient experimentally infected with rhinovirus develop cold and/or asthma symptoms and/or a significant decrease of their PEF/FEV_1 (33). The reasons for these differences are unknown and may relate to amount of rhinovirus inoculated, experimental conditions and intersubject variability in the immune/inflammatory response to rhinovirus.

iii) Cellular and molecular mechanisms of respiratory virus infections and asthma exacerbations

Despite mounting clinical evidence for a major role of respiratory viral infections in the pathogenesis of asthma exacerbations, the mechanisms of respiratory virus-induced airway inflammation during asthma exacerbations are relatively uncertain.

a) Pro-inflammatory and anti-inflammatory mediators released from epithelial cells

Respiratory viruses enter and replicate within epithelial cells lining the lower airways. The extent of epithelial cell destruction that they can cause varies according to the type of virus. Influenza virus typically causes extensive epithelial necrosis whereas rhinovirus causes little or only patchy epithelial damage (34). Death/damage of epithelial cells results in both an increase in epithelial permeability and increased penetration of irritants and allergens and exposure of the extensive network of sensory nerve fibres. These effects may contribute to the increased airway hyperresponsiveness induced by respiratory virus infection.

There is increasing evidence that the epithelium of the lower airway does not simply act as a physical barrier but has important regulatory roles on the immune/inflammatory response. Bronchial epithelial cells may act as antigen-presenting cells particularly during memory responses to respiratory viral infections. They express major histocompatibility complex (MHC) I and the costimulatory molecules CD80 (B7.1) and CD86 (B7.2), whose expression is upregulated *in vitro* by RV (35).

Bronchial epithelial cells also contribute to the inflammatory response that follows virus infection through the production of cytokines and chemokines. *In vitro* infection of these cells with rhinovirus induces the secretion of IL-1β, IL-6, IL-8, IL-11, tumour necrosis factor alpha (TNF-α), CCL-5 (RANTES), CCL11 (eotaxin-1), CCL24 (eotaxin-2), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (36).

These cytokines have profound effects on inflammatory cells involved in asthma exacerbations.

The function of IL-8 as a chemotactic factor for neutrophils and primed eosinophils indicates that it may play an important role in triggering the inflammation that leads to exacerbations of asthma. IL-8 has been detected in increased amounts in both nasal and sputum samples obtained during virus infections, and its levels correlated with induction of airway hyperresponsiveness (37) and neutrophilic bronchial inflammation (38).

Likewise, the pro-inflammatory cytokine IL-6 is induced by rhinovirus infection (39) and IL-6 is increased in the sputum of rhinovirus infected asthmatic subjects (40). Another cytokine which may be important in the pathogenesis of virus-induced asthma exacerbations is IL-11. IL-11 is secreted in very large amount in *vitro* and may directly increase airway responsiveness (41). Several respiratory viruses including RSV, parainfluenza viruses and rhinovirus strongly stimulate its production by lung fibroblasts. IL-11 is increased in nasal aspirates from children with upper respiratory infection were its levels are correlated with clinically detectable wheezing (42). Finally, also the eosinophil chemoattractant chemokines CCL-5 and CCL-3 are increased during upper respiratory viral infections associated to asthma exacerbations (43).

b) Neurogenic inflammation

Another mechanisms that can contribute to the development of asthma symptoms, airflow obstruction and airway hyperresponsiveness during acute respiratory viral illness are represented by the effects of virus infection on the neural networks of the airways.

Both animal and *in vitro* studies suggest that viral infections could stimulate sensory nerves, reflex parasympathetic bronchoconstriction and/or interfering with the function of nonadrenergic, noncholinergic (NANC) nerves, which produce bronchodilator mediators such as nitric oxide (NO), vasoactive intestinal peptide (VIP) and bronchoconstrictor mediators such as tachykinins (substance P and neurokinins) (44).

Virus-mediated damage to the epithelial layer can expose the dense subepithelial network of unmyelinated afferent sensory fibers, which may increase stimulation of sensory nerves by inhaled particles or pro-inflammatory mediators. Sensory nerves can directly release neuropeptides, such a tachykinins, that induce contraction of smooth muscle cells, or can trigger reflex bronchoconstriction by activating parasympathetic nerves. This reflex causes release of acetylcholine (ACh) and contraction of airway smooth muscle cells *via* stimulation of muscarinic M3 receptors. Normally, ACh inhibits its release trough the activation of muscarinic M2 receptors on the presynapse of the postganglionic parasympathetic (cholinergic) nerves (auto-inhibitory feedback). In animal models, respiratory viruses, directly and through the release of inflammatory mediators, such as major basic protein (MBP), can decrease the expression and/or the function of M2 ACh receptors and block this auto-inhibitory feedback, thus enhancing ACh release from cholinergic nerves and potentiating reflex parasympathetic bronchoconstriction (44).

To date, little information is available on the role in humans of neurogenic inflammation in the pathogenesis of virus-induced asthma exacerbations. Clinical studies using the dual tachykinin NK1/NK2 antagonist DNK333 have shown that this compound inhibits neurokinin A-induced bronchoconstriction in patients with stable asthma (45). Clinical trial testing the efficacy of this drug in asthma exacerbations are expected.

c) Adhesion molecules

Adhesion molecules are glycoproteins involved in inflammatory cell recruitment at sites of inflammation. One such molecule is ICAM-1 (CD54) that is expressed on the surface of epithelial and endothelial cells and is the natural ligand of the β2 integrin CD11a [lymphocyte function antigen 1 (LFA-1)] that is expressed on all leucocytes (46). ICAM-1 may play a

particular role as a pathogenetic link between virus infections and asthma exacerbations. ICAM-1 is the major receptor for rhinovirus binding to human cells. Approximately 90% of rhinovirus serotypes bind to and enter cells using cell surface ICAM-1. Rhinovirus infection of airway epithelial cells upregulates expression of ICAM-1 both *in vivo* and *in vitro* (47). Through this mechanism rhinoviruses are able to increase the expression of its own receptor on the epithelial surface, and this event can potentate viral attachment and entry in the host cell.

ICAM-1 has been proposed as a marker of persistent inflammation in allergic diseases (48). Indeed, in patients with atopic diseases (such as allergic conjunctivitis, allergic rhinitis and/or asthma) after spontaneous or experimental exposure to allergen there is an increased expression of ICAM-1 on the surface of the conjunctival and/or airway epithelial cells (49) that can promote the recruitment and the infiltration of eosinophils, neutrophils and lymphocytes at the sites of allergic inflammation. Th2 cytokines, such as IL-4, IL-5 and IL-13 induce *in vitro* increased ICAM-1 expression in bronchial epithelial cells (50). Bianco et al showed that IFN-γ in combination with each Th2-associated cytokine only slightly reduces, but does not override, the Th2-induced level of ICAM-1 expression on both uninfected and rhinovirus-infected airway epithelial cells (51). These data suggest that the effects of Th2-associated cytokines on ICAM-1 expression and on the recovery of rhinovirus are dominant over the effects of the Th1-associated cytokines such as IFN-γ. Since the airway mucosa in atopic asthma is predominantly infiltrated by Th2 lymphocytes, these results could partially explain both the increased susceptibility to human rhinovirus infection in asthmatic patients (52) and the associated exacerbation of asthma. Therefore allergic inflammation, by enhancing the ICAM-1 expression on airway epithelial cells may facilitate rhinovirus infection in atopic subjects. Conversely, rhinovirus infection by increasing the expression of its own receptor may potentially facilitate both epithelial infections

and leukocyte infiltration, which could contribute to the inflammatory cascade that leads to exacerbations.

A similar pathogenetic role could be referred to another adhesion molecule, vascular adhesion molecule-1 (VCAM-1; CD106). At variance with ICAM-1, VCAM-1 selectively recruits, among granulocytes, only eosinophils, since neutrophils do not possess the ligand for VCAM-1. VCAM-1 is present on the surface of the activated endothelial and epithelial cells and its expression is increased in airway mucosa by RV infection and allergen (53). This event could favour the recruitment of eosinophils into the airway mucosa and contribute to the development of the bronchial eosinophilia observed after experimental RV infection in human volunteers (27, 47).

d) Nitric oxide

Exhaled nitric oxide (NO) is increased during asthma exacerbations both in children and adults. Increased levels of exhaled NO are found in nonasthmatic volunteers following natural colds (54) as well as in asthmatic patients after experimental rhinovirus infection (55). In the latter study an inverse association between the NO increase and worsening of airway hyperresponsiveness was demonstrated arguing in favour of a protective role for this molecule. Increased NO production by infected epithelial cells of the lower airway may play an important role in lung defences against rhinoviruses (55-58).

Indeed, *in vitro* studies of rhinovirus-infected human bronchial epithelial cells have shown that NO can inhibit virus replication as well as rhinovirus-induced production of the pro-inflammatory cytokines IL-6, IL-8, GM-CSF (58).

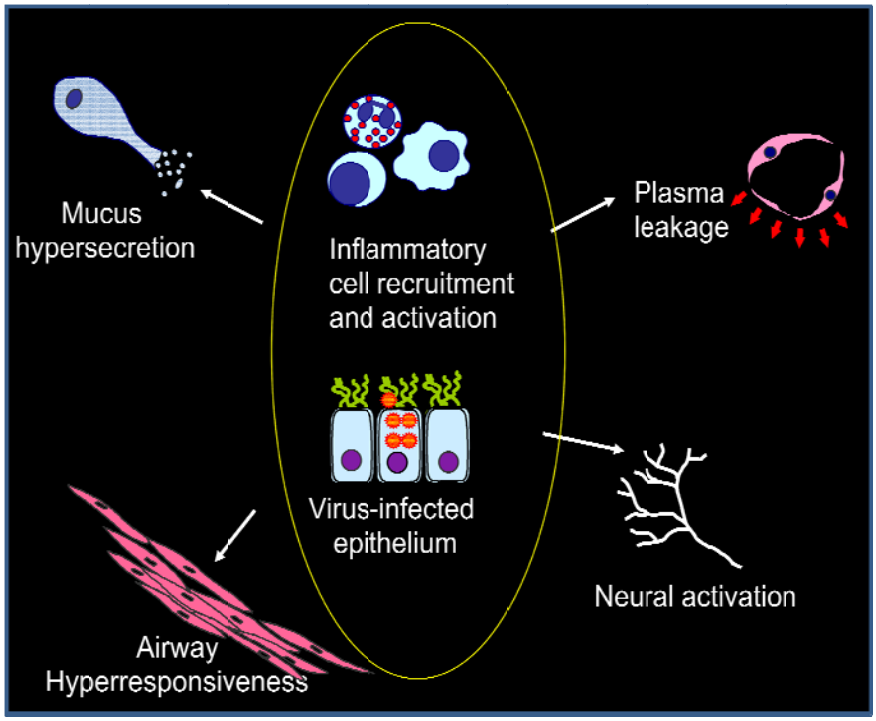

Figure 1: in vitro and in vivo experimental models in bronchial epithelial cells document that rhinovirus can induce several features of asthma exacerbation including induction of pro-inflammatory mediators, mucus secretion, plasma leakage, airway hyperresponsiveness and neural activation.

Infection in bronchial epithelial cells: pro-inflammatory intracellular signalling pathways

To date the specific intracellular signalling pathways involved in the production of pro-inflammatory mediators by bronchial epithelial cells during respiratory virus infections are not fully characterised.

Transcription factors bind to DNA-regulatory sequences (enhancers and silencers) and regulate the expression of many genes, including pro-inflammatory genes (59). Several studies have shown that the activation of pro-inflammatory gene transcription by transcription factors that regulate inflammatory mediator production is profoundly affected by respiratory virus infection.

The effects of rhinovirus infection on transcription factor activation have been particularly studied in the last few years. Activation of intracellular signalling pathways induced by rhinovirus in epithelial cells may be dependent on surface receptor binding (ICAM-1) or by intracellular products during viral replication such as double-stranded RNA (dsRNA). It has been shown that dsRNA can activate components of several signalling pathways including protein kinase R (PKR), NF-κB and p38 mitogen-activated protein kinase (MAPK). It is reasonable to hypothesise that this occurs through the binding of TLR3, a member of the toll-like receptors (TLR) that recognize molecular patters associated with microbial pathogens and induce antimicrobial immune response (60). However, to date no studies have investigated the role of these molecules in rhinovirus infection.

Virus induced activation of NF-κB leads to an increased expression of cytokines [IL-1, IL-6, granulocyte colony-stimulating factor (G-CSF), and GM-CSF], CXC chemokines (IL-8) and ICAM-1 (47, 61) whereas expression of VCAM-1 seems mediated by activation of NF-κB and GATA proteins (53).

It has been recently suggested that early activation of p38 MAPK pathway by rhinovirus infection, which induces the activation of many transcription factors, could a key event in the regulation of rhinovirus-induced cytokine transcription, and may provide a new target for inhibition of rhinovirus-induced asthma exacerbations (62).

While a cooperative interaction between NF-kB and AP1 occurs in RSV induced IL-8 production (63), an oxidant mediated activation of interferon regulatory factor (IRF) is required for RSV induced RANTES production (64).

Oxidants could represent a key intracellular mediator of virus-induced cellular activation. Interestingly, oxidative stress can induce activation of nuclear factor κB (NF-κB) and activator protein-1 (AP-1), two pivotal regulators of the inflammatory processes (65). Rhinovirus infection

induces increased production of superoxide anion in bronchial epithelial cells and this event is a crucial step for the activation of NF-κB and the following production of pro-inflammatory cytokines, chemokines and adhesion molecules (66). Reducing agents inhibit both rhinovirus-induced oxidant generation and inflammatory mediator production and release (66). These data suggest that the inhibition of oxidative stress may be a potential therapeutic target for treatment of virus-induced asthma exacerbations.

Antiviral immunity in asthmatic patients

Recent studies indicate that individuals with asthma are more susceptible to naturally occurring rhinovirus infection than normal individuals in that lower respiratory tract symptoms and changes in peak expiratory flow were both more severe and of longer duration in the asthmatic subjects than in the normal subjects (52). However, the reasons for this increased susceptibility in individuals with asthma have to date been largely unknown, but an emerging hypothesis supported by *in vitro* and *in vivo* experimental viral models is that asthmatic patients have a deficient innate and acquired antiviral defence against respiratory virus infections.

i) Decreased IFN-ß-production and apoptosis in asthmatic epithelial cells

A novel mechanism for increased susceptibility to rhinovirus infection in asthmatic subjects has been recently descibed. Asthmatic bronchial epithelial cells were found to have a defect in innate immune responses to rhinovirus infection, with profoundly impaired production of IFN-β and apoptosis in response to rhinovirus infection, resulting in greatly increased virus replication (67). In contrast normal bronchial epithelial cells rapidly produced IFN-β and underwent apoptosis, rendering them almost completely resistant to rhinovirus replication. Impaired responses in asthmatic cells were specifically restricted to innate immunity as

measured as IFN-β production and apoptosis while the production of inflammatory mediators (IL-6 and RANTES) was not affected (67). Impaired innate immune responses were observed in primary bronchial epithelial cells from both steroid naïve and steroid treated asthmatic patients (67), and in the presence of dexamethsone indicating that the defects in antiviral immunity were not a result of steroid treatment, and that steroids have no effects on apoptotic responses and on IFN-β production in bronchial epithelial cells after rhinovirus infection in accordance with the clinical observation of relative ineffectiveness of steroids in virus induced asthma exacerbations (68). Finally, exposure of bronchial epithelial cells from asthmatics to exogenous IFN-β restored innate immune responses and limited virus replication to levels observed in normals (67). Thus signalling pathways downstream of the type I interferon receptor that lead to activation of programmed cell death and interferon production are intact. This has important implications for therapy, indicating that pharmacologic replacement/augmentation of IFN-β to promote innate immune responses could be proposed as a novel approach to prevent and treat virus induced asthma exacerbations

ii) Decreased type 1 and increased type 2 NK cell responses in asthma

An interesting field of investigation in the innate immune responses to viral infection is represented by the antiviral actions exerted by NK cells. There are some data in the literature suggesting a role of NK2 cells in the pathogenesis of asthma. Indeed, it has been shown that in blood the percentage of IFN-γ-producing NK1 cells is much lower and the percentage of IL-4-producing NK2 cells is much higher in asthmatic patients as compared to healthy individuals (69). Therefore it may be proposed that, in an airway environment rich in type 2 cytokines, NK1 cell functions and effective antiviral innate immunity might be impaired. If this is the case, then a key component of the early immune response would be deficient and viral clearance would be

impaired. In addition, if NK2 function is favoured by the asthmatic microenvironment, production of type 2 cytokines by NK cells in response to virus infection might be one mechanism for amplification of type 2 and impaired of type 1 T cell responses. However there are no studies of NK cell function in the airway in asthma or in virus-induced asthma.

iii)Decreased type 1 T cell antiviral responses in asthma

In addition to innate immune responses, acquired immune responses are also important in contributing to antiviral immunity. Efficient T cell responses to virus infection are thought to be predominantly type 1. It has been suggested that in the lower airways of allergic asthmatics with a pre-existing Th2-type allergic inflammation microenvironment, the T cell responses to viral infection may be skewed towards inappropriate and potentially harmful type 2 responses. Indeed, in a murine model of asthma in transgenic mice expressing virus (lymphocytic choriomeningitis virus – LCMV)-specific $CD8^+$ T cells, the induction of a Th2 immune response to ovalbumin was able to switch the virus-specific $CD8^+$ T cell response to IL-5 production leading to accumulation of eosinophils in the lung (70). By contrast, the virus peptide-specific $CD8^+$ T cell-induced responses in non- or sham-immunized transgenic mice resulted in an accumulation of neutrophils, without any detectable eosinophil recruitment. Moreover virus peptide-specific lung $CD8^+$ T cells from OVA-immunized mice produced IL-5 and reduced levels of IFN-γ whereas $CD8^+$ T cells from sham-immunized mice secreted large amounts of IFN-γ and no detectable IL-5 (70). This data indicates that a Th2 environment in the lung can switch virus-specific $CD8^+$ T cell responses to IL-5 production leading to impaired secretion of IFN-γ and delayed clearance of the virus from the lung.

In vitro human data indicate that exposure of PBMCs to rhinovirus results in an up-regulation of IFN-γ, and IL-12 (type 1 cytokines produced by T cells and monocytes

respectively) production in both normal and atopic asthmatic subjects; however PBMCs from asthmatic subjects produced significantly lower levels of IFN-γ and IL-12, demonstrating a deficient type 1 cytokine production (71) IL-4 (type 2 cytokine produced by T cells) production in PBMC was induced by rhinovirus only in the atopic asthmatic group (71).

There is also evidence *in vivo* that imbalances in type 1/type 2 immune responses influence the outcome of rhinovirus infection. Gern et al. showed an inverse relationship between the ratio of IFN-γ/IL-5 mRNA in sputum and cold symptoms and time to virus clearance from sputum in asthmatics and atopics experimentally infected with rhinovirus, indicating that a stronger type 1 immune response is associated with less severe symptoms and faster viral clearance (36). Moreover it has been shown that the higher is the *in vitro* Th1 response to rhinovirus infection in PBMCs, measured as IFN-γ production or IFN-γ/IL-5 ratio, the lower is the bronchial hyperresponsiveness and the higher is the % predicted FEV_1 in stable asthmatic patients (72). These data suggest that an impaired Th1 antiviral immune response not only is a characteristic of asthmatic patients but it might indirectly reflect the severity of the disease.

Taken together these studies suggest that an increased levels of type 2 cytokines in asthmatic subjects are likely to be associated with deficient type 1 responses and that this may also play a role in the increased susceptibility of asthmatic patients to viral infections. Studies on airway T cell responses to rhinovirus infections in asthmatic and normal subjects will be required to confirm whether this is indeed the case.

A defective Th1 immune response has been implicated in the pathogenesis and severity of RSV bronchiolitis both in animal models and in humans. Mice with type 2 cytokine responses after RSV infection develop enhanced disease with pulmonary hemorrhage and eosinophilia, whereas those with type 1 responses have reduced immunopathology and enhanced viral clearance (73). Children with a deficient Th1 and a relative increased Th2 immune response in

both airway secretions and in peripheral blood after RSV upper respiratory tract infection, manifest acute bronchiolitis as compared to children with stronger type 1 responses, who develop mild clinical illness without bronchiolitis and clear virus more effectively (74). Several studies have documented that a relative increased Th2 cytokine profile during RSV bronchiolitis is associated not only with increased severity of the disease itself, but also with increased risk of wheezing during follow-up (most of which is likely to be caused by rhinovirus infections) (75, 76).

Taken together these data suggest that in asthmatic patients, characterised by a predominant Th2 inflammation in the airways, a deficient type 1 antiviral response occurs. A defective type 1 immune response to RVs may be implicated in the pathogenesis of virus induced exacerbations of asthma.

Figure 2: asthmatic patients are characterised by an impaired antiviral innate immune response. Following rhinovirus infection in bronchial epithelial cells an impaired apoptotic response and impaired type I and type III interferon production occur. These events lead to impaired inhibition of viral replication resulting in increased viral replication and enhanced inflammatory cascade.

Conclusions

Epidemiological data indicate that respiratory virus infections are the most important causes of asthma exacerbations, rhinoviruses being the most frequently identified virus type. Experimental infection of normal and asthmatic volunteers indicate that respiratory viruses can induce many of the features typical of asthma exacerbations, however until now no major differences in the airway responses to viral infection have been observed between asthmatic and normal subjects. Viral infections increase the production in the lower airways of several pro-inflammatory molecules (including cytokines, chemokines and adhesion molecules). Respiratory virus-induced activation of neurogenic inflammation is also an emerging field of investigation.

Collectively, molecular biology data suggest a critical role for several transcription factors, including NF-κB and AP-1, in the production of pro-inflammatory mediators following viral infection. Few studies have been conducted on the molecular mechanisms underlying the pro-inflammatory responses induced by respiratory viruses in human airways.

Results coming from *in vitro* and *in vivo* studies indicate that a deficient immune response to viral infection, both in the innate immune response and in the adaptive immune response, occurs in asthmatic patients (36, 67, 71). The mechanisms of this impaired antiviral immune response are unknown. In particular it is unknown whether this is a genetically induced impairment or/and whether it is due to the pre-existing asthmatic airway Th2 immune response able to inhibit the naturally occurring Th1 immune response that follows viral infection. Interestingly a recent *in vitro* study indicates that the asthmatic defective immune response to rhinovirus in bronchial epithelial cells can be restored by provision of exogenous IFN-β restoring innate immune responses and limiting virus replication to levels observed in normals (67). Further studies are needed to deeply evaluate the complex immunological events that follow viral

infection in asthmatic patients in order find out possible mechanisms responsible for the increased susceptibility of asthmatic patients to viral infection.

The relative importance of each cell, mediator, signalling pathway and transcription factor will hopefully be clarified in the next few years. Such knowledge will enable development of compounds able to block a specific cell/mediator signalling molecule and/or transcription factor and permit testing of new possible targets for the treatment of virus-induced asthma exacerbations.

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ROLE OF VIRUS INFECTIONS IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) EXACERBATIONS

COPD and exacerbations: definition

Chronic Obstructive Pulmonary Disease (COPD) is a preventable and treatable disease with some significant extrapulmonary effects that may contribute to the severity in individual patients. Its pulmonary component is characterized by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lung to noxious particles or gases (1).

The natural history of the disease is punctuated by recurrent exacerbations, i.e. acute events characterized by a change in the patient's baseline dyspnea, cough, or sputum beyond day-to-day variability and sufficient to warrant a change in management. Acute exacerbations are a common occurrence for many COPD patients. In addition to the morbidity and mortality attributable to the acute episode, exacerbations contribute to loss of lung function and impaired health status in COPD (1-3). Prevention or amelioration of exacerbations has become a major therapeutic outcome for the evaluation of existing and new therapeutic agents for COPD. There is no universally accepted definition of an exacerbation of COPD. The typical symptoms of exacerbation include breathlessness, cough, increased sputum production and purulence, wheeze and chest tightness. Although usually clinicians have little difficulty in diagnosing an exacerbation of COPD, establishing a universally accepted definition for epidemiological or interventional studies has proved difficult. The two most widely used approaches have been to use symptom-based definitions or event-based definitions, but both of these approaches have potential drawbacks. Symptom-based definitions rely on the patient reporting a worsening of

dyspnea and increases in sputum volume or purulence, but there are no symptoms or physiological measurements that are diagnostic of exacerbation. Symptoms are the primary concern of the patient and it is generally a worsening of symptoms that prompts patients to seek medical advice. However such a definition relies on a subjective assessment by the patient and there is great variability in the threshold at which patients perceive symptoms to be worse than their usual state. Event-based definitions define an exacerbation as an event that leads the patient to seek medical advice, requires hospitalisation or necessitates treatment (generally a requirement for oral steroids or antibiotics). Event-based definitions of exacerbations are widely used in interventional clinical trials, however data from a longitudinal study of a cohort of COPD patients has shown that up to 50% of episodes of worsening of symptoms identified by symptom diary cards were not reported to the study investigators (3). Therefore an event-based definition may miss a substantial proportion of exacerbations that do not lead to a request for medical advice or treatment. The debate regarding the definition of an acute COPD exacerbation continues and further research is required to develop physiological or biological markers that provide a more objective means of defining an exacerbation.

Epidemiology of COPD exacerbations

Most epidemiological data regarding COPD exacerbations is derived from hospital admission data or primary physician visits but these figures may be a considerable underestimate of the true incidence. Exacerbations are common events with a median of 1.2 to 2.4 exacerbations per patient per year (4), and the frequency of exacerbations increases with increased severity of the disease (5-7). Acute exacerbations of COPD are a major burden to health-care systems and their impact has increased over recent years. Since the mid-1990s emergency admissions for

COPD in the UK rose by 50% to a total of 98,000 in the year 2000 and one quarter of all hospital inpatient bed days used for treating acute respiratory disease are for COPD (8), amounting to nearly one million hospital bed days per year (9). In the USA there were over 660,000 hospital discharges for COPD in 1998. A survey of COPD patients in the USA found that one-third had been hospitalised with COPD at some point in their life and 20% had visited the Emergency Department at least once in the past year (10). 70% of the health-care costs of the disease were estimated to be due to unscheduled hospitalisations due to acute exacerbations. In Canada COPD is the 4th most common cause of hospitalisation among men and the numbers of hospitalisations attributable to the disease are expected to double by the year 2015 (11). A hospital admission due to an acute COPD exacerbation carries a high risk of mortality and re-admission. A study of COPD patients in the USA who were hospitalised for an acute exacerbation, half of whom required intensive care, reported an in-hospital mortality rate of 11%, and six-month and one-year mortality rates of 33% and 43%, respectively. There was a 50% rate of readmission within six months after discharge (12). Prevention of exacerbations has now been recognised as a major therapeutic outcome for the evaluation of existing and new pharmacological agents for COPD. New therapies are urgently needed that will have a major impact on the morbidity, mortality and costs associated with COPD exacerbations.

Aetiology of COPD exacerbations

Many exacerbations are associated with symptoms of infection of the tracheo-bronchial tree and bacteria have been considered the main infective cause of exacerbations (13). Determining the contribution of bacteria to exacerbations is difficult as COPD patients are often colonised with bacteria even when clinically stable (14). The proportion of patients with positive

bacterial cultures and an high bacterial load increases during exacerbations in most, although not in all studies (15-17). Newer molecular techniques have recently shown that colonisation is not a static condition and there is a frequent turnover of different strains of bacteria evoking specific host responses (18). Thus, it is likely that a change in the strain but not the organism may be responsible for the exacerbations. Therefore, previous studies lacking in the molecular characterisation of bacterial strains may have missed evidence of a new infection. Indeed, it has been documented that the acquisition of a new strain of colonising bacteria increases the risk of an exacerbation (19).

In the last few decades the use of highly sensitive diagnostic methods, such as polymerase chain reaction (PCR), to evaluate the association between respiratory virus infections and COPD exacerbations has shown that viruses are responsible for a much higher proportion of exacerbations than was previously realised. In a study of the East London COPD cohort, respiratory viruses were detected in 39% of exacerbations, the most common being rhinoviruses that accounted for 58% of viruses (20). A respiratory virus was detected in around 50% of patients with severe COPD exacerbation admitted to hospitals in Germany and in Italy, with rhinovirus again being the most common (16, 21). In patients with very severe COPD exacerbations requiring intubation and mechanical ventilation viruses were identified in 47% of patients (22). At variance with bacterial infections, the respiratory viruses more commonly found at exacerbations were virtually absent in stable state (16, 23) suggesting that they play a relevant role in the aetiology of the acute episodes.

A recent study has addressed the relative importance of viral versus bacterial infections, to the aetiology of severe (hospitalised) COPD exacerbations (Figure 1). Viral and/or bacterial infection was detected in 78% of COPD exacerbations, with viruses in 48.4% (6.2% when stable), bacteria in 54.7% (37.5% when stable). The more severe exacerbations were those in which viral and

bacterial co-infection was detected (16). Similar results have also been found in studies of COPD exacerbations in outpatients (24): if both bacterial and symptom of common cold were present, then the sputum inflammatory markers were higher and lung function impairment was greater. However, the relationship between viral and bacterial infection, especially when combined, needs to be further studied. In particular it needs to be established whether viral infection can pave the way for exacerbation by bacteria colonizing the lower respiratory tract of COPD patients.

Figure 1

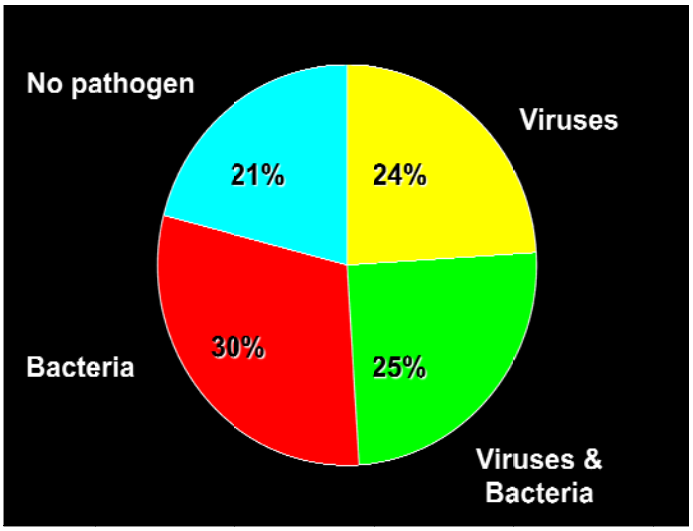

Figure 1: aetiology of COPD exacerbations.

Inflammatory response during virus induced COPD exacerbations

COPD exacerbations are associated with an increased number of activated inflammatory cells in the sputum and changed pattern of pro-inflammatory and anti-inflammatory mediators released into this compartment (25-27). Through influencing the inflammatory response

respiratory viral infections may enhance the pathological processes associated with cigarette smoking and contribute to the lung pathology and loss of lung function associated with COPD. The type and the degree of activation of the different inflammatory cells recruited to the lung during COPD exacerbations has not been well characterised.

CD8+ T lymphocytes, infiltrating both central (28) and peripheral airways (29) are a hallmark of COPD inflammation. These cells are further increased in the sputum during exacerbations (30).

Among the possible causes of CD8+ recruitment in the airways, chronic viral infection of the lungs has been suggested to be important. Particular attention has been given to the possible role of latent adenoviral infection in the pathogenesis of stable COPD. Adenoviral DNA sequences encoding the E1A protein are increased in the lung tissue of COPD patients as compared to smokers with normal lung function, suggesting latent adenoviral infection many occur in the lungs of COPD patients (31). Moreover, in COPD patients with emphysema the presence of adenoviral E1A protein in the alveolar epithelial cells was related to the severity of the emphysematous lesions and the number of CD8+ cells infiltrating the lung (32). These observations are consistent with the hypothesis that a persistent intracellular pathogen such as adenovirus may be capable of amplifying cigarette smoke-induced inflammation, possibly through interaction of viral proteins (such as E1A) with pro-inflammatory transcription factors [e.g. nuclear factor-κB (NF-κB) and activator protein (AP)-1)] (32). Recently it has also been documented that COPD patients in whom respiratory syncytial virus (RSV) was repeatedly detected in sputum over 2 years had faster lung function decline (33). Taken together these data indicate that in response to repeated/latent viral infections, an excessive recruitment of CD8+ T-lymphocytes may occur in the tracheobronchial tree. The CD8+ driven inflammation can damage the lung in susceptible smokers leading to COPD progression (34).

Enhanced neutrophilic inflammation and sputum purulence have been considered for many years markers of bacterial aetiology of COPD exacerbations. **T**he traditional association of sputum purulence with bacterial infection is supported by the higher rate of isolation of bacterial pathogens in individuals with purulent sputum at presentation, with one study identifying a positive bacterial culture in 84% of purulent sputum compared with 38% if sputum was mucoid (35). Self-reported assessment of sputum purulence has also been investigated: bacterial cultures performed on sputum samples and protected brush specimens showed that self-reported sputum purulence was associated with a high yield of potentially pathogenic microorganisms with a positive predictive value of 77% (36). Gompertz et al have shown that when purulent sputum exacerbations are treated with antibiotics, resolution of exacerbations is associated with progressive reduction of neutrophilic airway inflammation (37). A number of studies documented that higher bacterial loads were more frequently associated with increased sputum neutrophils, supporting the concept that when bacteria are present at exacerbation, airway inflammation is higher in those samples where bacterial load is higher (17, 24). IL-8 and TNF-α are potent neutrophilic chemoattractant chemokines with increased levels in sputum supernatants during COPD exacerbations. Although not in all studies, bacterial exacerbation have been associated with higher levels of sputum IL-8 and TNF-α , leading to enhanced neutrophil recruitment and activation (38, 39).

Virtually all the studies that have found a relationship between bacterial infection and increased markers of neutrophilic inflammation in sputum samples and/or increased sputum purulence during exacerbation, did not take into account viral and/or viral/bacterial coinfections. Whether enhanced neutrophilic inflammation in the airways of COPD patients during exacerbation is a marker of bacterial infection has been debated in the last few years. Indeed, in experimental condition rhinovirus infection induces peripheral blood and sputum netrophilia in smokers and

COPD subjects (40). A recent study showed increased number of neutrophils in sputum during exacerbations and the neutrophilic response occurred irrespective of the pathogen detected (bacteria vs viruses vs coinfection viruses+bacteria). The same study documented that purulent sputum at exacerbation was more frequent in infective exacerbations as compared to noninfective exacerbations but no difference was found between viral versus bacterial infections (16). Intriguingly a very recent study found that that COPD exacerbations associated with acquisition of new strains of bacteria, specifically *H. influenzae*, *S. pneumoniae*, *M. catarrhalis* and *P. aeruginosa* were associated with a more intense neutrophilic inflammatory response in the airway, as well as more intense systemic inflammation, compared to exacerbations not associated new strain acquisition and inflammatory cell profile at exacerbation (41).

One of the future major task in the field of exacerbation will be to identify inflammatory or specific biological markers able to tell us whether bacteria are in fact responsible for the ongoing exacerbation. This would be extremely helpful in addressing an appropriate use of antibiotic treatments. With this aim, several specific markers of bacterial infections have been tested in the last few years. A promising role of procalcitonin to define patients with a COPD exacerbation with a higher likelihood of bacterial infection has been recently suggested. Procalcitonin is a small (13-kD) protein normally undetectable in plasma that increases markedly in bacterial infections (42). Data from single-center, cluster-randomized, single-blinded study suggests that procalcitonin-guided therapy can be safely used to reduce antibiotic use in patients admitted to the hospital with COPD exacerbation at a low likelihood of bacterial infection (43).

Few studies have investigated the role of respiratory virus infection in COPD exacerbations, and very few have looked at the relationship between viral infections and airway inflammation at exacerbations.

One study found increased sputum IL-6 in viral associated acute episodes as compared to non viral exacerbations (23). Intriguingly, and only recently, it has been documented that plasma fibrinogen levels, a marker of systemic inflammation and a recognized independent risk factor for cardiovascular disease, are higher in viral associated COPD exacerbation (44). This indicates that respiratory viral infections are associated with an increased systemic inflammation and might predispose to an increased risk of cardiovascular disease. Bacterial and virus infection can synergistically interact to increase the severity of inflammatory response. Indeed, it has been shown that rhinovirus and *Haemophilus influenzae* coinfection at exacerbations is associated with increased levels of serum IL-6 (24). In this situations, i.e. viral and bacterial coinfection, exacerbations are more severe both in term of clinical and lung function parameters (16, 24). Similarly, levels of endothelin (ET)-1, a potent vasoconstrictor and bronchoconstrictor peptide with important pro-inflammatory activities in the airways, tend to be higher during COPD exacerbation associated with viral or chlamydial infection both in sputum and in plasma (45).

A recent study investigated the relative importance of viral and bacterial infection in COPD exacerbations and their relationship with airway inflammation (16). At variance with neutrophils, sputum eosinophils were significantly elevated at exacerbation only in the subgroups with viral infections (Figure 2). In this study, sputum eosinophils at exacerbation could predict a positive viral detection in sputum with a sensitivity of 0.82 and a specificity of 0.77 for eosinophil counts of 1.68 x 10^6/g or greater (best cut-off point). Similarly, sputum eosinophil count increases from stable conditions to exacerbation of 0.3 x 10^6/g or more predicted a virus-associated COPD exacerbation with a sensitivity of 0.87 and a specificity of 0.81. Sputum eosinophilic cationic protein (ECP) levels in the supernatant of samples obtained from exacerbations associated with viral infection, either with or without a bacterial coinfection, were significantly higher as compared with virus-free exacerbations. This data suggests that sputum

eosinophilia and not sputum neutrophilia can be a marker of viral infection during COPD exacerbations (16). Previous studies reported a prominent airway eosinophilia at exacerbation with a 30-fold increase in the number of eosinophils in the exacerbated group compared to the stable patients (46, 47).

Taken together this data indicates that noninvasive measurements could be used in clinical practice to provide clinically relevant etiologic information. Further studies are required to confirm and extend this pivotal observation.

Interestingly, increased sputum CD8+ T lymphocytes have been reported during COPD exacerbations with a relative reduction in the ratio of interferon (IFN)-γ/IL-4 expressing CD8+ T lymphocyte (30). Thus, a switch towards a T helper (Th)2-like immunophenotype during COPD exacerbations could trigger recruitment of eosinophils and might be activated by the immune response to some microbial pathogens.

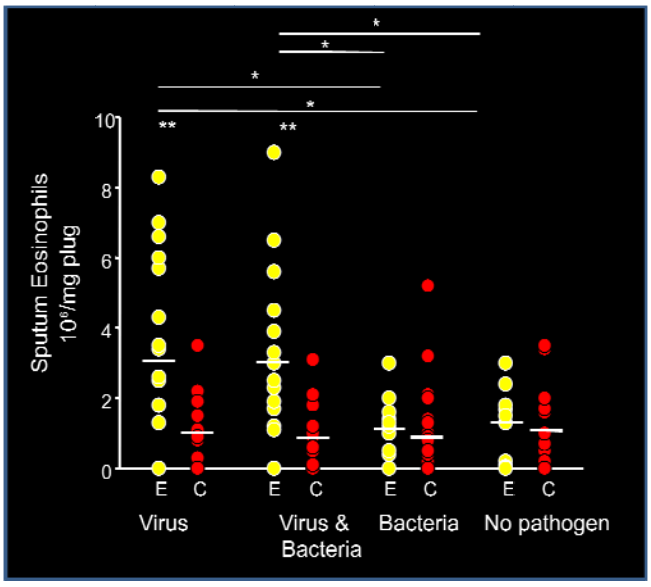

Figure 2: Sputum eosinophil counts in patients with COPD during severe exacerbation requiring hospitalization (E) and during stable convalescence (C). Subjects were grouped according to the presence of respiratory viruses alone, bacteria alone, both viruses and bacteria, or no pathogen in the sputum during exacerbation.

Mechanisms of virus induced COPD exacerbations

At variance with asthma in which several *in vitro* and *in vivo* studies have investigated the mechanisms that lead to exacerbation after viral infection of the airways (48, 49), very few data are available for COPD. By contrast, several mechanisms have been proposed to explain how bacterial infection can affect airway inflammation leading to COPD exacerbations. These include induction of mucus hypersecretion (50), reduction of ciliary beat frequency (51) and enhancement of neutrophilic inflammation (52). In particular, *H influenzae* can cause direct epithelial damage and its endotoxin has been shown to increase epithelial expression of the pro-inflammatory cytokines IL-6, IL-8, and TNF-α in vitro, providing potential mechanisms to upregulate inflammation and specifically neutrophilic inflammation (52). This is in line with *in vivo* data showing that COPD patients with a positive bacterial culture of potential pathogenic microbes have higher concentrations of TNF-α and increased neutrophils in the airways (38, 53).

Activated neutrophils degranulate, resulting in the release of elastases, oxidants, and proteases. Proteases and oxidants can damage the epithelium, reduce ciliary beat frequency, stimulate mucus secretion by mucus-secreting cells, and increase the permeability of the bronchial mucosa. These changes, especially in the small airways, may adversely affect airflow leading to increased dyspnoea, as well as increased mucus secretion and sputum purulence. In addition, neutrophil elastase and oxidants have been shown to increase epithelial mucin mRNA and protein gene expression *in vitro*. Neutrophil elastase is a major driver of lung injury, thus elevated levels of this enzyme seen in bacterial exacerbations could contribute significantly to the loss of lung function seen in COPD (54). Bacterial components, such as lipopolysaccharide (LPS), through their interaction with toll-like receptors can activate nuclear transcriptor factor κB (NF-κB) (55).

Activation of NF-κB leads to the production of several cytokines, chemokines and adhesion molecules involved in the inflammatory cascade that characterised COPD exacerbation (56). Interestingly, a recent study found increased NF-κB activation in sputum macrophages during COPD exacerbations (57).

Although i*n vitro* models can provide important insights into the molecular mechanisms of inflammatory and immune responses to bacterial infection, the *in vitro* data require validation *in vivo* models. Unfortunately, despite bacterial infections being considered the most important cause of COPD exacerbations there is still no *in vivo* model of bacteria induced COPD exacerbation.

Despite growing clinical evidence for a role of respiratory viral infections in the pathogenesis of COPD exacerbations, the precise mechanisms of interactions between respiratory virus and lower airway inflammation and of host resistance against respiratory viruses are poorly understood (56). Most of the data available relate to rhinovirus, *i.e.* the respiratory virus that appear to be more frequently involved in COPD exacerbations (5, 16, 21). The major group of rhinoviruses (accounting for 90% of total rhinovirus types) attaches to airway epithelium through intercellular adhesion molecule 1 (ICAM-1) (58). Interestingly, rhinovirus infection induces expression of its own receptor (ICAM-1) (59), which might promote inflammatory cell recruitment and activation. Indeed, since ICAM-1 is over-expressed in the bronchial mucosa of patients with stable chronic bronchitis (60), one of the possible mechanisms for increased susceptibility to infections and for increased airway inflammation is rhinovirus-induced ICAM-1 upregulation in bronchial epithelial cells. *In vitro* data documents that experimental rhinovirus infection of bronchial epithelial cells induces pro-inflammatory mediators including cytokines and chemokines such as IL-6, CXCL8, IL-11, GM-CSF, CCL5, 5-lipoxygenase (5-LO), 5-LO activating protein (FLAP) and cyclooxygenase (COX)-2, endothelin and adhesion molecules (56). Thus rhinovirus infection

of bronchial epithelial cells can lead to the production of inflammatory mediators able to induce both neutrophilc and eosinophilic inflammation, in line with clinical observations showing that neutrophilic and eosinophilic inflammatory responses can occur *in vivo* following rhinovirus infection. Interestingly, bronchial eosinophilia has been described some years ago in bronchial biopsies of healthy subjects experimentally infected with rhinovirus (61).

In vitro rhinovirus infection of human bronchial epithelial cells increases their MUC5AC (one of the major mucins in the sputum) synthesis and release (62). This could be relevant to explain the increased amount of sputum observed in some patients during COPD exacerbations.

Rhinovirus infection induces increased production of superoxide anion in bronchial epithelial cells and this event is a crucial step for the activation of NF-κB and the following production of proinflammatory cytokines, chemokines and adhesion molecules (63). Reducing agents inhibit both rhinovirus-induced oxidant generation and inflammatory mediator production and release (63). This data suggests that the inhibition of intracellular oxidative stress may be a potential therapeutic target for the treatment of virus induced COPD exacerbations.

It has been recently shown that early activation of mitogen-activated protein (MAP) kinase p38 is a key regulatory event of rhinovirus-induced pro-inflammatory transcription factor activation and cytokine/chemokines transcription both in bronchial epithelial cells and monocyte/macrophages (64, 65). These mechanisms could be a target for inhibition of airway inflammation associated with rhinovirus infection.

To validate *in vitro* observations, the development of an *in vivo* models is necessary. Thus the recent development of a human experimental model of rhinovirus-induced COPD exacerbations represents an innovative tool that will offer the opportunity to deeply elucidate the inflammatory and immunological mechanisms that lead COPD patients to exacerbate after respiratory virus infections and to identify novel possible pharmacological targets (66). This model shows that

experimental rhinovirus infection in COPD patients induces symptoms, lung function changes, increased peripheral blood total leukocyte count, increased peripheral neutrophil and increased sputum neutrophil number (40, 66) similar to that observed in naturally-occurring exacerbations. In COPD this is so far the only scenario in which a specific aetiology has been experimentally proven to induce an exacerbation.

Susceptibility to virus infections in COPD patients

There is solid evidence of impaired innate (67, 68) and possibly acquired (69, 70) immune responses to viral infection in asthmatic patients. Whether COPD patients are more susceptible to virus infection as compared to normal subjects is still debated. A recent study documented that patients with frequent (>2.5/year) COPD exacerbation have more frequent episodes of naturally occurring colds as compared to patients with infrequent exacerbations (71). These results suggest that COPD subjects with frequent exacerbations may represent a subgroup particularly susceptible to viral infections, but they do not determine whether this susceptibility relates to the general COPD population. Intriguingly patients experiencing frequent colds had a significantly higher exposure to cigarette smoke (71). Recently, using a murine model of cigarette smoke exposure, it was demonstrated that virus infections in combination with tobacco smoke decreased the number of dendritic cells in the lung and also altered the costimulatory molecule expression profile on these cells, that are believed to be fundamental to the initiation of adaptive immune response (72). In the same study it was also that mice exposed to smoke after adenovirus infection had a decreased T cell-mediated antiviral immune response, as documented by reduced infiltration of activated CD4 and CD8 T cells, in the lungs (72). Thus cigarette smoke, possibly

via alteration of both innate and adaptive immune responses, can contribute to the increased susceptibility of COPD patients to viral infections (73).

Another possible mechanism leading to increased susceptibility is related to up regulation of ICAM-1, the receptor for the major group of human rhinoviruses. Latent expression of adenoviral E1A protein in alveolar epithelial cells of patients with pulmonary emphysema may increases ICAM-1 expression and this could be a potential mechanism for a greater susceptibility to rhinovirus infection of COPD patients (32).

Finally, solid evidence exists that the surface of the bronchial mucosa of patients with COPD is chronically colonised with bacteria, and the bacterial load is related to the intensity of the airway inflammation and to disease progression (74). Moreover patients with a history of frequent exacerbations have a higher incidence of bacterial colonisation. Thus, given that viral infections are one of the most frequent causes of COPD exacerbation these results indicate that chronic bacterial colonisation could contribute to the increased susceptibility of COPD patients to viral infection, for example by increasing ICAM-1 expression on the surface of the bronchial epithelial cells (75). Further studies are required to investigate the interaction between chronic bacterial colonisation and respiratory viral infection and in particular whether chronic bacterial colonisation can increase viral infection susceptibility or alternatively that viral infection can increase the airway bacteria load leading to COPD exacerbations.

Conclusions

COPD is a major health problem worldwide with rising prevalence and mortality. The major morbidity, mortality and health care costs of COPD are due to exacerbations (13).

Thanks to the development of highly sensitive diagnostic tools, the interaction between respiratory viruses and bacteria has emerged as a leading cause of COPD exacerbations (16, 24).

However, the mechanisms responsible for the interaction between these airway pathogens and the inflammatory processes in causing COPD exacerbations are still largely unknown (56).

The recent development of the first human model of virus induced COPD exacerbation, is rapidly advancing our knowledge on these interactions (66). Improved understanding of the host-pathogen interaction in the lower airways in COPD patients will undoubtedly facilitate the identification of novel pharmacological targets that will provide opportunities to develop new treatments for COPD exacerbations.

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EXPERIMENTAL DATA

Innate immune response in asthmatic patients: role of deficient type III interferon-λ production in asthma exacerbations

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Summary and Aim

Acute exacerbations are the major cause of morbidity in asthma. The majority are precipitated by rhinovirus infections. Asthmatics have increased susceptibility to rhinovirus infections and risk of invasive bacterial infection. The mechanisms of this increased susceptibility are unknown.

The novel type III interferon-λs exhibit some properties similar to type I interferons but their activity in protecting against rhinovirus infections and in the pathogenesis of asthma exacerbations is unknown.

Here we investigated the role of interferon-λs in rhinovirus infection of human bronchial epithelial cells *in vitro,* their production in asthmatic and normal subjects in response to rhinovirus infection of primary bronchial epithelial cells and both rhinovirus & lipopolysaccharide (LPS) stimulation of bronchoalveolar lavage cells *ex vivo*. Finally we investigated the role of interferon-λ production in the pathogenesis of rhinovirus infection and asthma exacerbations *in vivo* using human experimental infections in asthmatic and normal subjects.

We show that interferon-λs are induced by and are anti-viral against rhinovirus infections *in vitro*. In asthma their induction by both rhinovirus infection and lipopolysaccharide is deficient. Finally deficient production correlated with severity of rhinovirus induced cold symptoms, lung function impairment and virus load *in vivo*. These data identify an important novel mechanism in the pathogenesis of asthma exacerbations and in increased susceptibility to viral and bacterial infections in asthma and suggest new approaches to prevention and/or treatment of asthma exacerbations.

Recently, deficient interferon-β production following *in vitro* rhinovirus infection of primary bronchial epithelial cells from asthmatic subjects has been reported (1). Although a novel observation providing some insight into mechanisms of increased susceptibility to virus infection in asthma, this study investigated only interferon-β, in a single cell type, bacterial stimuli were not studied and most importantly, relationship with clinical illness severity was not investigated. Here we have hypothesized that in asthma there could be deficiency in components of innate immune responses other than interferon-β production that would include other respiratory cells and be observed in response to both virus and bacterial infection. Finally we hypothesized that deficient production would be related to clinical illness severity *in vivo.*

The 13 α interferons and one β interferon are expressed on chromosome 9 and are classed as type I interferons as they signal through a common type I interferon receptor and have similar biologic properties in innate host defense. The single class II interferon is interferon-γ, which signals through the interferon-γ receptor and is important in both innate and acquired immunity. A novel class of interferons have recently been discovered and named type III interferons: interferon-λ1 (also known as interleukin [IL]-29) and interferon-λ2/3 (IL-28A/B) which are expressed on chromosome 19 and signal through a unique interferon-λ-specific receptor(2, 3). Similar to type I interferons, they induce interferon stimulated genes, signal via Jak/Stat pathways and have antiviral activity *in vitro*(2, 3). They also exhibited antiviral activity in vaccinia virus infected mice(4). However, their role in the pathogenesis of rhinovirus infection and in asthma is unknown.

We therefore investigated the role of interferon-λs in rhinovirus infection of human bronchial epithelial cells *in vitro,* their production in asthmatic and normal subjects in response to rhinovirus infection of primary bronchial epithelial cells and both rhinovirus &

lipopolysaccharide (LPS) stimulation of bronchoalveolar lavage cells *ex vivo*. Finally we investigated the role of interferon-λ production in the pathogenesis of rhinovirus infection and asthma exacerbations *in vivo* using human experimental infections in asthmatic and normal subjects.

Results

Interferon lambdas are induced by rhinovirus infection of bronchial epithelial cells, monocytes and macrophages

First, to determine whether interferon-λs are induced by rhinovirus infection, we investigated their expression in the BEAS-2B human bronchial epithelial cell line. We developed separate quantitative PCRs for human interferon-λ1 and -λ2/3 as they are only 80% homologous, but a single assay for interferon-λ2/3 as they are 96% homologous(2, 3). Interferon-λs were not expressed in uninfected cells, but rhinovirus-16 infection significantly induced mRNA expression for interferon-λ1 and interferon-λ2/3 peaking at ~4 logs 8 hours post infection and remaining significantly increased 24 & 48 hours after infection. Induction was dose responsive, increasing from ~2 logs at a multiplicity of infection (MOI) 0.4 to ~4 logs at a MOI of 10.

There are at least 100 different rhinovirus serotypes, separated into a major group (90%, including rhinovirus-16) which use ICAM-1 as cellular receptor(5) and a minor group using the LDL receptor(6). To determine whether interferon-λ induction was rhinovirus receptor and serotype independent, we performed similar experiments with another major group (rhinovirus-9) and a minor group (rhinovirus-1B) rhinovirus serotype. Infection with either induced interferon-λ1 and -λ2/3 to similar degrees to that observed with rhinovirus-16, indicating induction was both rhinovirus receptor and serotype independent. Also, UV-inactivation of all three serotypes

completely blocked induction of interferon-λ1 and -λ2/3, confirming that induction was rhinovirus replication dependent.

To confirm that rhinovirus induction of mRNA expression was accompanied by protein production, we developed an ELISA specific for interferon-λs and assessed protein production in the supernatants of the same experiments. Rhinovirus-16 infection induced interferon-λ protein production in a dose responsive manner.

Macrophages and/or dendritic cells also contribute to type I interferon production and therefore to the antiviral state in the airway(7). To investigate whether other cell types were important sources of interferon-λ production in response to rhinoviruses infections, we next investigated production in response to rhinovirus infection of peripheral blood mononuclear cells and observed similar rhinovirus induction of interferon-λ mRNA expression and protein production.

To determine whether induction was also observed in differentiated macrophages and was also replication dependent in non-epithelial cells, we investigated interferon-λ protein production in monocyte derived macrophages in response to UV-inactivated and live rhinovirus-16. Significant induction of interferon-λ protein production was only observed in cells infected with live virus, confirming replication dependent production in macrophages.

Interferon lambdas protect bronchial epithelial cells against rhinovirus infection *in vitro*

We next investigated whether interferon-λ is antiviral in the context of rhinovirus infection of bronchial epithelial cells. BEAS-2B cells were infected with rhinovirus-16 in the presence or absence of interferon-λ1 and virus replication assessed by quantifying rhinovirus viral RNA production in cell lysates and virus release into cell supernatants. Interferon-λ1 significantly suppressed viral RNA production and virus release (Figures 2), confirming anti-rhinoviral activity of this protein in bronchial epithelial cells.

Deficient interferon-λ production in human primary bronchial epithelial cells from asthmatic compared to normal subjects and relationship to rhinovirus replication *in vitro*

As we have previously reported deficient rhinovirus induced interferon-β production in asthmatic primary bronchial epithelial cells(1), we next wished to determine whether interferon-λ1 and interferon-λ2/3 induction occurred in response to rhinovirus infection of primary bronchial epithelial cells, and if so, whether this was deficient in asthmatic compared with normal cells.

First we investigated rhinovirus replication in these cells by assessing viral RNA production in cell lysates. As previously reported(1), at 8 hours after infection we found significantly (10 fold) increased rhinovirus replication in primary bronchial epithelial cells from asthmatic compared with normal subjects (Figure 3A). Rhinovirus induction of interferon-λ1 and interferon-λ2/3 mRNA expression in primary bronchial epithelial cells was confirmed in the same cell lysates in cells from both normal and asthmatic volunteers, however, induction of both interferon-λ1 and interferon-λ2/3 was significantly lower in asthmatic compared with normal subjects, confirming that rhinovirus induction of interferon-λs occurs in the relevant primary cells, but is deficient in asthma (Figure 3B).

To determine whether interferon-λ1 and interferon-λ2/3 production was related to virus replication in primary cells, we next investigated correlations between interferon-λ expression and virus replication. Expression of interferon-λ1 and interferon-λ2/3 mRNA were strongly inversely related to viral RNA copy number, indicating that interferon-λ induction in response to rhinovirus infection of primary bronchial epithelial cells is strongly related to virus replication. The deficient induction in asthmatic cells was also very clearly related to virus replication *in*

vitro, as the asthmatic and normal data were clearly separated, with asthmatics having greater virus load and lower interferon-λ expression.

Induction of interferon-λ1 and interferon-λ2/3 mRNA by rhinovirus infection of primary bronchial epithelial cells resulted in induction of interferon-λ protein production in cells from both normal and asthmatic volunteers. Protein production in supernatants of cells from asthmatic volunteers was also significantly impaired compared with normal volunteers. The relationship between interferon-λ protein production and rhinovirus replication was again investigated and found to be significantly inversely correlated and once again the asthmatic and normal data were clearly separated, with asthmatics having greater virus load and lower interferon-λ production (Figure 3C).

These data confirmed interferon-λ1 and interferon-λ2/3 are induced by rhinovirus infection of primary bronchial epithelial cells, that induction of both mRNA and protein is deficient in cells from asthmatic subjects and that induction is strongly related to rhinovirus replication *in vitro*.

Deficient rhinovirus induced interferon-λ production from bronchoalveolar lavage cells from asthmatic compared to normal subjects and relationship to illness severity and virus load *in vivo*

We next wished to investigate induction of interferon-λs and their relation to clinical illness severity *in vivo.* As the only useful animal model of rhinovirus infection is in chimpanzees (8, 9), these experiments were carried out using human experimental rhinovirus infections in a different group of normal and asthmatic subjects. Also, to determine whether deficient interferon production in asthma was restricted to epithelial cells, we wished to investigate whether interferon-λ induction in asthma was also deficient in cell types other than bronchial epithelial

cells. Having shown earlier that macrophages were robust producers of interferon-λ, we studied bronchoalveolar lavage cells, which are normally ~90% macrophages.

Bronchoalveolar lavage cells were obtained from asthmatic and normal volunteers and incubated *ex vivo*. Incubation with live rhinovirus-16 significantly induced interferon-λ protein production from both normal and asthmatic cells, this induction was virus-specific, as no induction was observed in cells incubated with medium or inoculum from which the virus had been removed by molecular weight filtration(10). We then compared induction of interferon-λ in cells from asthmatic and normal subjects and confirmed that, like bronchial epithelial cells, rhinovirus induction in bronchoalveolar lavage cells was also deficient in asthma (Figure 4A).

To determine whether rhinovirus induced interferon-λ production was related to clinical illness severity and virus load *in vivo*, the same volunteers were experimentally inoculated with rhinovirus-16 two weeks after the *ex vivo* studies. We observed a significant inverse correlation between interferon-λ concentrations in supernatants of bronchoalveolar lavage cells infected with rhinovirus *ex vivo*, and severity of cold symptoms following *in vivo* infection, confirming that rhinovirus induction of interferon-λ was related to symptom severity *in vivo*. A similar inverse correlation was observed between interferon-λ concentrations and bronchoalveolar lavage virus load during *in vivo* infection (Figure 4B).

A relationship between interferon-λ production and severity of rhinovirus induced asthma exacerbations was also demonstrated by a significant inverse relationship between interferon-λ concentrations and severity of reductions in lung function during *in vivo* infection (Figure 4C). Even with these *in vivo* data, the asthmatic and normal data were once again remarkably separated, with asthmatics having greater symptoms, virus load and lung function impairment and lower interferon-λ production.

Deficient LPS induced interferon-λ production from bronchoalveolar lavage cells from asthmatic compared to normal subjects and relationship to lung function *in vivo*

Asthmatics are at increased risk of invasive bacterial infection(11), through unknown mechanisms. Finally, to gain insight into possible mechanisms, bronchoalveolar lavage cells obtained from the same asthmatic and normal volunteers were incubated *ex vivo* with bacterial LPS and interferon-λ production assessed. These experiments were also designed to provide some initial insight to possible mechanisms of deficient interferon-λ production in asthma as Toll like receptor (TLR)3 mediates anti rhinovirus activity(12) as well as interferon-λ production(13) in bronchial epithelial cells, while LPS is a ligand for TLR4(14).

Significant induction of interferon-λ by LPS was observed in both normal and asthmatic subjects and induction was significantly impaired in asthmatic relative to normal subjects (Figure 5A). A relationship between LPS induced interferon-λ production and severity of rhinovirus induced asthma exacerbations was also demonstrated by the finding of a significant inverse relationship between interferon-λ concentrations in supernatants of bronchoalveolar lavage cells stimulated with LPS *ex vivo*, and severity of reductions in lung function *in vivo* (Figure 5B).

Discussion

Acute attacks of asthma are a serious and common medical problem poorly met by current medication(15-17). New therapies are needed, however although rhinovirus infection is the major precipitant(18-20), the pathogenesis is poorly understood(21). Innate immune responses are critical in host defense against viral infections and asthmatics have increased susceptibility to naturally occurring rhinovirus infections(20). The mechanisms of this increased susceptibility are unknown. Here we found that type III interferons were strongly induced by rhinovirus in

bronchial epithelial cells and macrophages and were antiviral against rhinovirus infection *in vitro*. We further demonstrated their induction in response to *ex vivo* rhinovirus infection of both primary bronchial epithelial and bronchoalveolar lavage cells and importantly, that this induction was deficient in both cell types derived from asthmatic subjects. We highlighted the importance of these interferons in host defence against rhinovirus infection by demonstrating strong relationships between interferon-λ production and rhinovirus replication *ex vivo*, and most importantly, with severity of clinical symptoms, reductions in lung function and virus load *in vivo*. These data identify a novel mechanism of increased susceptibility to rhinovirus infection in asthma and suggest replacement/augmentation of innate interferon production as a novel approach to treatment/prevention of asthma exacerbations.

The interferon-λs are already known to induce some interferon stimulated genes, signal via Jak/Stat pathways and have antiviral activity *in vitro*(2, 3) and in vaccinia virus infected mice(4). These data suggest they are likely important in host defense, however, their role in the pathogenesis of respiratory virus infections is poorly understood and in asthma, unknown. One previous study reported induction of interferon-λs in response to respiratory syncytial virus infection of an alveolar cell line and monocytes(22), we extend these observations to demonstrate their induction by rhinovirus infection of a bronchial epithelial cell line as well as primary human peripheral blood monocytes and monocyte derived macrophages. We then demonstrated that interferon-λ induced the ISGs CCL5 and CXCL10, which are implicated in the pathogenesis of rhinovirus infections(23, 24), and confirmed that interferon-λ had antiviral activity in suppressing rhinovirus replication in bronchial epithelial cells. Since rhinovirus infections are the major cause of acute exacerbations of asthma(18-20), these properties strongly suggest interferon-λ production is likely important in host defense against rhinovirus induced asthma exacerbations.

We next investigated the importance of interferon-λs in increased susceptibility to rhinovirus infection in asthma(20). Initially we investigated induction of interferon-λs in response to *ex vivo* rhinovirus infection of primary bronchial epithelial cells (from asthmatic and normal volunteers), as these are the natural host cell for rhinovirus infection in the lower respiratory tract(25). We confirmed rhinovirus induction of interferon-λs in both subject groups, confirming rhinovirus induction in the natural host cells *ex vivo*. As we had previously observed(1), rhinovirus replication was confirmed increased in the primary bronchial epithelial cells from asthmatic volunteers and induction of interferon-λs by rhinovirus infection of asthmatic primary bronchial epithelial cells was deficient compared to normal cells and was very strongly inversely related to rhinovirus replication *in vitro*. The strength of these relationships is impressive, despite the small numbers of subjects studied (9 in each group). However, most impressive is the complete separation between the asthmatic and normal subjects (open), as the asthmatics had uniformly lower interferon-λ production and higher virus loads and vice versa in the normals. Such clear separation in studies of human biology is rare and these data combined strongly attest to the likely biologic importance of these relationships and of deficient interferon-λ production in the pathogenesis of rhinovirus infections.

Our previous study of interferon-β(1) and our findings above with interferon-λs are restricted to primary bronchial epithelial cells, possibly suggesting a cell type-specific defect in interferon production in asthma. We therefore wished to determine whether deficient interferon-λ production was also observed in a different cell type also relevant to lung host defense. We initially demonstrated that rhinovirus induced interferon-λ in monocytes and macrophages *in vitro*. We then recruited a further group of asthmatic and normal subjects and confirmed that interferon-λ was induced by rhinovirus infection of primary bronchoalveolar lavage cells (~90%

macrophages) *ex vivo*. Interferon-λ induction by rhinovirus was also found deficient in bronchoalveolar lavage cells from asthmatic subjects, confirming that this deficiency was not restricted to bronchial epithelial cells.

We next wished to determine whether deficient interferon-λ production was related to clinical illness severity and virus load *in vivo*. One of the major obstacles to understanding the pathogenesis of rhinovirus infections has been the lack of a useful small animal model(8, 9). We therefore next investigated the role of interferon-λ *in vivo* in the same second group of asthmatic and normal volunteers, who were infected experimentally with rhinovirus-16 two weeks after their initial bronchoscopy and clinical illness severity assessed with symptom diary cards and lung function recording. A further bronchoscopy was carried out on day 4 of the experimental infection to determine virus load in the bronchoalveolar lavage during *in vivo* infection. Interferon-λ production was significantly inversely correlated with both common cold symptoms and virus load, confirming *in vivo*, the importance of interferon-λs in host defence against rhinovirus infection

To determine whether interferon-λs are important in the pathogenesis of rhinovirus induced asthma exacerbations, we also investigated the relationship between interferon-λ production and severity of reductions in lung function in response to rhinovirus infection. A significant correlation was observed. As with the *ex vivo* data, the strength and significance of the relationships with small subject numbers (4/5 asthmatic and 8 normal), as well as the separation between asthmatic and normal volunteers observed in all three relationships, was remarkable for human *in vivo* data. These data therefore very strongly implicate deficient interferon-λ production as an important novel mechanism in the pathogenesis of asthma exacerbations.

As asthmatic subjects have also been recently shown to be at increased risk of invasive bacterial infection(11), through unknown mechanisms, we then wished to determine whether interferon-λ production was deficient in asthma when studying a stimulus relevant to bacterial infections. These experiments confirmed interferon-λ production in response to LPS was also deficient in bronchoalveolar lavage cells from asthmatic compared to normal subjects and was inversely related to lung function impairment *in vivo*. With our previous study(1), these experiments indicate that the deficiencies in components of innate immune responses in asthmatic subjects are surprisingly broad and involve defective responses in two interferon families. In the present study, interferon-λ deficiency has also been observed in different lung cell types, as well as in response to both a bacterial stimulus and rhinovirus infection.

While replacement of interferons -β or -λ in asthma are possible new therapeutic options, in order to explore further therapeutic options, it is highly desirable to understand the mechanisms of deficient interferon-β and -λ production in asthma. While knowledge regarding interferon deficiency in asthma was restricted to interferon-β, polymorphism(s) in that gene represented an attractive possible explanation, however, with deficiency now involving 4 genes on two different chromosomes (9 for interferon-β and 19 for the lambdas(2, 3)), this explanation seem less likely.

There has been much recent interest in signalling pathways in innate anti-viral immunity. In response to viral infection, TLR3(12, 26-28), 7(29-32), 8(29-31) and 9(30, 33) and PKR (34-36) and RIG-I (29, 37, 38) have been implicated in induction of type I interferons. Much less is known about interferon-λ, however it has recently been demonstrated that TLRs 3, 4, 7, 8 and 9 mediate virus induction of interferon-λs, as well as α/β interferons(30). Induction of interferon-λs via TLRs 7-9 was shown to be mediated by the IL-1 receptor associated kinase-4 (IRAK-4), however, that via TLR3 and 4 was IRAK-4 independent(30). IRAK-4 deficiency is a novel

deficiency in TLR signalling associated with human disease(30), it may therefore seem an attractive possible mechanism to explain interferon-λ deficiency in asthma. However, LPS is a ligand for TLR4(14, 39, 40) and TLR3 mediates interferon-λ production(13) and anti-rhinoviral activity(12) in respiratory epithelial cells, while interferon-λ induction via TLR3 and 4 was IRAK-4 independent(30), thus IRAK-4 deficient signalling appears an unlikely explanation for the deficiencies we have observed in asthma.

TLRs activate downstream effectors through numerous other adaptors including MyD88, IRAK-1, Toll/IL-1 receptor domain-containing adaptor-inducing interferon-β (TRIF), TNF receptor-associated factor (TRAF) 6, as well as downstream kinases. TRAF3 has recently been shown to be an important component of both TLR-dependent and TLR-independent viral recognition pathways(41, 42). Deficiency in any of these signalling intermediates or of transcription factors involved in the induction of the interferon-β gene (involving NF-□B, IRF-3 and ATF/c-Jun transcription factors)(43-45) could potentially explain deficient production of interferon-β in asthma. However, with the exception of induction via TLR7-9 in blood cells and fibroblasts being known to be IRAK-4 dependent(30), the signalling intermediates and transcription factors mediating induction of the interferon-λs by virus infections are unknown, as are the mechanisms of induction of these interferons and interferon-β in response specifically to rhinovirus infection of bronchial epithelial cells. These mechanisms now require careful exploration.

Another possible explanation could involve impaired innate interferon responses in asthma being due to inadequate maturation of these responses consequent upon reduced exposure to infectious disease in early life. Little is known regarding development of type I and type III interferon responses but reduced exposure to infectious disease in early life has been implicated in the

increasing prevalence of asthma(46, 47). Related to this, wheezing is much more common in children than in adults(48), and more wheezing in children is related to virus infections(18-20). These data suggest that the deficiencies we have observed in adult asthma, could be even more profound in children and may help to explain the increased incidence of virus induced wheeze in children. Studies investigating these possibilities are required.

In conclusion, our findings identify a potentially important and novel mechanism in the pathogenesis of asthma exacerbations and in increased susceptibility to viral and perhaps bacterial infections in asthma, suggest new approaches to prevention and/or treatment of asthma exacerbations and open new avenues for investigation to increase our understanding of disease pathogenesis as well as reasons for its increasing prevalence.

Methods

BEAS-2B and virus culture

BEAS-2B bronchial epithelial cells (European Collection of Cell Cultures [ECACC]), were grown in RPMI 1640 media supplemented with Glutamax (Invitrogen, Paisley UK), with 10% foetal calf serum (FCS) buffered with 1% sodium bicarbonate and 2.5% HEPES (all Invitrogen) and grown in a humidified incubator at 37ºC with 5% CO_2. Major group rhinovirus serotypes rhinovirus-16 and rhinovirus-9 and minor group serotype rhinovirus-1B were grown in Ohio HeLa cells (ECACC) and stocks prepared as HeLa lysates at $1x10^7$ $TCID_{50}$/mL(10). The identities of each rhinovirus were confirmed by neutralisation using serotype specific antibody (ATCC). UV inactivation and filtration of virus were performed as previously described(10).

Rhinovirus infections and antiviral assay

BEAS-2B cells were seeded at $1.7x10^5$ cells/mL, then placed in RPMI 1640 media supplemented with 5% FCS (all Invitrogen) overnight, prior to infection with rhinovirus-16, rhinovirus-9 or rhinovirus-1B (multiplicity of infection [MOI] of 2 unless otherwise stated), for 1 hour at room temperature with shaking. Virus preparations were removed and 1 mL fresh medium added. Plates were incubated at 37ºC. Both cell lysates and supernatants were harvested at various time points and stored at –80°C. To investigate the antiviral effect of interferon-λ1, BEAS-2B cells were infected with rhinovirus-16 for 1 hour, washed and medium replaced with or without different doses of interferon-λ1 (Peprotech). Cells and supernatants were harvested at 24 hours. Viral RNA in cell lysates was measured by TaqMan® RT-PCR and infectious virus in supernatants measured by viral titration in Ohio-HeLa cells.

Biological effect of interferon-λ

To investigate the biological effects of interferon-λ1, BEAS-2B cells were seeded and treated for 24hrs with or without different doses of interferon-λ1 (Peprotech) and supernatants harvested for ELISA. To investigate the biological effects of interferon-λ1 with rhinovirus infection, BEAS-2B cells were seeded and treated with medium alone or infected with rhinovirus-16 for 1 hour, washed and medium replaced for 24hrs with or without different doses of interferon-λ1 (Peprotech) and supernatants harvested for ELISA.

Quantitative RT-PCR

Total RNA was extracted (RNeasy Kit, Qiagen) and 2μg used for cDNA synthesis (Omniscript RT kit, Qiagen). Quantitative PCR was carried out using specific primers and probes for rhinovirus (sense: 5'-GTG AAG AGC CSC RTG TGC T-3' 50nM, antisense 5'-GCT SCA GGG TTA AGG TTA GCC-3' 300nM, probe-5'-FAM-TGA GTC CTC CGG CCC CTG AAT G-

TAMRA-3' 175nM), interferon-λ1 (sense**:** 5'- GGA CGC CTT GGA AGA GTC ACT -3' 300nM, antisense: 5'- AGA AGC CTC AGG TCC CAA TTC -3' 900nM, probe 5'-FAM AGT TGC AGC TCT CCT GTC TTC CCC G TAMRA-3' 175nM), interferon- λ2/3 (sense: 5'- CTG CCA CAT AGC CCA GTT CA -3' 300nM, antisense: 5'- AGA AGC GAC TCT TCT AAG GCA TCT T -3' 900nM, probe: 5'-FAM TCT CCA CAG GAG CTG CAG GCC TTT A TAMRA-3' 175nM) and 18S rRNA (sense: 5'- CGC CGC TAG AGG TGA AAT TCT -3', antisense: 5'- CAT TCT TGG CAA ATG CTT TCG-3' 300nM each, probe: 5'-FAM ACC GGC GCA AGA CGG ACC AGA TAMRA-3' 175nM). Reactions consisted of 2 μL of cDNA (cDNA for 18S was diluted 1:100), 12.5μL 2x QuantiTect Probe PCR Master Mix (Qiagen). Reactions were performed on an ABI 7000 TaqMan® (ABI Foster City CA), interferon-λ and viral RNA expression were normalised to 18S rRNA and compared to standard curves and expressed as copies per μg RNA.

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells were obtained from whole blood from healthy volunteers by gradient centrifugation in lymphoprep (Histopaque 1077 – Sigma). They were washed and resuspended in RPMI-1640 medium with Glutamax (Life Technologies) containing 10% FCS and antibiotics (penicillin, streptomycin) at a final concentration of $2x10^6$/mL in 6-well plates. Rhinovirus-16 was added at final a concentration of 5 MOI. After 1 hour of shaking at room temperature, cultures were placed in a humidified 5% CO_2 incubator at 37°C. Supernatants were harvested at various time points, clarified by centrifugation, and stored at –80°C until assayed. Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. This study was approved by St Mary's NHS Trust Ethics Committee and all subjects gave informed consent.

Monocyte-derived macrophages

Monocytes, isolated from peripheral blood mononuclear cells by positive selection using anti-CD14 magnetic beads (MACS), were differentiated into monocyte derived macrophages by culture in 12-well plates for 7 days in macrophage serum-free-medium (M-SFM) (Invitrogen) supplemented with 10ng/mL GM-CSF (Biosource). Macrophages were exposed to rhinovirus-16 at a MOI of 2 or UV inactivated virus with continuous shaking. After 1 hour cells were washed extensively and 1ml of M-SFM was added to each well. After 24 hours supernatants were harvested and stored at -80°C for analysis.

Primary bronchial epithelial cell tissue culture

Primary bronchial epithelial cells from 9 atopic asthmatic and 9 normal volunteers were obtained by fibreoptic bronchoscopy and cell culture was performed as previously described(1). Normal subjects had no history of respiratory disease, were non-atopic and had no bronchial hyperresponsiveness (PC_{20} histamine >8 mg/mL). Among the 9 asthmatic patients all were atopic (skin prick test positive) and with bronchial hyperresponsiveness (PC_{20} histamine $\leq$8 mg/mL), 2 were steroid naïve and seven were on regular inhaled corticosteroids and 2 had mild intermittent, 4 mild persistent, 2 moderate persistent and 1 severe persistent asthma according to international guidelines(1). Demographic details and clinical characteristics of included subjects are given in Table 1. All were non-smokers. Primary cultures were established by seeding freshly brushed bronchial epithelial cells into hormonally supplemented bronchial epithelial growth medium (BEGM; Clonetics, San Diego, USA) containing 50U/mL penicillin and 50µg/mL streptomycin. At passage 2 cells were seeded onto 12 well trays and cultured until 80% confluent(1) before

exposure to rhinovirus-16 at an MOI of 2. Cell lysates were harvested after 8 hours incubation and total RNA extracted using RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Supernatants were harvested at 48 hours post infection, clarified by centrifugation, and stored at –80°C until assayed. This study was approved by Southampton University Hospitals Ethics Committee and all subjects gave informed consent.

Experimental rhinovirus infection of asthmatic and normal subjects

Outline of experimental design

Six atopic asthmatic and 8 non-atopic normal, rhinovirus-16 serum neutralising antibody negative subjects were recruited to this study, all were non smokers, demographic details and clinical characteristics are provided in Table 1. All asthmatic and no normal subjects had bronchial hyperresponsiveness. Subjects underwent bronchoscopy with bronchoalveolar lavage at baseline and bronchoalveolar lavage cell pellets were incubated with rhinovirus-16 or bacterial LPS to determine interferon-λ production. Two weeks later, the same subjects underwent experimental rhinovirus inoculation. One asthmatic patient withdrew before experimental rhinovirus inoculation. Diary card recordings were performed daily from screening to 2 weeks after infection to document common cold symptoms. Daily spirometry was performed from screening to 2 weeks to document reductions in lung function measured as forced expiratory volume in 1 second (FEV_1). Bronchoalveolar lavage was repeated on day 4 after the acute infection and virus load determined by quantitative PCR. Subjects were free of common cold symptoms for 6 weeks before commencing the study. This study was approved by St Mary's NHS Trust Ethics Committee and all subjects gave informed consent. Full details of the experimental design and validation of this human experimental model of acute exacerbations of asthma are described elsewhere (SDM, manuscript in preparation).

Experimental infection with rhinovirus-16

Experimental infection was induced using 10,000 tissue culture infective doses 50% ($TCID_{50}$) of a safety tested rhinovirus-16 inoculum (49) by nasal spray, with a DeVillbiss 286 atomizer. 2 aliquots of 500μL (each 2,500 $TCID_{50}$) were applied to each nostril. Infection was confirmed by culture of post inoculation nasal lavage in Ohio HeLa cells and/or by positive serology (a four fold rise in neutralizing antibody titer between pre-inoculation sample and convalescent sample taken at 6 weeks post infection). Cultured viruses were confirmed as rhinovirus-16 by neutralisation of virus with rhinovirus-16 specific antiserum (ATCC).

Recording of common cold symptoms

A total common cold score for the 2 week period post virus inoculation was derived from diary card recordings maintained by the subject from screening. This score was corrected for baseline symptoms and for the effects of bronchoscopy. Symptoms recorded daily were sneezing, headache, malaise, chilliness, nasal discharge, nasal obstruction, sore throat, cough, fever, each graded 0-3 for absent, mild, moderate or severe.

Lung Function Testing

Subjects recorded daily morning FEV_1 on a portable microDL spirometer (MicroMedical) at home from screening. Data was analysed using Spida software. Reductions in lung function were expressed as the maximum fall in FEV_1 during the 2 weeks post virus inoculation expressed as the % change in FEV_1 from the mean obtained during the baseline stage, corrected for the effects of bronchoscopy. Full details of the derivation of total cold scores and lung function assessment of this human experimental model of acute exacerbations of asthma are described elsewhere (SDM, manuscript in preparation).

Bronchoscopy

Bronchoscopies were performed using a Keymed P100 bronchoscope. Bronchoalveolar lavage was performed by instillation of sterile normal saline (room temperature) into the right middle lobe bronchus in 8x30mL aliquots. Bronchoalveolar lavage was collected in a single plastic chamber and transferred immediately to polypropylene tubes on ice for transport to the laboratory for determination of rhinovirus virus load by quantitative PCR (as described above) in unprocessed lavage. One asthmatic volunteer recorded symptoms and lung function but did not have a successful bronchial lavage, so no virus load was determined for this subject.

Bronchoalveolar lavage cell cultures

Cells from the bronchoalveolar lavage obtained at baseline bronchoscopy were washed and resuspended in RPMI-1640 medium with Glutamax (Life Technologies, Uxbridge, UK) containing 10% FCS and antibiotics (penicillin, streptomycin), at a final concentration of $2x10^6$/mL. Cells were incubated with 5 MOI rhinovirus-16, with inoculum from which the virus had been removed by molecular weight filtration(10) or with 0.1μg/mL of *E. Coli* 026:B6 LPS (Sigma). After 48 hours supernatants were harvested and stored at -80°C for ELISA for interferon-λ.

Statistical analysis

Data for multiple comparisons were first analysed by ANOVA. If significant ($p<0.05$), differences between groups/conditions were then analysed using paired or unpaired *t*-tests as appropriate. Linear regression analysis was used when appropriate and the Pearson test to investigate correlations. All the analyses were performed using GraphPad Prism 4.0 for Windows. Data are expressed as mean and standard error of the mean.

Table 1. Demographic data for patients entering the clinical studies

	Asthma	Healthy controls	p-value
Primary bronchial epithelial cell study			
Number	9	9	NA
Sex (percent male)	55.5	66.6	p=0.3
Mean age (range)	31.2 (21-50)	27.8 (23-31)	p=0.4
Mean $FEV_{1\%}$ predicted (SEM)	84.1 (2.3)	108 (4.4)	p<0.001
Mean dose of ICSs, BDP μg/day (SEM)	411.1 (118.4)	0	NA
Experimental rhinovirus infection study			
Number	6	8	NA
Sex (percent male)	16.7	50	p=0.2
Mean age (range)	23.2 (19-31)	27.8 (23-31)	p=0.5
Mean $FEV_{1\%}$ predicted (SEM)	104.3 (3.9)	100.4 (7.1)	p=0.6
Histamine PC_{20} mg/mL (SEM)	3.1 (0.9)	13.9 (0.9)	p<0.001

FEV_1: forced expiratory volume in 1 second

SEM: standard error of the mean

ICS: inhaled corticosteroids

BDP: beclomethasone dipropionate equivalents

PC_{20}: provocative concentration causing a 20% fall in FEV_1

Figures and legends

Figure 1

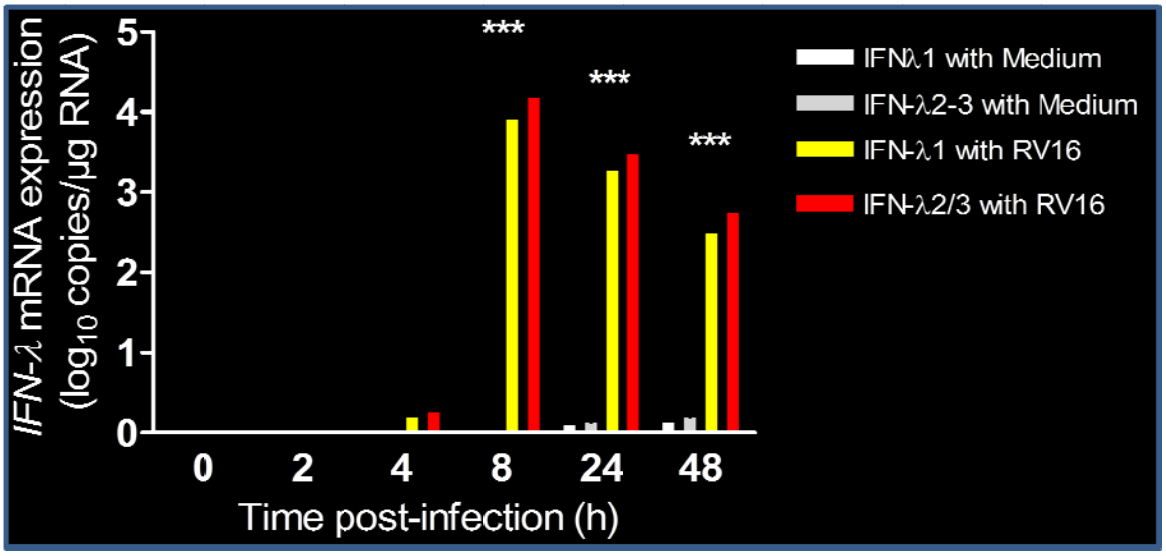


Figure 1 Interferon lambdas are induced by rhinovirus infection of bronchial epithelial cells

Time course of rhinovirus induction of interferon-λ mRNA expression in BEAS-2B cells. BEAS-2B bronchial epithelial cells were infected with rhinovirus (RV)-16 at time zero and the time course of induction of interferon-λ1 and interferon-λ2/3 studied in cell lysates at indicated time points post infection by quantification of mRNA expression by quantitative PCR. Rhinovirus significantly induced expression of each interferon subtype peaking at 8 hours post infection and induction remained significant at both 24 and 48 hours. Data are mean and standard error of 5 experiments. *** indicates $p<0.001$ for both interferon-λ1 and interferon-λ2/3 compared with medium control.

Figure 2

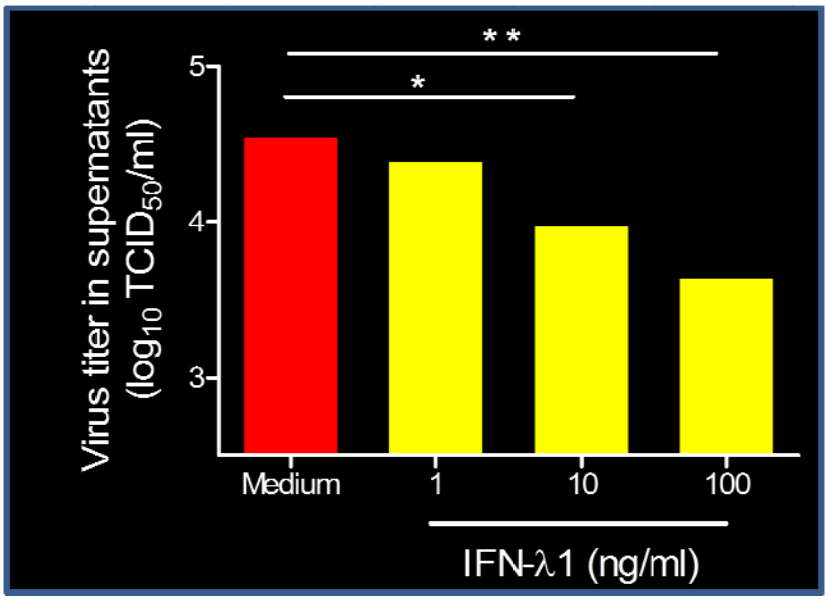
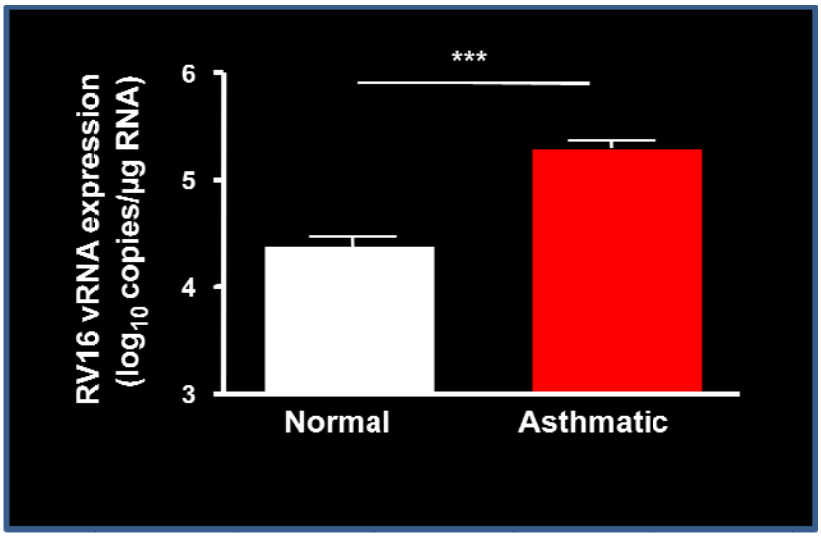


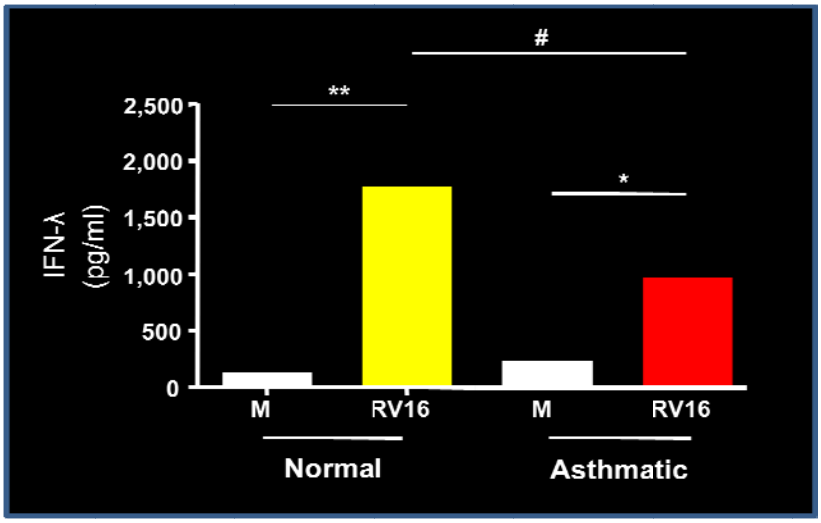
Figure 2 Interferon lambdas induce protect bronchial epithelial cells against rhinovirus infection *in vitro*

Interferon-λ1 inhibits rhinovirus RNA production in BEAS-2B bronchial epithelial cells. BEAS-2B cells were pre-treated for 24 hours with indicated doses of interferon-λ1 or medium control before infection with rhinovirus-16. Viral RNA in cell lysates was measured by quantitative PCR at 24 hours after infection. Data are mean and standard error of 4 experiments. * indicates $p<0.05$ and ** $p<0.01$ compared with medium control.

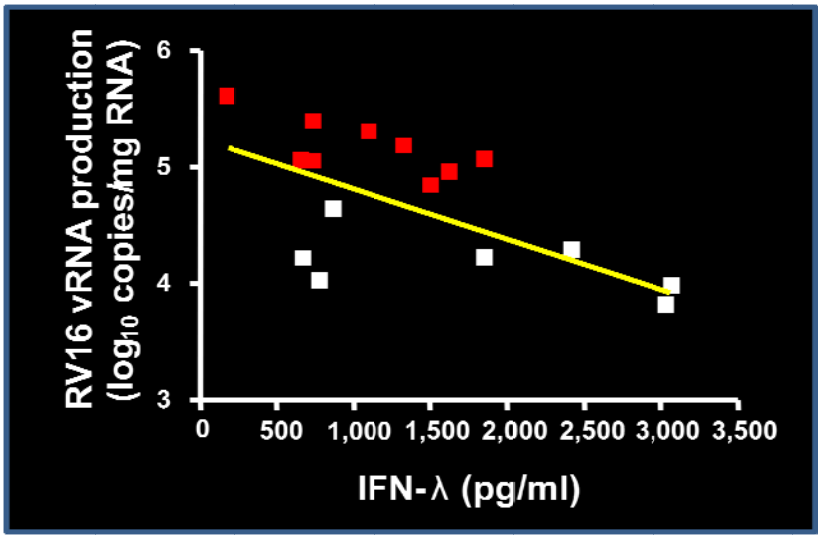
Figure 3


***
RV16 vRNA expression
(log10 copies/µg RNA)
6
5
4
3
Normal
Asthmatic

A


#
**
*
IFN-λ
(pg/ml)
2,500
2,000
1,500
1,000
500
0
M
RV16
M
RV16
Normal
Asthmatic

B



C

Figure 3 Deficient interferon-λ production in primary human bronchial epithelial cells from asthmatic compared to normal subjects and relationship to increased rhinovirus replication *in vitro*

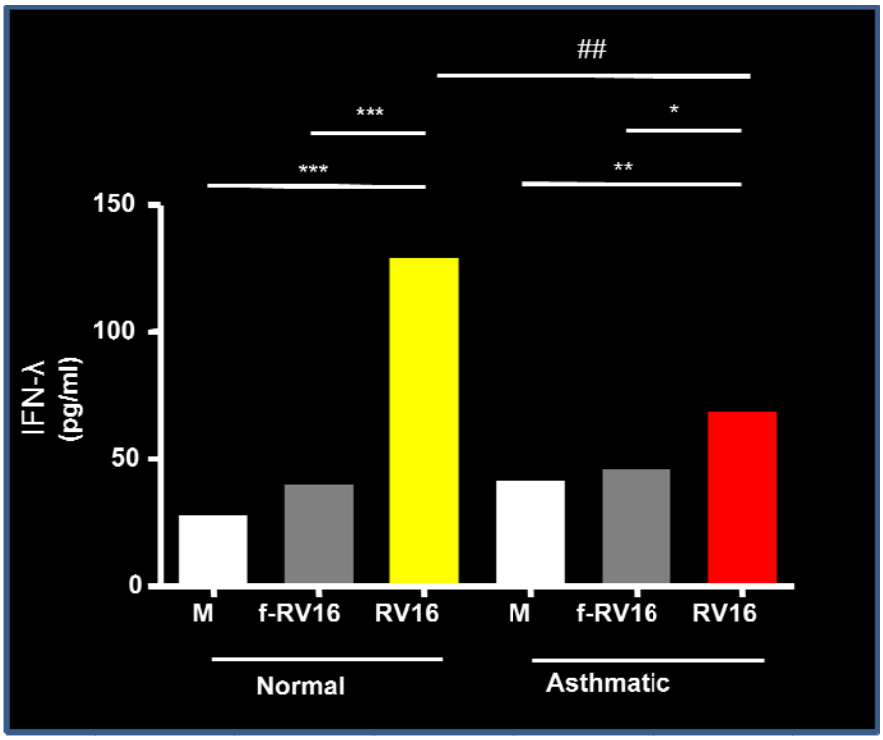
(a) Rhinovirus replication is increased in primary bronchial epithelial cells from asthmatic compared to normal subjects. Primary bronchial epithelial cells from asthmatic and normal subjects were infected with rhinovirus-16 and viral RNA expression determined by quantitative PCR at 8 hours after infection. As previously reported {Wark, 2005 #682}, virus replication was significantly increased in cells from asthmatic subjects. ***$p<0.001$ compared to normal subjects.

(b) Rhinovirus infection induces interferon-λ1 and interferon-λ2/3 mRNA expression in primary bronchial epithelial cells and induction of both is deficient in asthmatic compared to normal subjects. Primary bronchial epithelial cells from asthmatic and normal subjects were infected with rhinovirus-16, UV-inactivated virus or medium control (M) and interferon-λ1 and interferon-λ2/3 mRNA expression determined by quantitative PCR at 8 hours after infection. Expression of both interferon-λ subtypes was significantly increased in infected cells in a

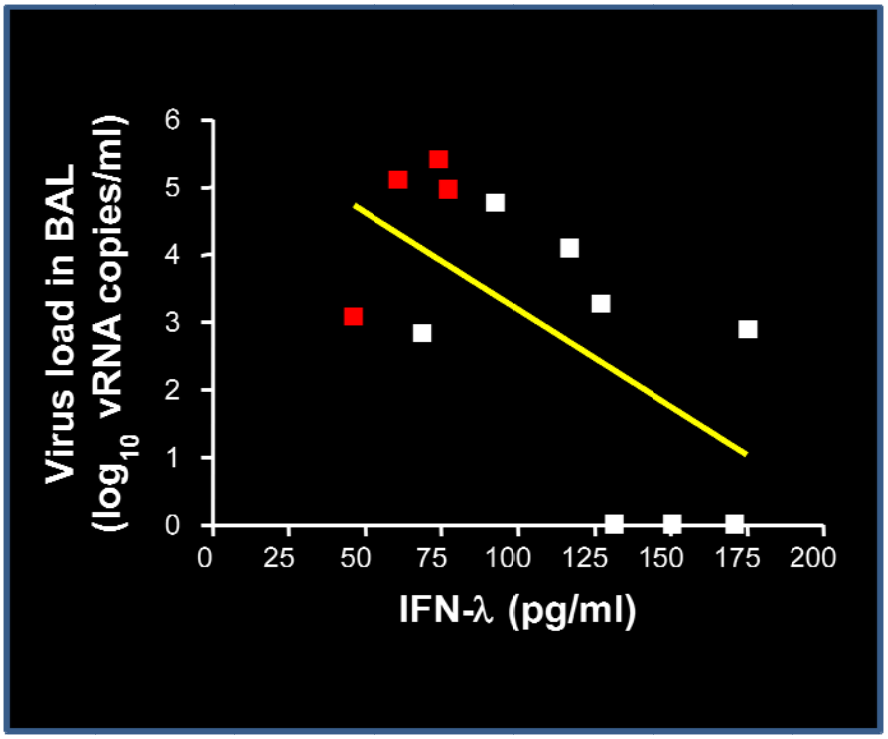
replication dependent manner in both normal and asthmatic subjects. However levels of expression of both interferon-λ subtypes induced by infection were significantly lower in cells from asthmatic subjects. ***$p<0.001$ compared to medium control and UV-inactivated virus. ###$p<0.001$ compared to normal subjects.

(C) Rhinovirus induction of interferon-λ protein is strongly related to rhinovirus replication in primary bronchial epithelial cells. Levels of interferon-λ protein production induced by rhinovirus infection of primary bronchial epithelial cells was strongly inversely related ($r^2=0.46$, $p<0.01$) to levels of viral RNA in the cell lysates from the same experiments. Red squares = asthmatic and white = normal subjects.

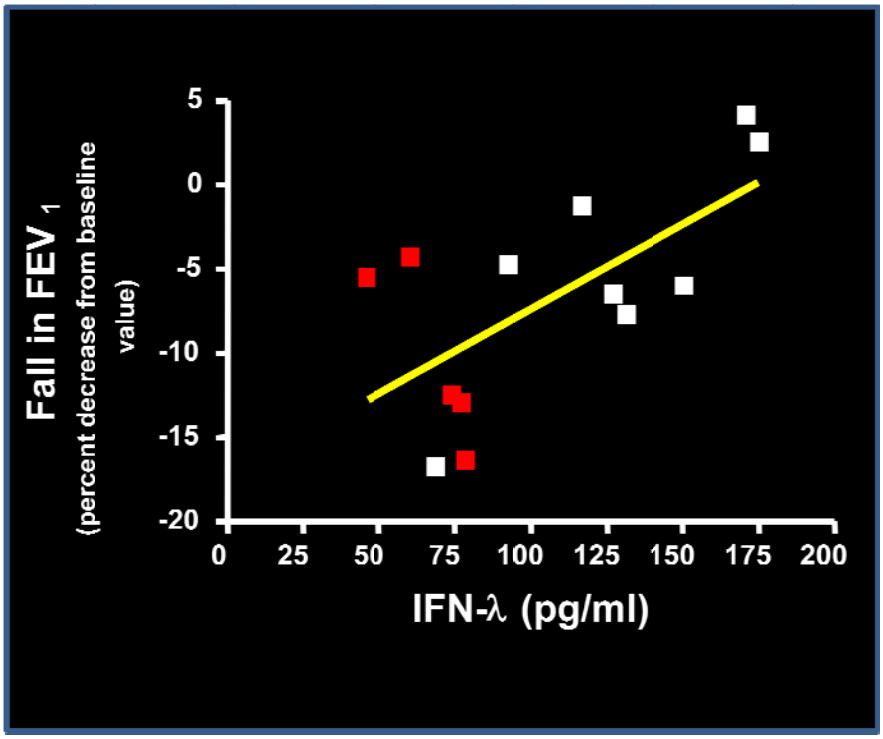
Figure 4


##
***
*
***
**
150
100
50
0
IFN-λ (pg/ml)
M
f-RV16
RV16
M
f-RV16
RV16
Normal
Asthmatic

A


Virus load in BAL (log10 vRNA copies/ml)
6
5
4
3
2
1
0
0
25
50
75
100
125
150
175
200
IFN-λ (pg/ml)

B



C

Figure 4 Deficient rhinovirus induced interferon-λ production from bronchoalveolar lavage cells from asthmatic compared to normal subjects and relationship virus load *in vivo*

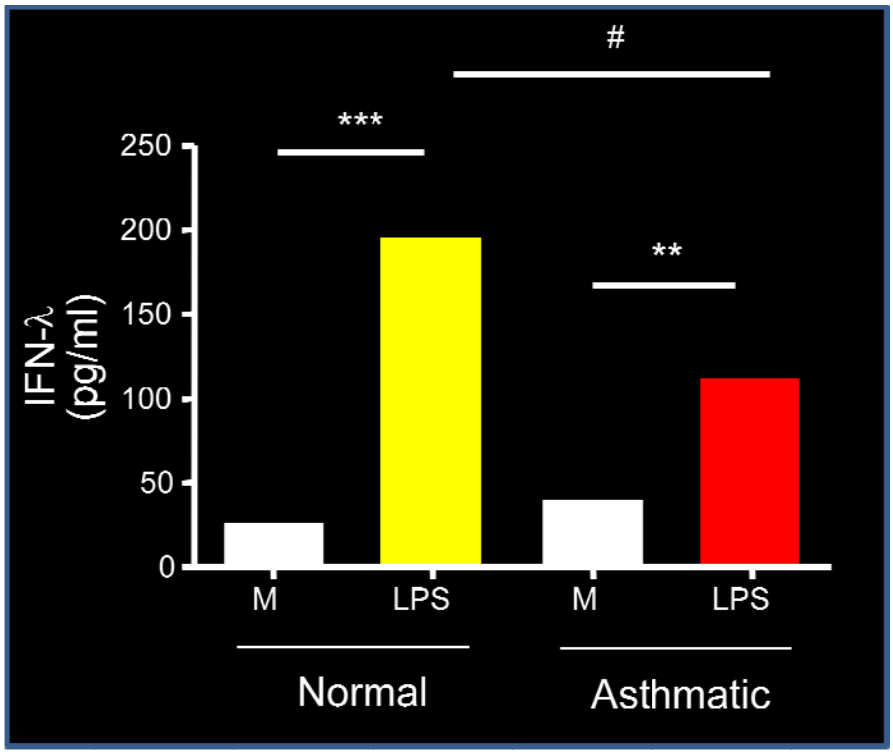
(a) Rhinovirus infection induces interferon-λ protein production in bronchoalveolar lavage cells and induction is deficient in asthmatic compared to normal subjects. Bronchoalveolar lavage cells from asthmatic and normal subjects were infected with rhinovirus-16 or inoculated with virus stock from which virus had been removed by molecular weight filtration (f-RV16) or medium control (M) and interferon-λ protein production determined in supernatants by ELISA at 48 hours after infection. Production of interferon-λ was significantly increased in infected cells in both normal and asthmatic subjects. However production of interferon-λ induced by infection was significantly lower in cells from asthmatic subjects. ***p<0.001, **p<0.01 and *p<0.05 compared to medium control or f-RV-16. ##p<0.01 compared to normal subjects.

(B) Rhinovirus induction of interferon-λ protein by bronchoalveolar lavage cells is strongly inversely related to virus load on subsequent *in vivo* rhinovirus experimental infection.

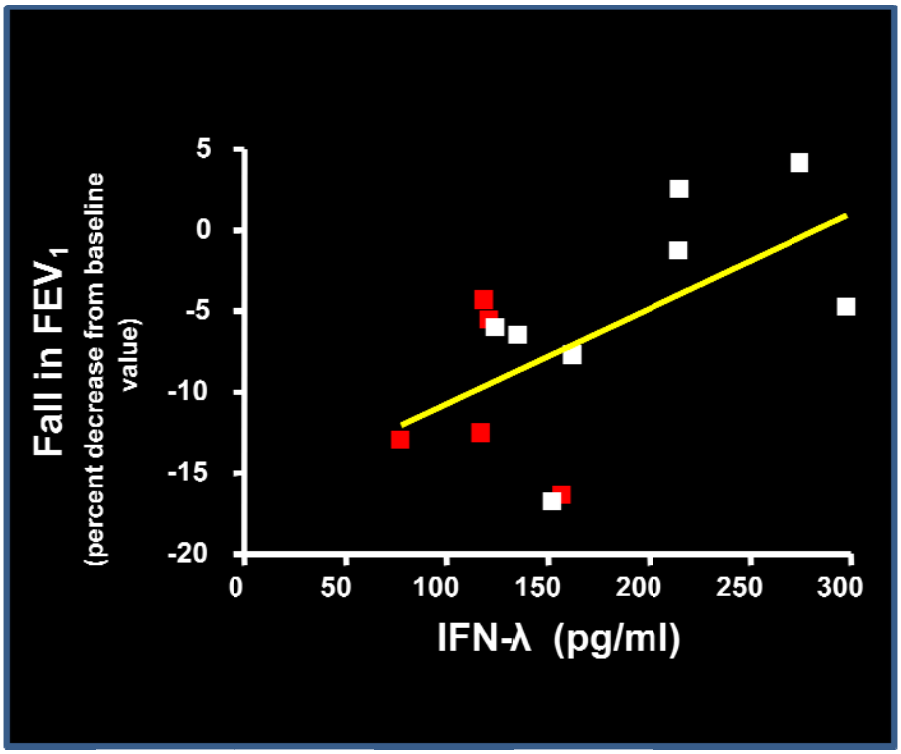
Interferon-λ protein production in bronchoalveolar lavage cells infected *in vitro* with rhinovirus-16, was significantly inversely correlated with bronchoalveolar lavage virus load determined on day 4 of the acute infection period when subjects were subsequently experimentally infected with rhinovirus-16 *in vivo* (r^2 0.44, $p<0.05$). Red squares = asthmatic and White = normal subjects.

(C) Rhinovirus induction of interferon-λ protein by bronchoalveolar lavage cells is strongly related to severity of reduction in lung function on subsequent *in vivo* rhinovirus experimental infection. Interferon-λ protein production in bronchoalveolar lavage cells infected *in vitro* with rhinovirus-16, was significantly inversely correlated with severity of maximal reduction from baseline in FEV_1 recorded over the 2 weeks infection period when subjects were subsequently experimentally infected with rhinovirus-16 *in vivo* (r^2 0.43, $p<0.05$). Red squares = asthmatic and White = normal subjects.

Figure 5


#
***
**
250
200
150
100
50
0
IFN-λ (pg/ml)
M
LPS
M
LPS
Normal
Asthmatic

A


Fall in FEV1
(percent decrease from baseline value)
5
0
-5
-10
-15
-20
0
50
100
150
200
250
300
IFN-λ (pg/ml)

B

Figure 5 Deficient LPS induced interferon-λ production from bronchoalveolar lavage cells from asthmatic compared to normal subjects and relationship to lung function *in vivo*

(a) LPS induces interferon-λ protein production in bronchoalveolar lavage cells and induction is deficient in asthmatic compared to normal subjects. Bronchoalveolar lavage cells from asthmatic and normal subjects were stimulated with LPS or medium control (M) and interferon-λ protein production determined in supernatants by ELISA at 48 hours after infection. Production of interferon-λ was significantly increased in cells stimulated with LPS in both normal and asthmatic subjects. However production of interferon-λ induced by LPS was significantly lower in cells from asthmatic subjects. ***$p<0.001$ and **$p<0.01$ compared to medium control. #$p<0.05$ compared to normal subjects.

(b) LPS induction of interferon-λ protein in bronchoalveolar lavage cells is strongly related to severity of reduction in lung function on subsequent *in vivo* rhinovirus experimental infection. Interferon-λ protein production in bronchoalveolar lavage cells stimulated *in vitro* with LPS, was significantly inversely correlated with severity of maximal reduction from baseline in FEV_1 recorded over the 2 weeks infection period when subjects were subsequently experimentally infected with rhinovirus-16 *in vivo* (r^2 0.40, $p<0.05$). Red squares = asthmatic and White = normal subjects.

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Respiratory virus production of alpha-, beta- and lambda-interferons in bronchial epithelial cells: comparison of patterns of induction

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Background and Aims

Respiratory viruses, are the major cause of asthma exacerbations. Impaired production of interferon-β and of interferon-λs is implicated in asthma exacerbation pathogenesis. Thus replacement of deficient interferon is a candidate new therapy for asthma exacerbations. Rhinoviruses and other respiratory viruses infect bronchial epithelial cells (BECs), but their relative capacities for α-, β- and λ-interferon production are unknown.

Aim: To provide guidance regarding which interferon type is the best candidate for development for treatment/prevention of asthma exacerbations we investigated respiratory virus induction of α-, β- and λ-interferon in BECs by reverse transferase-polymerase chain reaction and enzyme-linked immunosorbent assay (ELISA).

Asthmatic individuals are more susceptible to naturally occurring rhinovirus (RV) infection than normal individuals (1) and it has been recently reported that primary bronchial epithelial cells (BECs) from asthmatic subjects exposed to RV in vitro had profoundly impaired production of interferon (IFN)-β compared to normal bronchial epithelial cells (2) . In these studies, normal BECs were almost completely resistant to RV infection, and impaired IFN-β production in asthmatic cells was strongly correlated with increased RV replication. Restoring IFN-β responses with exogenous IFN-β in asthmatic cells restored antiviral activity and rendered asthmatic cells as resistant to infection as normal ones (2) . These studies suggest IFN-β could be an effective therapy for RV-induced asthma exacerbations. We have recently demonstrated that type III IFN-λs are also produced by human BECs on infection with RV and are antiviral against RVs in vitro (3) . Further, they are also produced by RV-infected bronchoalveolar lavage (BAL) cells (~90% macrophages) and production by both BAL cells and BECs was deficient in asthmatic compared to normal subjects (3) . Importantly, IFN-λ production was strongly inversely correlated with severity of clinical illness, and with virus load and airway inflammation, when subjects were experimentally infected with RV in vivo (3) . These data strongly implicate type III IFN deficiency in the pathogenesis of asthma exacerbations.

These studies combined suggest that administration of IFN-β or IFN-λs, is likely to be an attractive approach to prevention and/or therapy of virus induced asthma exacerbations. However, little is known about the types of IFNs produced by virus infected BECs, nor about the relative contributions of the different types of IFNs in response to rhinoviruses. We have recently shown that BECs induces both IFN-β (2) and IFN-λ (3) .

We have therefore investigated respiratory virus induction of type I and type III IFNs in the BEAS-2B BEC line and in primary BECs. We elected to study RVs as the most common virus type implicated in asthma exacerbations, and influenza virus type A as a respiratory virus known

to suppress type I IFN responses, as the patterns of induction could be different for this virus type.

Materials and methods

Primary BECs and BEAS-2B tissue culture

All cells were cultured at 37°C in 5% CO2. Primary BECs obtained from three different donors were purchased from Cambrex, USA. Primary cultures were established by seeding bronchial epithelial cells into supplemented bronchial epithelial growth medium (BEBM) according to the manufacturer_s instructions (Cambrex). Cells were seeded onto 12 well trays and cultured until 80% confluent before exposure to virus. The human bronchial epithelial cell line BEAS-2B (ECACC) was cultured in RPMI-1640 supplemented with 10% FCS (Invitrogen). BEAS-2B cells were cultured in 12-well tissue culture plates (Nalge Nunc) for 24-h before being placed into 2% FCS RPMI medium for a further 24-h prior to infection.

Viral stocks

Rhinovirus serotypes 16 and 1B obtained from the Medical Research Council Common Cold Unit were grown in Ohio HeLa cells and prepared as previously described (4) . Viral stocks were used at $1 \cdot 10^7$ TCID50/ml. The identities of all RVs were confirmed by titration on HeLa cells and neutralization using serotype-specific antibodies (ATCC). Ultraviolet (UV) inactivation was performed as previously described (5) . Influenza A Victoria 75/3 (gift from Peter Morley, GSK, Stevenage) was propagated in MDCK cell cultures (gift from Peter Morley). At 80% confluence cell cultures were washed twice with sterile PBS and then growth medium replaced with serum free eagle minimum essential medium (Invitrogen). 0.5 ml of influenza stock was added to each

flask and incubated for 1 h. Cells were then washed to remove any non-adherent virus and resuspended in serum free medium for culture for 48 hours. Two days after infection, supernatants were collected from flasks, aliquotted and stored at -80°C for future use. Viruses were titrated on MDCK cells to determine TCID50/ml. Stock was assessed as being at 10^7 TCID50/ml.

Infection of cells with RV and influenza virus

BEAS-2B cells were seeded in 12 well plates (Nunc, Roskilde, Denmark) at $1.7 \cdot 10^5$ cells/ml, allowed to attach and grow for 24 h. BEAS-2B were then placed in RPM1 1640 + 2% FCS (infection media) overnight. Cells were then treated with RV16 or RV1B [multiplicity of infection (MOI) of 1] for 1 h at room temperature with shaking. Cells supernatants and RNA lysates were harvested at the times indicated. Supernatants and lysates were stored at -80°C until required. The same protocol was used for infecting cells with Influenza A Victoria 75/3 virus. Virus was used at a MOI of 1.

RNA extraction, reverse transcription and TaqMan real-time PCR

RNA was extracted from cells using the RNeasy method (RNeasy Mini Kit; Qiagen) following the manufacturer_s instructions, including the optional DNaseI digestion of contaminating DNA (Dnase (Rnase free Dnase); Qiagen). cDNA was synthesized using Omniscript RT and components as directed by the manufacturer (Qiagen). Primers specific for IFN-αs, IFN-β, IFN-λ1, IFN-λ2/3 were purchased from Invitrogen and probes from Qiagen (Table 1). TaqMan analysis of -α, -β and -λ interferon mRNA was normalized with respect to 18s rRNA and presented as $\log_{10}$-fold induction relative to medium control. Reactions consisted of 12.5 µl 2 x QuantiTect Probe PCR Master Mix (Qiagen) and 300 nM of forward primer and 900 nM of

reverse primer for IFN-α.1, 300 nM of each primer for IFN-α.2, 300 nM and 900 nM for IFN-β, IFN-λ1 and IFN-λ2/3, 300 nM of each primer for 18s; 175 nM of each specified probe was used. Two microliters of cDNA (18s diluted 1/100) was made up to 25 ll with nuclease-free water (Promega). The reactions were analysed using an ABI7000 Automated TaqMan (Applied Biosystems). The amplification cycle consisted of 50°C for 2 min, 94°C for 10 min and 40 cycles of 94°C for 15 s, 60°C for 15 s.

Enzyme-linked immunosorbent assay to evaluate IFN-α, IFN-β and IFN-λ

Interferon-α and IFN-β proteins were quantified by enzyme-linked immunosorbent assay (ELISA) in supernatants from untreated and infected cell cultures collected and stored at -80°C using commercially available paired antibodies and standards, following the manufacturer's instructions using a high sensitivity IFN-α human Biotrak ELISA (Amersham Biosciences), and a human IFN-β ELISA kit (Biosource). All the measurements were done according to manufacturer‘s instructions. The detection limits for described assays are 0.63 pg/ml for IFN-α, 2.5 pg/ml for IFN-β. To measure interferon-λs we developed an assay using a monoclonal anti-human IL-29/IFN-λ1 antibody as capture, a polyclonal anti-IL-29 antibody as secondary and biotin conjugated donkey anti-goat IgG as third antibody (all R&D Systems) followed by streptavidin conjugated HRP (Biosource). Recombinant human IL-29 (Peprotech) was used as standard. The sensitivity of the assay was 25 pg/ml. The assay for IFN-lambda detection detects IL-29 protein but also detects some IL-28 as there is 25% cross-reactivity with IL-28.

Statistical analysis

Data are presented as mean (SEM). For time course experiments data were analysed using one-way anova for repeated measures followed by paired t-tests between baseline and individual time

points where appropriate. For other comparisons paired t-tests and Bonferroni's multiple comparison post hoc test were used. Data were accepted as significantly different when $p<0.05$.

Results

Rhinovirus induction of type I and type III interferons in BEAS-2B BECs

The time course of expression of type I and type III IFN mRNAs was studied during infection of BEAS-2B cells with RV-16. There was no significant induction by RV-16 of IFN-α subtypes 1, 6 or 13 detected by IFN-α.1 at either 4, 8 or 24 h, however, subtypes 2, 4, 5, 8, 10, 14, 17 and/or 21, detected by IFN-α.2, were significantly induced in comparison to medium at 8 h [2.74 (0.3) log10-fold induction compared to medium, $P < 0.001$], but not at 4 or 24 h . IFN-β mRNA expression was induced by RV-16 only at 24 h [3.22 (0.33), $P < 0.001$], while IFN-λ1 mRNA expression was statistically significantly increased by RV-16 at 8 h [3.1 (0.75), $P < 0.01$] and further increased at 24 h [4.84 (0.24), $P < 0.001$]. We next investigated whether the induction of IFN mRNAs by RV-16 infection of BEAS-2B cells leads to production of detectable levels of type I and type III IFN proteins at 24 h after infection. We detected no significant induction of IFN-α protein, but IFN-β protein production was significantly increased [19.8 (2.3) pg/ml, $P < 0.01$, Fig. 1C] while IFN-λ was produced at greater levels [99.89 (2.03) pg/ml, $P < 0.001$, Fig. 1D] in RV-16 infected BEAS-2B cells in comparison to non-infected cells.

Rhinovirus induction of type I and type III interferons in primary BECs.

Similar to BEAS-2B cells, there was no significant induction by RV-16 of IFN-α subtypes detected by IFN-α.1 at 4, 8 or 24 h, however, subtypes detected by IFN-α.2 were significantly induced in comparison to medium at 8 h [3.06 (0.24) log10-fold induction compared to medium,

P < 0.001] and remained elevated at 24 h, though at 24 h this was not statistically significant. Also similar to BEAS-2B cells, IFN-β mRNA expression was induced by RV-16 only at 24 h [4.1 (0.44), P < 0.001]. IFN-λ2/3 mRNA expression paralleled the increase in IFN-λ1, both being induced by RV-16 at 8 h [IFN-λ2/3 2.75 (0.23), P < 0.001 and IFN-λ1 2.2 (1.23), P = NS] and further induced at 24 h [IFN-λ2/3 4.2 (0.12), and IFN-λ1 5.03 (0.21), both P < 0.001]. These levels of mRNA induction were not sufficient to lead to production of detectable levels of type I and type III IFN proteins at 24 h after infection, as each of α, β, and λ IFNs were undetectable.

Rhinovirus induction of type I and type III interferons in primary BECs is receptor independent and replication dependent

To determine whether RV induction of type I and type III IFNs in primary BECs is receptor restricted or not we next investigated the effect of RV-1B. Similar to RV-16, there was no significant induction by RV-1B of IFN-α subtypes detected by IFN-α.1 at 4, 8 or 24 h, however, subtypes detected by IFN-α.2 were significantly induced in comparison to medium at 8 h [2.96 (0.29) log10-fold induction compared to medium] though these remained significantly elevated at 24 h [3.1 (0.4), both P < 0.05]. Also similar to RV-16, IFN-β mRNA expression was induced by RV-1B only at 24 h [4.12 (0.25), P < 0.01]. IFN-λ2/3 mRNA expression again paralleled the increase in IFN-λ1, both being induced by RV-1B at 8 h [IFN-λ2/3 3.76 (0.19), P < 0.05 and IFN-λ1 1.83 (0.65), P = NS] remaining induced at 24 h [IFN-λ2/3 3.6 (0.2) P < 0.05, and IFN-λ1 5.01 (0.38), P < 0.001,]. Again, these levels of mRNA induction were not sufficient to lead to production of detectable levels of type I and type III IFN proteins at 24 h after infection. We next investigated the effects of live and UV-inactivated RV-16 to determine whether IFN induction was replication dependent. In these experiments UV-inactivated RV-16 induced no type I or type

III IFN mRNA , indicating that RV-induced expression of type I and type III IFNs has a replication dependent mechanism.

Influenza virus induction of type I and type III interferons in primary BECs

Influenza viruses are important respiratory pathogens also implicated in asthma exacerbations (6, 7) , they are also known to suppress type I IFN responses (8-11). We therefore next investigated the effects of influenza virus type A on type I and type III IFNs in primary BECs. Similar to RV induction, there was no significant induction by influenza virus of IFN-α subtypes detected by IFN-α.1 at 4, 8 or 24 h, however, subtypes detected by IFN-α.2 were significantly induced in comparison to medium at 24 h [2.46 (0.16) log10-fold induction compared to medium, $P < 0.01$] though not at earlier time points. Also similar to RV induction, IFN-β mRNA expression was induced by influenza virus only at 24 h [4.16 (0.14), $P < 0.001$] and IFN-λ2/3 mRNA expression again paralleled the increase in IFN-λ1, both being induced by influenza virus at 8 h [IFN-λ2/3 3.48 (0.04), and IFN-λ1 3.13 (0.36), both $P < 0.001$] and remaining elevated at 24 h [IFN-λ2/3 3.84 (0.17) $P < 0.001$, and IFN-λ1 4.97 (0.09), P = NS]. Again, all IFN proteins were undetectable at 24 h in influenza virus infected primary BECs.

Discussion

Here we show that the BEC line BEAS-2B and primary BECs expressed increased levels of mRNAs of α, β, and λ-IFNs in response to RV infection, but that λ-IFNs were the most strongly induced both early and during sustained induction (Figure 1). RV induction of IFN proteins were mostly below detection limits, but when proteins were detected in BEAS-2B cells, IFN-λs were detected at the highest levels. Similar results were obtained with influenza A virus infection of

primary BECs (Figure 2). Respiratory epithelial cells are the major site of RV replication as replication has been confirmed in both BEC lines (4, 5) and primary BECs (2, 3, 12) in vitro, and RV has been detected by in situ hybridization and immunochemistry within the BEC cell layer in bronchial biopsies in vivo. BEC innate responses to RV infection are therefore likely of major importance in protection against RV infections. This interpretation is supported by the recent evidence implicating impaired BEC innate responses in the pathogenesis of rhinovirus induced asthma exacerbations (2, 3) . Our studies indicate both in a cell line and in primary cells, that IFN-λs are likely the most abundant IFN type induced in response to RV infection of BECs. These data would support the development of IFN-λs as candidate therapy for asthma exacerbations, as well as perhaps other viral diseases where BECs are the major site of virus replication. We were unable to detect IFN proteins at 24 h in RV infected primary BECs. Both IFN-α and IFN-β mRNAs were expressed only at low levels (~3–4 logs), protein levels may thus have been below the detection limits of the assays. We believe that the levels of induction of IFN mRNA in BEAS2B cells or primary BECs were not sufficient to generate detectable levels of protein production using the assays available. For both α and β-IFNs it is likely that IFN proteins are quickly taken up by the IFN-αb receptor as the affinity of the IFN AR2 subunit for IFN-α2 alone is high and increased up to 20-fold when complexed with IFN AR1 (13). The affinities of the IFN-λ receptor subunits for the IFN-λs are not known and the assay for IFN-λs was considerably less sensitive (25 pg/ml) than those for type I IFNs (0.63 and 2.5 pg/ml for a and b, respectively), potentially explaining why the IFN-λs were not detectable despite mRNAs being induced to higher levels than the type I IFNs.

Our data indicate that in the context of RV infection of their natural lower respiratory tract host cells (BECs), induction of IFN-λs is likely important in both early induction events, as well as sustained induction consequent upon autocrine/paracrine signalling, however, further studies on

RV infection in BECs and the interplay between type I and type III IFNs will be required to dissect these relationships further. We also investigated influenza virus type A induction of IFNs in primary BECs as this virus type is known to suppress type I IFN production via its NS1 protein (8-11) and influenza induction may therefore differ from RV. The pattern of IFN-λ responses observed with influenza infection of BECs was similar to that observed with RV. However induction of IFN-α was different, as there was no early induction - both IFN-β and subtypes of IFN-α detected by primer/probe IFN-α.2 were only induced late at 24 h. It is likely the lack of an early IFN-αβ response is a consequence of the known action of influenza NS1 in suppressing type I IFN production (8-11). Influenza virus suppresses type I IFN production by inhibition of the transcription factors NF-κB (11, 13-15) and IRF3 (10) which are essential for activation of αβ IFN promoters. Influenza virus is also thought to inhibit type I IFN production by inhibiting double-stranded-RNA (dsRNA)-activated protein kinase (PKR) (8). The early induction of IFN-λs by influenza suggests these IFNs may be less susceptible to the same mechanisms of viral suppression of IFN induction. Further work will be needed to investigate these possibilities.

We conclude that both type I and type III IFNs are induced by RV and influenza infection of BECs, but IFN-λs appear to be the principal IFNs involved in responses to respiratory viruses in BECs.

Table 1

Primer and probe set	Subtypes detected	Sequence of primers and probes	References
IFN-α1	1,6,13	Forward – 5'-CAG AGT CAC CCA TCT CAG CA-3' Reverse – 5'-CAC CAC CAG GAC CAT CAG TA-3' Probe – 5'-FAM ATC TGC AAT ATC TAC GAT GGC CTC GCC TAMRA-3'	25
IFN-α2	2, 4, 5, 8, 10, 14, 17, 21	Forward – 5'-CTG GCA CAA ATG GGA AGA AT-3' Reverse – 5'-CTT GAG CCT TCT GGA ACT GG-3' Probe – 5'-FAM TTT CTC CTG CCT GAA GGA CAG ACA TGA TAMRA-3'	25
IFN-β	IFN-β	Forward – 5'-CGC CGC ATT GAC CAT CTA-3' Reverse – 5'-GAC ATT AGC CAG GAG GTT CTC A-3' Probe – 5'-FAM TCA GAC AAG ATT CAT CTA GCA CTG GCT GGA TAMRA -3'	14
IFN-λ1	IL-29	Forward – 5'-GGA CGC CTT GGA AGA GTC ACT-3' Reverse – 5'-AGA AGC CTC AGG TCC CAA TTC-3' Probe – 5'-FAM AGT TGC AGC TCT CCT GTC TTC CCC G TAMRA-3'	19
IFN-λ2/3	IL-28A/B	Forward – 5'-CTG CCA CAT AGCCCA GTT CA-3' Reverse – 5'-AGA AGC GAC TCT TCT AAG GCA TCT T-3' Probe – 5'-FAM TCT CCA CAG GAG CTG CAG GCC TTT A TAMRA-3'	19
18S	18S	Forward – 5'-CGC CGC TAG AGG TGA AAT TCT-3' Reverse – 5'-CAT TCT TGG CAA ATG CTT TCG-3' Probe – 5'-FAM ACC GGC GCA AGA CGG ACC AGA TAMRA-3'	37

Table 1. Sequences of primers and probes used for detection of IFN-α, IFN-β, IL-29, IL-28A/B and 18S

Figure 1

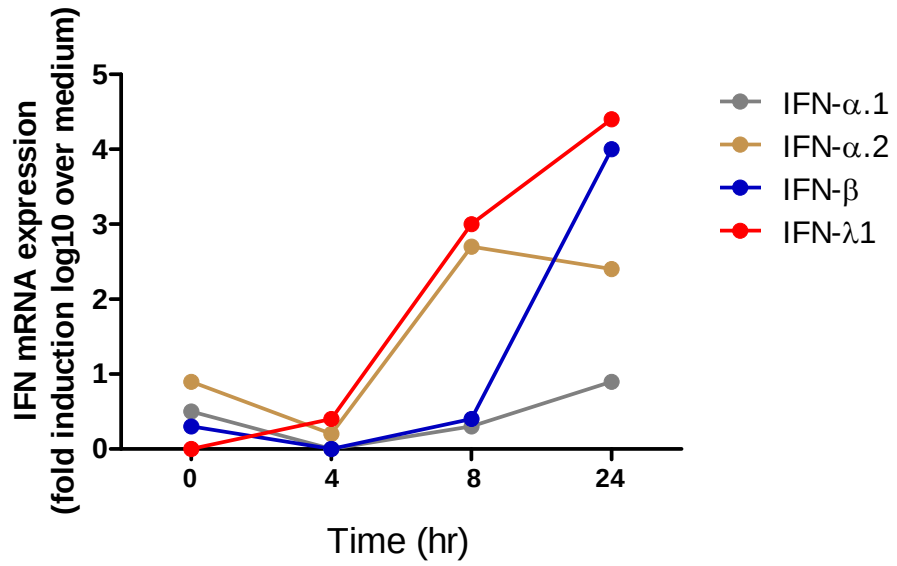

Figure 1. Time course of RV-16 induction of type I and type III IFNs in primary BECs. The expression of IFN mRNAs was assessed by Taqman PCR. IFN-αs detected by IFN-α.1 primer pair were not induced by rhinovirus 16. With IFN-α.2 statistically significant induction was observed by 8 h ($n = 4$, $P < 0.01$ compared to time point 0). Significant induction of IFN-β mRNA by RV-16 was detected only at 24 h ($n = 4$, $P < 0.001$ compared to time point 0). IFN-λ1mRNA expression was up-regulated at 8 and 24 h ($n = 4$, $P < 0.01$ and $P < 0.001$ respectively compared to time point 0).

Figure 2

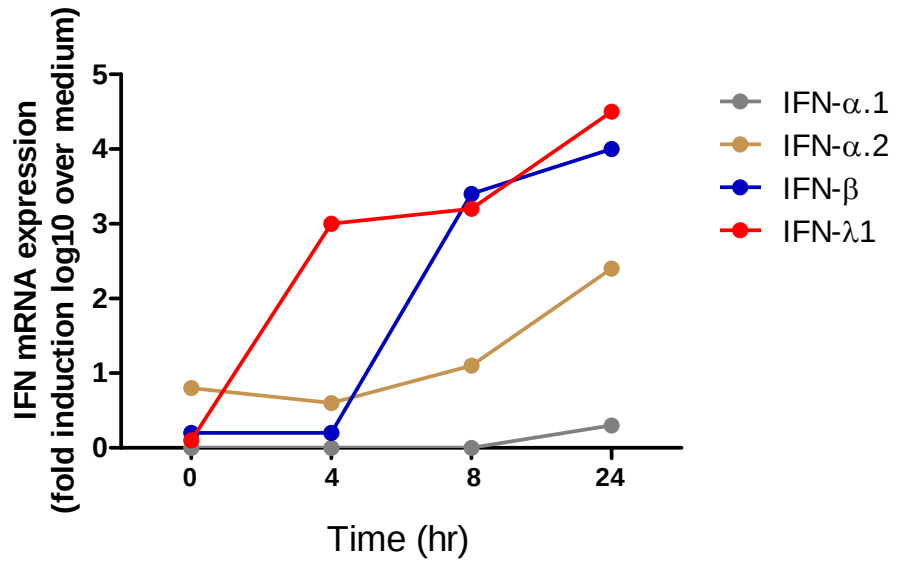

Figure 2. Time course of influenza virus induction of type I and type III IFNs in primary BECs. The expression of IFN mRNAs was assessed by Taqman PCR. IFN-α subtypes detected by primer/probe IFN- α.1 were not induced by rhinovirus 16. IFN- α.2 primer/probe detected significant induction at 24 h ($n = 4$, $P < 0.01$ compared to time point 0). Significant induction of IFN-β mRNA by influenza virus was detected at 24 h ($n = 6$, $P < 0.001$ compared to time point 0). IFN-λ1 mRNA was significantly induced by influenza virus at 8 h ($n = 4$, $P < 0.001$ compared to time point 0) and 24 h ($n = 4$, $P < 0.001$ compared to time point 0).

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Mechanisms of rhinovirus induced inflammation in respiratory epithelial cells: role of xanthine-oxidase activation

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Background and Aims

Rhinoviruses are believed to directly infect airway epithelium inducing pro-inflammatory cytokine production (1-3). This leads to recruitment and activation of inflammatory cells, resulting in airway inflammation (4, 5).

Increased oxidative stress is implicated in induction of the acute airway inflammation during exacerbations of asthma and COPD (6). Oxidants are directly involved in inflammatory responses via signaling mechanisms, including the redox-sensitive activation of transcription factors such as NF-kB (7, 8).

Recent data indicate that rhinovirus and other respiratory viruses can alter cellular redox homeostatic balance towards a pro-oxidative condition (9-11). The molecular pathways responsible for such disequilibrium are virtually unknown. In a previous study we documented that rhinovirus infection induces a rapid increase of intracellular superoxide anion (O_2^-), which occurs within 15 min after infection. This early pro-oxidative response was found to induce NF-kB activation and downstream pro-inflammatory molecule production (12).

O_2^- is a product of cellular metabolism and mainly originates from the activity of two enzyme systems: NADPH oxidase and xanthine dehydrogenase/xanthine oxidase (XD/XO) (13).

Here we studied the molecular mechanisms by which rhinovirus induces rapid O_2^- production in respiratory epithelial cells. We also analyzed the mechanisms by which reducing agents can abolish rhinovirus induced O_2^- production and thus can stabilize the intracellular redox state in respiratory epithelial cells following infection. Finally, we demonstrated that blocking the activity of the system responsible for rhinovirus triggered O_2^- generation inhibited rhinovirus induced inflammatory mediator production in respiratory epithelial cells.

Experimental procedures

Cell culture- Ohio HeLa cells were obtained from the MRC Common Cold Unit, Salisbury, UK, and A549 cells, a type II respiratory cell line, were obtained from the American Type Culture Collection (ATCC), (Rockville, MD). Primary human bronchial epithelial cells (HBEC) were obtained by bronchial brushing from healthy volunteers, and cultured as previously described (12, 14, 15).

Virus stocks- Rhinovirus type 16 (RV16 - a major group rhinovirus) was obtained from the MRC Common Cold Unit, Salisbury, UK. Viral stocks were prepared by infection of sensitive cell monolayers (Ohio HeLa, O'HeLa) as described elsewhere (12, 15). $TCID_{50}$/ml values were determined and rhinovirus serotype was confirmed by neutralization with serotype specific antibodies (ATCC) (16). Filtered virus stocks were used as negative control. Virus at multiplicity of infection (MOI) of 1 was used for all the experiments.

Infections, harvesting of cells, preparation of cell homogenates and preparation of membrane and cytosolic fractions- Confluent A549 or HBEC cells were exposed to rhinovirus, medium alone, or filtered virus (f-RV) inoculum for different time intervals (20 min - 8 hr). Cell layers were thereafter washed three times in cold phosphate-buffered saline (PBS) before harvesting by scraping. Harvested cells were centrifuged and the cell pellet was resuspended in phosphate buffer (10 mM, pH 7,2). Cell lysis was obtained by repeated (three times) freezing and thawing. For preparation of cytosolic fractions, the cell homogenate was then ultracentrifuged at 20,000 g for 30 mins, the cell fragments were pelleted and the supernatant (cytosol) collected. Where indicated, to obtain membrane fraction, the cell homogenates were centrifuged at 800 g for 10

min to separate nuclei from cell membranes. Supernatants were harvested and were again centrifuged at 2,000 g, supernatants discarded and membrane pellets diluted in 0.1 M sucrose solution. A final centrifugation at 11,000 g for 20 min was performed at 4 °C. Pellets containing membranes were diluted in 100 µl of PBS buffer. Protein content was determined photometrically using the Bio-Rad protein assay (Bio-Rad).

Protease and XO inhibition- In selected experiments cells were pretreated, before infection, as follows: 12 hr [0.25 to 10 mM] GSH (Sigma), or 12 hr [0.25 to 2.5 mM] SH_2 (Acqua Breta, Riolo Terme, Ravenna, Italy) or 4 hr [20 µM] oxypurinol (4, 6-dihydroxyprazol (3,4-d) pyrimidine - Sigma), a permanent inactivator of xanthine oxidase (17). Where indicated, a 4 hr pretreatment with antiproteases ([0.625 µM] serine protease inhibitor phenylmethyl sulphonyl fluoride (PMSF), or [1.25 µM] serine and cysteine protease inhibitor leupeptin (Leu), or [1.25 µM] aspartic protease inhibitor pepstatin (Pep), or [1.25 µM] serine protease inhibitor aprotinin (Apr), or [1.25 µM] metalloprotease inhibitor phenanthroline (Phe) or cysteine protease inhibitor E-64 - all from Sigma) or diluent alone was performed (18-23). Diluent was made of phosphate buffer pH 7.2 maximal final concentration 0.4%. PMSF only was previously resuspended in ethanol before dilution in PBS. Antiproteases were removed immediately before infection.

Cytochrome c reduction kinetics- The intracellular production of O_2^- was spectrophotometrically evaluated by superoxide dismutase (SOD)-inhibitable cytochrome c reduction kinetics, as previously described (12, 24). Kinetics were carried out in 2 ml quartz cuvettes at 37°C for 20 min in a Uvikon 860 (Kontron Instruments) spectrophotometer in the presence or in the absence of superoxide dismutase (SOD, 500 IU/ml, Sigma). Concentration of cytochrome c from beef heart (Sigma) was 10^{-5} M. Absorbance readings were taken at 550 nm (peak of reduced cytochrome). Newly generated O_2^- was measured in each sample and expressed as µM, according to standardized procedures (13). Measurements were based on absorbance differences in the

presence or absence of SOD, after 5 min of kinetics, when the kinetic slope of cytochrome c reduction was steepest. Data were normalized per mg of protein.

Uric acid kinetics- Uric acid kinetics were performed at 1 hr infection to evaluate the involvement of XD/XO in rhinovirus induced O_2^- generation, as uric acid represents the other end-product of xanthine degradation by XO. Uric acid kinetics were spectrophotometrically monitored at 293 nm in a UviKon spectrophotometer (Kontron), according to standard procedures (25, 26), on 500 µl supernatant collected in PBS, pH 7.4, after addition of xanthine (0.1 mM, Sigma). After 15 min of kinetics, uricase (1.0 units/ml) was added to evaluate the amount of uric acid produced.

NADPH oxidase assay- NADPH oxidase assay was performed at different time intervals (20 min - 3 hr) to evaluate the involvement of this system in rhinovirus induced O_2^- generation. Cell homogenates were centrifuged as described above to separate nuclei from cell membranes. To reconstitute NADPH oxidase, supernatants containing membranes were centrifuged again at 40,000 g. The reaction mixture contained 200 µl supernatant and 50 µl of diluted membrane pellet. After 2 min, 200 µM NADPH and 5 mM $MgCl_2$ were added in the presence or in the absence of the specific inhibitor of NADPH oxidase diphenylene iodonium chloride (DPI [0.92 µg/ml], Sigma) (27). Cytochrome c was added to a concentration of 0.1 mM and PBS (pH 7.2) to a final volume of 0.5 ml, and the reduction kinetics was monitored for 15 min at 37 °C as previously described.

Western blot analysis for xanthine dehydrogenase/oxidase. Whole cell proteins were extracted from A549 cells as previously described (28). At least 50 µg/lane of whole-cell proteins were subjected to a 4-12% tris-glycine gel electrophoresis, and transferred to nitrocellulose filters by blotting. Filters were blocked for 45 minutes at room temperature in Tris-buffered saline (TBS),

0.05% Tween 20, 5% non-fat dry milk. The filters were then incubated with rabbit anti human XD/XO (LS-C26419; from LifeSpan Biosciences) for 1h at room temperature in TBS, 0.05% Tween 20, 5% non-fat dry milk at dilution of 1:500. Filters were washed three times in TBS, 0.5% Tween 20 and after incubated for 45 minutes at room temperature with goat anti-rabbit antibody conjugated to horseradish peroxidase (Dako) in TBS, 0.05% Tween 20, 5% non-fat dry milk, at dilution of 1:4000. After further three washes in TBS, 0.05% Tween 20 visualization of the immunocomplexes was performed using the ECL as recommended by the manufacturer (Amersham Pharmacia Biotech). As an internal control we reprobed each filter with an anti-human actin antibody (Santa Cruz Biotechnology). The 145kDa and 85 kDa bands of the XD/XO system [(full length XD and the post cleavage fragment containing the active site respectively (29, 30)] and the 43kDa (actin) band were quantified using densitometry with VisionWorks®LS software (UVP) and expressed as the ratio with the corresponding actin optical density value of the same lane.

Knockdown of xanthine dehydrogenase expression. RNA interference was used to specifically suppress expression of XD in A549 cells. Cells were transfected in 6 well plates with small interfering RNA (siRNA) using siPORT^{TM} *NeoFX*^{TM} Transfection Agent (Applied Biosystem), as described by the manufacturer. The following siRNA (all from Ambion) were used: siRNAs for XD (s14918; target sequence: sense: GCAUCGUCAUGAGUAUGUAtt, antisense: UUUAUAGCAUCCUCAAUUGtg), siRNA for GAPDH (4390849) and nonsilencing siRNA (4390843). Total mRNA was extracted by using RiboPureTM kit (Ambion) as per manufacturer's instructions. 1 μg of mRNA was used to perform reverse transcription assay with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem). XD mRNA expression was monitored by Real Time RT-PCR by using TaqMan® Gene Expression Assay (Applied Biosystem) specific for XD (Cat. n. Hs00166010_m1) following the manufacturer's

recommendations. The reaction was carried out in a Rotor-Gene^{TM} 6000 instrument (Corbett Life Science**).** Results were normalized to 18S rRNA (sense: 5'- CGC CGC TAG AGG TGA AAT TCT -3', antisense: 5'- CAT TCT TGG CAA ATG CTT TCG-3' 300nM each, probe: 5'-FAM ACC GGC GCA AGA CGG ACC AGA TAMRA-3' 175nM) and expressed as XD mRNA relative levels as compared to nonsilencing siRNA transfected cells by using the RotorGene software (Corbett Research) and the two standard curve method for relative quantitation (31).

High performance liquid chromatography (HPLC) analysis of intracellular GSH- A549 cells were cultured at 85% confluence, incubated with RV16, medium alone or f-RV for different periods (20 min to 1 hr) then trypsinized, collected and harvested in cryovials with 1.2 ml 3% metaphosphoric acid in sterile conditions to avoid GSH oxidation and finally frozen in liquid nitrogen until used. Cell homogenate was obtained and protein content was determined as previously described. Intracellular GSH concentration was evaluated by HPLC in a Kontron Instruments apparatus (Milan, Italy) equipped with a C18 hydrophobic column (5 um particle size, 4.6 x 250 mm), a 420 pump (range 0.005-10 ml/min), a 425 gradient former and injection valve with 20-µl sampling loop. Elution was carried out at room temperature in isocratic gradient (75% methanol and KH_2PO_4 buffer, pH 3, 1 ml/min speed). GSH was analyzed at 200 nm by a 432 UV detector (Kontron) with IBM integrated software PC Pack. Homogenates of cells were centrifuged at 40,000 g for 20 min at 4 °C and the supernatant collected and concentrated on Amicon Ultra 10,000 centrifugal filter devices (Millipore, Bedford, MA) to a final volume of about 300 µl. Samples were analysed without derivatisation against standards of pure lyophilised GSH (Biomedica Foscama), diluted in 1 ml normal saline solution. The final concentration was obtained by serial dilutions of the lyophilized product. In selected experiments cells were pretreated, before infection, as previously described, for 12 hr with GSH, SH_2 (0.25 to 2.5 mM) or 4 hr oxypurinol (20 µM). Where indicated, a 4 hr pretreatment with protease inhibitors (PMSF

[0.625 μM]; Leu [1.25 μM]; Pep [1.25 μM]; Apr [1.25 μM]; Phe [1.25 μM]; E-64 [1.25 μM]) or diluent was performed. All protease inhibitors were removed immediately before infection.

ELISA assays for chemokines- Subconfluent A549 cells were pretreated for 4 hr with oxypurinol (20 μM) before RV16 or f-RV inoculum or medium alone treatment. After 1 hr infection unbounded virus was removed and fresh medium added. Supernatants were harvested at 4 hr and levels of IL-8, and GRO-α were assessed using commercially available ELISA kits (R&D System) following manufacturer's instruction. Detection limits for IL-8 and GRO-α ELISA assay were approximately 10 pg/ml and 15 pg/ml respectively.

NF-kB transcription factor activation. Nuclear extracts were prepared from A549 cells using the Nuclear Extract Kit (Active Motif). NF-kB activation was assessed in A549 cell nuclear extracts using the TansAM p65 Transcription Factor Assay Kit (Active Motif) following manufacture's recommendations. Nuclear extract of Jurkat cells provided by the manufacture (Active Motif) were used as positive controls.

Statistical Analysis- Group data were expressed as mean and standard error (SEM). Analysis of variance was used to determine differences between groups. Paired or unpaired Student's t tests were performed after the analysis of variance when appropriate. All experiments were carried out at least 5 times. Bonferroni adjustment was applied where indicated. A probability value of <0.05, was considered significant.

Results

Rhinovirus induced O_2^- production in respiratory epithelial cells is cytosolic not membrane associated. O_2^- production was evaluated by superoxide dismutase (SOD)-inhibitable cytochrome

c reduction kinetics. Since other workers had implicated NADPH oxidase in RV induction of reactive oxygen species (32) and since NADPH oxidase is a membrane bound system, we first sought to identify the cellular site of O_2^- production. In the search of cellular sources of RV16 induced O_2^- production, we first confirmed our previous findings of rapid induction of O_2^- by RV16 in membrane free cytosolic fractions (Figure 1). To investigate membrane production, directly O_2^- production was evaluated in cell membranes obtained from A549 epithelial cells infected with RV16. No induction was observed (P=0.82 vs unstimulated controls) at any time point (20, 60, 120, 180, 240, 300, 360, 420, 480 min), indicating that the cytosol, not the membrane, is the site of RV16 induced O_2^- production.

Rhinovirus induction of O_2^- is independent of the NADPH oxidase system. We investigated whether rhinovirus induced O_2^- production could derive from the activity of NADPH oxidase, in experimental conditions where both the cytosolic and membrane fractions of the homogenates were pooled together. The membrane fractions were added, together with the substrate of NADPH oxidase, NADPH, in the presence and absence of an inhibitor of the NADPH enzyme system, diphenylene iodonium chloride (DPI) (27). NADPH addition would increase O_2^- production if production is NADPH oxidase-dependent. We found that both the addition of NADPH and of DPI (Figure 2A) to the reaction mixture did not change O_2^- production induced by RV16 infection, indicating that the NADPH oxidase system is not relevant to early O_2^- generation induced by RV16.

Rhinovirus induces O_2^- production via the XD/XO enzyme system. To confirm that O_2^- is generated via the XD/XO enzyme system, we next investigated RV induction of uric acid, the

other product, besides O_2^-, of XO degradation of the purine base xanthine. These experiments were conducted at 1 hr after infection, i.e. at the peak of O_2^- generation. Uric acid was detected only in RV16 infected samples. Its identification was confirmed by addition, after 15 min of the kinetic assay, of uricase, which degrades uric acid to allantoin (25, 26). As expected, uric acid was rapidly degraded by uricase confirming the XD/XO system was activated by rhinovirus infection. Similar findings were observed in homogenate samples obtained from HBEC, in the same experimental conditions , confirming rhinovirus activation of XD/XO in primary cells. To further confirm the role of XD/XO enzyme system in O_2^- production we performed experiments in presence of oxypurinol, a permanent inactivator of the oxidase form of the enzyme (XO), the only form of the enzyme able to produce O_2^- (17). At 1 hr infection RV16 induced cytosol O_2^- production was completely quenched when A549 cells were pretreated with oxypurinol (Figure 2B). Similar findings were observed in homogenate samples obtained from HBEC in the same experimental conditions as in both cases oxypurinol reduced O_2^- production to levels observed with medium or diluent alone or inactivated virus.

Rhinovirus induces proteolytic activation of XD/XO enzymatic system. In other experimental systems, the XD/XO enzymatic system is able to produce O_2^- when is converted to the oxidase form by proteolytic activity, exerted by a serine protease, which partially hydrolyzes the enzyme to its active form (29, 33). To investigate the mechanisms of rhinovirus induction of XD/XO, cells were next infected in the presence or absence of serine protease inhibitors, or with cysteine or metalloprotease inhibitors as controls. RV16 infection (20 min to 1 hr) failed to generate O_2^- in the cytosol when epithelial cells were pretreated for 4 hr with protease inhibitors phenylmethyl sulphonyl fluoride (PMSF), or leupeptin (Leu), pepstatin (Pep), aprotinin (Apr), which all act as serine protease inhibitors (Figure 3A). Serine protease involvement was confirmed as neither the

metalloprotease inhibitor phenanthroline (Phe – Figure 3B) nor the cysteine protease inhibitor E-64 (Figure 3B) (18-23) had any effect on rhinovirus induction of O_2^-. This data confirm the involvement of specific proteolytic mechanisms mediated by serine proteases in rhinovirus induced activation of XO. To directly evaluate the effects of RV infection on XD/XO proteins, we performed western blotting analysis using an antibody able to recognize XD/XO expression. The 145 kDa band corresponding to full length XD was significantly reduced at 30 min (2 fold; $p<0.05$) and at 1 hr (4 fold; $p<0.01$) after RV16 infection as compared to uninfected control cells ($p<0.05$). Such a reduction was paralleled by 2 fold-increased expression of the 85 kDa band, containing the active site, at 1 hr infection ($p<0.05$). By measuring XD/XO ratio we found a progressive significant reduction at 30 min and 1 hr after RV16 infection as compared to uninfected control cells (3 fold; $p<0.05$ and 10 fold; $p<0.01$, respectively). Moreover 4hr serine-protease inhibitor Apr, but not 4 hr cysteine-inhibitor E64 pretreatment, abolished XD cleavage.

XD knockdown reduces rhinovirus induced O_2^- production. To further confirm the role of the XD/XO system in rhinovirus-induced O_2^- production, we performed experiments in which XD expression in A549 cells was knocked down by siRNA. Transfection of XD siRNAs resulted in marked suppression of XD mRNA expression as compared to scrambled siRNA transfected cells, with a peak of inhibition at 24hr. At this time point, XD knockdown suppressed RV16-induced O_2^- production spectrophotometrically measured by SOD-inhibitable cytochrome c reduction kinetics (Figure 4). Control siRNAs had no effect either on XD mRNA expression (Fig 4D) or on RV16-induced O_2^- production (Figure 4).

Rhinovirus depletes intracellular reduced gluthathione (GSH). To investigate the consequences of RV-induced O_2^- production on intracellular redox equilibrium, we evaluated whether the

concentration of the intracellular reducing agent GSH is modified by RV16 infection. In A549 cells, as the duration of RV16 infection increased, endogenous stores of GSH were progressively reduced and complete depletion was observed at 1 hr after infection (Figure 5 left panel). As observed with O_2^- induction, RV16 induced depletion of intracellular GSH at 1 hr after infection was completely inhibited when A549 cells were pretreated with either oxypurinol or serine protease inhibitors, but not with metalloprotease inhibitor phenanthroline (Phe) or cysteine protease inhibitor (E-64).

Increasing intracellular GSH inhibits rhinovirus induced intracellular GSH depletion and O_2^- production. Since reduction of intracellular reducing power is "per se" a known mechanism of activation of XO (34, 35), we next investigated whether by increasing intracellular GSH with the reducing agents SH_2 or exogenous GSH, we could block the activation of XO induced by RV infection. We first showed that pretreatment of A549 cells with SH_2 and exogenous GSH increased intracellular GSH levels in a dose-dependent manner. A549 cells were then pretreated for 12 hr with 2 mM SH_2 to enhance intracellular GSH and then infected for 1 hr with RV16. No reduction of endogenous GSH was observed after 1 hr RV16 infection, confirming that enhancing intracellular GSH protected cells against virus induced GSH depletion. Similar protection was observed with 10 mM exogenous GSH (data not shown) treatment before the infection. Pretreatment with either 2 mM SH_2 or 10 mM exogenous GSH, not only increased intracellular GSH levels but also completely inhibited RV16 induced O_2^- production at 1 hr after infection, as assessed by SOD-inhibitable cytochrome c reduction assay (Figure 5 right panel). In these experiments uric acid production was similarly suppressed.

XO inhibition reduces rhinovirus induced chemokine production. RV infection of bronchial epithelial cells induces the expression of several proinflammatory cytokines, including many that are involved in neutrophil chemoattraction and activation [i.e interleukin(IL)-8 and Gro-α]. Neutrophil inducing cytokines are not effectively suppressed by the currently available asthma therapies, i.e. steroids or long acting beta agonists (2). Since the induction of these mediators occurs through oxidative sensitive pathways [e.g. NF-kB signaling activation (6)], we evaluated whether oxypurinol inhibition of XO mediated O_2^- generation affects rhinovirus induced IL-8 and Gro-α production in A549 respiratory epithelial cells. Significant induction of IL-8 and Gro-α was apparent at 4 hr post RV16 infection. Oxypurinol pre-treatment significantly reduced both IL-8 and Gro-α RV16-induced production, while diluent had no effect.

Effect of rhinovirus infection on NF-kB activation. Modulation by oxypurinol. To assess whether rhinovirus infection activates NF-kB and whether this is mediated by O_2^-production, we measured activated NF-kB in nuclear extracts in A549 cells following RV16 infection with or without 4hr oxypurinol pretreatment. p65 nuclear concentration was 40.7 ± 6.3 pg/µl in unstimulated conditions. In accordance with previous data (12, 15) we found that 30 min RV16 infection significantly induced p65 nuclear translocation (2-fold vs unstimulated; $p<0.05$). Four hr pre-treatment with 20µM oxypurinol significantly inhibited RV16 induced NF-kB activation ($p<0.05$ vs RV16 infected cells not pretreated).

Conclusion

In this study we found that O_2^- generation induced by rhinovirus infection is initiated by proteolytic activation of the XD/XO enzyme system. Consequently, newly generated O_2^- leads to progressive depletion of intracellular GSH storage, a condition that can further activate the XD/XO system. Inhibition of XO activity completely abolished rhinovirus induced O_2^- production and intracellular GSH depletion, as did a variety of serine protease inhibitors. We also found that by enhancing intracellular GSH storage with exogenous SH_2 or GSH rhinovirus infection was rendered unable to induce O_2^- production and to affect intracellular GSH levels.

Several studies have described oxidant generation following respiratory virus infection both in vitro and in vivo (9, 36). In some studies a role for virus induced oxidants in the production of inflammatory responses/mediators has been identified (37). However the molecular mechanisms regulating generation of oxidant species by viruses in biological systems have never been fully investigated.

The findings of the present study indicate a complex mechanism of oxidant induction following activation of XO induced by rhinovirus infection (Figure 6). A “vicious circle” would represent the final scenario where activation of XO, initiated immediately after infection via proteolysis of XD to XO, is thereafter implemented via a non-proteolytic mechanism mediated by oxidative consumption of intracellular reducing capacity via depletion of GSH stores. Depletion of intracellular reducing agents is a known mechanism of activation of XO and O_2^- production (34, 35). The involvement of rhinovirus induced oxidants in GSH consumption was confirmed by the finding that, when XO activation was inhibited, rhinovirus infection did not result in GSH depletion. The sequence of events represented in Fig. 9 is supported by the timing of the different steps involved, with O_2^- production being rapidly induced 20 min after infection (Fig. 1), while GSH depletion is undetectable 20 min after infection and thereafter progressively increases for 40 min, being complete at 60 min after infection (Fig. 5 A & B). The fact that in a reducing

environment no uric acid was produced and that the intracellular concentration of GSH was unchanged after rhinovirus infection confirms the inverse relationship between GSH intracellular concentration and XO activation. The involvement of a NADPH oxidase-like enzyme in rhinovirus induced oxidative stress has been previously described (32). In contrast to the study by Kaul and colleagues, our study focuses strictly on the early oxidative events occurring within cells immediately after rhinovirus infection (with a peak at 1 hr after infection). Also, by using a method able to specifically detect newly generated O_2^-, i.e. the SOD-inhibitable cytochrome c reduction assay (38), we directly evaluated O_2^- intracellular production, and not oxidative stress in general. Moreover, at variance with Kaul and colleagues, we employed oxypurinol, which completely inactivates XO by direct binding to the enzyme active site (17), and not allopurinol, which only partially inhibits the enzyme. Treatment with oxypurinol completely abolished rhinovirus induced O_2^- generation, thus confirming the specificity of our findings on XO activation. These and other difference between the two studies, in particular different samples for analyses (intracellular vs extracellular compartments) and different timing are likely explanations for the different results reported.

Despite the effort and resources expended, no antiviral drugs are currently marketed for the prevention or treatment of rhinovirus infection (39). In the absence of effective anti-viral therapies, development of therapies that blocked the inflammatory responses to infection would be a major advance. Our demonstration of the molecular mechanisms by which rhinovirus activates oxidant generation, a crucial step in the complex inflammatory response to infection, (12, 32) could open new possibilities in the search for therapeutic targets for future intervention. In particular, the documentation that: a) exogenous interventions able to increase intracellular reducing agent storage can block rhinovirus-mediated activation of the vicious circle that leads to sustained production of O_2^-, and b) inhibition of XO, with protease inhibitors or specific

inhibitors (oxypurinol) inhibits O_2^- and pro-inflammatory mediator production, indicate promising options for the development of treatment for rhinovirus induced diseases including the common cold and exacerbations of asthma and COPD.

Figure 1

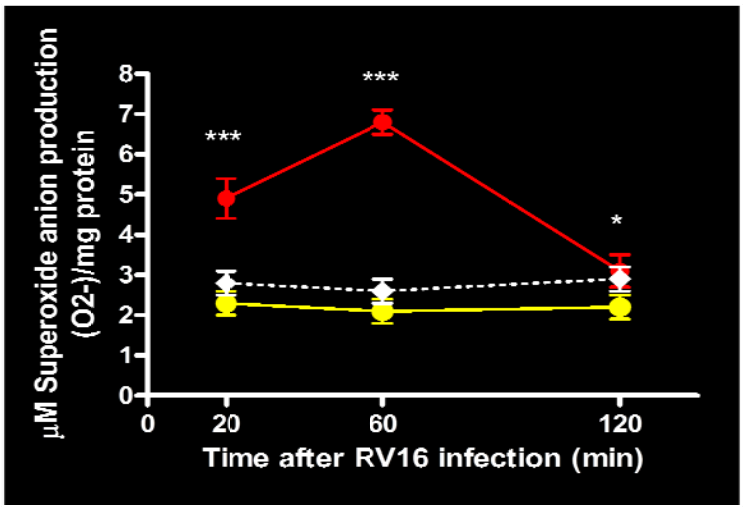
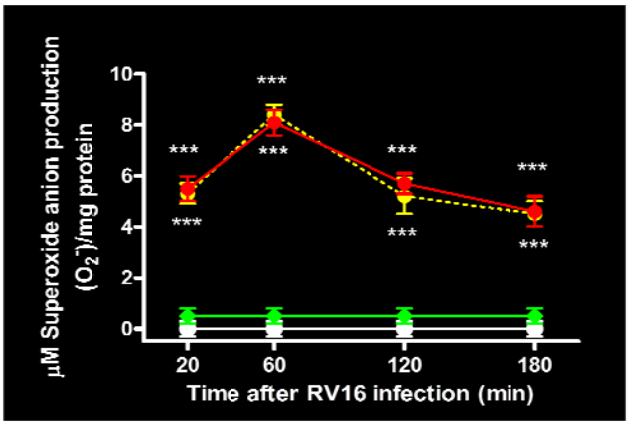
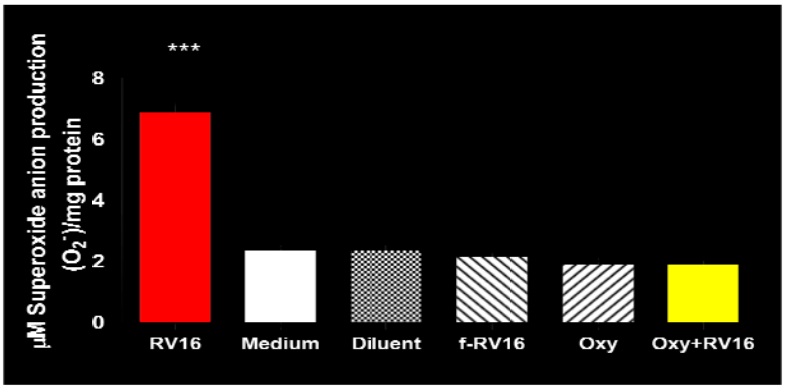


Figure. 1. Rhinoviruses-induced production of superoxide anion (O_2^-) in human primary bronchial epithelial cells (HBEC).

Figure 2



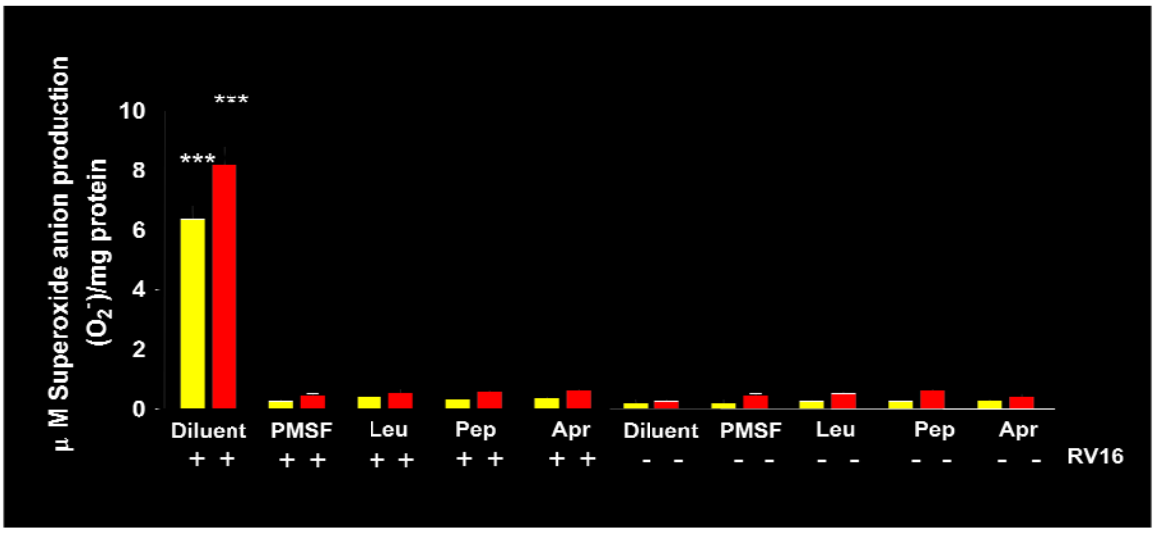
A



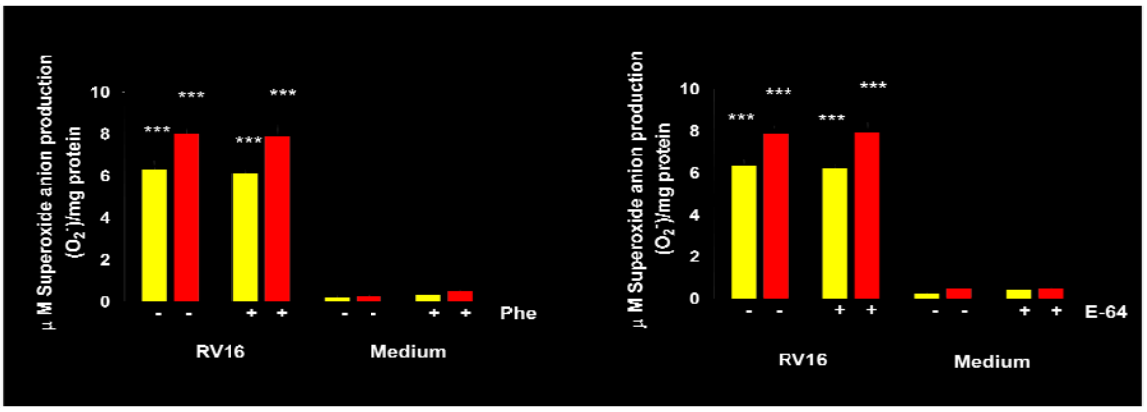
B

Figure 2: superoxide anion (O_2^-) production is independent of the NADPH oxidase system and Xanthine oxidase (XO) involvement in rhinovirus induced cytosolic superoxide anion (O_2^-) production. (A) O_2^- production was not changed when a specific inhibitor of NADPH oxidase (DPI) was added to the reaction mixture [RV16 physically removed by filtration (open circles), medium alone (diamonds) or life RV16 infection (black circles), without (continuous line) or with (dashed line) NADPH] confirming lack of involvement of NADPH oxidase (N=5). (B) HBEC were exposed for 1 hr to life RV16 (RV16), RV16 physically removed by filtration (f-RV16), medium alone (Medium) or diluent alone (Diluent). Where indicated cells were 4 hr pretreatment before the infection with oxypurinol and than exposed for 1 hr to medium alone (Oxy) or RV16 (Oxy+RV16).

Figure 3



A



B

Figure 3: Effects of protease inhibitors on rhinovirus induced cytosolic superoxide anion (O_2^-) production. Effects of serine protease inhibitors phenylmethyl sulphonyl fluoride (PMSF), leupeptin (Leu), pepstatin (Pep), aprotinin (Apr) on RV16 induced cytosolic O_2^- production in A549 cells (N=5). Effects of metallo-protease inhibitor Phenanthroline (Phe) (B) or cysteine protease inhibitor E-64 (C) on RV16 induced cytosolic O_2^- production in A549 cells. Where indicated (+) cells were 4 hr pretreated with protease inhibitors, then exposed, for 20 min or 1 hr RV16 or medium alone. (In panel A *** $p<0.001$ vs all other conditions, in panels B *** $p<0.001$ vs medium alone treated cells with or without inhibitors pre-treatment).

Figure 4

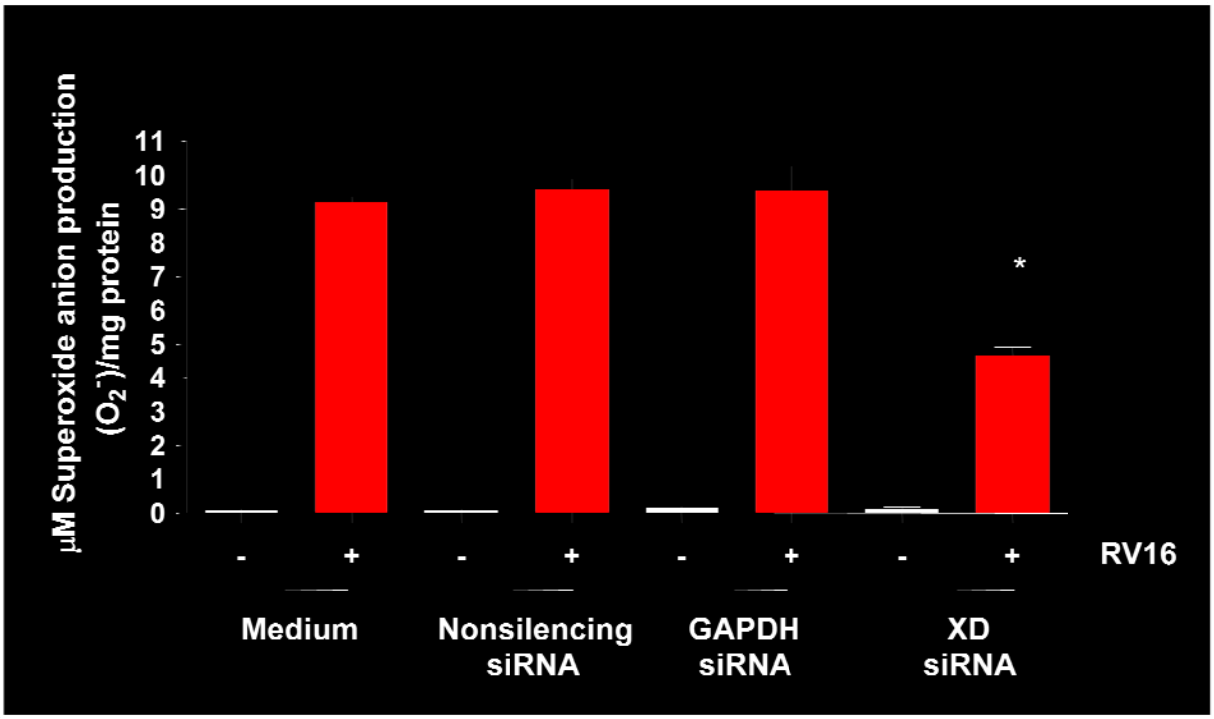


Figure 4: Effect of XD knock-down on rhinovirus induced superoxide anion production. Superoxide anion production following 1 hr RV16 infection in A549 cells transfected with medium alone, nonsilencing siRNA, GAPDH siRNA and XD siRNA. (N= 3, *$p<0.05$ vs RV16 infected cells in all other experimental conditions.).

Figure 5

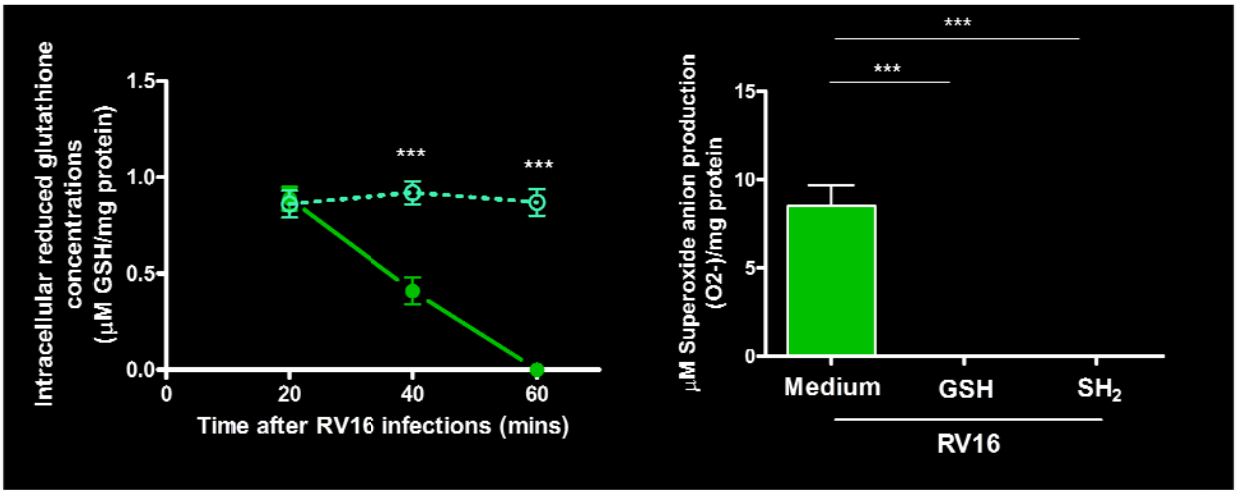


Figure 5: (Left panel) Effect of rhinovirus (RV) infection on intracellular reduced glutathione (GSH) concentration in A549 cells. Intracellular GSH was evaluated by high performance liquid chromatography (HPLC) in A549 cells incubated with RV16 (continuous line), or medium alone (dashed line) for 20 min to1 hr. (N=5, *** $p<0.001$ vs control samples). (Right panel) Effects of exogenous SH_2 and GSH pre-treatment on RV16 superoxide anion production.

Figure 6

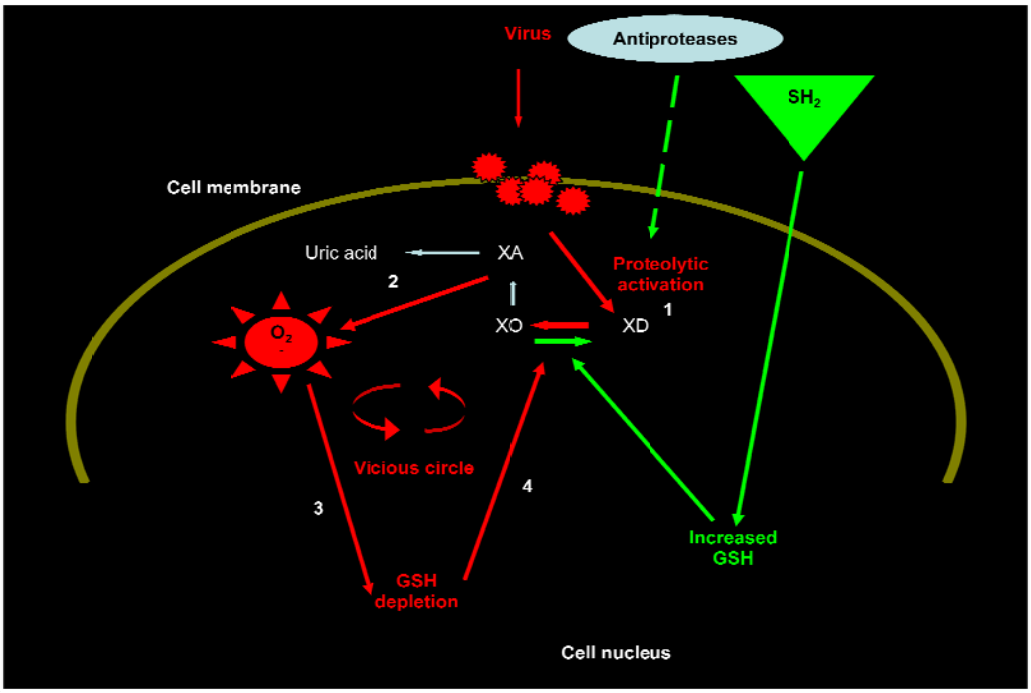


Figure 6: Proposed mechanisms of rhinovirus induced cytosolic superoxide anion (O_2^-) production. Following rhinovirus infection xanthine-oxidase is activated via proteolysis of xanthine-dehydrogenase (1). O_2^- and uric acid are thereafter produced from the enzyme substrate xanthine (2). Intracellular oxidant production results in the depletion of intracellular reducing capacity (including reduced glutathione depletion) (3), which in turns induces xanthine-oxidase activation via a non-proteolytic mechanism (4). Antiprotease shield and exogenously induced increased intracellular reducing capacity (green pathways) respectively block the two distinct mechanisms of xanthine oxidase activation initiated by rhinovirus infection (O_2^- = superoxide anion; XO = xanthine-oxidase; XD = xanthine-dehydrogenase; XA = xanthine; GSH = Reduced glutathione).

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Human model of rhinovirus induced asthma exacerbations

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Background, Aims and Summary

Acute exacerbations are the major cause of asthma morbidity, mortality and health care costs. Exacerbations are poorly treated and prevented by current therapies. New therapies are urgently needed. The great majority of asthma exacerbations are associated with viral infection. Rhinovirus is the commonest virus identified (1-3).

There is no useful animal model of rhinovirus-induced asthma exacerbations. Experimental rhinovirus infection in human volunteers has been used to investigate mechanisms of virus-induced exacerbations, however no previous study has demonstrated differences between asthmatic and normal subjects in lower respiratory physiologic or pathologic responses to rhinovirus infection. Here we use experimental rhinovirus infection to investigate lower airway physiology, virus load and immunopathology in 10 asthmatic and 15 normal volunteers.

We found that rhinovirus infection induced significantly greater lower respiratory symptoms, reductions in lung function, increases in bronchial hyperresponsiveness and lower airway inflammation in asthmatic compared to normal subjects. Virus load was strongly related to severity of clinical symptoms, changes in lung function, bronchial hyperresponsiveness and lower airway inflammation. Moreover, these virologic and clinical outcomes in asthmatic subjects were strongly related to deficient IFN-γ responses and augmented Th2 cytokine responses

This model provides evidence supporting a causal relationship between rhinovirus infection and asthma exacerbation, reproduces many features of naturally occurring exacerbations and could be used to investigate mechanisms of disease and to test new candidate treatments.

Methods

Study design

RV16 experimental infections were induced in RV16 seronegative atopic asthmatic and normal non-atopic adult subjects. Clinical and atopic status were defined by questionnaire, skin prick testing, serum IgE and lung function testing including PEF, FEV1, forced vital capacity (FVC) and histamine challenge. Normal subjects were taking no medication, asthmatics inhaled short acting 2 agonists only. Subjects were free of common cold symptoms for 6 wks before starting the study. All were non smokers. Baseline, acute infection and convalescent samples of blood, nasal lavage, induced sputum and bronchoalveolar lavage (BAL) were taken. Baseline samples were taken 14 days prior to infection and diaries kept to record symptoms and home lung function throughout the study. All subjects gave informed consent and the study was approved by St Mary's NHS Trust Research Ethics Committee.

Experimental infection with RV16

Infection was induced using 10000 TCID50 RV16 on day 0 by nasal spray, as described (4). Following inoculation, subjects returned home.

Assessment of blood and airway inflammation and T cell cytokine production

Blood Coulter counts were performed. Absolute and differential induced sputum and BAL cell counts were determined on cytospins. Fresh PBMC and BAL cells were stained for CD4 and CD8 T cells and NK and B cells and intracellular type 1 (IFN-γ) and type 2 (IL-4, -5, -13) cytokines and IL-10 were determined in CD4+ T cells by 3 and 4 colour flow cytometry (BD LSR flow cytometer) as described (5).

Bronchoalveolar lavage cell cultures

Cells from the BAL obtained at baseline bronchoscopy were incubated with medium alone, RV16, LPS or PHA. After 48 hours supernatants were harvested and stored at -80°C for cytokine analysis by Luminex.

Statistical analysis

Symptom scores and lung function were corrected for differences at baseline and effects of bronchoscopy. Except where otherwise stated data are presented as median and interquartile range. Differences during infection from baseline and convalescence were analysed using Friedman's test and if significant Wilcoxon tests. Differences between normal and asthmatic groups were analysed using Mann Whitney tests and correlations using Spearman's rank correlation. Results of stimulation of BAL cells were analysed within groups by paired and between groups by un-paired parametric or non parametric tests as determined by data distribution.

Results

Recruitment and baseline characteristics of subjects

11 atopic asthmatic and 17 non atopic normal volunteers were recruited into the study. One asthmatic and 2 normal volunteers did not continue following the baseline phase. There were no serious adverse events and no subject required inhaled or oral steroids for their asthma.

Confirmation of RV16 infection

Successful experimental RV16 infection was confirmed in all subjects. These were similar in both groups and characteristic of an acute infection, with undetectable virus in most volunteers on day 1 following inoculation, followed by a rapid increase in virus loads on day 2 following inoculation with a peak around days 2-4. Virus was also detected in the lower airway in both groups of subjects. Virus loads in both upper and lower respiratory tracts were between 0.5 and 2 Logs greater in the asthmatic compared with the normal subjects, however these differences were not statistically significant.

RV induction of chest symptoms

Both groups also had significantly increased chest symptoms during infection. Daily chest scores peaked later than cold symptoms for both groups at day 6. Relative to the mean score on days –4 to 0 there were significant increases in daily chest scores for asthmatic subjects on days 1 and 3-7 but for normal subjects only on day 6. Daily chest scores were significantly greater in the asthmatic group than the normal group on day 3 (p=0.018), day 4 (p=0.002) and day 7 (p=0.021). Peak chest score was higher in the asthmatic group [asthmatic 3 (1.5,7.5) v normal 1 (0,3) p=0.05] (Figure 1) as were total chest symptoms in the asthmatic subjects [13 (5,25)] compared with normal subjects [2(-1,7)], p=0.017.

RV induced reductions in lung function

RV infection induced no significant changes in PEF or FEV1 in the normal group on any study day (p=NS on all days), in the asthmatic group there were significant falls in FEV1 on day 3 and 5-14 (not shown) and in PEF on days 1-8 and 12-14 (figure 2). In addition the median maximum % fall was significantly greater in the asthmatic group for both FEV1 [12.5 (5.1,17.9) v 3.8 (0.3,6.8), p=0.027] and PEF [10.8 (9.2,30.6) v 4.5 (0.8,9.6) p=0.003].

RV induction of bronchial hyperreactivity

For the asthmatic group there was a significant reduction in both the PC20 [-0.6 doubling dilutions (-0.6,-1.3) p=0.038] and the PC10 histamine [-0.9 (0.1,-1.5) p=0.05] at day 6 relative to baseline (Figure 3A). In contrast, there was no significant change in PC10 [0.2 (-0.9, 0.7) p=0.9] for the normal group (Figure 3B). As a consequence, at infection, the asthmatics had significantly greater bronchial hyperreactivity [day 6 PC10 for asthmatic subjects 0.3 mg/mL vs 6.4 mg/mL for normal subjects $p<0.001$].

Relationship between clinical illness severity and virus load

There was a strong significant correlation between total lower respiratory symptoms and nasal virus load in the asthmatic group [r=0.79, p=0.01], but none in normal subjects (Figure 4).

Relationship between immunopathology and clinical illness severity and virus load

Lower airway inflammation was related to clinical illness severity in asthmatic subjects as both BAL neutrophils [r=-0.74, p=0.021] and eosinophils [r=-0.7, p=0.049] were significantly related to maximal fall in PEF and sputum eosinophil counts on day 3 correlated with total lower respiratory symptoms [r=0.83, p=0.042] and on day 7 with fall in PC10 histamine [r=-0.87, p=0.05] , there were no such relationships in normal subjects (p=NS).

Relationship between Th1/2 immune responses and clinical illness severity and virus load

We investigated responses in the lower airway and their relation to lower respiratory outcomes and found that IFN-γ production by BAL CD4+ T cells was associated with protection, as

stronger responses were strongly related to less severe falls in PEF [(r=0.85, p = 0.004)] (Figure 5A), while in contrast, BAL CD4+ T cell production of the Th2 cytokines IL-4, IL-5 and IL-13 were all associated with adverse outcomes, as stronger responses were related to more severe lower respiratory symptom scores [(IL-4 r=0.7, p = 0.03; IL-5 r=0.75, p = 0.02; IL-13 r=0.68, p = 0.045)] (Figure 5B).

Conclusions

We have used experimental RV infection in asthmatic and normal volunteers to investigate the pathogenesis of virus induced asthma exacerbations. We found that asthmatic subjects had markedly increased lower respiratory symptoms compared with normal subjects who developed only minimal lower respiratory symptoms. The total lower respiratory tract symptom score in asthmatics was >6 times greater than that recorded by the normal volunteers. In addition, only asthmatic subjects had reductions in lung function and increases in bronchial hyperreactivity in response to infection. These observations confirm that RV infection in asthma is associated with greatly increased lower respiratory clinical illness severity. In conclusion, we have demonstrated clear differences between the responses of asthmatic and normal subjects to RV infection: in terms of clinical symptoms and airway physiology. We have related these changes to virus load and both clinical outcomes and virus load with airways inflammation. Further, we demonstrate that Th1 cytokines are associated with protection from exacerbation while Th2 cytokines were associated with increased disease severity. These observations provide compelling evidence supporting an important role for RV induced lower airway inflammation in precipitating asthma exacerbations, perhaps through impaired Th1and augmented Th2 responses.

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Figure 1

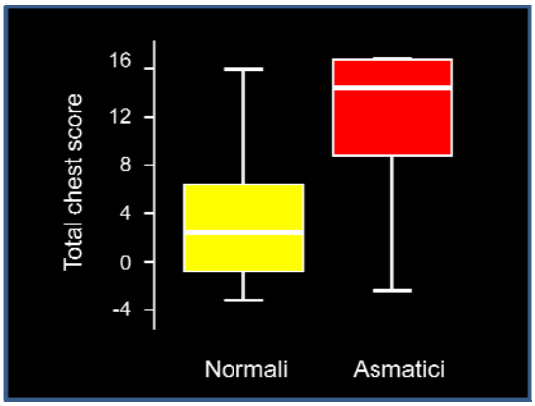


Figure 1: Lower respiratory symptom scores during RV16 infection

Lower respiratory (chest) symptom scores were determined following RV16 inoculation in the asthmatic and normal subject groups. Chest symptoms were more severe in the asthmatic group.

Figure 2

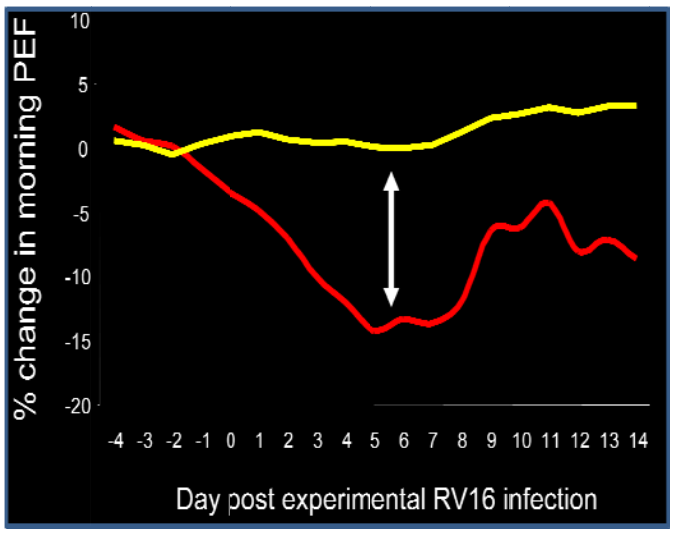
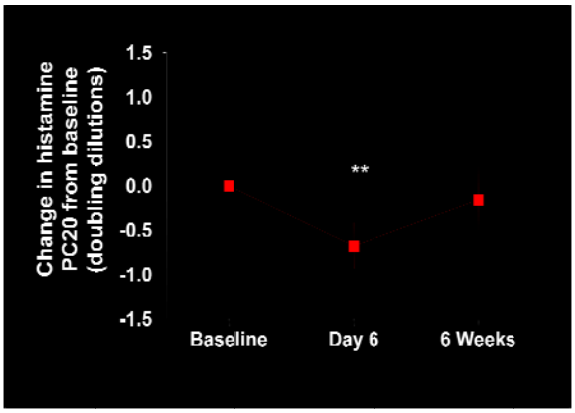


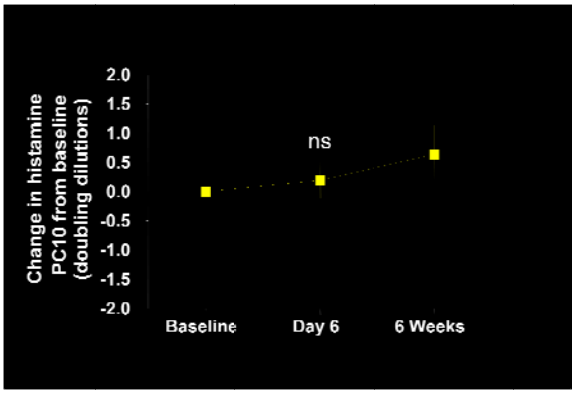
Figure 2: lung function (PEF) during RV16 infection

Daily PEF are expressed as the % change in morning PEF from baseline. PEF group median % change (asthmatics solid line, normals dotted line) for days –4 to 14. In the normal group no significant change in PEF was seen on any of the days after inoculation. In contras in the asthmatic group there were significant falls in PEF on days 1-8 and 12-14.

Figure 3



A



B

Figure 3: Changes in bronchial reactivity during

Histamine PC10 and PC20 (in asthmatics only) were measured at baseline, day 6 and at convalescence. In the asthmatic group (A) there were significant falls in both PC20 and PC10 (not shown). In contrast in the normal group (B) there was no change in histamine PC10 after inoculation.

Figure 4

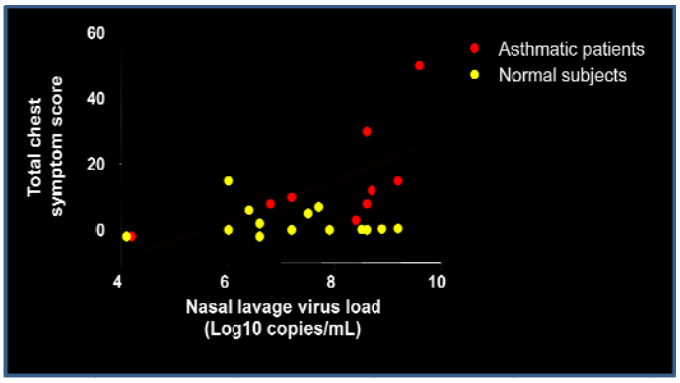
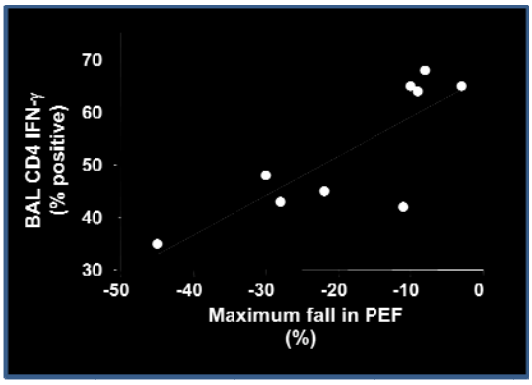


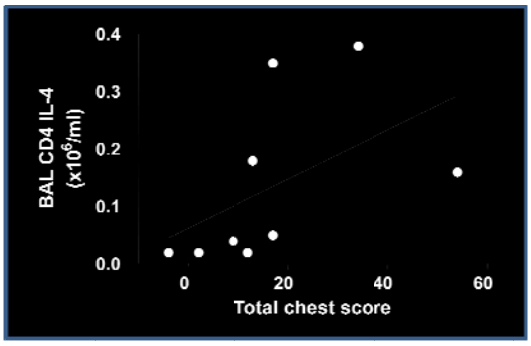
Figure 4: Relationships between virus load clinical illness severity

In the asthmatic group (red circles) there was a significant correlation between peak virus load after inoculation and total chest symptom score.

Figure 5



A



B

Figure 5: Relationships between BAL CD4+ IFN-γ and IL-4 production and reductions in lung function and lower respiratory symptoms in asthmatic subjects.

Bronchoalveolar lavage (BAL) CD4+ IFN-γ and IL-4 production were assessed at baseline prior to experimental RV infection by intracellular cytokine staining. In the asthmatic group there was a significant inverse correlation between the maximum fall in PEF and CD4 IFN-γ production and significant positive correlations between CD4+ IL-4 production and total chest symptom score.

Development of a human model of rhinovirus-induced COPD exacerbations

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Abstract and Aim

Viral infections are the most frequent cause of COPD exacerbations. The recent development of a human experimental model of rhinovirus-induced COPD exacerbations represents an innovative tool with the potential to increase our understanding of the inflammatory and immunological mechanisms that lead COPD patients to exacerbate after respiratory virus infections. Moreover this model will provide the opportunity to test, in a carefully controlled setting, novel pharmacological compounds with potential for treating and preventing COPD exacerbations. In this chapter we will discuss preliminary reports regarding the development of this human model of virus induced COPD exacerbation.

In vitro data document that respiratory virus infection can lead to COPD exacerbation via the production of several proinflammatory molecules that are relevant to the pathogenesis of COPD exacerbation (1-4). However, although i*n vitro* models can provide important insights into the molecular mechanisms of inflammatory and immune responses to viral and bacterial infections, the *in vitro* data require validation using *in vivo* models.

Bacterial infections have been considered important causes of COPD exacerbations for a long time. Nevertheless, while several mechanisms [e.g. induction of mucus hypersecretion (5), reduction of ciliary beat frequency (6) and enhancement of neutrophilic inflammation (7)] have been proposed to explain how bacterial infection can trigger COPD exacerbations, no animal model of bacteria induced COPD exacerbation is available. Until recently this was also true for viral exacerbations.

Carrying out studies of naturally occurring COPD exacerbations has proved difficult for a number of reasons including non reporting of exacerbations by patients, lack of baseline data before exacerbations, wide variation in aetiology, variation in timing of sampling relative to onset of exacerbation and finally carrying out invasive airway investigations in acutely unwell patients is difficult and may jeopardize their health. One way to overcome these obstacles is the development of a human experimental model that would allow studies to take place under controlled conditions. The first step towards development of such a model has been recently realised (8) with the reporting of the first study evaluating the effects of an experimental rhinovirus infection in COPD patients.

In this pilot study mild COPD patients were selected for experimental infection with the purpose a) of evaluating whether the procedure is safe in patients with mild COPD (mean FEV_1 was 74.8% predicted) and b) of providing preliminary data on whether experimental rhinovirus infection in COPD patients is per se sufficient to trigger an exacerbation. As safety was the prime

concern, a very carefully designed rhinovirus dose escalation study was performed in a small (n=5) group of subjects - to determine the minimum dose of virus able to induce clinical colds in 80% (4 out of 5) of the inoculated subjects. Surprisingly, all of the first 4 patients exposed to the initial lowest dose of rhinovirus inoculum experienced not only cold symptoms but also lower respiratory tract symptoms characteristic of a COPD exacerbation associated with a significant fall in lung function (8) as occurs with naturally occurring exacerbations (9). The severity of the exacerbations induced was graded mild to moderate, thus achieving the primary aim of the study – to show that experimental rhinovirus infection could be safely carried out in mild COPD patients.

This model also showed that experimental rhinovirus infection can cause exacerbation in COPD patients and that this model has the potential to provide a valid model of naturally occurring COPD exacerbation. In COPD this is so far the only scenario in which a specific aetiology has been experimentally proven to induce exacerbation.

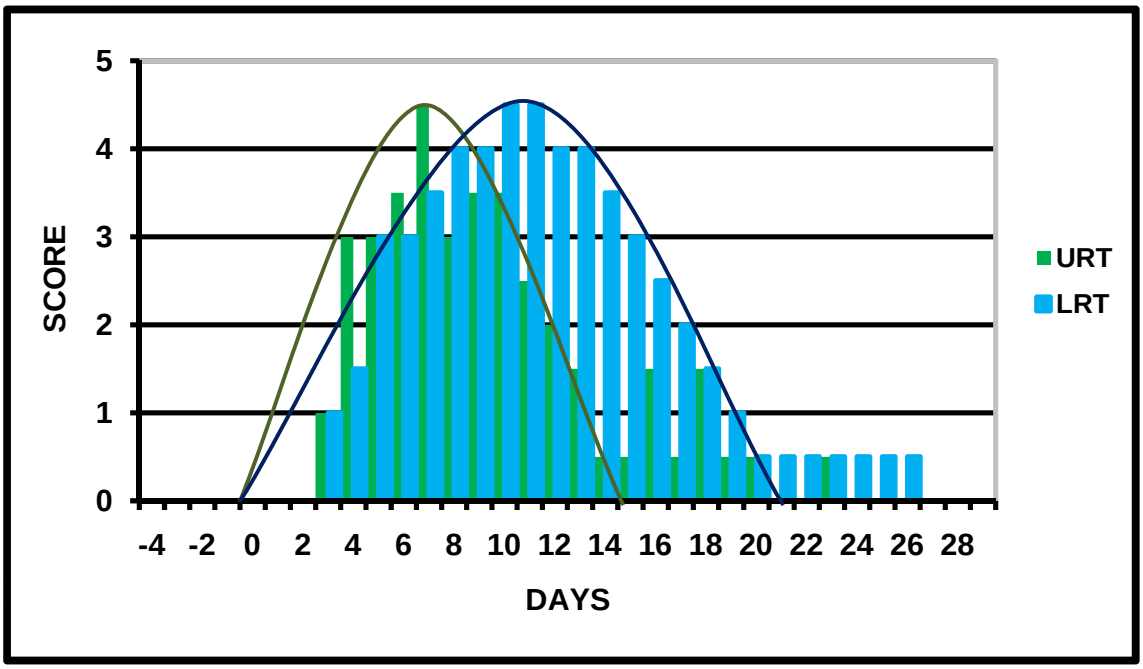
Two potentially important preliminary observations arose from this study: i) COPD patients developed colds and exacerbations with 100- to 1,000-fold lower doses of virus than used in previous studies in asthmatic and normal volunteers; ii) there was a 3 to 4 days gap between the peak of cold symptoms and the peak of lower respiratory symptoms (8) (figure 1). These data suggest that COPD patients may be highly susceptible to virus infection and that if an effective antiviral or anti-inflammatory treatment could be given at the onset of cold symptoms, this could possibly change the clinical outcome of the viral infection in COPD. These preliminary findings clearly require confirmation in a larger study of COPD patients.

In a similar experimental model it has been recently documented in asthmatic patients that the severity of the virus-induced exacerbations, both in terms of symptoms, lung function reduction and inflammation in the airways, was inversely related to the production of a novel

class of interferons called IFN-λ (10). An impaired innate immune response can therefore be one of the mechanisms of increased susceptibility of asthmatic patients to respiratory viral infections. The new model in COPD will give the opportunity to test whether similar or different immune deficiencies are present in COPD, and if so, whether modulation and/or restoring of the immune response could represent a plausible pharmacological approach to treat/prevent COPD exacerbations.

To further validate the results achieved in the *in vivo* experimental model in COPD and to investigate more deeply the mechanisms of viral induced COPD exacerbations, a larger study has been recently undertaken and preliminary findings published in form of an abstract (11). In this study 21 subjects (10 mild COPD patients and 11 age- and smoking-matched controls) have been enrolled. The results confirm that the model is feasible and safe. Symptomatic colds were accompanied by lower respiratory tract symptoms in both groups with breathlessness increasing significantly only in the COPD group. Interestingly, at variance with smokers with normal lung function, sputum neutrophils significantly increased in COPD patients following experimental infection. These data indicate that experimental RV infection in COPD induces symptoms, lung function changes and systemic and airway inflammation similar to that observed in naturally-occurring exacerbations supporting, for the first time, a causal relationship between rhinovirus infection and COPD exacerbations. The development of such an experimental model in which causation is clearly defined and in which detailed clinical studies on mechanisms of disease can be carried out, will offer an invaluable tool to increase our understanding of the specific immunological and inflammatory events that lead COPD patients to exacerbate after viral infection. Moreover this model will offer the possibility to highlight and to test novel pharmacological targets able to treat and/or prevent viral induced COPD exacerbations.

Figure 1



Upper (URT) and lower (LRT) respiratory tract symptoms of rhinovirus in vivo experimentally infected COPD patients. A 3 to 4 days gap between the peak of cold symptoms and the peak of lower respiratory symptoms was documented (8). This data suggests that if an effective antiviral or anti-inflammatory treatment could be given at the onset of cold

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Appendix

Pubblication list (2005-2008)

Peer-reviewed journals

- **Contoli M**, Caramori G, Mallia P, Johnston S, Papi A. Mechanisms of respiratory virus-induced asthma exacerbations. *Clin Exp Allergy* 2005; 35: 137-45.

- Giovannini M, Braccioni F, Sella G, **Contoli M**, Perri G, Frati F, Incorvaia C. Comparison of allergen immunotherapy and drug treatment in seasonal rhinoconjunctivitis: a 3-years study. *Allerg Immunol* 2005; 37: 69-71

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- **M. Contoli**, S. D. Message, V. Laza-Stanca, M. R. Edwards, P. A. B. Wark, N. W. Bartlett, T. Kebadze, P. Mallia, L. A. Stanciu, H. L. Parker, L. Slater, A. Lewis-Antes, O. M. Kon, S. T. Holgate, D. E. Davies, S. V. Kotenko, A. Papi, S. L. Johnston. Role of deficient type III interferon-λ production in asthma exacerbations. *Nature Medicine* 2006; 12 (9): 1023-26.

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- Simon D Message, Vasile Laza-Stanca, Patrick Mallia, Hayley L Parker, Jie Zhu, Tatiana Kebadze, **Marco Contoli**, Gwen Sanderson, Onn M Kon, Alberto Papi, Peter K Jeffery, Luminita A Stanciu, Sebastian L Johnston. Rhinovirus induced lower respiratory illness is increased in asthma and related to virus load and Th1/2 cytokine

and IL-10 production. *Proceedings of the National Academy of Sciences (PNAS).* 2008, 105 (36): 13562–13567.

- Musa R. Khaitov, Vasile Laza-Stanca, Michael R. Edwards, Ross P. Walton, Gernot Rhode, **Marco Contoli**, Alberto Papi, Luminita A. Stanciu, Sergei V. Kotenko and Sebastian L. Johnsto**n.** Respiratory Virus Induction of Alpha-, Beta- and Lambda-interferons in Bronchial Epithelial Cells and Peripheral Blood Mononuclear Cells. *Allergy*. 2008, in press.

Book chapters

- **Marco Contoli**, Luminita A. Stanciu, Simon Message, Alberto Papi, Sebastian L. Johnston. *Susceptibility to asthma exacerbations – antiviral immunity and protection against asthma exacerbations.* In: Exacerbations of asthma. Editors: Sebastion L. Johnston and Paul O'Byrne. Published by: Informa Healthcare, 2 Park Square, Milton Park, Abingdon, Oxfordshire OX14 4RN, UK. 2007: 167-185.

- **Marco Contoli**, Gaetano Caramori, Patrick Mallia, Alberto Papi, Sebastian L. Johnston. *A human model of rhinovirus-induced COPD exacerbations.* In: Models of Exacerbations in COPD and Asthma. Edited by U. Sjöbring and J.D. Taylor. A volume in the series 'Contributions to Microbiology'. 2007;14:101-12.

- **Marco Contoli**, Gaetano Caramori, Brunilda Marku, Anita Pandit, Alberto Papi. *Interactions of Airway Pathogens and Inflammatory Processes.* In: Exacerbations of chronic obstructive pulmonary disease (COPD). Edited by: Wisia Wedzicha (UK) Fernando Martinez (USA). Published by: Informa Healthcare, 52 Vanderbilt Avenue New York, NY USA, 2008 in press

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- **M. Contoli**, V. Laza-Stanca, M. Edwards, C. Hewson, S. Kotenko, A. Papi, S.L. Johnston. Rhinovirus induces IFN-λs in bronchial epithelial cells: new cytokines with anti-viral activity against rhinovirus. *American Thoracic Society Annual Conference 2005*, poster number 708, page A583

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