Rhinovirus Induced Airway Inflammation in Naïve and Allergic Mouse Models

by

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List of Abbreviations

ANOVA, analysis of variance; **APC**, antigen presenting cell; **Arg**, arginase; **ATCC**, American Type Culture Collection; **BAL**, bronchoalveolar lavage; **CD**, cluster of differentiation; **COPD**, chronic obstructive pulmonary disease; **CysLT,** cysteinyl leukotriene; **DC**, dendritic cell; **DPP-I**, dipeptidyl protease I; **ECP**, eosinophil cationic protein; **Fizz**, found in inflammatory zone; **FN**, fibronectin; **FEV_1,** forced expiratory volume/ 1 second; **ELISA**, enzyme-linked immunosorbent assay; **ENA**, epithelial neutrophil attractant**;** **ELR**, Glu-Leu-Arg; **GAPDH**, Glyseraldehyde-3-phosphate dehydrogenase; **GM-CSF**, granulocyte macrophage colony stimulating factor; **GRO**, growth related oncogene**;** **HSV**, herpes simplex virus; **INOS**, inducible nitric oxide synthase; **IGF**, insulin like growth factor; **ICAM**, intercellular adhesion molecule**;** **IFN-γ**, interferon gamma, **IL,** interleukin**;** **IP**, intra-peritoneal; **IP-/CXCL-10**, interferon gamma inducible protein-10; **IN**, intranasal; **KC,** keratinocyte chemoattractant; **LDL-R**, low density lipoprotein receptor; **LPS**, lipopolysaccharide; **MBP**, major basic protein**;** **MCP,** monocyte chemotactic protein**;** **MIP**, macrophage inflammatory protein**;** **MMP,** matrix metalloproteinase; **MPO,** myeloperoxidase; **MRC**, mannose receptor-C; **NE,** neutrophil elastase; **NK**, natural killer; **OVA**, ovalbumin; **PBS**, phosphate-buffered saline; **RANTES,** regulated upon activation normal T-cell expressed and secreted**;** **RSV**, respiratory syncytial virus, **RT-PCR**, reverse transcriptase polymerase chain reaction; **RV**, Rhinovirus; **Th-2,** T-helper (h)-2**;** **TGF,** transforming growth factor; **TNF**, tumor necrosis factor; **UV**, ultraviolet.

Chapter 1

Introduction

Rhinovirus as a lower respiratory pathogen

Rhinovirus (RV) is a small, positive-stranded RNA virus of the *Picornaviridae* family, responsible for majority of the common colds. Respiratory viruses, including RV, are responsible for 80% of asthma exacerbations in children and roughly 50% of exacerbations in adults (1, 2). Of these, RV accounts for the most virus induced asthma exacerbations (3). There are over 100 serotypes of RV. The major group serotypes (approximately 90%), for example RV14, 16 and 39, bind to intercellular adhesion molecule (ICAM)-1 (4). Minor group viruses, such as RV1B, bind to low density lipoprotein family receptors (LDL-R) (5). Finally, a third group of previously unrecognized RVs has been shown to cause respiratory illness in infants (6, 7).

Upon binding, endocytosis, and endosomal acidification, RV undergoes uncoating subsequent release of viral RNA into the cytosol. The virus RNA dependent RNA polymerase 3D then forms the corresponding negative strand which serves as a template for synthesizing the positive strand. A full cycle of replication occurs in roughly 8 hours at an optimal temperature range from 33º to 35º C, the temperature present in the nasal passages. On this basis, RV was not believed to be a lower respiratory tract pathogen. However more recently, RV RNA was detected in lower airway cells of infected subjects

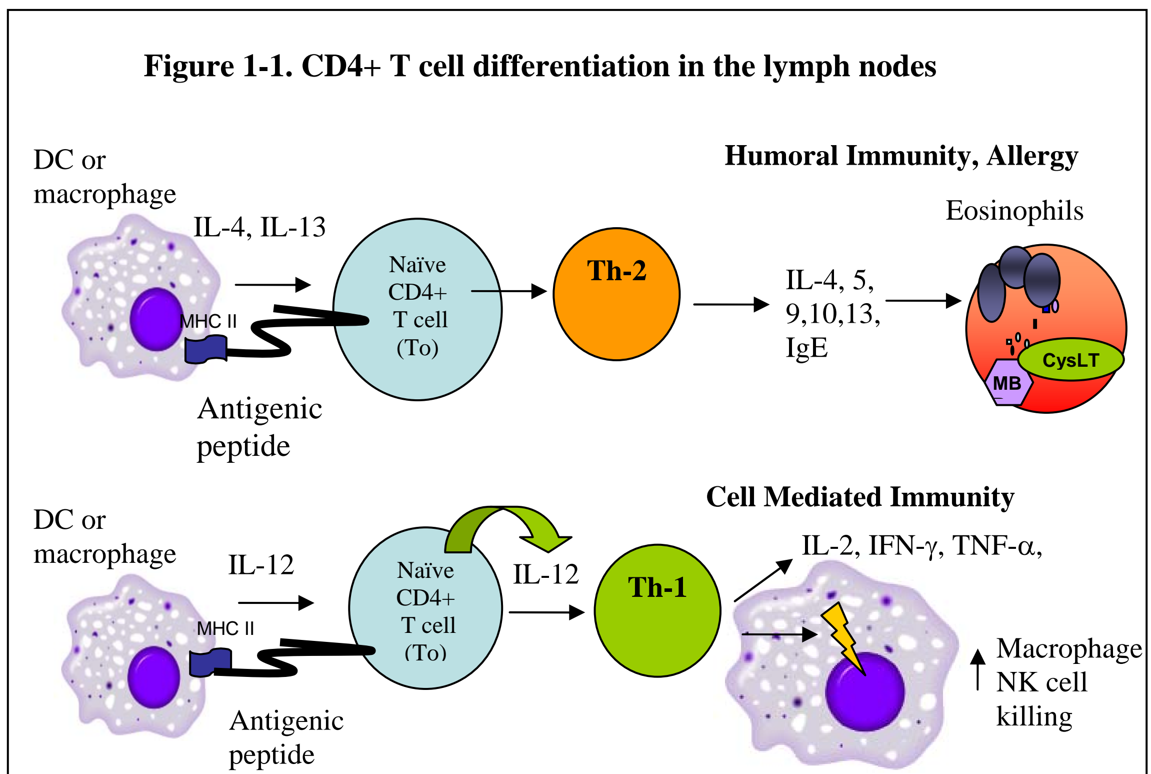
following bronchoalveolar lavage (BAL) (8, 9), and RV capsid was present in bronchial biopsies after viral inoculation (10). Finally RV RNA has been detected in bronchial biopsies of asthmatic subjects long after their symptoms of exacerbations had cleared (11). These studies suggest that RV may infect the lower airways *in vivo* and contribute to airway inflammation, although the extent of viral replication is not clear.

Asthma and the Th-2 paradigm

Asthma is a chronic disease characterized by airway inflammation, mucus overproduction, and bronchoconstriction which together result in airflow obstruction (12). Roughly 7 % of the US population suffers from asthma, and eleven million asthma exacerbations occur each year driving up the costs of hospitalization to over ten billion dollars annually. As noted above, viral infections are the most common triggers of asthma exacerbations, though allergens, and environmental irritants can also elicit attacks (13). Classically, asthma is considered a T-helper (h)-2 cluster of differentiation (CD)4+ T cell driven allergic airways disease. Upon contact with an allergen, antigen presenting cells (APC), including dendritic cells (DC) and macrophages residing in the tissues process the antigen and present its peptide via major histocompatibility complex (MHC) II molecules present on the their surface to naive CD4+ T cells in the lymph nodes. In the presence of pro-allergic cytokines IL-4 and IL-13, these naïve CD4+ cells (T_o) then differentiate into CD4+ Th-2 cells which secrete more IL-4, IL-13, pro-eosinophil growth factors IL-5, and granulocyte macrophage colony stimulating factor (GM-CSF) (14) (Figure 1-1). IL-13-overexpressing mice show increased airway hyperreactivity and Th-2 cytokine production suggesting that IL-13 plays a critical role in

exacerbating allergic airway inflammation (15) and hyperresponsiveness. In humans, IL-4, IL-5, and IL-13, have been detected in bronchial biopsy specimens from asthmatics (16, 17). Thus chronic asthma is directed in a large part by the Th-2 cell-derived cytokines IL-4 and IL-13, which in turn promote eosinophil infiltration, mucus secretion (18, 19) and contribute to overall airway inflammation and airflow obstruction. In the absence of IL-4 and IL-13, and the presence of Th-1 cytokines IL-12, naïve CD4+ T cells differentiate into CD4+ Th-1 directed cells which have been shown to trigger cell-mediated immunity via activation of cytotoxic T cells and natural killer cells, reviewed in (14). Finally, neutrophils are also an important feature of asthma and in fact some severe asthmatics have predominantly neutrophilic airway inflammation, which is typically unresponsive to treatment (20, 21).

Figure 1-1. CD4+ T cell differentiation in the lymph nodes



Chemokines and their role in inflammation

Chemokines are a family of secreted cytokines that form a chemotactic gradient which triggers the migration of leukocytes including neutrophils, eosinophils, and lymphocytes to the site of inflammation or injury. They are also involved in important physiological outcomes including fibrosis, angiogenesis, and hematopoiesis. Chemokines are comprised of 70 to 90 amino acids and are 8-12 kDa in molecular weight. They are classified into 4 supergene families based on the arrangements of N-terminal cysteine repeats and are designated as C, CC, C-X-C, and CX_3C. Chemokines play critical roles in the chronic disease conditions including asthma and COPD (22).

Inflammatory cells and chemokines implicated in asthma: Eosinophils and Eotaxin

Typically, eosinophils, neutrophils, and macrophages are derived from myeloid progenitor cells from the bone marrow. Eosinophils mature in response to growth factors including IL-3, IL-5, and GM-CSF (23). Eosinophil degranulation can lead to the release of bronchoconstrictors including cysteinyl leukotriene (CysLTs) and major basic protein (MBP) which act on the airways to increase smooth muscle contraction. CysLTs and MBP are upregulated in eosinophils elicited from asthmatics (24, 25). Eosinophils have been closely associated with disease pathology in asthma, reviewed in (23, 26) and correlate with decreased airflow (27). Indeed sputum eosinophil counts have been associated with more frequent exacerbations (28) although considerable variability exists between the level of eosinophil inflammation in asthmatics. In addition eosinophils themselves can release inflammatory cytokines including IL-4, IL-5, and IL-13 which can potentiate airway inflammation (23, 26, 29). Th-2 directed severe eosinophilic

asthma is also associated with the presence of airway remodeling due in part to the production by eosinophils of transforming growth factor (TGF)-β (20).

Chemokines which bind eosinophils and cause migration into the airways include eotaxin-1/CCL11, eotaxin-2/CCL24, eotaxin-3/CCL26, macrophage inflammatory protein (MIP)-1α, and regulated upon activation normal T-cell expressed and secreted (RANTES) (30-32). Among these, the eotaxins, which bind only the CCR3 receptor on eosinophils, are eosinophil-specific chemokines, therefore recruiting a distinct cell population. Furthermore eotaxin-1 levels negatively correlate with lung function in patients with asthma (33-35).

In animal models**,** eotaxin-1 and eosinophils have been shown to play a role in eosinophil recruitment in allergic airway inflammation as well as airway hyperreactivity (19, 30, 31, 36). Recently, eotaxin-1 has been shown to regulate eosinophil accumulation in the tissues while eotaxin-2 directs eosinophil accumulation in the airway (37). Studies involving double knockout of both genes show a cooperative regulation of eosinophilic inflammation in response to a Th-2 environment (38). Furthermore targeted siRNA interference of the eosinophil growth factor, IL-5, after ovalbumin sensitization and challenge reduced airway eosinophilic inflammation, eotaxin levels, and airway resistance to methacholine underscoring the relationship between eosinophils and airway responses (39). In addition to migration, eotaxin has also been demonstrated to play a role in eosinophil activation and degranulation via activation of mitogen activated protein (MAP) kinases (40). In this study, inhibition of the MAP kinase signaling reduced eotaxin-induced release of the cytotoxic mediator eosinophil cationic protein (ECP), suggesting that eotaxin may be involved in more direct pathways of cell injury and

inflammation than merely chemotaxis. These studies suggest a critical role of eosinophils and eotaxin in augmenting airway inflammation and bronchoconstriction.

Neutrophils and C-X-C chemokines

Non-Th-2 cell types including neutrophils are also found in patients with asthma. However the role of neutrophils in the pathogenesis of asthma exacerbations is not clearly understood. Neutrophils and their granule products including neutrophil elastase (NE) and matrix metalloproteinase (MMP)-9, which can cause damage to the tissues, have been found in the lungs of severe asthmatics (20, 21, 41, 42). In addition, C-X-C chemokines including IL-8, which serve as chemotactic factors for neutrophils, are increased in the induced sputum of asthmatics (43). In patients with asthma, neutrophilic inflammation has been associated with reductions in pre- and post-bronchodilator forced expiratory volume/ 1 second (FEV_1) (44) suggesting an important correlation between neutrophils and airflow limitations. Murine CXCR2 is widely expressed on neutrophils and serves as a receptor for the IL-8 homologs keratinocyte chemoattractant (KC) and macrophage inflammatory protein (MIP)/CXCL-2. In mice, CXCR2 is responsible for the recruitment of neutrophils to the lungs in various mouse models of airway inflammation (45, 46). In fact, CXCR2 antagonists have been used in animal models to reduce neutrophil influx and mucus hypersecretion, indicating a therapeutic effect of CXCR2 blockade (47). In other studies, CXCR2 has been implicated in respiratory syncytial virus (RSV)-induced lung inflammation; although this effect was mediated by macrophages, not neutrophils (48). In animal models of Th-17 mediated neutrophilic airway inflammation, neutrophils play a causal role in airway hyperreactivity in mice

exposed to ovalbumin (OVA) and lipopolysaccharide (LPS) (46). In another study, doxycycline-induced overexpression of KC/CXCL-1 caused increased neutrophil influx and hyperreactivity in a fungal model of asthma and depletion of neutrophils reduced airway responses to methacholine (49). In addition to releasing tissue-damaging agents, neutrophils can also release several pro-inflammatory mediators which promote airway inflammation and obstruction, including TNF-α, IL-1β and serine proteases (50-53). Many cytokines, including IL-1β and TNF-α are shown to be expressed at higher levels in patients with asthma (54, 55). TNF-α is a potent cytokine that is secreted by macrophages, monocytes, lymphocytes and fibroblasts, and it is known inducer of airway inflammation (54, 56). Increased levels of TNF-α are found in the bronchoalveolar lavage fluid of patients with asthma, and inhalation of TNF-α has been shown to increase airway hyperresponsiveness and sputum neutrophils (54). In a murine model of Sendai virus induced airway inflammation, knockout of the neutrophil-derived protease dipeptidyl protease (DPP)-I reduced lung pro-inflammatory cytokine expression of TNF-α, IL-1β, and MIP-2 (57); reconstitution of DPP-I-sufficient neutrophils via adoptive transfer upregulated these cytokines. Taken together, these studies point towards a causal role of neutrophils and neutrophil mediated inflammation in airway responses in asthma.

Classical and alternative activation of macrophages and their relationship to airways disease

Macrophages have been long considered to have anti-microbial properties and were conventionally regarded as protective in response to invading pathogens (58, 59). Classically-activated M1 macrophages, which are stimulated by interferon gamma (IFN-

γ), express typically anti-bacterial and anti-viral responses by upregulating pro-inflammatory cytokines including TNF-α, IL-6, IL-12, inducible nitric oxide synthase (iNOS) and interferon gamma inducible protein, IP/CXCL-10. Alternatively, when stimulated with Th-2 cytokines IL-4, and IL-13, which can both bind to IL-4Rα, these cytokines generate an "anti-inflammatory" or an M2 response by inhibition of the M1 pathway and augmentation of a "wound-healing" phenotype characterized by induction of arginase (Arg)-1, insulin like growth factor (IGF)-1, transforming growth factor (TGF)-β, fibronectin (FN) and mannose receptor C (MRC)-1 which can contribute to collagen deposition, hypertrophy of the airway smooth muscle, and pro-fibrotic growth (Figure 1-2).

In recent studies, cluster of differentiation (CD)-68 positive macrophages have been seen in bronchial biopsies of subjects with asthma and chronic obstructive pulmonary disease (COPD), and associated with long term airway pathology (60-63). M2 markers, Arg-1 and TGF-β have been detected in airways of asthmatics (20, 64). In COPD smokers, classical activation markers were markedly downregulated whereas certain M2 markers including MMP-2 and MMP-7 were induced, suggesting a polarization of these cells (60) in disease states. In animal models, IFN-γR- deficient mice infected with herpes simplex virus displayed severe pulmonary fibrosis and the presence of alternatively activated macrophages, indicating that inhibition of the M1 pathway may lead to an M2-directed airways disease (65), and that the balance between M1 and M2 states of macrophages is essential for lung immune homeostasis. M2 markers including Ym-1, macrophage galactose N-acetyl-galactosamine specific lectin (MGL)-1 and (MGL)-2 have also been noted in mouse models of allergic asthma

utilizing parasitic infection, and are expressed in response to Th-2 cytokine IL-4 (66, 67). In one study, macrophage depletion of chicken egg antigen, ovalbumin (OVA) sensitized and challenged mice reduced bronchial hyperresponsiveness (68). It is therefore conceivable that exposure to Th-2 cytokines IL-4 and IL-13 alters macrophage activation state so as to induce a shift from M1 to M2, releasing mediators that cause airway inflammation and remodeling. The dichotomy between M1 and M2 pathways, however, may not be absolute as, in the same study, neutralization of M1 marker IFN-γ also reduced bronchial hyperreactivity, implying that certain M1 mediators may also contribute to airway sensitivity. Finally little is known about macrophage response to respiratory viruses in the context of already present allergic inflammation. Their response to stimuli *in vitro* appears to be contingent on the cytokine milieu in which they suspended (69). For instance, exposure to IL-4 induced more M2 cytokine MCP-1/CCL2 production than exposure to IL-4 followed by M1 cytokine IFN-γ, revealing complex interactions underlying cytokine production within the same cell.

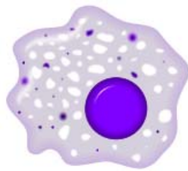


Figure 1-2. Documented macrophage activation markers in mice

M1 markers (IFN-γ stimulated)- Classical Activation

- TNF-α
- IL-6
- IL-12
- IP/CXCL-10
- IFN-γ
- RANTES

Pro-inflammatory, anti-viral, anti-bacterial responses which promote airway inflammation

M2 Markers (IL-4, IL-13 stimulated)- Alternative Activation

- Arg-1, Ym-1, MGL-2, Fizz-1
- MRC-1
- IGF-1
- FN-1
- TGF-β
- IL-10

Anti-inflammatory, wound healing, but pro-fibrotic response, collagen deposition, increased airway responses in chronic diseases including fibrosis, COPD, and asthma

RV induction of chemokines and their role in inflammation

In vitro, binding of RV to airway epithelial cell ICAM-1 receptor triggers the induction of neutrophil-attracting C-X-C chemokines with a Glu-Leu-Arg (ELR) motif including IL-8/CXCL8, epithelial-neutrophil activating peptide (ENA)-78/CXCL5 and growth related oncogene (GRO)-α/CXCL1 (70-74). Minor group serotypes such as RV1B produce a similar pattern of inflammatory cytokines upon receptor engagement (75, 76). In humans, C-X-C chemokines including IL-8, granulocyte chemotactic protein (GCP)-2, and GRO-α bind to the G protein-coupled seven transmembrane receptor

CXCR1 and CXCR2 (77-79). Notably, in a human bronchial epithelial cell line, 16HBE 14o-, tumor necrosis factor (TNF)-α, a pro-inflammatory cytokine that is upregulated in asthmatics (80) and induced upon experimental RV infection in allergic subjects (81), potentiates epithelial cell ICAM-1 expression as well as IL-8 secretion (74). These studies illustrate the cooperative effect of RV and cytokines which may exacerbate airway neutrophilia. Additionally, RV infection of a bronchial epithelial cell line (BEAS-2B) induces expression and production of the eosinophil specific chemokines eotaxin-1/CCL11 and eotaxin-2/CCL24, consistent with the notion that the cytokine response to RV infection recruits eosinophils to the airways (82). Accordingly both neutrophils and eosinophils have been reported in normal and asthmatic subjects in response to RV infection (83, 84) and have been implicated in the pathophysiology of asthma exacerbations (20).

RV as a trigger for asthma exacerbations

RV is responsible for the most virus-induced asthma exacerbations (3). IL-8 and neutrophils are found in the nasal secretions, sputum and bronchoalveolar lavage fluid of allergic subjects undergoing experimental RV infection (83-88). Furthermore, the number of neutrophils correlates with the level of IL-8 (86, 88). After RV16 infection, asthmatic patients show increased IL-8 production in nasal lavages which correlates with the level of airways responsiveness (85), in contrast to unaffected individuals in whom IL-8 does not increase (55). Thus RV infection of airway epithelial cells may potentiate pre-existing inflammation by enhancing the production of neutrophil chemoattractants and neutrophilic airway inflammation. On the other hand, eosinophils, eotaxin, and ECP

have also been detected in the airways of subjects following experimental RV infection (83, 84, 89, 90). In asthma patients experimentally inoculated with RV, eosinophils recruited in the BAL fluid were significantly higher than normal subjects and correlated with decline in lung function (83). Furthermore, in allergic patients infected with respiratory viruses including RV, eosinophils persist in the airways long after the infection has cleared (90).

According to the current theory, chemokines produced by RV-infected airway epithelial cells recruit cytokine-producing inflammatory cells, thereby increasing airway responses. However, it has recently been shown that mice infected with Sendai virus showed IL-13 production in macrophages that was dependent on natural killer (NK)-T cell interactions (91). Thus, it is conceivable non-Th-2 cell types, including macrophages, produce Th-2 cytokines in response to a viral stimulus, thereby aggravating existing allergic inflammation. In Chapter 3, we will explore this new paradigm in order to explain poorly-understood viral exacerbations of asthma.

Current animal models of RV infection.

Species differences in ICAM-1 represented the main challenge in developing an animal model of a human major group RV infection. However, recently we (76) and others (92) have shown that minor group serotype RV1B infects C57BL/6 and Balb/c mice thereby providing a model to study RV induced airway inflammation. We have shown that human RV1B replicates in mouse lungs, as evidenced by: 1) the presence of negative-strand viral RNA in the lungs of inoculated mice; 2) transmissibility of RV infection from the lung homogenates of inoculated mice to cultured HeLa cells; and 3)

the induction of a robust lung interferon response (76). Replication-deficient UV-irradiated virus has none of these effects. Furthermore, RV infection was accompanied by a significant increase in IL-8 homologs KC and MIP-2 as well as an influx of airway neutrophils. RV infection caused a moderate increase in airway resistance to methacholine, suggesting a role of RV induced airway inflammation in airway hyperreactivity.

The airways response to a major group virus, RV16, was recently studied using a transgenic mouse expressing humanized ICAM-1 (92). Effects of RV16 were indistinguishable from those of RV1B. Further, major and minor group viruses induce nearly identical patterns of gene expression in cultured airway epithelial cells (93). Finally, recent analysis of all known HRV genomes revealed that HRV1 and HRV16 are highly homologous and respond similarly to small-molecule antiviral compounds (94). Thus, the distinction between at least some major and minor group strains may not be clinically relevant. We therefore believe that these mouse models of human RV infection hold promise for the study of RV-induced exacerbations of chronic airways diseases such as asthma.

Significance

Asthma exacerbations are characterized by increased airway inflammation including neutrophils, eosinophils, and lymphocytes, excessive mucus production, and bronchoconstriction which together result in airflow obstruction. Viral infections cause 80% of asthma exacerbations in children and roughly 50% of exacerbations in adults (1, 2). RV is responsible for the most virus induced asthma exacerbations (3) although the

specific mechanisms of RV triggered asthma exacerbations are unknown. From our previous studies we have determined that the primary response to RV infection is the infiltration of neutrophils and lymphocytes along with the expression of C-X-C chemokines KC and MIP-2. We hypothesized RV induction of C-X-C ligands, augments airway inflammation and hyperresponsiveness by recruiting inflammatory cells including neutrophils to the airway. We further hypothesized that the response to RV infection of "asthmatic" mice is qualitatively different from the response to infection in naïve mice. Identification of these differences would help us identify why a common cold causes moderate changes in airflow in normal subjects but induces sizeable airflow limitations in individuals with asthma. Thus, we attempted to generate an allergic model of Th-2 directed eosinophilic inflammation combined with RV infection. We sought to examine the mechanisms of RV induced airway inflammation in naïve and allergic mice via the following specific aims:

1. Determine the contribution of RV-induced airway neutrophils in airway inflammation and hyperresponsiveness in naïve mice (Chapter 2)
2. Determine the role of eosinophils in RV inflammation and hyperresponsiveness in a mouse model of asthma (Chapter 3).

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Chapter 2

CXCR2 is required for neutrophilic airways inflammation and hyperresponsiveness in a mouse model of human rhinovirus infection

Summary

Human rhinovirus (RV) infection is responsible for the majority of virus-induced asthma exacerbations. Using a mouse model of human RV infection, we sought to determine the requirement of CXCR2, the receptor for ELR-positive CXC chemokines, for RV-induced airway neutrophilia and hyperresponsiveness. Wild-type and CXCR2 -/- mice were inoculated intranasally with RV1B or sham HeLa cell supernatant. Following RV1B infection, CXCR2 -/- mice showed reduced airway and lung neutrophils and cholinergic responsiveness compared to wild-type mice. Similar results were obtained in mice treated with neutralizing antibody to Ly6G, a neutrophil-depleting antibody. Lungs from RV-infected, CXCR2 -/- mice showed significantly reduced production of tumor necrosis factor (TNF)-α, MIP-2/CXCL2 and KC/CXCL1, and lower expression of MUC5B, compared to RV-treated wild-type mice. The requirement of TNF-α for RV1B-induced airways responses was tested using TNF receptor (TNFR)-1 -/- mice. TNFR1 -/- mice displayed reduced airways responsiveness to RV1B, even when exogenous MIP-2 was added to the airways. We conclude that CXCR2 is required for RV-induced neutrophilic inflammation, and that TNF-α release is required for airways hyperresponsiveness.

Introduction

Viral infections trigger 80% of asthma exacerbations in children and nearly 50% in adults, with human rhinovirus (RV) being the most common virus identified. In addition, a large number of patients with chronic obstructive pulmonary disease (COPD) experience RV-induced exacerbations (1). Consistent with the notion that RV causes exacerbations of asthma, experimental RV infection has been shown to increase airway hyperreactivity in asthmatic subjects (2-4). RV has also been shown to increase maximal responses to methacholine in normal subjects (5, 6).

The major group serotypes, including RV16 and 39 bind to intercellular adhesion molecule (ICAM)-1 (7). Binding of RV to airway epithelial cell ICAM-1 triggers the induction of C-X-C chemokines including IL-8, ENA-78/CXCL5 and GRO-α/CXCL1 (8-11). Minor group viruses, such as RV1B, bind to low density lipoprotein family receptors (12). Minor group serotypes such as RV1B produce a similar profiles of chemokine induction *in vitro* (13, 14). ELR (+) C-X-C chemokines, which cause migration of neutrophils to the site of infection, bind to the G protein-coupled seven transmembrane receptor CXCR1 and CXCR2.

Allergic subjects undergoing experimental RV infection experience increased IL-8 and neutrophils in the nasal secretions, sputum or bronchoalveolar lavage fluid (6, 15-18) and the level of neutrophil infiltration correlates with the level of IL-8 indicating that IL-8 may recruit neutrophils to the airways following inoculation with RV. (16, 17). In asthmatic patients, RV infection increases IL-8 in nasal lavage fluid which correlates with the level of airways responsiveness (3) suggesting that IL-8 may affect airflow limitation, possibly via neutrophil chemotaxis. Together, these data suggest that RV

infection of airway epithelial cells may augment already present inflammation by enhancing the production of neutrophil chemoattractants and neutrophilic airway inflammation. Upon stimulation, activated neutrophils release a variety of pro-inflammatory mediators including cytokines such as TNF-α and IL-1β, superoxide, myeloperoxidase and various proteases which could promote airway inflammation and responsiveness (19-23). Among these, IL-1β and TNF-α are shown to be expressed at higher levels in patients with asthma (24, 25). However, the requirement of IL-8 and other CXCR2 ligands, or of airway neutrophils, for RV-induced airway responses has not been studied.

RV1B, a minor group virus, binds to mouse airway epithelial cells (26). Accordingly, a mouse model of human RV1B infection has recently been developed. We have shown in C57BL/6 mice that intranasal inoculation of high-dose RV1B, but not sham HeLa cell supernatant or UV-irradiated virus, induces migration of neutrophils and lymphocytes to the airways, as well as robust lung cytokine, chemokine and interferon production (14). The influx of inflammatory cells is also accompanied by moderate airways hyperresponsiveness to methacholine, which is present both 24 and 96 h post-infection. Inoculation with high-dose RV1B but not UV-irradiated virus also induces airway inflammation and interferon production in BALB/c mice (27). In the present study, we sought to determine the requirement of CXCR2 ligands for RV-induced airway responses, employing a CXCR2 -/- mouse strain that is impaired in neutrophil recruitment. We found that CXCR2 is required for neutrophilic airway inflammation following RV infection. Further, the reduction in airway neutrophils was accompanied by a reduction in airway responsiveness 24 h post infection. Finally, airways

responsiveness was also decreased in TNFR -/- mice, suggesting that neutrophil derived TNF-α release is contributes to RV-induced airways hyperresponsiveness.

Methods

Animals

Wild type and CXCR2-/- BALB/c mice, and TNFR1 -/- C57BL/6 mice, were purchased from Jackson Laboratories (Bar Harbor, MA). Mice were housed in a specific pathogen-free area within the animal care facility at the University of Michigan. All mice were 8 wk old females. This study was approved by the Institutional Animal Care and Use Committee.

Generation of RV stocks

RV1B was generated from an infectious cDNA clone, as described (26). Viral stocks were generated as previously described (11). Briefly, HeLa cells were infected with RV until 80% of the cells were cytopathic. HeLa cell lysates were harvested and cellular debris pelleted by centrifugation (10, 000 x *g* for 30 min at 4°C). RV in HeLa cell lysates was concentrated and partially purified by centrifugation with a 100,000 MW cutoff Centricon filter (2,000 rpm at 4°C for 8 h; Millipore, Billerica, MA) (28). Virus was titered by infecting confluent HeLa monolayers with serially diluted RV (range: undiluted to 10^{-9}) and assessing cytopathic effect five days after infection. Fifty percent tissue culture infectivity doses ($TCID_{50}$) values were determined by the Spearman-Karber method.

RV1B exposure

Mice were anesthetized by intraperitoneal injection with ketamine (40 mg/kg) and xylazine (5 mg/kg) and intranasally inoculated with 45 μl of $1x10^8$ $TCID_{50}$/ml RV1B or equal volume sham HeLa cell lysate, as previously described (14). Mice were euthanized 1 or 4 days post infection.

Bronchoalveolar lavage (BAL) and tissue inflammation

BAL was performed by exposing and intubating the trachea using a 1.7-mm OD polyethylene catheter, and instilling PBS containing 5 mM EDTA in 1-ml aliquots. Cytospins prepared from BAL cells and stained with Diff-Quick (Dade Behring, Newark, DE) and differential counts were determined by counting 200 cells. To quantify the number of inflammatory cells in the tissues, lung digests were performed by mincing the lungs with scissors and suspending the tissue in 30 mg collagenase type IV (Gibco Invitrogen, Carlsbad, CA) in 5 ml serum free RPMI for one h. Cells were isolated by straining through a 70 μm nylon mesh (BD Falcon, San Jose, CA), spun at 1500 g, and the resultant pellet treated with red blood cell lysis buffer (BD Pharmingen, San Diego, CA). Finally, leukocytes were enriched by spinning the cells through 40% Percoll (Sigma-Aldrich, St. Louis, MO), decanting the supernatant and resuspending the pellet in PBS (29). The total cell count was determined on a hemocytometer.

Flow cytometry

In selected experiments, BAL fluid was examined for the number of TNF-α-expressing neutrophils. $1x\ 10^6$ cells were blocked with brefeldin A (3 μg/ml) and

incubated in low attachment polystyrene plates for 5-6 hours. Cells were then stained with Pacific Blue-conjugated antibody against the neutrophil cell surface marker LY6G (BD Pharmingen) and FITC-labeled anti-TNF-α (E-Bioscience, San Diego, CA). IgG antibodies were used as isotype controls. Finally, cells were fixed in 1% formaldehyde, covered with foil and refrigerated until flow cytometry was performed the following day.

Histology

Lungs were fixed in 10% formalin overnight, and then transferred to 70% ethanol and paraffin embedded. H&E staining was performed on a 5 μm section of each lung.

Cytokine/chemokine expression

Lung RNA was extracted using Trizol reagent (Sigma-Aldrich) and analyzed for the presence of MIP-2, TNF-α, GM-CSF, MUC5AC and MUC5B by quantitative two-step real time PCR using specific primers and probes. Primer probe mixes for TNF-α, GM-CSF and MIP-2 expression were purchased from Applied Biosystems (Foster City, CA). MUC5AC and MUC5B primers were from IDT (Coralville, IA) and employed FAM as the fluorescent tag and TAMRA as a quencher. For MUC5AC, the sequences were: forward primer, 5’ AAA GAC ACC AGT AGT CAC TCA GCA A 3’; reverse primer, 5’ CTG GGA AGT CAG TGT CAA ACC A/ 3BHQ_1/-3’; and probe, 5'- /56-FAM/5’ TCA CAC ACA ACC ACT CAA CCA GTG ACC A /36-TAMSp/ -3.’ For MUC5B, the sequences were: forward primer, 5’ GAG CAG TGG CTA TGT GAA AAT CAG 3’; reverse primer, 5’ CAG GGC GCT GTC TTC TTC AT-3’; and Taqman probe,

5'-/56-FAM/ ATC CGC, CTA GTC CTC ACC TTC CTG TGG/ 3 BHQ_1/3.' The signal was normalized to GAPDH and expressed as fold-increase over sham.

Cytokine production

Lungs were homogenized in 1 ml PBS , spun for 15 minutes at 1500 g, and the supernatant assayed for murine homologs of IL-8 including MIP-2/CXCL2, KC/CXCL1, and the proinflammatory cytokines TNF-α and GM-CSF by ELISA (R&D systems, Minneapolis, MN).

Neutralizing antibody preparation

In some experiments, mice were injected intraperitoneally with 30 μg of neutralizing antibody to Ly6G (clone mAb RB6-8C5) or an equivalent dose of rat anti-mouse IgG, inoculated simultaneously with RV1B, and euthanized 24 h post infection. Ascites for anti-Ly6G, an anti-mouse granulocyte neutralizing antibody (30), was obtained from the University of Michigan Vector Core and stored at -20ºC. A 20 mL volume was then thawed overnight and centrifuged at 3000 rpm for 15 min. Debris was removed and the clear suspension transferred to a fresh tube. The ascites fluid was then clarified by ultracentrifugation at 40,000 rpm for 1 h, followed by removal of any lipid masses. Five mL clear ascites was then purified on a Protein G bead column (Millipore) at 4ºC. The ascites was diluted in a 1:2 ratio with binding buffer containing 0.01M sodium phosphate and 0.15 M sodium chloride adjusted to pH 7.0, and loaded onto the Protein G column. The eluate was reapplied to the column and washed 3 times with 50 mL binding buffer to remove any non specific proteins. Bound IgG was then eluted with

20 mL 0.1 M glycine hydrochloride, pH 2.6. The antibody was then stored in 10 tubes containing 0.5 ml 1.0 M Tris-HCL, pH 9.0. Each of the 10 fractions of eluate was measured at A280, and the peak fractions were pooled and dialyzed overnight with 3 changes of PBS. The final concentration of the RB6-8C5 antibody was 1.75 mg/mL.

MIP-2 administration

In some experiments, wild type C57BL/6 and TNFR1-/- mice were administered MIP-2 (1 μg/ml intranasally, R&D Systems) immediately following sham or RV1B infection. Mice were harvested for BAL fluid and airway resistance measured 24 h post-treatment.

Presence of viral RNA

RNA was extracted from lungs of mice using Trizol reagent (Sigma-Aldrich, St. Louis, MO) and analyzed for the presence of viral RNA by reverse transcriptase-PCR. Quantitative one-step real time PCR for positive-strand viral RNA was conducted using RV-specific primers and probes for RV (forward primer: 5'-GTG AAG AGC CSC RTG TGC T-3'; reverse primer: 5'-GCT SCA GGG TTA AGG TTA GCC-3'; probe: 5'-FAM-TGA GTC CTC CGG CCC CTG AAT G-TAMRA-3' (31). Copy numbers of positive strand viral RNA were normalized to 18S RNA, which was similarly amplified using gene-specific primers and probes.

Gene arrays

Lung RNA from sham and RV-treated wild-type and CXCR2 -/- mice was subjected to a targeted PCR array examining mouse inflammatory cytokines (SA Biosciences, Frederick, MD).

Measurement of respiratory system resistance. Mice were anesthetized with sodium pentobarbital (50 mg/kg mouse, intraperitoneal injection) and intubated via cannulation of the trachea with a 20-gauge stub adapter cannula (Becton-Dickinson, Sparks, MD). Mechanical ventilation was performed using a FlexiVent ventilator (Scireq, Montreal, Quebec, Canada) at 150 breaths/min with a tidal volume of 10 ml/kg body weight. Airway responsiveness was assessed by measuring respiratory system resistance in response to increasing doses of nebulized methacholine, as described (14).

Data analysis

SigmaStat computing software (SPSS, Chicago, IL) was used for data analysis. Data are represented as mean±SEM. Statistical significance was assessed by one- or two-way analysis of variance (ANOVA). Differences identified by ANOVA were pinpointed by the Student Newman-Keuls' multiple range test. For gene arrays, unpaired t tests were used to establish differences between groups.

Results

RV infection of BALB/c mice

BALB/c mice were inoculated with 3 x 10^8 $TCID_{50}$ RV1B intranasally or sham equivalent, and lung digests and BAL performed 24 h and 96 h respectively post-infection. The initial (24 h) response to RV infection in BALB/c mice was primarily neutrophilic in character (Figures 2-2A,C). By 96 h, BAL neutrophils were significantly reduced (Figure 2-2E). To further characterize the time course of the neutrophilic response, we examined lung neutrophil counts 2, 8, 16 and 24 hours post-infection (Figures 2-2 A). We observed maximum neutrophil infiltration 24 h post-exposure. Significant differences in sham- and RV1B-treated mice were noted as early as 8 h after infection.

CXCR2 -/- mice exhibit significantly reduced lung neutrophils following RV infection.

To determine the requirement of ELR (+) CXC chemokines for the observed neutrophilic inflammation, we examined the response of CXCR2 -/- mice to RV infection. CXCR2 serves as the receptor for the murine chemokines KC/CXCL1 and MIP-2/CXCL2, the homologs of human IL-8. Histological sections demonstrated overall reduced inflammation in the CXCR2 -/- mice after RV1B infection (Figure 2-1). Compared to their wild type controls, CXCR2 -/- mice showed a significant reduction in lung neutrophils 24 h post RV1B infection (Figure 2-2C). By 96 h, the neutrophilic response was reduced, although RV1B-treated CXCR2 -/- mice still exhibited significantly lower BAL neutrophils compared to wild-type mice. Together, these data imply that the CXCR2 ligands are the major neutrophil chemoattractants elaborated following RV infection. Surprisingly, CXCR2 deficient mice displayed significantly increased lung lymphocytes at 24 and 96 hours post infection compared to wild type RV infected mice

(Figure 2-2D and F), suggesting a compensatory response. Notably, CXCR2 deficient mice did not display reduced infiltration of macrophages in response to RV 24 hours post infection compared to wild type mice, indicating that the reduced responsiveness to methacholine may have been due to an impairment of neutrophil recruitment.

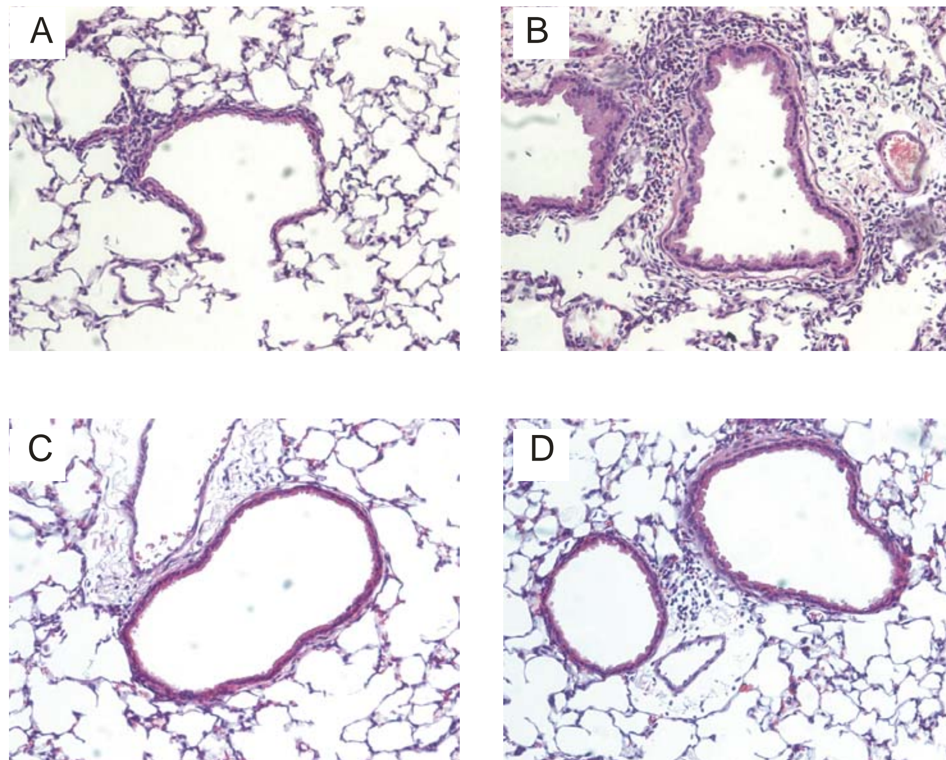

Figure 2-1. Hematoxylin and eosin-stained lung sections from RV1B-infected wild-type BALB/c mice and CXCR2 -/- mice. A-B Airway inflammation in wild-type mice ranged from mild (left panel, A) to severe (right panel, B). Inflammation was attenuated in CXCR2 -/- mice and ranged from minimal (left panel, C) to moderate (right panel, D). These results are typical of the five mice studied in each group. (Original magnification, 160X.)

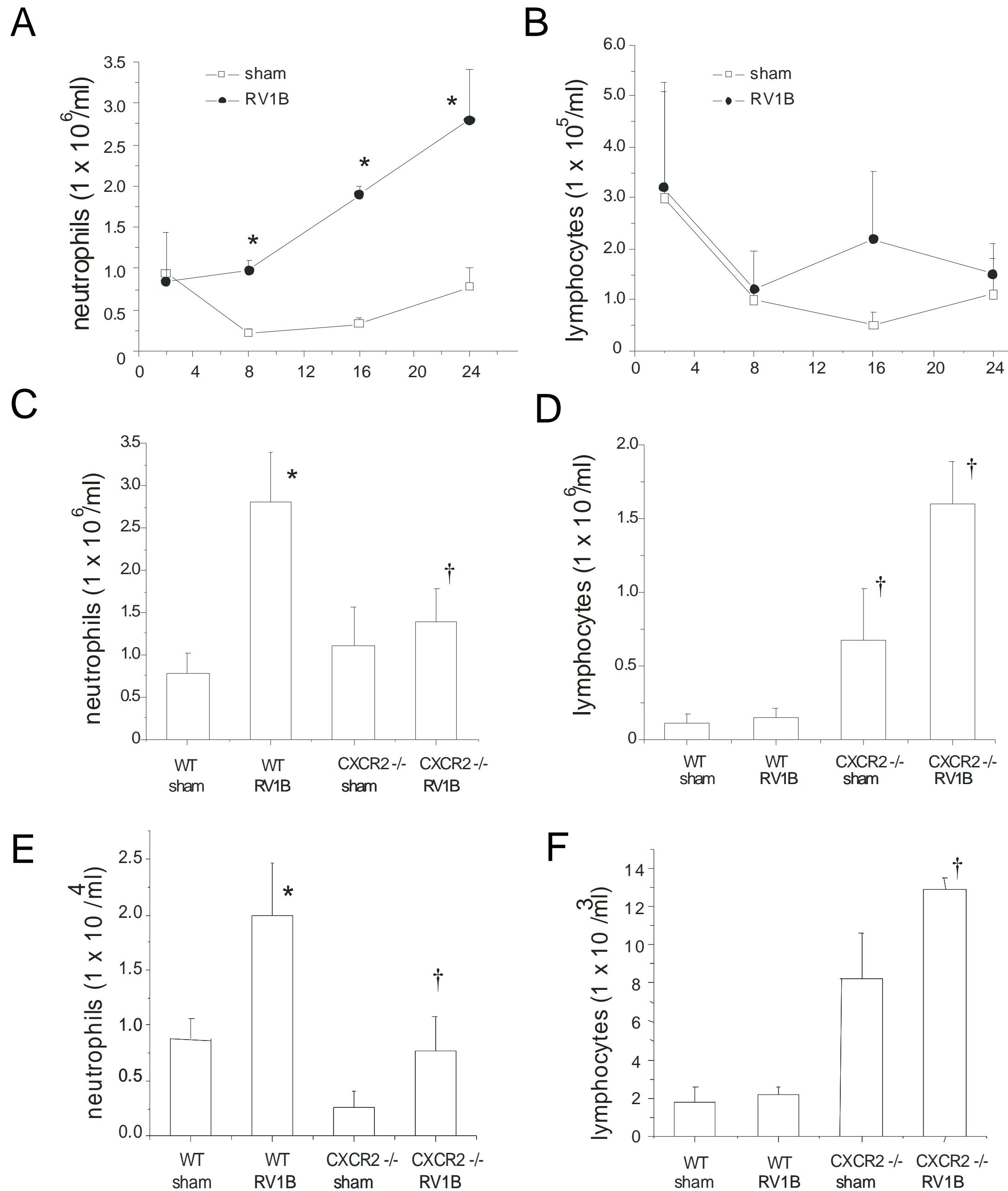

Figure 2-2. Effects of CXCR2 knockout on lung inflammatory cells in sham and RV-treated mice. A-D. Mouse lungs were isolated, minced, and then digested in collagenase type IV for 1 h. Leukocytes were enriched following RBC lysis treatment, and counted for the presence of neutrophils (A, C) and lymphocytes (B, D). Panels A and B show the early time course of neutrophil and lymphocyte influx in wild type mice. Panels E and F represent BAL neutrophils and lymphocytes in wild type and CXCR2 deficient mice 96 hours post infection. (*N* = 5 mice per group, bars represent mean±SEM, *different from respective sham group, p<0.05, ANOVA; †different from RV1B-treated wild- type mice, p<0.05, one-way ANOVA.)

CXCR2 -/- display significantly reduced lung expression of pro-inflammatory cytokines.

To determine the induction of pro-inflammatory cytokine and mucin gene expression, lungs were homogenized in Trizol reagent, cDNA was synthesized using reverse transcriptase, and subjected to quantitative real time PCR employing a Taqman probe. Inductions in TNF-α and MUC5B expression following RV infection were significantly higher in wild-type mice compared to CXCR2 -/- animals (Figure 4). Levels of MIP-2/CXCL2, GM-CSF and MUC5AC expression were comparable in RV-infected wild type and CXCR2 -/- mice.

We then subjected lung mRNA samples to gene array analysis focused on 84 inflammatory cytokines and receptors. RV infection induced a statistically significant, >2-fold increase in the expression of 26 genes, and a significant, >2-fold decrease in the expression of 6 genes (Table 2-1). In addition to TNF-α, genes increasing in expression included those encoding KC/CXCL1, ENA-78/CXCL5, IP-10/CXCL10, IL-1α, IL-1β, TARC/CCL17 and LARC/CCL20. We also computed the ratio of gene expression in RV1B-treated CXCR2 -/- mice compared to RV1B-treated wild-type BALB/c mice. CXCR2 knockout mice demonstrated a statistically significant, >2-fold increase in the expression of 11 genes, and a significant, >2-fold decrease in the expression of 8 genes (Table 2-2). In addition to TNF-α, CXCR2 -/- mice inoculated with RV1B showed significantly lower expression levels of KC/CXCL1, ENA-78/CXCL5, IL-1α, IL-1β, TARC/CCL17, LARC/CCL20 and eotaxin-2/CCL24. In contrast, RV1B-infected CXCR2 -/- mice showed an increase in the lymphocyte chemokine IP-10/CXCL10, perhaps explaining the observed increase in lung and BAL lymphocytes 24 and 96 h after infection respectively.

We measured lung protein levels of TNF-α, GM-CSF and the IL-8 homologs MIP-2/CXCL-2 and KC/CXCL-1 in wild type and CXCR2-/- deficient mice and found significantly lower levels of TNF-α , MIP-2/CXCL-2 and KC/CXCL-1 (Figure 2-4). No significant difference could be observed in the production of GM-CSF.

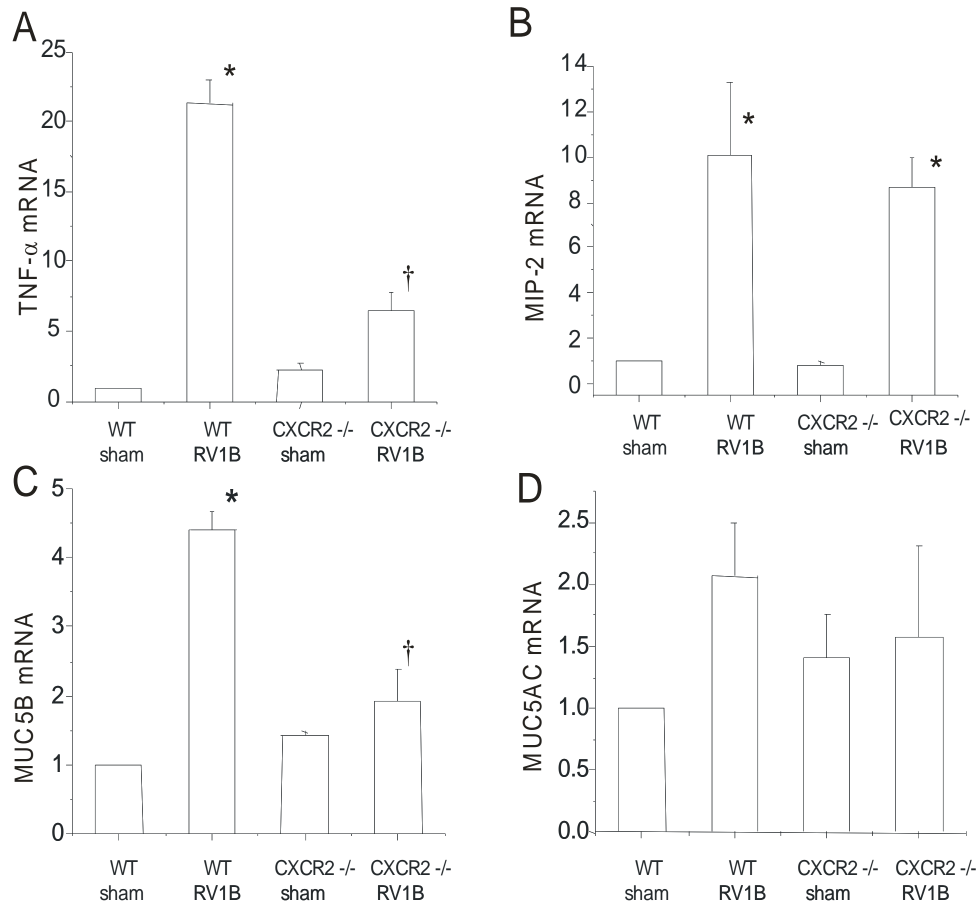

Figure 2-3. CXCR2-/- mice show reduced TNF-α and MUC5B mRNA expression 24 h post-RV1B exposure. A-D. Wild type and CXCR2-/- mouse lungs were homogenized in Trizol reagent. RNA was extracted and analyzed for the presence of TNF-α, MIP-2, MUC5B, MUC5AC by quantitative two-step real time PCR using specific primers and probes. (*N*=4-5 mice per group, bars represent mean±SEM, *different from respective sham group, $p < 0.05$, †different from wild type-RV1B treated group, $p<0.05$, one-way ANOVA.)

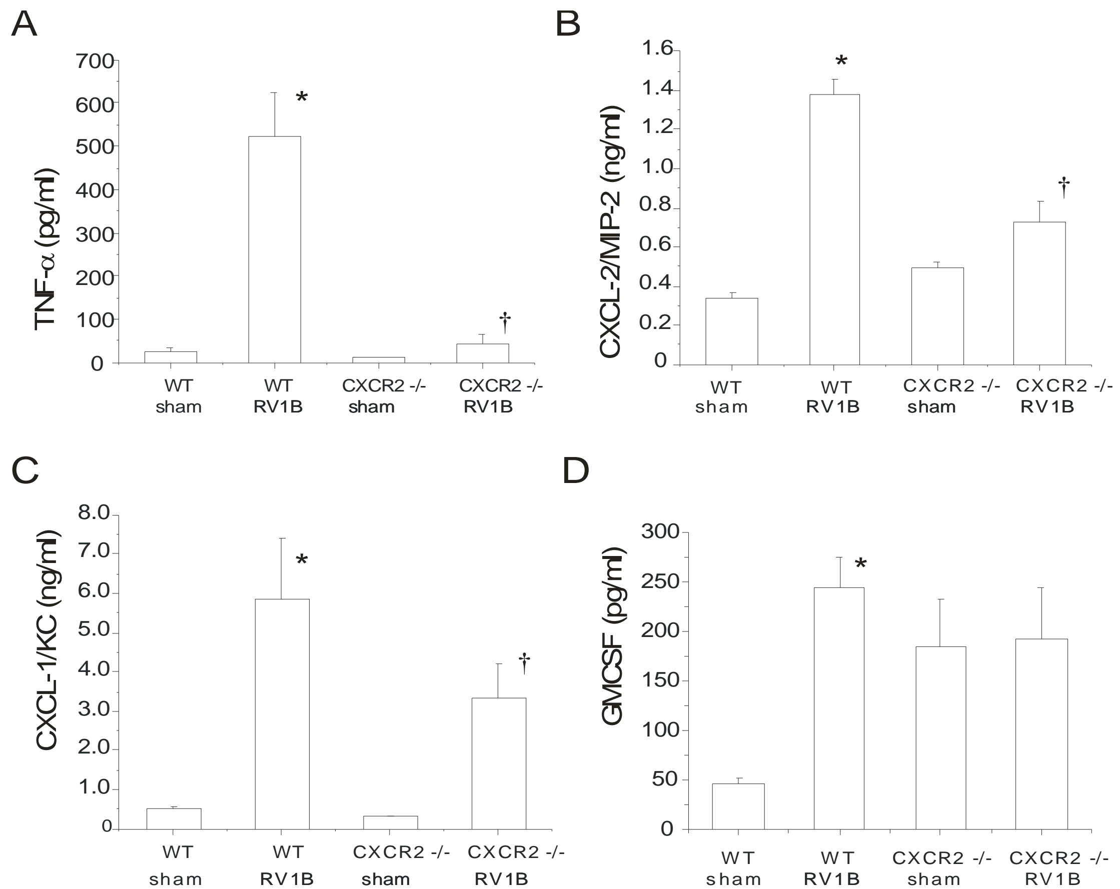

Figure 2-4. **CXCR2-/- mice show deficient cytokine and chemokine responses to RV1B infection 24 h post infection.** TNF-α, MIP-2, CXCL-1, levels were significantly lower in the knockout mice as opposed to wild type RV1B infected mice. (N=5 mice per group, bars represent mean±SEM, *different from respective sham group, $p<0.05$, †different from wild type-RV1B treated group, $p < 0.05$, one-way ANOVA.)

Table 2.1. Effect of RV infection on the expression of inflammatory cytokines and receptors. RV infection induced a statistically significant, >2-fold increase in the expression of 26 genes, and a significant, >2-fold decrease in the expression of 6 genes.

GeneBank	*Symbol*	*t-statistic*	*p-value*	*fold change*
NM_011332	*Ccl17*	7.25	0	14.82
NM_011333	*Ccl2*	4.83	0.001	17.50
NM_016960	*Ccl20*	4.81	0.001	6.45
NM_013654	*Ccl7*	4.81	0.001	12.90
NM_011338	*Ccl9*	5.22	0.001	5.42
NM_008176	*Cxcl1*	5.07	0.001	13.45
NM_008361	*Il1b*	5.4	0.001	9.84
NM_009137	*Ccl22*	4.48	0.002	3.43
NM_009139	*Ccl6*	4.53	0.002	3.38
NM_021274	*Cxcl10*	4.53	0.002	15.56
NM_009141	*Cxcl5*	4.6	0.002	13.83
NM_010554	*Il1a*	4.52	0.002	3.11
NM_009778	*C3*	4.05	0.003	2.15
NM_019494	*Cxcl11*	4.11	0.003	10.05
NM_018866	*Cxcl13*	4.24	0.003	4.08
NM_008599	*Cxcl9*	3.91	0.004	9.18
NM_013693	*Tnf*	3.9	0.004	4.95
NM_011331	*Ccl12*	3.81	0.005	5.38
NM_009912	*Ccr1*	3.69	0.006	3.34
NM_009917	*Ccr5*	3.66	0.006	3.03
NM_010555	*Il1r2*	3.59	0.007	19.02
NM_008401	*Itgam*	3.55	0.007	3.97
NM_009915	*Ccr2*	3.49	0.008	2.84
NM_011337	*Ccl3*	3.41	0.009	5.42
NM_009914	*Ccr3*	2.88	0.02	2.77
NM_013652	*Ccl4*	2.39	0.043	3.16
NM_007551	*Cxcr5*	-4.44	0.002	0.33
NM_011339	*Cxcl15*	-3.79	0.005	0.30
NM_013653	*Ccl5*	-3.53	0.007	0.27
NM_008370	*Il5ra*	-3.59	0.007	0.18
NM_021704	*Cxcl12*	-2.9	0.019	0.48
NM_009913	*Ccr9*	-2.68	0.027	0.48

Table 2-2. Effect of CXCR2 knockout on the expression of inflammatory cytokines and receptors. CXCR2 -/- mice demonstrated a statistically significant, >2-fold increase in the expression of 11 genes, and a significant, >2-fold decrease in the expression of 8 genes.

GeneBank	*Symbol*	*t-statistic*	*p-value*	*fold change*
NM_007551	*Cxcr5*	4.87	0.001	2.81
NM_013653	*Ccl5*	3.88	0.004	3.41
NM_011339	*Cxcl15*	3.68	0.006	2.73
NM_008370	*Il5ra*	3.56	0.007	4.29
NM_021443	*Ccl8*	3.5	0.008	3.56
NM_021274	*Cxcl10*	3.51	0.008	6.32
NM_008357	*Il15*	3.45	0.008	2.79
NM_013652	*Ccl4*	3.16	0.013	3.71
NM_009913	*Ccr9*	3.13	0.014	2.07
NM_019494	*Cxcl11*	2.86	0.02	4.03
NM_008360	*Il18*	2.67	0.027	2.55
NM_016960	*Ccl20*	-5.23	0.001	0.17
NM_011332	*Ccl17*	-4.15	0.003	0.26
NM_010554	*Il1a*	-4.11	0.003	0.41
NM_019577	*Ccl24*	-3.49	0.008	0.27
NM_008176	*Cxcl1*	-3.4	0.009	0.22
NM_009141	*Cxcl5*	-2.87	0.02	0.24
NM_013693	*Tnf*	-2.63	0.029	0.39
NM_008361	*Il1b*	-2.51	0.035	0.40

CXCR2 -/- mice show reduced airway responsiveness to methacholine 24 h following RV1B infection.

In order to determine the contribution of neutrophils to RV1B induced airway responsiveness, CXCR2 -/- and wild type BALB/c mice were tested for responsiveness to the bronchoconstrictor agonist methacholine 24 and 96 h after RV1B or sham treatment. Compared to sham-infected mice, RV1B infection was also associated with moderate but significant airways cholinergic hyperresponsiveness which persisted up to 96 h after treatment (Figure 2-5A). At 24 h, RV-infected CXCR2 -/- mice demonstrated significantly lower airways responses than wild-type mice ($p<0.001$, two-way ANOVA). However, RV1B-treated CXCR2 -/- mice still showed a significantly higher maximum methacholine response compared to sham-infected CXCR2 -/- mice. These data suggest that CXCR2 and airway neutrophils are required for maximal RV-induced methacholine responsiveness, but do not completely account for the RV response.

At 96 h, the airways responsiveness of RV-infected CXCR2 -/- mice was no longer different than RV1B-treated wild type mice (Figure 2-5B). However, the response to RV infection in CXCR2 -/- mice appeared attenuated, as there was no difference in airways responsiveness between RV-infected and sham-treated CXCR2 -/- mice. These data suggest that neutrophils play a lesser role in the determination of airway responses at later time points. We therefore focused further experiments on the 24 h time point. First, we examined lung mRNA for viral load. There was no difference in RV copy number between the lungs of infected wild-type and CXCR2 -/- mice (Figure 2-5C).

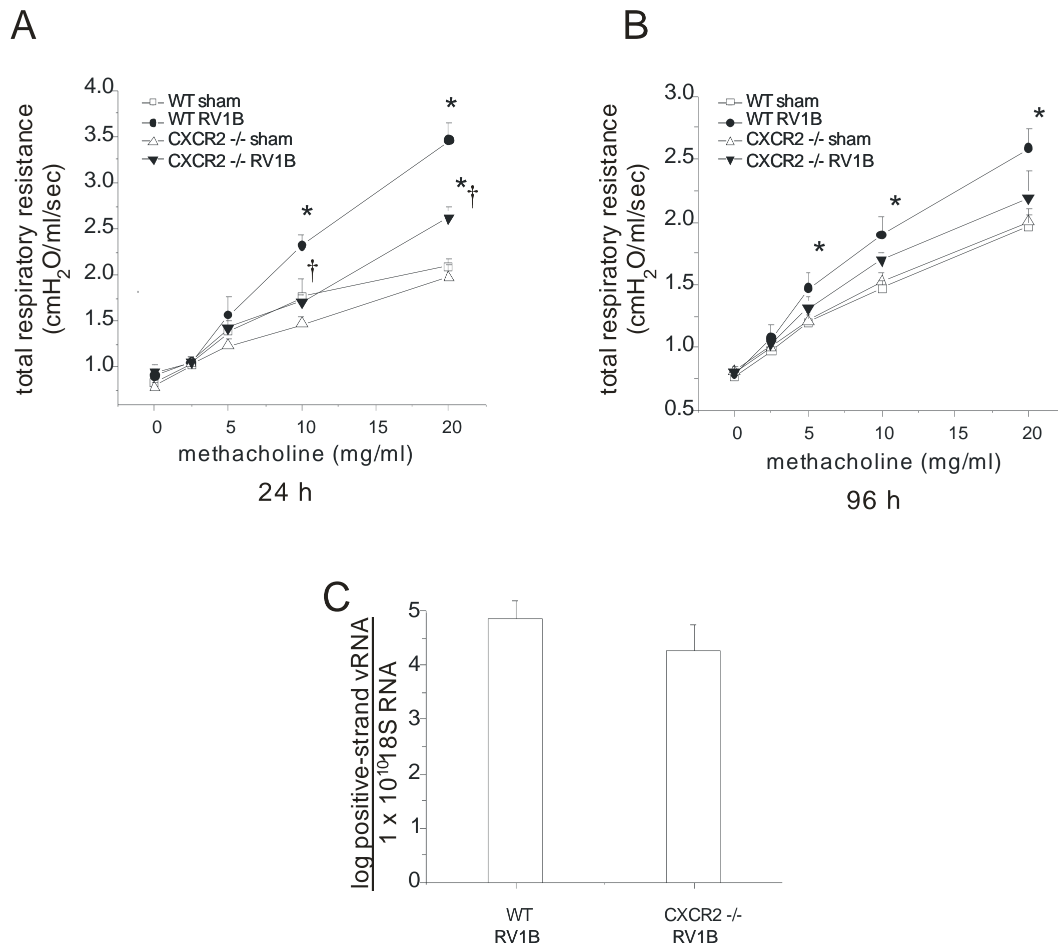

Figure 2-5. CXCR2-/- mice show reduced airways cholinergic responsiveness 24 h post-infection. Following anesthesia and endotracheal intubation, changes in respiratory system resistance to nebulized methacholine were measured using the FlexiVent system (Scireq, Montreal, CA). Mice were studied either 24 h (A) or 96 h post-viral exposure (B). (*N*=5 mice per group. Open squares, sham-treated wild-type mice; closed circles, RV1B-treated wild-type mice; open triangles, sham-treated CXCR2 -/- mice; closed triangles, RV1B-treated CXCR2 -/- mice; error bars represent ±SEM, *different from respective sham group, †different from wild type RV1B- treated group, p<0.05, two-way ANOVA.) C. RV1B-infected CXCR2-/- mice show comparable viral loads compared to RV1B-treated wild type mice 24 h post infection. Wild type and CXCR2-/- mouse lungs were homogenized in Trizol reagent 24 h post exposure. RNA was extracted and analyzed for the presence of positive strand RV RNA. Viral copy number was normalized to the quantity of 18S RNA present in mouse lungs. (*N*=6 mice per group, bars represent geometric mean±SEM.)

Anti-Ly6G treated BALB/c mice exhibit reduced airway neutrophils and methacholine responsiveness 24 h after RV1B treatment.

Although CXCR2 is classically expressed on neutrophils (32), it may also be expressed on monocytes, macrophages and lymphocytes (33-35). To confirm that RV-induced airways hyperresponsiveness was due to the contribution of granulocytes, we examined the effect of granulocyte depletion using the RB6-8C5 monoclonal antibody to Ly6G, an antigen expressed widely on granulocytes, including neutrophils (30). Mice were injected with 30 μg of neutralizing antibody to mouse Ly6G or the corresponding isotype IgG control, and inoculated intranasally with RV1B or sham. Treatment with anti-Ly6G significantly attenuated neutrophil numbers 24 h following RV infection compared to RV1B/IgG-treated animals (Figure 2-6A). Finally, depletion of neutrophils in the RV1B/anti-LY6G group was associated with a partial but statistically significant reduction in maximal methacholine response compared to RV1B/anti-IgG group (Figure 2-6B). Neutrophil depletion was also accompanied by a partial but significant reduction in lung TNF-α levels (Figure 2-6C). These data suggest that neutrophils are required for maximal airways hyperresponsiveness 24 h post-RV1B infection but do not constitute for all of the airway responses at this time point.

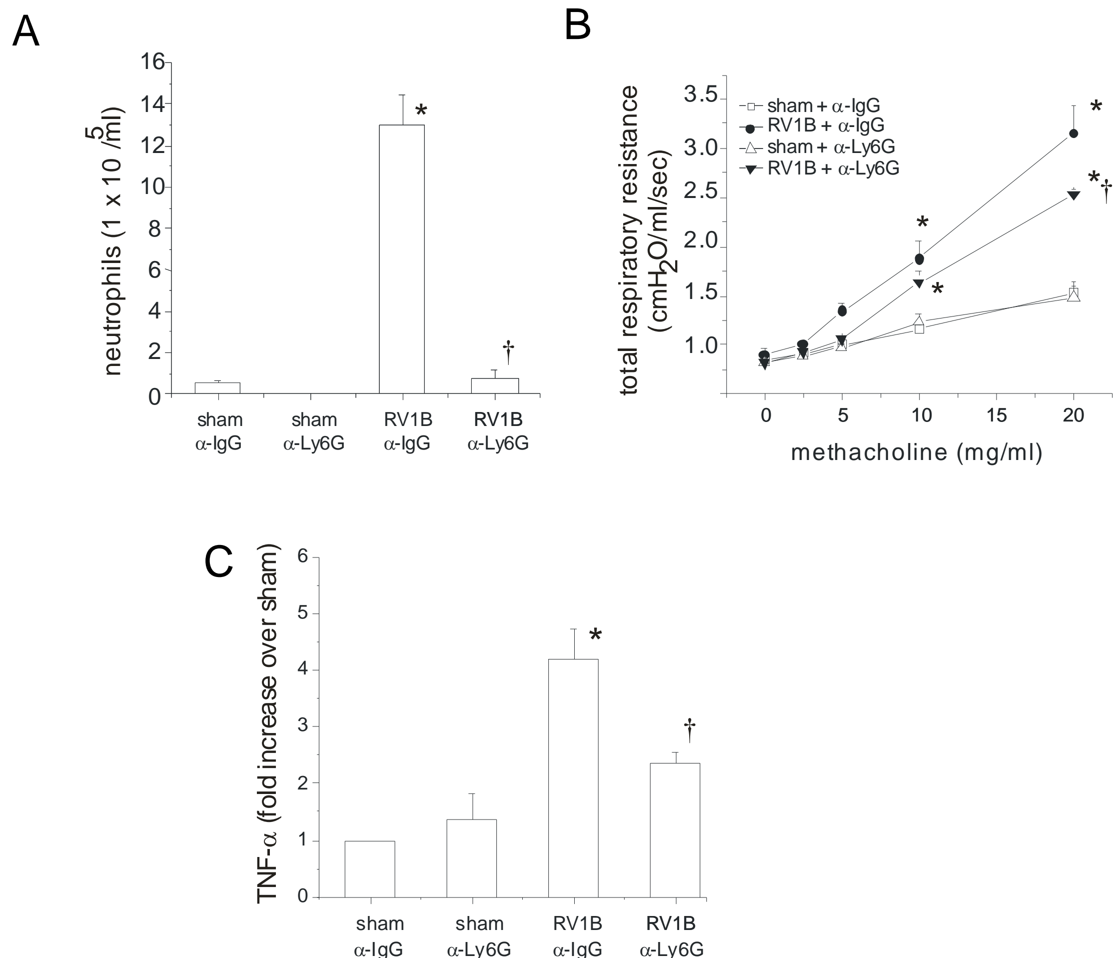

Figure 2-6. Anti-Ly6G treated BALB/c mice exhibit reduced airway granulocytes including neutrophils, methacholine responsiveness and lung TNF-α expression 24 h after RV1B treatment. Mice were injected intraperitoneally with 75 μg of anti-LY6G antibody or isotype conrol anti-IgG antibody. A. RV1B/ anti-LY6G mice showed a significant reduction in BAL neutrophils 24 h post-infection. (*N*=5 mice per group, bars represent mean±SEM, *different from respective sham group, $p<0.05$, † different from wild-type-RV1B treated group, $p<0.05$, one-way ANOVA. B. RV1B/anti-LY6G treatment reduced maximal airway responsiveness to methacholine compared to RV1B/anti-IgG treated mice. (*N*=3-4 mice per group, error bars represent SEM, *different from respective sham group, †different from IgG-treated RV1B group, $p<0.05$, two-way ANOVA. C. RV1B/anti-LY6G treatment reduced lung TNF-α expression compared to RV1B/anti-IgG treated mice. (*N*=5 mice per group, error bars represent SEM, *different from respective sham group, †different from IgG-treated RV1B group, $p<0.05$, one-way ANOVA.

TNFR1 -/- mice show reduced airway neutrophils and methacholine responsiveness following RV1B infection.

Based on the reduced TNF-α mRNA expression found in RV1B-infected CXCR2 -/- mice, we wondered whether neutrophil-derived TNF-α could be responsible for the airways cholinergic hyperresponsiveness observed 24 following RV infection. TNF-α has been demonstrated to increase the responsiveness of airway smooth muscle to contractile agonists (36-38). First, we measured the number of TNF-α-expressing neutrophils in the BAL of sham and RV1B-inoculated BALB/c mice by flow cytometry. RV-infected mice demonstrated a 16-fold increase in the number of LY6G- and TNF-α-positive cells. Next, to test the requirement for TNF-α, wild type C57BL/6 mice and TNFR1 -/- mice were inoculated with RV1B or sham, and airway inflammation and resistance measured 24 h post infection. RV-treated TNFR1 -/- mice showed a partial but significant reduction in methacholine response relative to wild-type RV treated mice (Figure 2-7A), consistent with the notion that TNF-α signaling is required for maximum RV-induced airway responsiveness.

TNFR1 -/- mice also showed a significant reduction in BAL neutrophils after RV treatment compared to wild type mice (Figure 2-7B). It is therefore conceivable that the observed reduction in airways responsiveness in TNFR -/- mice is secondary to neutrophil diminution, rather than a defect in TNF-α signaling. To test this, BAL neutrophils were restored in RV-treated TNFR1-/- mice by intranasal administration of the neutrophil chemokine MIP-2. MIP-2 administration dramatically increased airway neutrophils in sham and RV1B-inoculated TNFR-/- mice (sham, $15.1 \pm 5.4 \times 10^5$ cells/ml; RV1B, $21.7 \pm 6.3 \times 10^5$ cells/ml, n=5). However, airway hyperresponsiveness in MIP-2-

treated RV1B-infected TNFR1-/- mice was not restored (Figure 2-7C). Taken together, these data demonstrate that CXCR2, airway neutrophils, and intact TNFR1 receptor are required for RV1B-induced airways hyperresponsiveness.

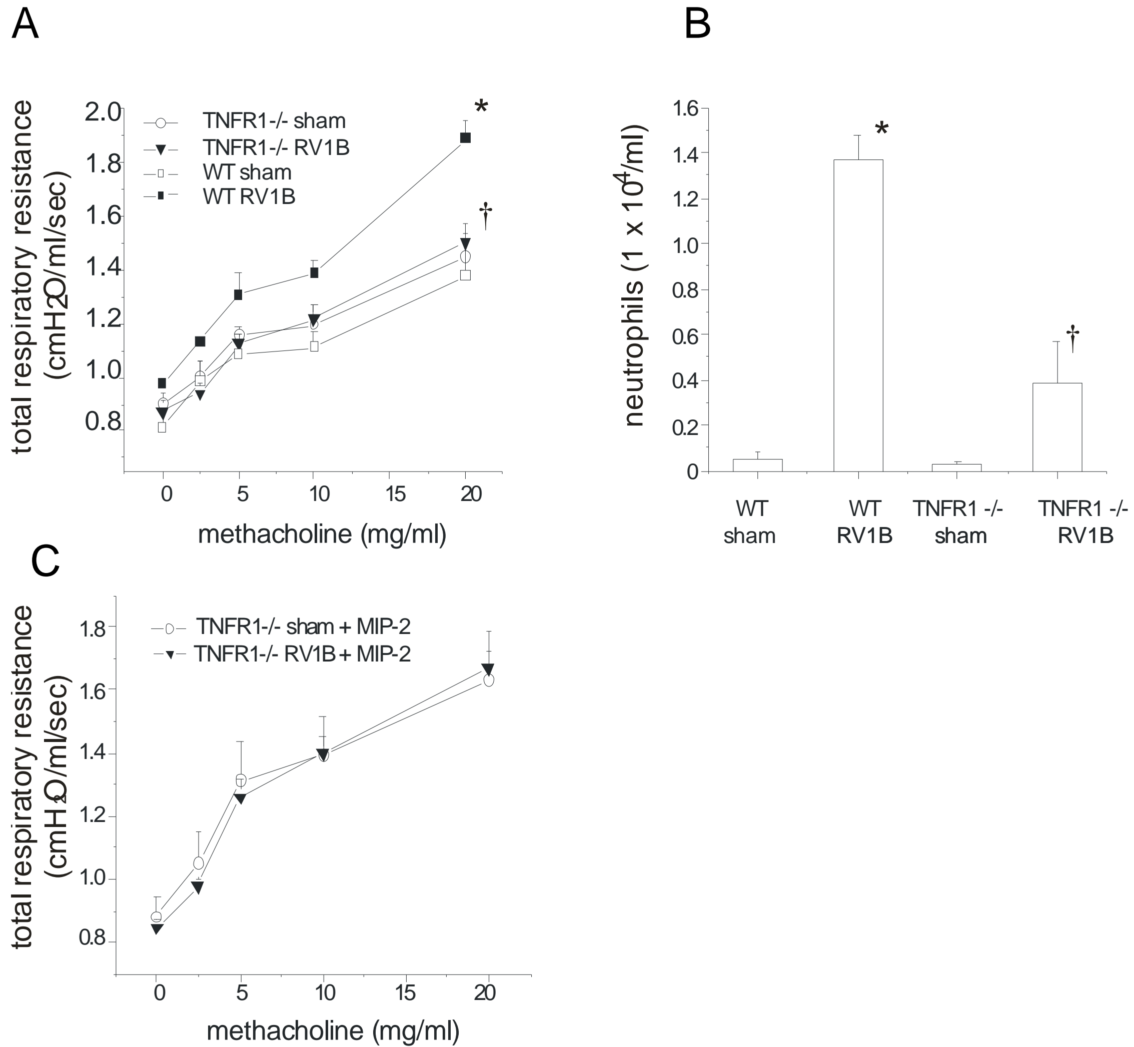

Figure 2-7. TNFR1-/- mice show reduced airway responsiveness and BAL neutrophils 24 h post-RV1B infection. TNFR1-/- and wild type C57BL/6 mice were inoculated with sham or RV1B and examined 24 h after infection. A. Changes in respiratory system resistance to nebulized methacholine were measured using the FlexiVent system. B. BAL was performed with 0.9% NaCl containing 5mM EDTA. C. Intranasal administration of MIP-2 fails to restore airways hyperresponsiveness in RV-infected TNFR1 -/- mice. (N=6 per group. Data represent mean±SEM, *different from respective sham group, †different from wild type-RV1B treated group, $p<0.05$, one- or two-way ANOVA, as appropriate.)

Discussion

RV is responsible for the majority of the common colds and approximately 50% of asthma exacerbations. We and others have shown that RV1B infects mouse airway epithelial cells (14, 26). Further, infection of C57BL/6 and BALB/c mice induces airway neutrophilia and hyperresponsiveness to methacholine challenge 24 h post infection (14, 27) We therefore sought to determine the contribution of ELR(+) CXC chemokines and neutrophils to RV1B-induced airway inflammation and hyperreactivity. Upon stimulation, activated neutrophils release a variety of pro-inflammatory mediators including cytokines such as IL-8 and TNF-α, superoxide, myeloperoxidase and various proteases which could promote airway inflammation and obstruction (19-22). Experimental RV infection has been shown to increase airway neutrophilic inflammation in asthmatic subjects (6, 15-18). Finally, RV infection has been shown to increase airway neutrophils (18) and maximal responses to methacholine (5, 6) in normal subjects.

In addition to ELR (+) CXC chemokines, other neutrophil chemoattractants include complement activation products such as C5a, lipid mediators such as leukotriene B4 and platelet activating factor, and host derived peptides such as N-acetyl-Pro-Gly-Pro, a degradation product of the extracellular matrix (39-41). To test for the contribution of ELR(+) CXC chemokines to RV-induced airways hyperresponsiveness, we employed a CXCR2 -/- mouse strain. CXCR2 serves as the receptor for the neutrophil chemoattractants and IL-8 homologs KC/CXCL1, MIP-2/CXCL2 and ENA-78/CXCL5. Twenty-four h after infection, CXCR2 -/- mice demonstrated significantly fewer airway neutrophils. CXCR2 -/- mice also showed lower lung mRNA and protein levels of the neutrophil chemokines KC/CXCL1 and MIP-2/CXCL-2, and lower expression of ENA-

78/CXCL5 than RV-infected wild-type BALB/c mice. These data demonstrate that CXCR2 ligands are the main chemoattractants mediating RV-induced neutrophilic airway inflammation. In addition, they suggest that, in the context of RV infection, lung neutrophils intensify granulocyte infiltration of the airways by secreting their own chemokines. Previous studies have demonstrated the ability of neutrophils to express pro-inflammatory cytokines and chemokines, including IL-8/CXCL8, ENA-78/CXCL5 and GRO-α/CXCL1 (23). Finally, RV-infected CXCR2 -/- mice showed significantly reduced methacholine responsiveness compared to wild type RV1B-infected mice, consistent with the notion that airway neutrophils contribute to maximal RV-induced airways responses.

As noted above, stimulated neutrophils produce a large number of pro-inflammatory substances which could promote airway inflammation and obstruction, including TNF-α, IL-1β and neutrophil elastase (19-22). In human airway smooth muscle cells loaded with fura 2, TNF-α enhances thrombin- and bradykinin-evoked elevations of intracellular Ca^{2+} (36). TNF-α increases the Ca^{2+} sensitivity of myofilaments by activating the RhoA signaling pathway, which in turn leads to an inhibition of myosin light chain phosphatase and an increase in myosin light chain phosphorylation (37). Recently, TNF-α-enhanced contractile responses in cultured airway smooth muscle cells were found to depend on activation of CD38, a multifunctional ectoenzyme involved in cell adhesion, signal transduction and calcium signaling (38).

In the present study, we observed a consistent reduction in TNF-α expression in CXCR2-deficent mice, as well as mice treated with a granulocyte-depleting antibody,

anti-LY6G. On this basis, we employed TNFR1-/- mice to determine the contribution of TNF-α signaling to RV1B-induced airway inflammation and hyperresponsiveness. TNFR1-/- mice exhibited a significant reduction in the maximal response to methacholine 24 h after RV1B exposure, consistent with the notion that TNF-α is required for airways hyperresponsiveness following RV infection. However, our analysis was complicated by a significant reduction in airway neutrophils in TNFR1 -/- mice; hence, we could not distinguish the contribution of TNF-α signaling from the previously uncovered neutrophil requirement described above. TNF-α has been shown to mediate recruitment of neutrophils to the airways following allergen sensitization and challenge (42). Despite reconstitution of the neutrophil response with exogenous MIP-2, RV-infected TNFR1-/- mice remained less responsive to methacholine than wild type mice, indicating that a functional TNFR1 receptor is required for RV-induced airways hyperresponsiveness. Taken together with our previous results, these data suggest that neutrophils, attracted to the airways by CXCR2 ligands, induce a state of hyperresponsiveness by elaboration of TNF-α.

It is possible that other mechanisms play a role in RV-induced airways hyperresponsiveness. For example, neutrophil elastase induces airway constriction and hyperresponsiveness, as well as airway mucus production (20, 22). In our study, MUC5B gene expression was highly induced in the wild-type mice 24 h after RV1B infection, but not in the CXCR2-/- mice, suggesting that neutrophils play a role in mucin gene expression following RV infection. We also observed a significant reduction in airway lymphocytes and the lymphocyte chemotactic factors TARC/CCL17 and LARC/CCL20.in CXCR2-/- mice 24 h after RV1B infection. It is therefore conceivable

that lymphocytes also play a role in the observed RV-induced airway hyperresponsiveness. Consistent with this, airway neutrophils are reduced 10-fold at 96 h following RV infection, yet airways hyperresponsiveness persists. However, CXCR2 -/- RV-infected mice with attenuated airway cholinergic responses exhibited significantly higher airway lymphocytes compared to the wild type mice. Since phagocytic neutrophils are ordinarily briskly recruited to the lung upon infection, the heightened lymphocytic infiltration of the airways in CXCR2 -/- animals may represent a compensatory response to the functional immunodeficiency of these mice.

In this study, we did not examine the effect of RV on mice with pre-existing airways inflammation. These initial studies in control animals are necessary to assess the disease-specific mechanisms of virus-induced asthma exacerbations. For example, our unpublished data indicate that airway inflammatory and constrictor responses in RV-infected ovalbumin sensitized- and -challenged mice are qualitatively and quantitatively different than those in normal mice. Also, we did not rule out the possibility that RV infection of airway smooth muscle cells directly influences contractile responses. However, as far as we are aware, there is no evidence that RV infects airway smooth muscle cells *in vivo*, and immunohistochemical stains of RV1B-infected mice have shown infection to be limited to the airway epithelium and perhaps airway inflammatory cells (14). Finally, we did not examine the requirement of CXCR2 for major group RV responses. Recent studies suggest that minor group viruses are more cytotoxic (43) and stimulate higher IFN-β production (44) in bronchial epithelial cells. On the other hand, we have shown that major and minor groups viruses stimulate similar levels of IL-8 and Akt phosphorylation (11), and major and minor groups viruses have been shown to

stimulate nearly identical patterns of mRNA expression in primary human bronchial epithelial cells (45). Also, we (14) and others (27) have found similar effects of RV1B and RV16 infection in wild-type and human ICAM-1 transgenic mice, respectively.

In summary, we have demonstrated that, in naïve mice, CXCR2, neutrophils and TNF-α play a causal role in RV-induced airways hyperresponsiveness. Following RV infection, airway neutrophils release factors that regulate neutrophil chemotaxis, mucin expression and airway smooth muscle responses. Further studies using mouse models of RV1B infection may elucidate mechanisms underlying exacerbations of asthma, COPD and other chronic airways diseases.

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Chapter 3

Rhinovirus infection of allergen-sensitized and -challenged mice induces eotaxin release from functionally polarized macrophages

Summary

Human rhinovirus is responsible for the majority of virus-induced asthma exacerbations. To determine the immunologic mechanisms underlying rhinovirus-induced asthma exacerbations, we combined mouse models of allergic airways disease and human rhinovirus infection. We inoculated ovalbumin-sensitized and challenged BALB/c mice with rhinovirus serotype 1B, a minor group strain capable of infecting mouse cells. Compared to sham-infected, ovalbumin-treated mice, virus-infected mice showed increased lung infiltration with neutrophils, eosinophils and macrophages, airway cholinergic hyperresponsiveness, and increased lung expression of cytokines including eotaxin-1/CCL11, IL-4, IL-13 and IFN-γ. Administration of anti-eotaxin-1 attenuated rhinovirus-induced airway eosinophilia and responsiveness. Immunohistochemistry showed eotaxin-1 in the lung macrophages of virus-infected, ovalbumin-treated mice, and confocal fluorescence microscopy revealed co-localization of rhinovirus, eotaxin-1 and IL-4 in CD68-positive cells. RV inoculation of lung macrophages from ovalbumin-treated, but not PBS-treated, mice induced expression of eotaxin-1, IL-4, and IL-13 *ex*

vivo. Macrophages from ovalbumin-treated mice showed increased expression of arginase-1, Ym-1, Mgl-2 and IL-10, indicating a shift in macrophage activation status. Depletion of macrophages from ovalbumin-sensitized and -challenged mice reduced eosinophilic inflammation and airway hyperreactivity following RV infection. We conclude that augmented airway eosinophilic inflammation and hyperresponsiveness in RV-infected mice with allergic airways disease is directed in part by eotaxin-1. Airway macrophages from mice with allergic airways disease demonstrate a change in activation state characterized in part by altered eotaxin and IL-4 production in response to RV infection. These data provide a new paradigm to explain RV-induced asthma exacerbations.

Introduction

Viral infections trigger 80% of asthma exacerbations in children and nearly 50% in adults (1, 2), with human rhinovirus (RV) being the most common virus identified. While RV infections were once thought to be restricted to upper airway tissues (3), it is now clear that infections of the upper respiratory tract are accompanied by the entry of virus into lower respiratory tract cells (4-7), though the quantity of viral replication is not known.

In normal subjects, RV causes airway narrowing in response to methacholine as well as increased airway neutrophils and submucosal CD3+ cells (8, 9). In theory, RV infection of airway cells elicits the production of chemokines, subsequently inducing recruitment of inflammatory cells to the airways. Inflammatory cells, in turn, elaborate cytokines and mediators capable of increasing airways responsiveness. This paradigm,

however, does not explain why asthmatic subjects experience exacerbations of lower airways disease following respiratory tract infection while control subjects do not.

Numerous clinical studies suggest a role for interleukin (IL)-8/CXCL8 in the pathogenesis of RV-induced asthma exacerbations. IL-8 and neutrophils are found in the nasal secretions, sputum or bronchoalveolar lavage fluid of allergic subjects undergoing experimental RV infection (9-14). After RV16 infection, asthmatic patients show increased levels of IL-8 in their nasal lavage which correlates with the level of airways responsiveness (11), in contrast to unaffected individuals in whom IL-8 does not increase (15). Eosinophils and eosinophil cationic protein have also been detected in the airways following experimental RV infection (10, 14, 16). Asthmatics undergoing experimental RV infection demonstrate greater eosinophilic inflammation than RV-infected control subjects (14). Together, these data suggest that patients with asthma experience a different response to viral infection than controls.

We recently showed that inoculation of C57BL/6 mice with RV1B, a minor group virus which binds to proteins of the highly conserved low-density lipoprotein receptor family, induces airway neutrophilic inflammation and methacholine hyperresponsiveness (17). In contrast, replication-deficient UV-irradiated virus did not cause lasting hyperresponsiveness. We also found positive and negative-strand viral RNA in the lungs up to 4 days after infection, suggesting replication of RV *in vivo*. It was recently shown that RV infection of BALB/c mice induces similar airway changes (18). Infection of ovalbumin (OVA)-sensitized and -challenged mice increased bronchoalveolar neutrophils, eosinophils and lymphocytes compared with allergen-challenged mice treated with UV-inactivated virus. However, the mechanism by which eosinophils are

attracted to the airways following RV infection, and the requirement of eosinophils for the development of RV-induced airway hyperresponsiveness, were not examined.

In the present study, we show that, in OVA-sensitized and challenged BALB/c mice, RV1B infection increased production of pro-inflammatory cytokines including eotaxin-1/CCL11, Th-2 cytokines IL-4, IL-13. Bronchoalveolar and lung neutrophils, eosinophils, and macrophages, as well as airways responsiveness, were elevated in the RV-infected, OVA-treated mice. Neutralization of eotaxin-1/CCL11 blocked both airway eosinophilia and hyperresponsiveness. Eotaxin-1 and IL-4 were localized to RV-infected airway macrophages. Finally, macrophages from OVA-treated, but not PBS-treated, mice expressed eotaxin-1, IL-4, IL-13 in response to RV infection *ex vivo*, as well as the alternative activation markers arginase-1, Ym-1, MGL-2, and IL-10. Finally, depletion of macrophages from OVA/RV treated mice significantly decreased eosinophil infiltration and airway responses compared to non-depleted controls. These results suggest that allergen sensitization and challenge skews a predominantly neutrophilic RV response in naïve mice to a Th-2-dominant eosinophil response that is augmented, at least in part, by alternatively activated macrophages.

Methods

Generation of RV

RV1B (ATCC, Manassas, VA) was concentrated, purified and titered as described previously (19, 20). Fifty percent tissue culture infectivity doses ($TCID_{50}$) were determined by the Spearman-Karber method. RV1B was UV-irradiated using a CL-1000 crosslinker (UVP, Upland, CA).

OVA sensitization/challenge and RV exposure

This study was approved by the Institutional Animal Care and Use Committee. Animal usage followed guidelines set forth in the "Principles of Laboratory Animal Care" (National Society for Medical Research). Female 8 wk-old BALB/c mice (Jackson Laboratories, Bar Harbor, MA) were injected intraperitoneally with 200 μl of a 5 mg/ml solution of alum and endotoxin-free OVA or PBS (Sigma-Aldrich, St. Louis, MO) on days 1 and 7 and treated intranasally with 50 μl of a 20 mg/ml solution of OVA or PBS on days 14, 15 and 16. Immediately following the last OVA or PBS treatment, mice were inoculated intranasally with 45 μl of $1x10^8$ $TCID_{50}$/ml RV1B, UV-irradiated RV or an equal volume sham HeLa cell lysate (17).

Bronchoalveolar inflammatory cells and macrophage culture

Bronchoalveolar lavage (BAL) was performed using 1 ml PBS aliquots. Cytospins were stained with Diff-Quick (Dade Behring, Newark, DE) and differential counts determined from 200 cells. BAL fluid from PBS- and OVA-treated mice was seeded in 12 well plates. To partially purify macrophages, cells were allowed to adhere for 90 min and non-adherent cells removed by suction. Diff-Quick staining showed adherent cells to consist of >90% macrophages, with the rest of the cells being neutrophils. Remaining cells were resuspended in RPMI (Invitrogen, Carlsbad, CA), stimulated for 2 h with sham or RV1B (multiplicity of infection, 5.0), and RNA harvested 8 h after infection. In selected experiments, cells were pre-treated with 30 ng/ml IL-4 and IL-13 (Peprotech, Rocky Hill, NJ).

Lung inflammation

To quantify inflammatory cells, lung digests were obtained by mincing the tissue, proteolysis in collagenase type IV (Gibco Invitrogen, Carlsbad, CA) and straining through a 70 μm nylon mesh (BD Falcon, San Jose, CA), as described (21). The resulting pellet was treated with red blood cell lysis buffer (BD Pharmingen, San Diego, CA) and leukocytes were enriched by spinning the cells through 40% Percoll (Sigma-Aldrich). Lung leukocyte cytospins were stained and counted as described above.

Cytokine/chemokine expression

Lung RNA was extracted with Trizol (Sigma-Aldrich) and analyzed for cytokine and mucin gene expression by quantitative real time PCR using specific primers and probes. Signals were normalized to GAPDH and expressed as fold-increase. In some experiments, BAL fluid was spun for 15 min at 1500 g, and the supernatants were analyzed for cytokine protein by multiplex immune assay (Biorad, Hercules, CA).

Respiratory system resistance.

Airway responsiveness was assessed by measuring changes in respiratory system resistance in response to increasing doses of nebulized methacholine (17).

Macrophage depletion.

Depletion of alveolar macrophages was accomplished by intratracheal instillation of liposomes containing clodronate (dichloromethylenediphosphonic acid, disodium salt), as previously described (22). PBS-containing liposomes were used for control

experiments. Briefly, 8 mg cholesterol and 86 mg phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) were dissolved in chloroform and slowly evaporated. The filmy layer was resuspended in 10 ml of PBS or 0.6 M clodronate. The mixture was exposed to N_2 gas and incubated for 2 h at room temperature with gentle shaking. The mixture was then sonicated and incubated for another 2 h to allow liposome swelling. The solution was centrifuged at 10,000 x *g* for 15 min, and the liposomes were collected and washed twice with sterile PBS. Liposomes were kept at 4°C under N_2 until use. Depletion was performed 24 h after the last OVA challenge by introducing 60 µl of clodronate- or PBS-containing liposomes intratracheally. 24 h later, mice were infected with RV1B, as described above. Differential cell counts were performed on lung digests and respiratory resistance to methacholine was measured.

Immunohistochemistry and confocal fluorescence microscopy

Lungs were fixed with 10% formaldehyde overnight and paraffin embedded. Blocks were sectioned at 500 µm intervals at a thickness of 5 µm and each section was deparaffinized, hydrated and stained with goat anti-mouse eotaxin-1 (Santa Cruz Biotechnology, Santa Cruz, CA). For immunohistochemistry, sections were incubated with biotinylated secondary goat-IgG, ABC reagent (Vector Laboratories, Burlingame, CA), diaminobenzidine (DAB, Sigma-Aldrich) and Gill's hematoxylin (Fisher Scientific, Kalamazoo, MI). For fluorescence microscopy, slides were incubated with Alexa Fluor (AF)-conjugated donkey anti-goat IgG (Molecular Probes, Portland, OR) and rat anti-mouse CD68 (AbD Serotec, Raleigh, NC) or isotype control IgG. In selected experiments, sections were co-stained with antiserum against RV1B (ATCC). Antiserum

was partially purified by incubation with nitrocellulose-bound HeLa cell proteins and passing through an affinity resin containing non-denatured mouse lung protein. Repurified antibody was directly conjugated to AF. Nuclei were stained with Hoescht 33258. Images were visualized using a Zeiss LSM 510 confocal microscope and Axiovert 100M inverted microscope. CD68-, eotaxin-1/CCL11-positive cells were counted at 500 μm intervals and expressed as the number per field.

Data analysis

Data are represented as mean±SEM. Statistical significance assessed by one- or two-way analysis of variance (ANOVA), as appropriate. Differences were pinpointed by Student Newman-Keuls' multiple range test.

Results

RV infection of OVA-sensitized and -challenged mice further increases airway inflammation.

We previously showed that RV1B infection of naïve C57BL/6 mice induces a state of modest airways hyperresponsiveness which lasts at least four days after viral inoculation (17). Hyperresponsiveness was associated with a short-lived increase in bronchoalveolar neutrophils. In the present study, we infected OVA-sensitized and -challenged BALB/c mice. Tissue eosinophils, macrophages and neutrophils were elevated in OVA-treated mice up to four days after RV inoculation (Figure 3-1), with maximal recruitment of macrophages and eosinophils occurring two days post-infection. In terms of absolute numbers, macrophages were the cell type most heavily recruited to

the tissues. Lung neutrophil recruitment was a relatively brisk event, with a significant increase in OVA/RV mice on day 1 after infection and a dramatic decrease on days 2 and 4. Although OVA treatment significantly increased lung lymphocytes compared to naïve mice, there was no significant difference in lymphocyte counts between OVA/RV and OVA/sham mice. In the BAL, RV infection increased neutrophils, eosinophils and lymphocytes in both naïve and OVA-sensitized and -challenged mice (Figure 3-2). As in the lung tissue, the largest absolute increase in BAL inflammatory cells following RV infection of OVA-sensitized and -challenged mice was observed in the macrophage line.

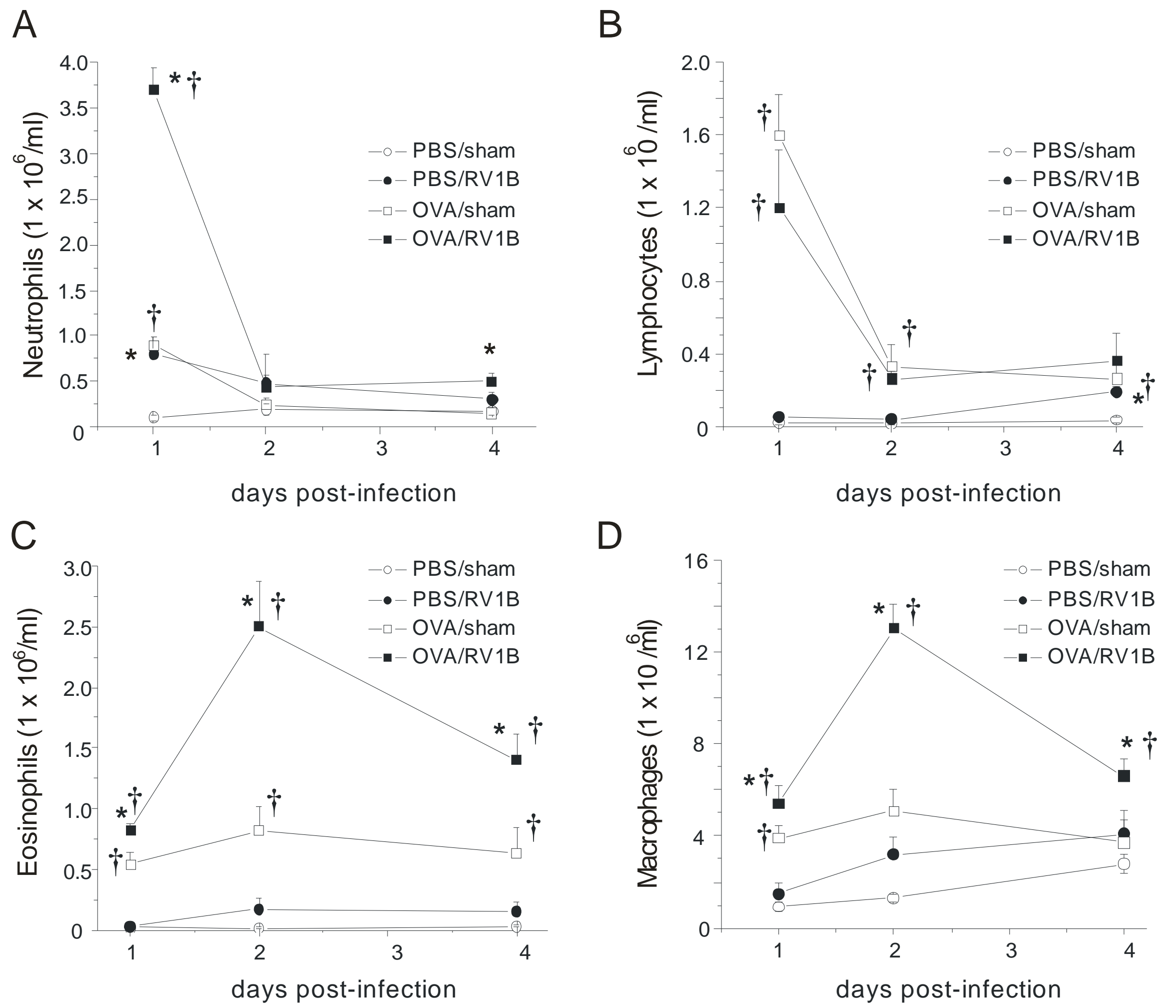

Figure 3-1. OVA/RV treated mice show increased tissue eosinophils and macrophages in response to RV infection. Wild type BALB/c mice were sensitized intraperitoneally with endotoxin-free OVA and alum on days 1 and 7, and challenged intranasally on days 14, 15, and 16 with OVA. Controls were treated with PBS. Mice were inoculated with RV1B or sham (HeLa cell supernatant) on day 16. Mouse lungs were harvested 1, 2 and 4 after infection. Lungs were digested for 1 h in Type IV collagenase in serum free RPMI. Strained cells were treated with RBC lysis buffer, spun and enriched for leukocytes with 40% Percoll. Resulting pellets were resuspended in PBS and total cell count determined. Cytospins of leukocytes were stained with Diff-Quik and differential cell count determined for 200 cells. Time courses for tissue neutrophils (A), lymphocytes (B), eosinophils (C) and macrophages (D) are shown. (N=4-5 mice per group, bars represent mean±SEM, *different from respective sham group, †different from respective PBS group, P<0.05, one-way ANOVA.)

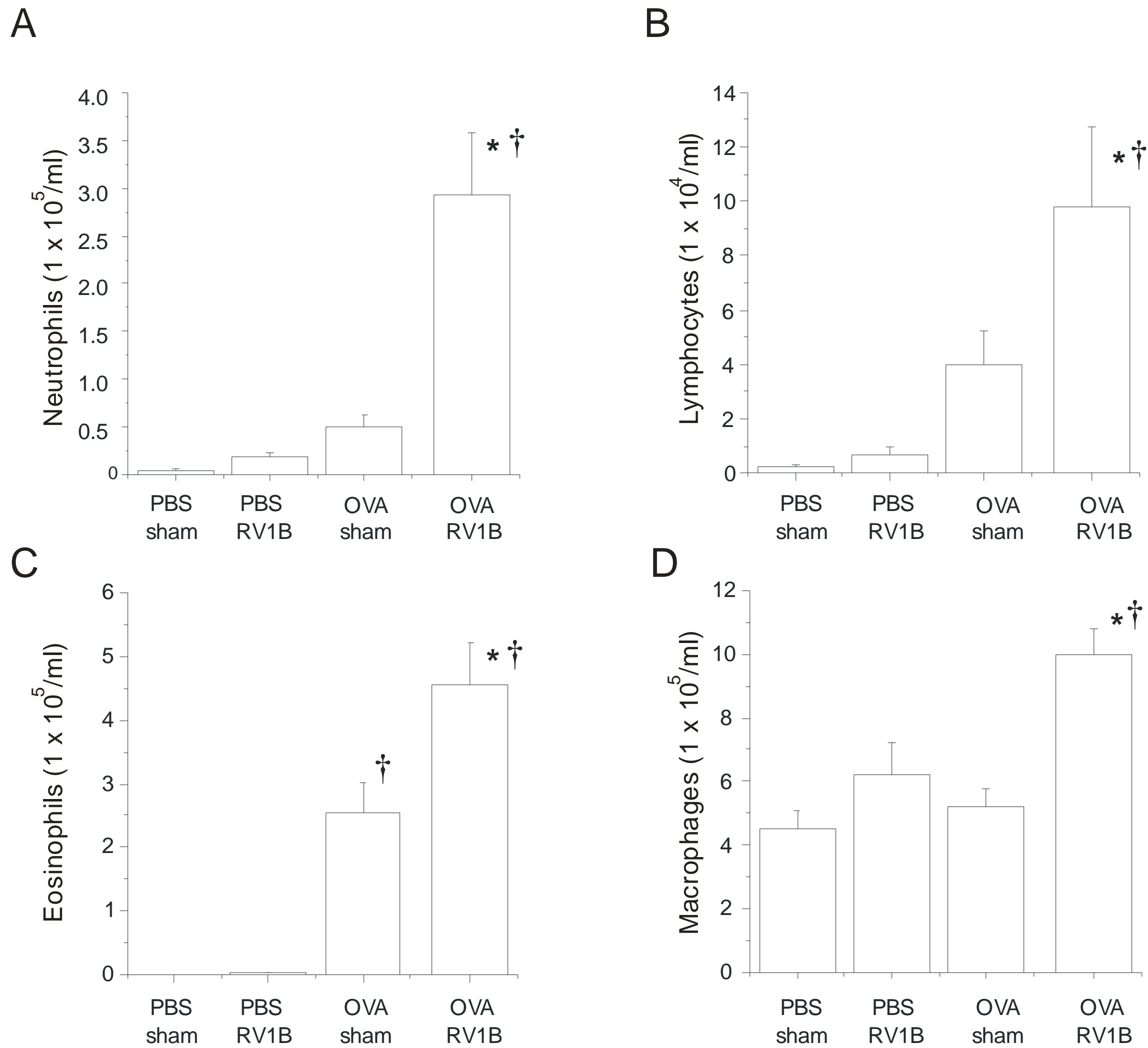

Figure 3-2. OVA-sensitized and -challenged mice show increased airway inflammation in response to RV infection 4 days after infection. Bronchoalveolar lavage was performed with saline containing 5mM EDTA. BAL neutrophils (A), lymphocytes (B), eosinophils (C) and macrophages (D) are shown. (*N* = 4-6 mice per group, bars represent mean ± SEM, *different from respective sham group, p<0.05; †different from respective PBS group, *P*<0.05 one-way ANOVA.)

Effects of RV infection on lung pro-inflammatory cytokines.

In order to determine changes in pro-inflammatory cytokines that might be responsible for the observed increase in eosinophilic inflammation, we measured lung cytokine levels by multiplex immunoassay. Compared to OVA-treated mice, OVA/RV mice demonstrated significantly higher levels of eotaxin-1/CCL11, IL-4 and IL-13 one day after infection (Figures 3-3). In contrast, there was no effect of RV infection on lung IL-5 levels. On day 4, lungs from RV-infected OVA-treated mice showed a sustained increase in eotaxin-1 and IL-4 levels (Figure 3-4). Eotaxin mRNA was elevated in the OVA/RV treated groups on day 1, 2, and 4 post infection.

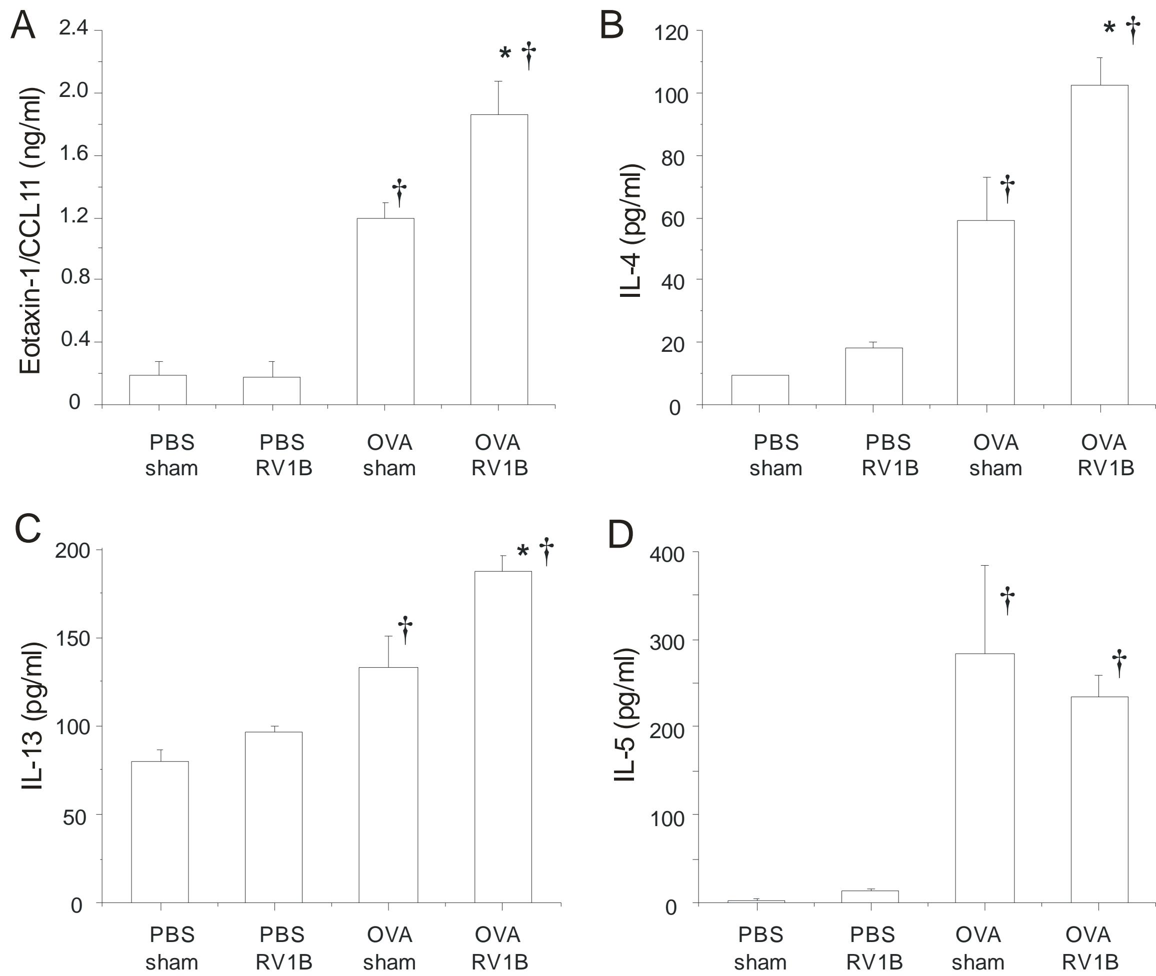

Figure 3-3. RV infection of OVA-sensitized and -challenged mice increases cytokine production. Twenty-four h after sham or RV infection, lung BAL fluid was centrifuged at 1500g and the resulting supernatant subjected to multiplex immunoassay. Results are shown for eotaxin-1/CCL-11 (A), IL-4 (B), IL-13 (C) and IL-5 (D). (*N*=5 mice per group, bars represent mean ± SEM, *different from respective sham group, $p<0.05$; †different from respective PBS group, $P<0.05$ one-way ANOVA.)

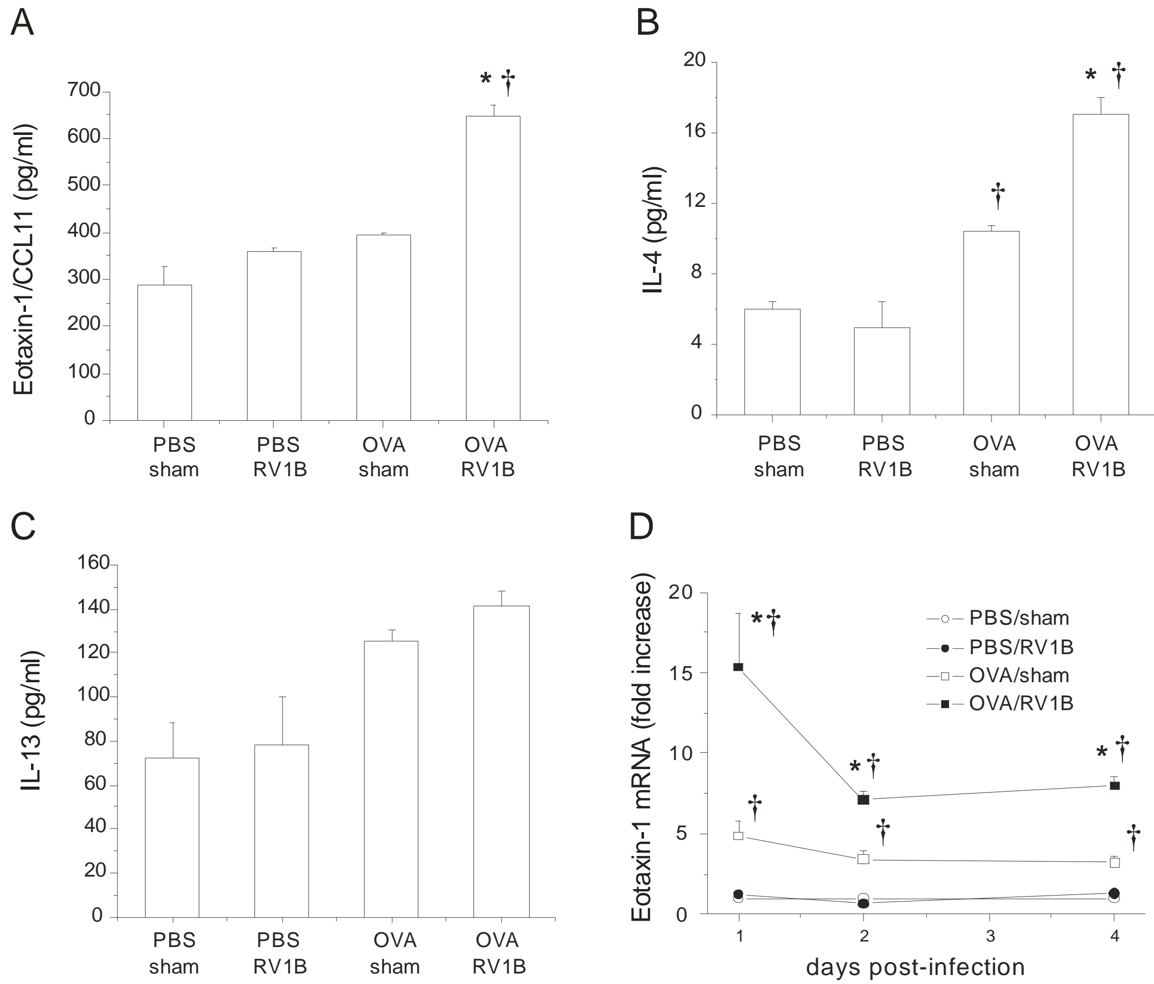

Figure 3-4. RV infection of OVA-sensitized and -challenged mice increases cytokine production four days after infection. Lung BAL fluid was centrifuged at 1500g and the resulting supernatant subjected to multiplex immunoassay. Results are shown for eotaxin-1/CCL11 (A and D), IL-4 (B) and IL-13 (C). cDNA for eotaxin-1/CCL-11 was synthesized using reverse transcriptase and subjected to quantitative real time PCR employing a Taqman probe. (*N*=5 mice per group, bars represent mean ± SEM, *different from respective sham group, p<0.05; †different from respective PBS group, *P*<0.05 one-way ANOVA.)

RV infection increases the airways responsiveness of OVA-sensitized and -challenged mice.

In order to determine whether the observed airway inflammation was functionally significant, all groups were tested for responsiveness to a bronchoconstrictor agonist four days after sham or RV1B treatment. Methacholine (0-20 mg/ml) was administered by nebulization and total respiratory system resistance values recorded. As expected, OVA treatment increased airway cholinergic responsiveness (Figure 3-5A). However, RV-infected OVA mice demonstrated significantly higher airways responses, with significant differences noted at methacholine doses of 10 and 20 mg/ml ($p < 0.05$, two-way ANOVA). In contrast, UV-irradiated, replication-deficient RV had no effect on airways responses in OVA-sensitized and -challenged mice. To determine whether the observed elevated airway responses to methacholine were due to a higher viral load, we measured lung positive-strand RV RNA. Surprisingly, vRNA levels were significantly lower in the OVA/RV treatment group (Figure 3-5B), suggesting that allergen sensitization and challenge increases viral clearance. As shown previously (17), viral copy numbers were negligible by four days after infection, consistent with the notion that airways hyperresponsiveness persists after viral clearance in this model.

Eotaxin-1 is required for RV-induced eosinophilic airway inflammation and hyperresponsiveness in OVA mice.

In OVA-treated mice, RV infection increased lung eosinophils (Figure 3-1) and the protein level of eotaxin-1/CCL11 (Figures 3-3 and 3-4), an eosinophil-specific chemokine. We therefore sought to examine the contribution of eosinophils to RV-

induced airway responsiveness by administering neutralizing antibody to mouse eotaxin-1. To ensure the suppression of augmented eosinophilic inflammation in RV-infected mice, a subset of OVA-treated mice was given two systemic injections of rabbit antiserum, the first on the day of RV inoculation and the second two days later. Control mice were treated with the isotype control. We found that, compared to IgG, anti-eotaxin treatment significantly reduced lung eosinophils in OVA-treated, RV-infection mice, but not OVA-treated, sham-inoculated mice (Figure 3-5C). Anti-eotaxin-1 neutralizing antibody did not reduce the infiltration of neutrophils, macrophages or lymphocytes (data not shown), suggesting that the antibody specifically targeted eosinophils. Further, administration of anti-eotaxin to OVA/RV mice significantly reduced responsiveness to methacholine compared to IgG (Figure 3-5D), suggesting that eotaxin-1 and eosinophils are required for maximal airway responses in RV-infected allergen-sensitized and -challenged mice. Anti-eotaxin had no effect on lung vRNA one day post infection (data not shown).

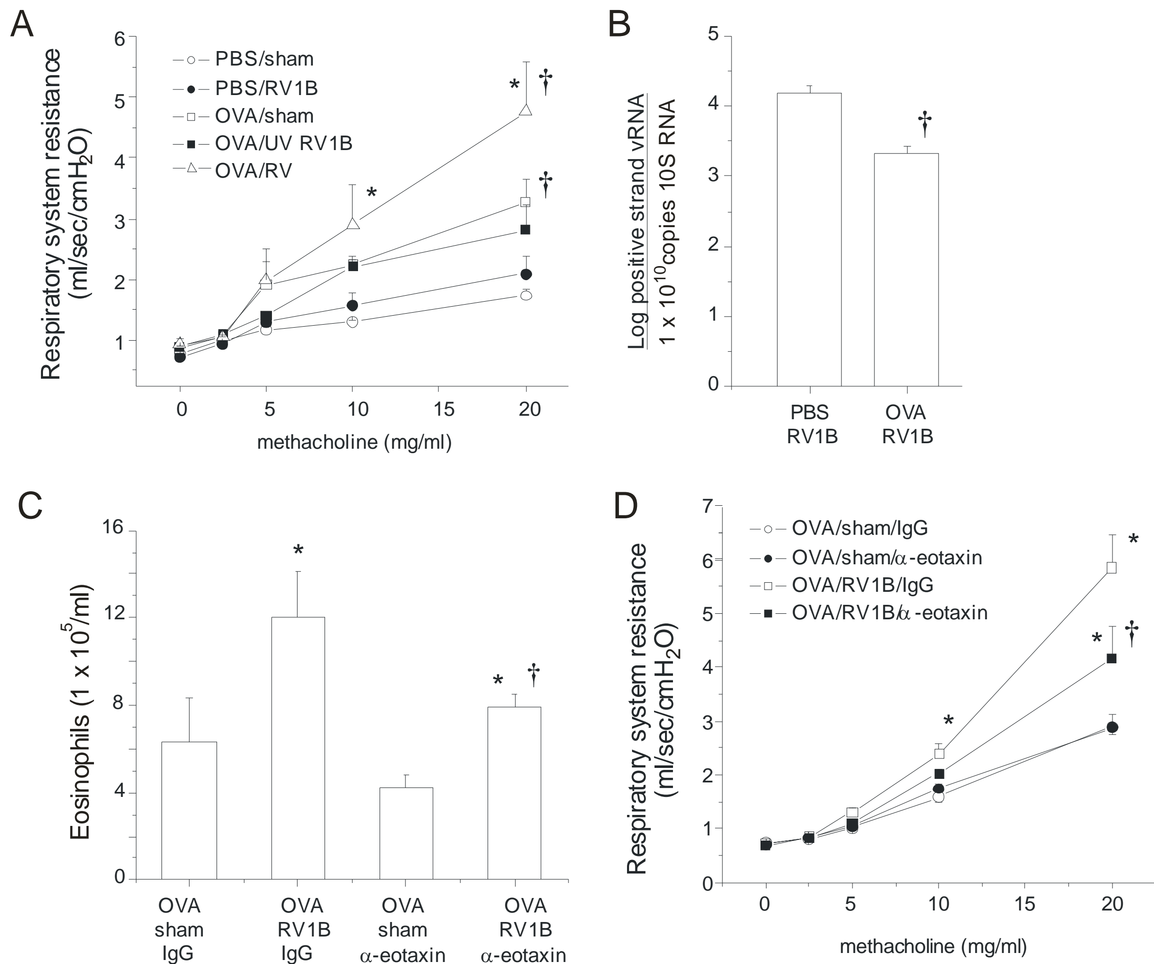

Figure 3-5. RV infection of OVA-sensitized and -challenged mice induces eotaxin-1-mediated airways cholinergic responsiveness. A. Mice were anesthetized and endotracheally intubated, and changes in respiratory system resistance to nebulized methacholine measured using the FlexiVent system (Scireq, Montreal, CA). Four days after infection, RV-infected OVA mice demonstrated significantly higher airways responses than all other groups at methacholine doses of 10 and 20 mg/ml. B. Measurement of viral copy number from lungs of PBS/RV and OVA/RV treated mice 1 day post infection. OVA/RV treatment significantly reduced viral copy number by 1 log. (*N*= 5 mice per group, bars represent mean ± SEM, *different from respective sham group, p<0.05; †different from respective PBS group, *P*<0.05 one-way ANOVA.) C. Selected RV-infected, OVA-sensitized and -challenged mice were given two systemic injections of rabbit anti-mouse eotaxin-1. Additional mice were treated with the isotype control antibody. Mice given anti-eotaxin displayed reduced tissue eosinophils 4 days after infection. D. Neutralizing antibody and isotype control-treated mice were administered increasing doses of aerosolized methacholine. Treatment with anti-eotaxin-1 significantly reduced airway cholinergic responsiveness compared to the IgG-treated group. (Bars represent mean ± SEM, *different from respective sham group, †different from IgG group, *P*<0.05, one-way ANOVA.)

Eotaxin-1 is mainly localized to CD68-positive macrophages.

We examined lungs of PBS-and OVA treated mice for eotaxin-1 localization (Figure 3-6 A-D). Fluorescence confocal microscopy with anti-eotaxin-1 and anti-CD68 showed intense yellow-orange staining, consistent with colocalization of CD68 and eotaxin-1 in lung macrophages[1] (Figure 3-6D). When CD68+/eotaxin-1/CCL11 positive cells were counted, OVA/RV mice showed a significantly higher number of cells per field compared to the other groups (panel E). Immunohistochemical analysis of lungs from RV-infected OVA-treated mice showed abundant eotaxin-1-staining which appeared to be localized to airway and submucosal macrophages. There also appeared to be a small number of eotaxin-positive eosinophils as well as limited staining in the airway epithelium (Figure 3-7A). We also found colocalization of RV1B in CD68-, eotaxin-1/CCL11-positive cells (Figure 3-7, panels B-F), suggesting that RV infection initiates cytokine expression and/or secretion in airway macrophages. Most RV-infected macrophages were located in the submucosa of large airways, but others were found in the airways and epithelium (Figure 3-8, panel A). A minor amount of RV1B and eotaxin staining was also found in airway epithelial cells. Finally, we found co-localization of RV1B, IL-4 and CD68 in the lungs of OVA-treated (Figure 3-8, panels C-G) but not IgG antibody treated sections (panel B) or PBS-treated mice (data not shown), indicating that, following exposure to an allergic environment, lung macrophages produce Th2 cytokines in response to RV infection *in vivo.*

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[1] Dr. John Bentley and Marisa Linn assisted with these experiments.

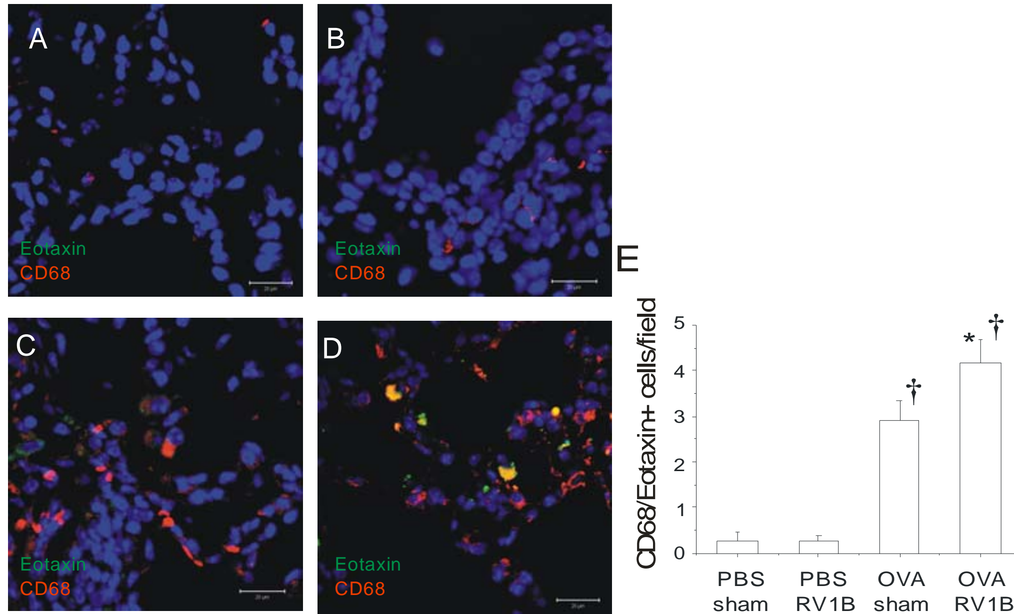

Figure 3-6. Eotaxin-1 is produced by alveolar macrophages from OVA-sensitized and -challenged mice, but not cells from PBS-treated mice. Lungs sections were stained with donkey-anti-mouse eotaxin-1 for 30 min and AF488-labeled donkey anti-goat IgG secondary overnight. Sections were costained with AF594-conjugated rat anti-mouse CD68 or isotype control IgG overnight. Lungs from PBS/sham (A), PBS/RV (B), OVA/sham (C) and OVA/RV (D) mice are shown. Intense yellow staining is seen in OVA/RV sections indicating colocalization of CD68 (red) and eotaxin-1 (green), while OVA/sham lungs show less intense staining. Sections incubated with secondary antibody alone showed no staining. E. OVA/RV lungs have increased CD68+/eotaxin-1+ cells per field compared to all other groups. (N= 3, bars represent mean ± SEM, *different from respective sham group, P<0.05; †different from respective PBS group, P<0.05 one-way ANOVA.)

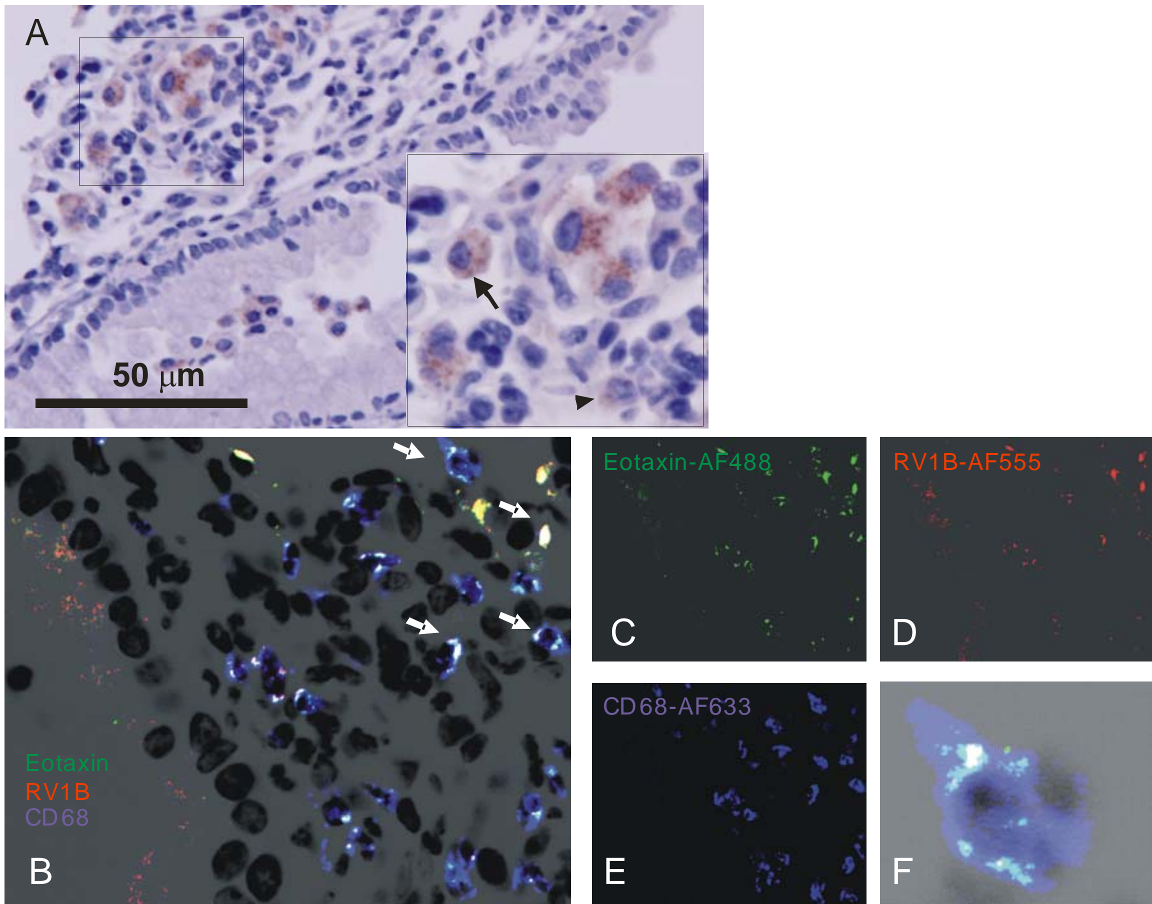

Figure 3-7. RV colocalizes with CD68+ macrophages, eotaxin-1 in OVA-sensitized and -challenged mice. A. Lungs were formaldehyde fixed overnight, paraffin embedded, sectioned at 5 µm and incubated with a 1:2500 dilution of donkey-anti-mouse eotaxin-1 (Santa Cruz Biotechnology, CA) or isotype control IgG. Eotaxin was identified by DAB staining. Following OVA/RV treatment, eotaxin-1 localization is noted in macrophages (arrows) and eosinophils (arrowheads) but not in the airway epithelium (line segment = 50 µm). B-E. OVA/RV lung sections were co-stained with antiserum against RV1B which was directly conjugated to AF-594 (red), while CD68 was conjugated to AF-633 (far red, shown in blue). Secondary antibody to eotaxin-1 was conjugated to AF-488 (green). Cells with colocalization (white) are designated by arrows and a high magnification view is shown in panel F. Original magnification, 600X.

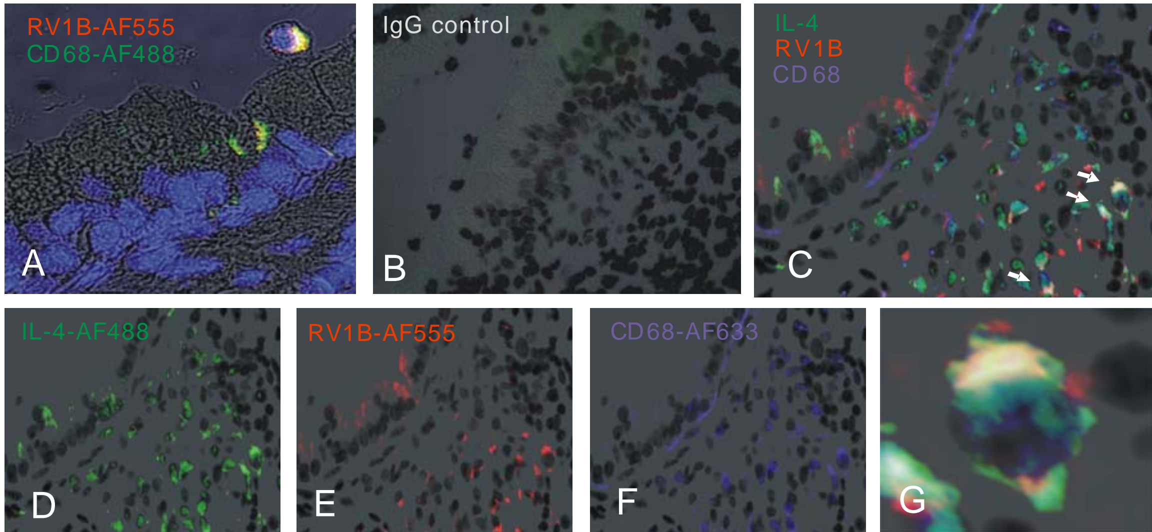

Figure 3-8. RV colocalizes with CD68+ macrophages, IL-4 in OVA-sensitized and -challenged mice. A. RV infection of CD68-positive cells in the airway lumen and epithelial layer. In this panel, RV anti-serum was directly conjugated to AF-594 (red), while CD68 was conjugated to AF488 (green). Colocalization is yellow. Colocalization in the epithelium suggests infiltration by a macrophage. B. Sections incubated with secondary antibodies alone showed no staining. C-G. RV co-localizes with IL-4 in CD68-positive cells. RV and CD68 were conjugated with AF-594 (red), and AF-633 (shown in blue) respectively. There is some blue background staining of elastin in the epithelial basement membrane. IL-4 was directly conjugated to AF-488 (green). G. Co-localization of RV, IL-4, and CD68 is white. Original magnification, 400X.

Macrophages are required for RV-induced eosinophil infiltration and airway hyperresponsiveness in OVA-sensitized and -challenged mice.

We delivered clodronate- or PBS-containing liposomes to OVA-treated mice intratracheally[2]. 24 h later, mice were inoculated with sham or RV1B. As expected, clodronate treatment depleted total lung macrophages (Figure 3-9A). Differential cell counts revealed a markedly lower eosinophil influx in clodronate treated mice (Figure 3-9B). No significant differences in neutrophil or lymphocyte accumulation were observed (Figures 3-9 C-D), OVA/RV mice receiving clodronate liposomes demonstrated a strikingly reduced response to methacholine compared to mice receiving PBS liposomes (Figure 3-9E). Finally, we examined the expression of IL-4, IL-5, and IL-13 in the liposome treated animals. Our pilot data shows a significant reduction in IL-13, and eotaxin-1 in the OVA/RV/clodronate treated group compared to the OVA/RV/PBS liposome treated group (Figure 10, panels A, B). Together, these data suggest that macrophages play an essential role in eosinophil infiltration and airway hyperreactivity in RV-infected mice with allergic airways disease.

[2] Dr. John Bentley and Emily Bowman assisted with these experiments

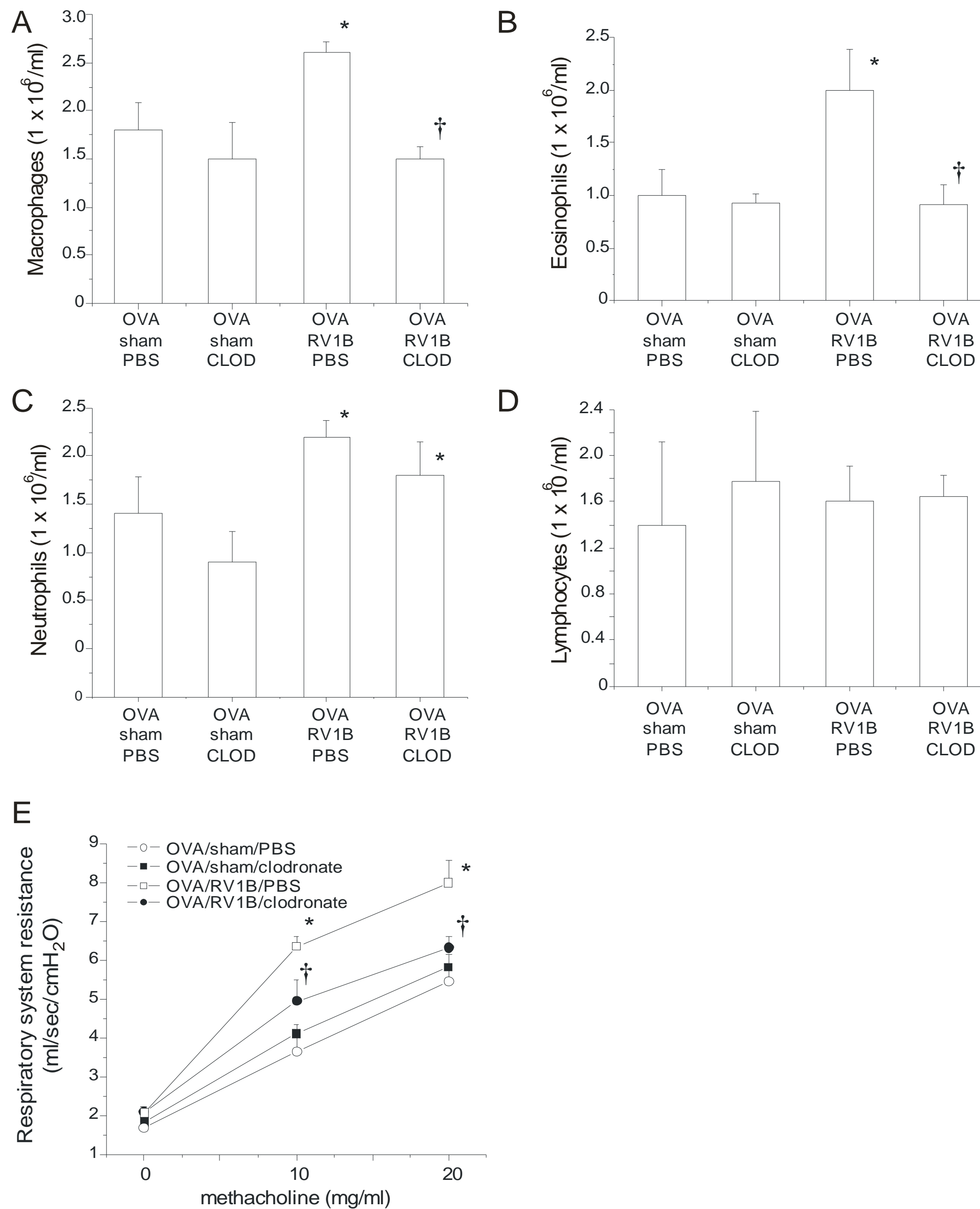

Figure 3-9. Macrophage-depleted OVA-treated mice show reduced airway eosinophils and hyperresponsiveness following RV infection. Clodronate or PBS-containing liposomes were instilled into the trachea 24 h after the last OVA challenge. 24 h following macrophage depletion, mice were inoculated with sham or RV and harvested 24 h after infection. Lung digests were performed as described in Figure 1. Differential counts were determined. Cytospins of leukocytes were stained with Diff-Quik and differential cell count determined for 200 cells. Macrophages (A), eosinophils (B), neutrophils (C) and lymphocytes (D) are shown. E. Airway resistance for each group was measured following treatment with 0, 10, and 20 mg/ml methacholine. (*N*=3-4 mice per group, bars represent mean±SEM, *different from respective sham group, †different from OVA/RV/ PBS group, *P*<0.05, one-way ANOVA.)

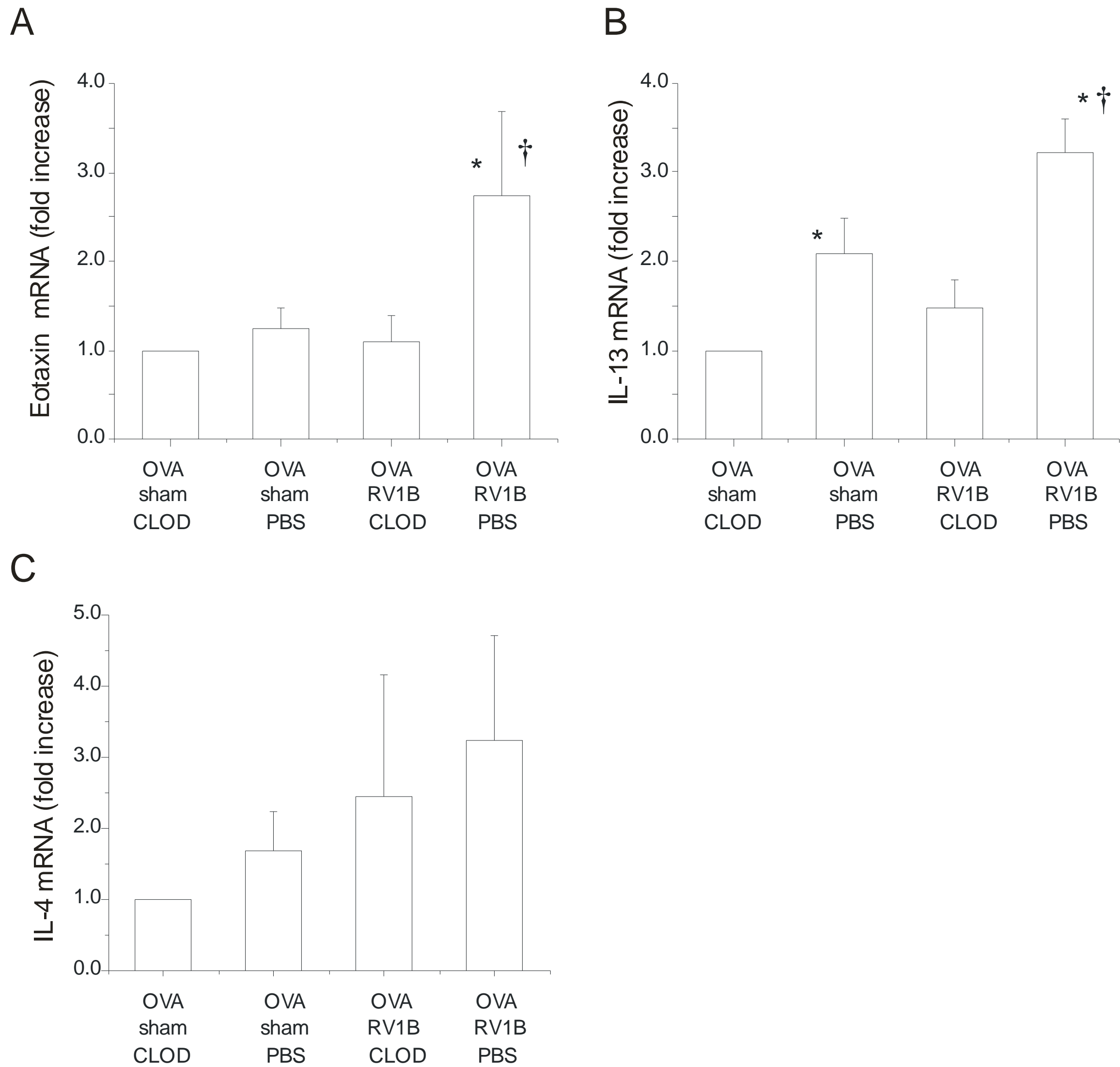

Figure 3-10. Clodronate treatment of OVA/RV mice decreases eotaxin and IL-13 expression. cDNA was synthesized using reverse transcriptase and subjected to quantitative real time PCR employing a Taqman probe. (*N*=4-5 mice per group, bars represent mean ± SEM, *different from clodronate-liposome treated group, $p<0.05$; †different from respective PBS-liposome group, $P<0.05$ one-way ANOVA.)

OVA sensitization and challenge alters macrophage pro-inflammatory cytokine expression in response to RV and upregulates markers of alternative activation.

In order to determine the combined effects of allergen sensitization and RV on macrophage responses, adherent BAL cells were studied. Cells from PBS- or OVA-sensitized and -challenged mice were then stimulated with sham HeLa cell lysate (sham) or RV1B *ex vivo*. 8 h after sham or RV exposure, cells were harvested for total RNA. Cytokine expression was determined by quantitative real time PCR. Macrophages from control mice produced no eotaxin-1 *ex vivo*, either at baseline or in response to RV (Figure 3-11). However, macrophages exposed to an allergic environment *in vivo* expressed eotaxin-1 mRNA, and this level was significantly increased following RV1B stimulation. In addition, RV treatment of macrophages from OVA sensitized mice induced expression of IL-4, IL-13, IL-10 and IFN-γ. UV-irradiation of RV abrogated the eotaxin, IL-10 and IFN responses, indicating that expression is dependent on viral replication. mRNA expression of IL-4 and IL-13 appeared to replication-independent. Finally, in contrast to the above cytokines, the TNF-α response to RV infection was significantly decreased in macrophages isolated from OVA-sensitized and -challenged mice.

We also measured the production of selected cytokines in cell supernatants following ex vivo RV stimulation, (Figure 12). IL-4 production was significantly increased in RV-stimulated macrophages from OVA-treated mice (panel B). We detected a small but significant increase in eotaxin-1/CCL11 production in macrophages from OVA mice exposed to RV 8 h post-infection (panel A). It is conceivable that, in contrast to eotaxin mRNA expression, the release of eotaxin-1 requires the coordinated

action of other mediators which may not be present *in vitro*. In contrast, macrophages from OVA-sensitized and -challenged mice showed reduced levels of TNF-α and p70 IL-12 production after RV stimulation (panels C and D).

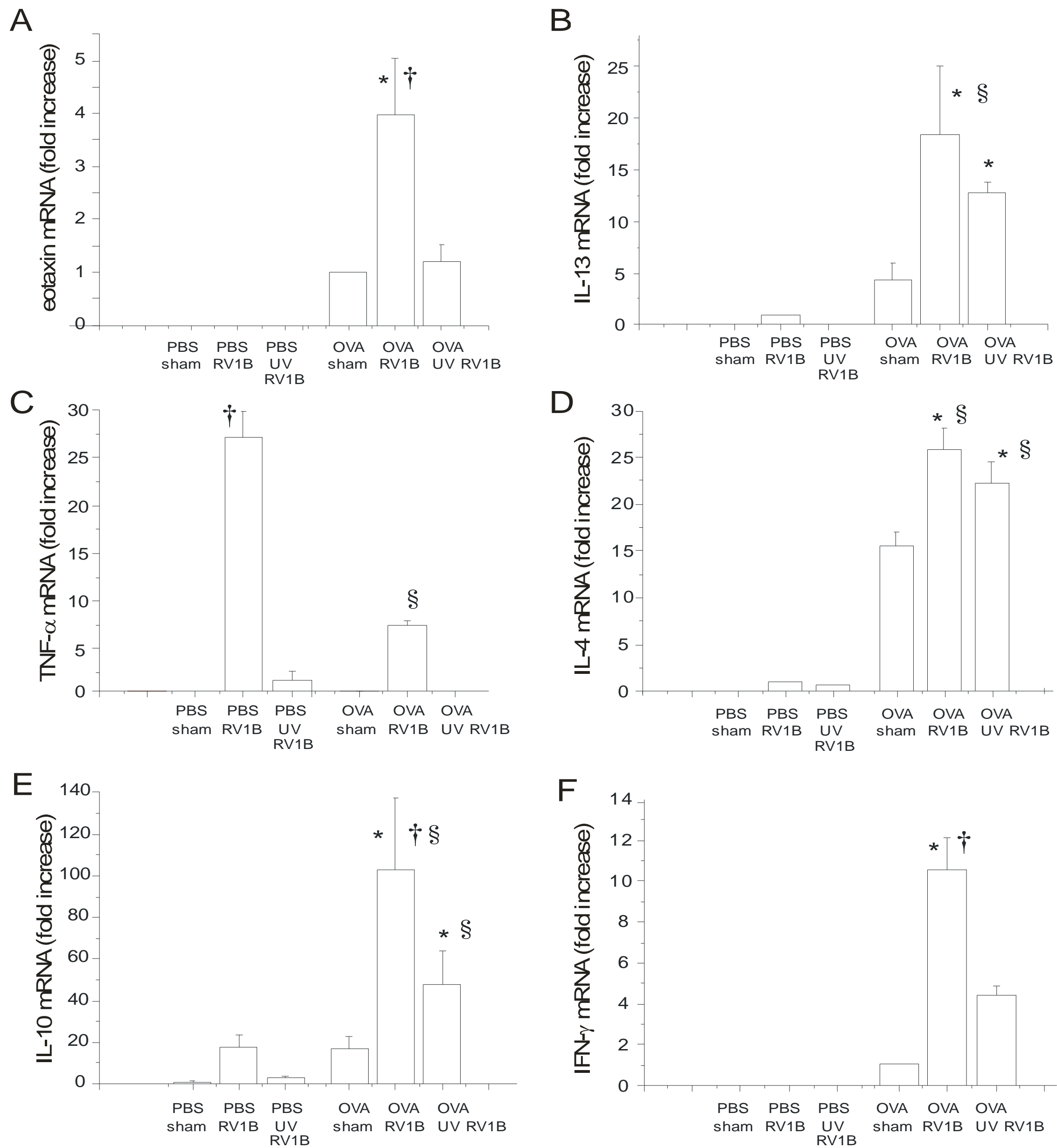

Figure 3-11. Macrophages from OVA-sensitized and -challenged mice show increased cytokine mRNA expression after RV stimulation *ex vivo*. BAL fluid was extracted from PBS-treated and OVA-sensitized and -challenged mice and seeded in 12-well plates. Cells were allowed to adhere to plates for 90 minutes. Adherent cells were subsequently infected with RV1B, or sham or media (controls). A . Eotaxin-1 expression was observed in adherent BAL cells from OVA-treated but not PBS-treated mice. Eotaxin-1 significantly increased following RV stimulation. B. IL-13. C. TNF-α. D. IL-4. E. IL-10. F. IFN-γ. (*N*=3-4, bars represent mean±SEM. Because some treatment conditions yielded no detectable mRNA expression, data were normalized to the condition with the lowest detectable mRNA signal. *different from respective sham group, †different from respective UV RV group, §different from respective PBS group, $P<0.05$, one-way ANOVA.)

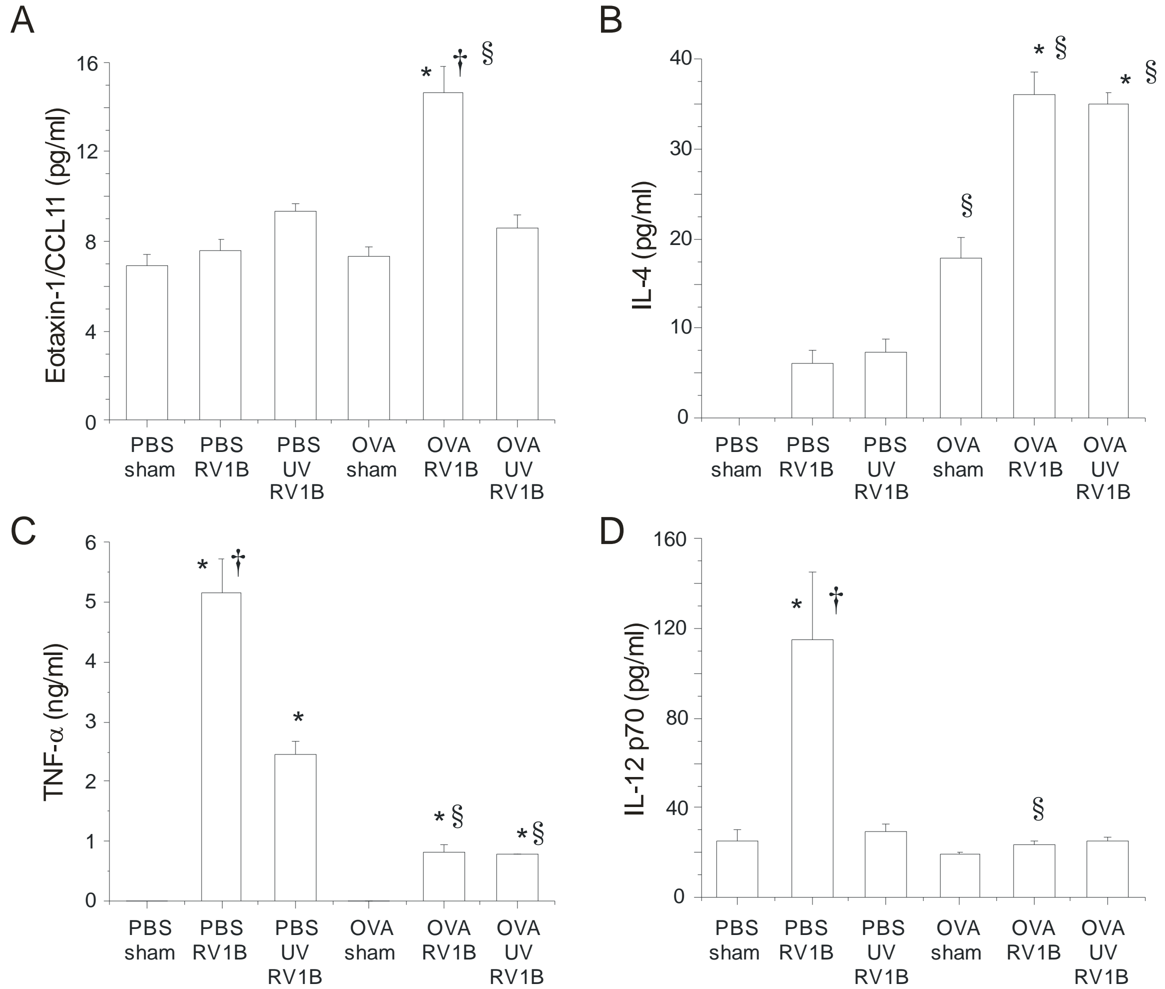

Figure 12. Macrophages from OVA-sensitized and -challenged mice show increased cytokine secretion after RV stimulation *ex vivo*. Cell supernatants were spun and subjected to bioplex immuneassay or ELISA. A. Eotaxin-1. B IL-4. C. TNF-α. D. IL-12 p70. (*N*=3, bars represent mean±SEM, *different from respective sham group, †different from respective UV RV group, §different from respective PBS group, $P<0.05$, one-way ANOVA.)

Based on the pattern of increased Th2 cytokine expression, we hypothesized that allergic sensitization induces macrophages to deviate from their classical pattern of activation, and instead exhibit a functionally polarized phenotype. To test this hypothesis, we measured markers of macrophage activation in cells isolated from PBS- and OVA-treated mice. We also found significant upregulation of the M2 polarization markers Arg-1, MGL-2, Ym-1 (Figure 3-13A) and, as noted above, IL-10.

IL-4 and IL-13 treatment has been shown to shift classically activated M1 macrophages to an M2 alternative activation phenotype (23-25). We therefore tested the effect of these cytokines on eotaxin mRNA expression in macrophages from naïve mice. *In vitro* exposure of macrophages from PBS-treated mice to the Th2 cytokines IL-4 and IL-13 significantly increased RV-induced eotaxin mRNA expression (Figure 3-13B). Taken together, these data suggest that allergen sensitization and challenge alters the activation state and augments the cytokine response of lung macrophages to RV infection, contributing to enhanced recruitment of eosinophils to the airways.

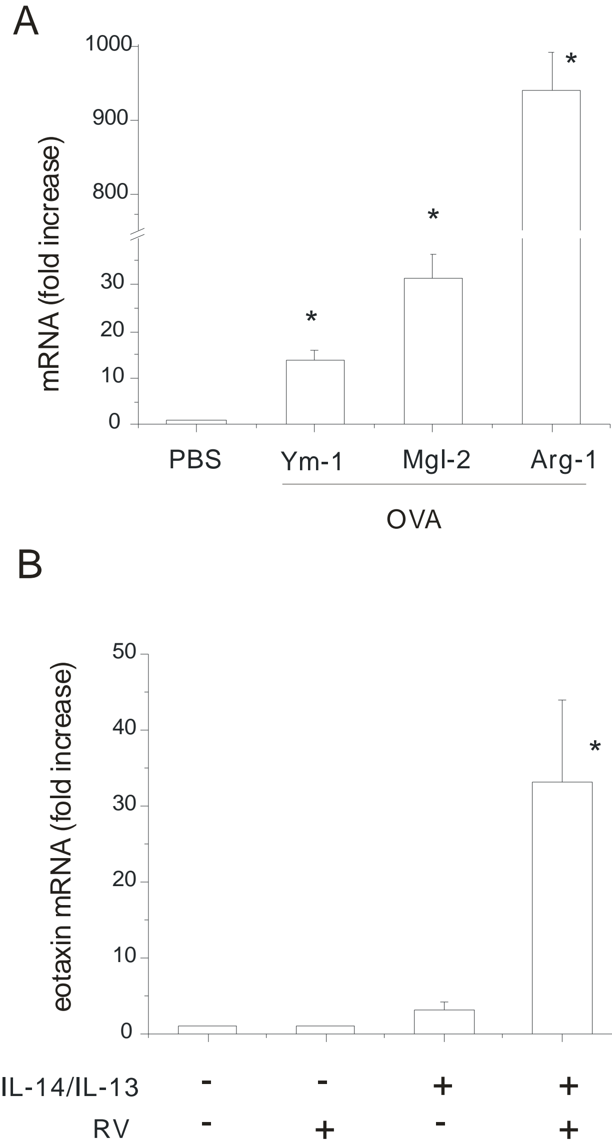

Figure 13. Effect of Th2 environment on macrophage polarization. A. OVA sensitization and challenge alters the mRNA expression of macrophage activation markers. Data are fold-increase compared to macrophages from PBS-treated mice (*N*=3-4, bars represent mean±SEM).. B. Effect of IL-4/IL-13 incubation on the eotaxin response to RV infection in macrophages from PBS-treated naïve mice. (*N*=3, bars represent mean±SEM.of fold increase in mRNA expression compared to control cells, mean±SEM, **P*<0.05, one-way ANOVA).

Discussion

RV is responsible for majority of the common colds and approximately 50% of asthma exacerbations (1, 2). Previous studies have demonstrated that neutrophils are the predominant inflammatory cell in the airways of patients with acute asthma exacerbation (26-28). Experimental RV infection has been shown to increase airway neutrophilic inflammation in normal and asthmatic subjects (9-14). Eosinophils and cationic protein have also been detected in the airways following experimental RV infection (10, 14, 16). However the precise mechanism of RV-induced asthma exacerbations is not well understood. After experimental RV16 infection, asthmatic patients show increased levels of IL-8 in their nasal lavage which correlates with the level of airways responsiveness (11), in contrast to unaffected individuals in whom IL-8 does not increase (15). In a recent study, asthmatics undergoing experimental RV infection demonstrated greater neutrophilic, lymphocytic and eosinophilic inflammation than RV-infected control subjects, though only the number of eosinophils achieved statistical significance (14). Together, these data suggest that patients with asthma experience a different response to viral infection than control subjects.

Previously, we showed that RV infection of naïve mice induces airway inflammation marked predominantly neutrophils and lymphocytes (17). RV infection also induced moderate airways hyperresponsiveness to methacholine. In the present study, we delineate the response to RV in the context of allergic inflammation. We found that, following RV infection of allergen-sensitized and -challenged mice, the largest populations of cells elicited in the BAL fluid were, in fact, eosinophils and macrophages.

The increase in eosinophils was associated with a concomitant rise in expression of the eosinophil chemoattractant eotaxin-1/CCL11, which was significantly greater in OVA/RV mice in comparison to all other groups. Eosinophil infiltration was also accompanied by a synergistic increase in the Th-2 cytokines IL-4 and IL-13, each of which were both significantly higher in the OVA/RV-treated mice compared to all other groups. It is worth noting that RV infection alone failed to significantly increase airway eosinophils, eotaxin-1, IL-4, IL-13. RV infection also enhanced airways responsiveness in allergen-sensitized and -challenged mice, with hyperresponsiveness persisting at least 4 days after infection. These data confirm and extend a recent report (18), and are consistent with the notion that the allergic environment qualitatively alters the response to RV.

We measured viral copy number in the lungs of infected PBS- and OVA-treated mice. Viral load was not increased in allergen-sensitized and -challenged mice, demonstrating that the augmented airway inflammation and responsiveness was not due to an increase in the susceptibility to RV. Indeed, RV copy number was unexpectedly decreased in mice with allergic airways disease. These data are consistent with a previous report examining parainfluenza infection of OVA-treated animals (29), and suggest that inflammatory cells play a role in viral clearance. More importantly, these data demonstrate an uncoupling of viral load and airway inflammation. While this may seem surprising, viral infection may set off a pro-inflammatory cascade that outlasts the presence of live virus. Consistent with this, we previously found that RV-infected, naïve mice demonstrate airways hyperresponsiveness four days after RV infection, when viral copy number is decreasing (17). We also found that replication-deficient virus is

sufficient to induce moderate neutrophilic inflammation and airways responsiveness one day after inoculation.

In OVA-treated mice, RV infection increased lung eosinophils and expression of eotaxin-1/CCL11. Eosinophils and the eotaxin/CCR3 axis are known to play a critical role in chronic experimental allergic airway inflammation (30-32). To test for the requirement of eotaxin-1 for enhanced eosinophilic inflammation and airways hyperresponsiveness in allergic, RV-infected mice, we targeted eotaxin-1 production by administering an anti-mouse eotaxin-1 neutralizing antibody following the last OVA challenge and RV infection. Anti-eotaxin-1 significantly reduced the number of airway eosinophils, but not the neutrophils or lymphocytes, demonstrating that eotaxin-1 is required for homing of eosinophils to the airway following RV infection. Further, administration of anti-eotaxin-1 blocked RV-induced airways hyperresponsiveness in allergen-sensitized and -challenged mice. While we did not determine the precise mechanism by which eosinophils increase airways responses, eosinophils are a known source of bronchoconstrictor agonists including major basic protein (MBP) and cysteinyl leukotrienes. When guinea pigs are sensitized to OVA and subsequently infected with parainfluenza, virus-induced hyperresponsiveness and M2 receptor dysfunction are blocked by depletion of eosinophils with antibody to IL-5 or antibody to MBP (29). RV infection has also been shown to increase 5-lipoxygenase and cyclooxygenase-2 in bronchial biopsy specimens from nonatopic subjects (33).

To determine the cell(s) responsible for the observed increase in eotaxin-1 expression in response to RV, we performed immunohistochemistry on OVA/RV mice. Though previous reports demonstrated production of eotaxin-1/CCL11 by RV-infected,

cultured airway epithelial cells (34-38), eotaxin-1 was only minimally localized to the airway epithelium. Instead, eotaxin-1 protein abundance was readily apparent in alveolar macrophages. These data are consistent with previous reports showing that, in airway inflammatory cells from asthmatic patients, eotaxin-1 immunoreactivity is colocalized predominantly to macrophages, with a lesser contribution from eosinophils (39-41). Further, macrophages isolated from allergen-sensitized and -challenged mice demonstrated a significant eotaxin-1 response to RV stimulation *ex vivo*, in contrast to cells from naïve mice, which showed no response. RV has previously been shown to induce cytokine responses in alveolar macrophages *in vitro.* Production of monocyte chemoattractant protein (MCP)-1/CCL2 and IP-10/CXCL10 is replication-independent (42-45), whereas production of tumor necrosis factor-α may be replication-dependent (44). In the present study, we show for the first time that *ex vivo* macrophage responses to RV are augmented following allergen-sensitization and -challenge, and that macrophages produce cytokines in response to airway RV infection *in vivo.* However, we cannot tell from our images whether colocalization represents true replicative infection, endocytosis of virus, or phagocytosis of RV1B by airway macrophages.

Eotaxin production in response to RV infection has not been previously demonstrated *in vivo.* In the one study of which we aware examining eotaxin-1 expression in response to natural or experimental RV infection in asthmatic subjects, mRNA transcripts for eotaxin-1 were not expressed at consistently detectable levels in induced sputum (46). However, our preliminary studies examining nasal washes from asthmatic children show that natural viral infection, as detected by PCR, is associated with a 6-fold increase in eotaxin-1 protein abundance compared to virus-negative weeks

(T. Lewis, T. Henderson, M. Hershenson, unpublished data).

In addition to eotaxin-1, we found that the combination of OVA treatment and RV infection increased production of IL-4 from alveolar macrophages, both *in vivo* and *ex vivo.* Macrophages from OVA-treated mice also expressed higher levels of IL-13 in response to RV *ex vivo*. The notion that a non-T cell source of Th2 cytokines may also act to enhance allergic inflammation by secreting IL-4 or IL-13 has not been well-studied. IL-13 production has been noted in lung macrophages from Sendai virus-infected C57BL/6J mice (47). The role of macrophages in the pathogenesis of asthma and allergic inflammtion is unresolved. Macrophage subsets are recruited into the lung following OVA sensitization and challenge of Balb/cJ mice, and transfer to naïve mice increased airways responsiveness, eosinophilic inflammation and in Th-2 cytokine secretion (48). On the other hand, transfer of alveolar macrophages from OVA-exposed Sprague-Dawley rats protects against the development of airways hyperresponsiveness in macrophage-depleted OVA-treated Brown-Norway rats (49). In our study, depletion of macrophages resulted in a significant amelioration of eosinophil infiltration and airway responsiveness, suggesting for the first time that RV-induced asthma exacerbations may be directed by lung macrophages.

Our data demonstrating increased production of eotaxin-1 and IL-4 from alveolar macrophages, both *in vivo* and *ex vivo*, suggests an alteration in the phenotype of tissue macrophages in response to allergen sensitization and RV infection. In addition, RV treatment of macrophages from OVA sensitized mice, but not PBS-treated mice, induced expression of IL-13, IL-10 and IFN-γ. In contrast, TNF-α and p70 IL-12 were significantly decreased. Shift of classically activated M1 macrophages to an M2

alternative activation phenotype, under the influence of the Th2 cytokines IL-4 and IL-13, has been associated with an altered secretory repertoire and pattern of phagocytic receptors (reviewed in (25)). IL-4 and IL-13 have been shown to induce alternative macrophage activation *in vitro* (23) and *in vivo* (24). In the latter study, IL-13-overexpressing transgenic mice infected with *C. neoformans* showed the presence of alternatively-activated macrophages expressing Arg-1, macrophage mannose receptor and Ym-1, as well as lung eosinophilia, goblet cell metaplasia, elevated mucus production and enhanced airway hyperreactivity. Consistent with this, we found significant up-regulation of M2 markers in OVA sensitized and challenged mice including Arg-1, MGL-2, Ym-1, Fizz-1 and IL-10. Modulation of Arg-1, Ym-1, Fizz-1, MGL-1 and MGL-2 expression was previously noted following OVA sensitization and challenge (50). Upregulation of Arg-1 may be of particular physiologic importance. In patients with asthma, Arg-1 mRNA expression is increased in submucosal inflammatory cells (51). Arginase expression is increased in the lungs of allergen-sensitized and challenged mice, and inhibition attenuates methacholine responsiveness in OVA-sensitized and challenged mice (52).

In addition to changes in macrophage receptor and cytokine expression typically associated with M2 polarization including Arg-1, MGL-2, Ym-1 and IL-10, we also found that OVA treatment increased expression of the classical activation marker IFN-γ. Patterns of macrophage gene expression may not display a strict dichotomy between type 1 and type 2 responses. For example, it has been reported that exposure of macrophages to IL-4 prior to LPS stimulation strongly enhances inflammatory activity (TNF-α, IL-12 production) as well as Arg-1 expression (53). These data suggest the possibility that

exposure to a Th2 environment induces a functional phenotypic change in airway macrophages which does not strictly fit the M1/M2 model, leading to increased secretion of both type I and type II cytokines in response to RV stimulation.

In cultured macrophages, UV-irradiation of RV abrogated the eotaxin, IL-10 and IFN responses. The reduced cytokine expression following treatment with UV-irradiated virus is consistent with the notion that RV causes a replicative infection in macrophages. *In vitro* studies have noted attachment of HRV to peripheral blood monocytes and airway macrophages, with subsequent secretion of numerous pro-inflammatory cytokines, chemokines and IFNs (42-45, 54, 55). A small amount of viral replication has been noted in HRV-infected peripheral blood monocyte-derived macrophages, but not in bronchoalveolar lavage (BAL)-derived macrophages (42, 44). (42)

In conclusion, we have shown in allergen-sensitized and -challenged mice that lung macrophages participate in RV-enhanced airway eosinophilic inflammation and hyperreactivity. Macrophages from allergen-sensitized and -challenged mice, but not control animals, produce eotaxin-1 and IL-4 in response to RV infection, both *in vivo* and *ex vivo.* The altered response to RV infection is driven by a functional change in macrophage polarization state, likely a response to Th2 cytokines in the allergic environment. These data provide a new paradigm to explain RV-induced asthma exacerbations, and identify the macrophage as a potential therapeutic target for the treatment of viral-induced exacerbations of chronic airways disease.

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Chapter 4

Summary, Limitations, and Future Directions

Summary

RV is responsible for the most virus-induced asthma exacerbations in children and at least half of the exacerbations in adults (1, 2). While RV was until recently considered an upper respiratory pathogen, recent studies have revealed RV localization in the lower airways, although the extent of replication has not been determined (3-6). The underlying goal of this thesis was to examine why RV causes only a moderate change in airway hyperreactivity in normal subjects, while causing severe airflow limitations in asthmatic subjects. In order to achieve these objectives, we first employed a mouse model of RV infection which we have developed in our laboratory (7) and examined the pro-inflammatory changes observed after RV inoculation in naïve mice. Next, we extended this line of study to a mouse model of asthma characterized by Th-2 driven airway inflammation. We found that, compared to naïve mice, RV infection causes a qualitatively different response in mice with allergic airways disease. Perhaps more importantly, our studies identify the CD68-positive lung macrophage as a key player in viral-induced airway inflammation, changing the current paradigm explaining viral-induced asthma exacerbations.

RV infection *in vitro* has been associated with ELR (+) CXC chemokines GRO-α/CXCL1, ENA-78/CXCL5, and IL-8/CXCL8, and *in vivo* with murine homologs of IL-8, KC/CXCL1 and MIP-2/CXCL-2 (7-10). In addition, experimental RV16 infection of normal subjects induces IL-8 release, neutrophil infiltration, and increased airway hyperreactivity to bronchoconstrictor agonists (11-15). In asthmatic patients, RV infection increases IL-8 in nasal lavage fluid which correlates with the level of airways responsiveness (16) suggesting that IL-8 may affect airflow limitation, possibly via neutrophil chemotaxis. Since CXC chemokines including IL-8 bind to CXCR1 and CXCR2 receptors in humans, we hypothesized that CXC chemokines and CXCR2 were required for RV induced airway inflammation and hyperreactivity in naïve mice. We observed that, as in C57BL/6 mice, naïve wild type Balb/c mice respond to RV infection primarily with increased neutrophils on day 1 post infection along with increased airway hyperreactivity. In addition, CXCR2 -/- mice displayed reduced neutrophil infiltration and hyperresponsiveness compared to wild type mice 1 day post infection, suggesting a pathological role for CXC chemokines and neutrophils in the early stages of RV induced airway inflammation. Furthermore, CXCR2-/- mice demonstrated reduced pro-inflammatory cytokine induction including TNF-α and IL-1β, suggesting that CXCR2 response is required for the production of RV induced cytokines and lung neutrophilia. RV infected, neutrophil depleted mice, also showed a significant reduction in airway responsiveness to methacholine.

Finally, the lungs of CXCR2-/- mice showed reductions in expression of TNF-α and IL-1β. We therefore hypothesized that RV induced TNF-α expression was required for airway responses. Surprisingly, TNFR1-/- mice showed a marked reduction in both

airway neutrophils as well as a complete abrogation of airway hyperreactivity. Reconstitution of neutrophils with exogenous MIP-2 failed to restore airways responsiveness, demonstrating an absolute requirement of TNFR1 signaling for RV-induced airway responses. It is important to note that there was negligible infiltration of eosinophils in response to RV in the naïve mice (data not shown), suggesting that neutrophils are the predominant cell types recruited in normal subjects following inoculation in normal subjects.

In our mouse model of allergic inflammation characterized by Th-2 cytokines, RV infection of OVA-sensitized and challenged mice caused a pronounced increase in lung macrophages, eosinophils, and neutrophils. Both eosinophils and neutrophils have been detected in asthmatics following experimental RV16 infection (15). While the infiltration of neutrophils in OVA/RV treated groups was short-lived as in naïve mice, macrophages and eosinophils persisted 4 days post infection. Th-2 cytokines IL-4, IL-13, and eotaxin were also elevated in OVA/RV treated groups compared to all other groups, suggesting RV exacerbates Th2-driven eosinophilic airway inflammation. Airway inflammation was accompanied by airway hyperresponsiveness. Neutralization of eotaxin-1/CCL11 markedly decreased RV induced tissue eosinophils and airway hyperresponsiveness, suggesting that eotaxin production and eosinophil chemotaxis are closely associated with airway hyperreactivity implicating eosinophils in RV induced exacerbations of asthma.

Eotaxin is expressed in epithelial cells in response to RV (17); hence we hypothesized that we would find abundant localization in the airway epithelium of OVA/RV mice. Instead, we found co-localization of RV and eotaxin in CD68+ cells and our *ex vivo* studies suggested that lung macrophages were important cellular sources of

eotaxin following OVA/RV treatment. IL-4 was also detected in RV/CD68+ cells suggesting that other Th-2 cytokines may also be produced by macrophages in response to RV *in vivo*. Furthermore our *ex vivo* studies implicated a functional polarization of macrophages such that the priming of these cells in an IL-4/IL-13 environment triggered an exacerbated Th-2 cytokine response. Indeed IL-4, IL-13, IL-10, eotaxin expression markedly increased in macrophages from OVA mice following RV stimulation. Moreover cytokines that are characteristically M1 (TNF-α, IL-12 p40) were down-regulated in the OVA cells 8 h after RV inoculation. Finally, we tested the requirement of macrophages in RV induced airway inflammation and hyperrreactivity. We found that following OVA -sensitization and -challenge, clodronate depletion of macrophages caused significant reductions in RV induced airway hyperresponsivness and lung macrophages and eosinophils, compared to PBS liposome treated RV infected animals. Reduced expression of eotaxin and IL-13 were also detected in the clodronate treated, RV infected animals, implicating a causal role for these cytokines in the pathology of the OVA/RV treated mice.

While some studies have suggested that asthmatics are susceptible to RV due to a deficient interferon response in epithelial cells (18, 19), this evidence has not been replicated by others (20) and there is now increasing evidence that RV may in fact infect more cell types than just the airway epithelium (21-24). Our observations in OVA-sensitized and -challenged macrophages could provide an explanation for why RV causes exacerbations despite limited and patchy localization in the airway epithelial cells (3).

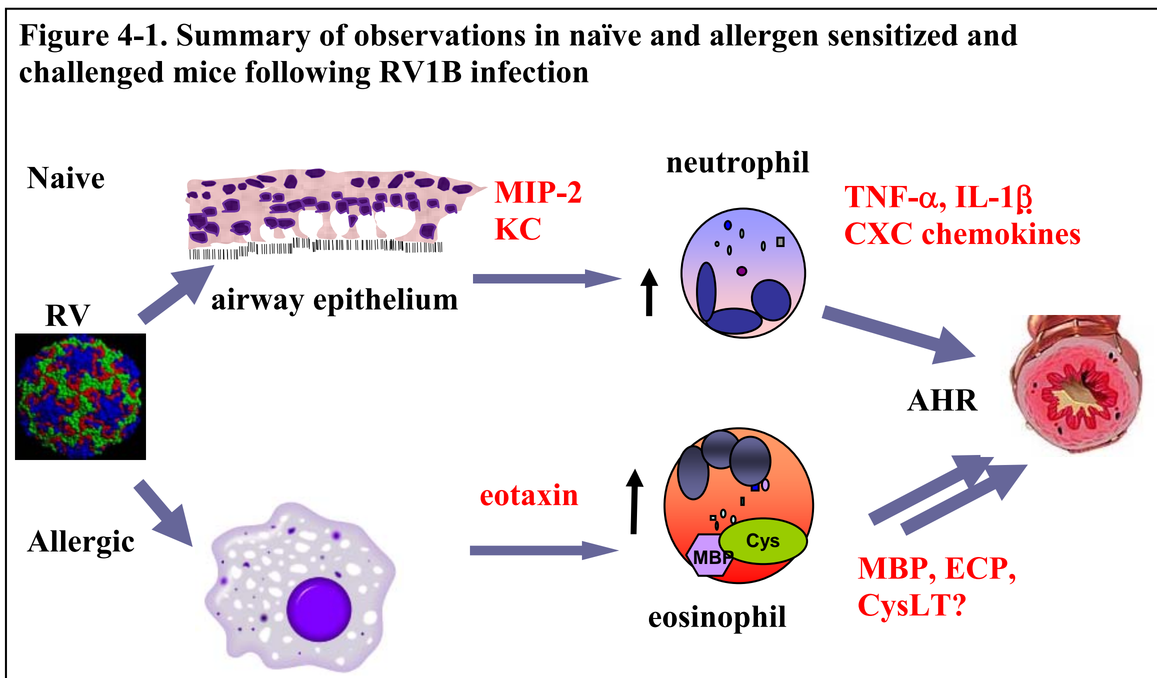

Figure 4-1. Summary of observations in naïve and allergen sensitized and challenged mice following RV1B infection

Limitations and Future Directions

Mouse model of RV infection

We have previously infected C57BL/6 mice with minor groups serotype RV1B and shown increased CXC chemokines, increased neutrophils, and moderate changes in airway hyperreactivity which were in part abrogated in UVRV (replication deficient virus) treated mice (7). In addition, RV inoculation of naïve mice induces a strong interferon response pattern which is double-stranded viral RNA dependent. We have also shown that lung homogenates from RV infected mice when overlayed on Hela cell monolayers produce cytopathic effects up to 4 days following RV inoculation, consistent with the presence of a live virus. We are therefore confident that is minor group RV mouse model will be useful in further investigating RV induced airway inflammation *in vivo*.

However, some concerns with our model remain. First, studies in cultured airway epithelial cell lines have noted differences in cell pathology after infection with RV1B, a minor group virus which binds to LDL-R, and major group viruses including RV16 and RV39 which bind to the receptor to ICAM-1 (25). However, similar patterns of cytokine including CXC chemokine induction have been noted across major and minor group serotypes (7, 26, 27). Major and minor group viruses induce nearly identical patterns of gene expression in cultured airway epithelial cells (28). Finally, recent analysis of all known HRV genomes revealed that HRV1 and HRV16 are highly homologous and respond similarly to small-molecule antiviral compounds (29), implying that the distinction between some major and minor group strains may not be clinically relevant.

Also, species differences between mouse and human continue to restrict viral replication of human RV in mice. In our model, aside from a shallow peak 18 h after infection, viral titer decreases gradually with time. Hence, our RV model does not exactly reproduce human infection, in which viral replication plateaus for several days. In future studies we could employ serotype RV1A which is currently being developed as a mouse-adapted serotype with a sustainable viral titer in mice (Vincent Raccanielo, unpublished data).

RV induced inflammation in naïve and CXCR2 -/- mice

RV infection of CXCR2 deficient mice, showed reduced accumulation of neutrophils and airway hyperrreactivity compared to naïve mice. However CXCR2-/- mice infected with RV still showed higher airway responses to methacholine compared to the sham infected groups, despite minimal neutrophil influx. In addition RV infected,

LY6G treated animals showed small but significant reductions in airway hyperreactivity.. These results indicate that while neutrophils are required for the maximal airway responses to RV. Additional experiments with TNFR1 knockout mice demonstrated the requirement of TNF-α for RV-induced airway hyperresponsiveness. We did not test the precise mechanism by which TNF-α causes airway narrowing. TNF-α has been reported to enhance airway smooth muscle contraction both directly, by increasing calcium influx, and indirectly by increasing calcium sensitivity (30-32). Intra-tracheal delivery of TNF-α also increases mucus secretion *in vivo* (33) which could result in airway occlusion.

It is also conceivable that other chemokines play a role in RV-induced neutrophil chemotaxis and airway hyperresponsiveness. Our gene array demonstrated that in addition to CXC chemokines, cytokines including CCL17, CCL20, and IL-1β were also expressed at significantly lower levels in the CXCR2-/- mice infected with RV. Antibodies to CCL17 diminish OVA induced airway inflammation in mice (34), while CCL20 has been associated with dendritic cell recruitment, allergic airway inflammation and airway responses (35). Overexpression of IL-1β in the lung causes neutrophil and macrophage chemotaxis and emphysema-like airway pathology in adult mice (36). In future studies, neutralizing antibodies to the above mediators could help elucidate more inflammatory mechanisms which RV infection may trigger in naïve mice.

Differential expression of CXCR2

Another limitation of this study is that CXCR2 has been detected in more cell types than just neutrophils. For instance, in RSV-induced lung inflammation, CXCR2 was expressed on macrophages, not neutrophils, in response to infection (37). Future

studies could involve clodronate depletion of macrophages in naïve mice to examine whether macrophages constitute part of the inflammatory response to RV. CXCR2 is also expressed on smooth muscle cells (38), thus it is conceivable that the RV induced methacholine responsiveness is due to the activation of CXCR2 and initiation of contraction signaling. In this regard, *in vitro* studies have shown that major group RV16 can increase airway smooth muscle contractility (39). Therefore we could perform an *in vitro* study to see if RV induced airway smooth muscle contractility is impaired after treatment with CXCR2 antagonist. However, there has been no evidence in either humans or animal models showing that RV infects airway smooth muscle *in vivo*.

OVA -sensitization and -challenge protocol

The most obvious limitation of our allergen -sensitization and -challenge protocol is our choice of allergen. While OVA, is a chicken-egg antigen extensively used in studies in order to generate a Th-2 environment, it is not a reagent that naturally simulates an allergic response in murine lungs, nor does exposure to OVA typically cause asthma in humans. We utilized aluminum hydroxide (alum) as an adjuvant which helps process and present the antigen. Further studies could involve protease-containing cockroach egg and dust mite antigens, which do not require adjuvants and which also evoke a robust Th-2 response. Secondly our mode of delivery through intranasal treatments do not reflect a realistic exposure to airborne allergens which are typically inhaled in humans; thus our experimental regimen would be further enhanced by aerosolized delivery of the antigen and hence would demonstrate a more natural model of allergen sensitization and challenge. Finally we have employed a large dose of ovalbumin (1 mg/ml) in order to

sensitize and challenge the mice. Humans are exposed to allergens in order of micrograms—a far smaller dose than our OVA treatment. Finally, some patients with asthma demonstrate neutrophilic rather than eosinophilic inflammation (see below), suggesting the need for a non-allergic model of asthma. Future studies will entail optimizing our experimental regimen to meet these standards.

Strain limitation and translational research

The Balb/c strain of mice is particularly skewed towards a Th-2 driven response. Thus it is plausible that the changes we see after OVA treatment and RV infection are far more robust and physiologically relevant in these mice compared to other strains or even humans. However previous studies in C57BL/6 mice which are not predisposed to allergic inflammation, have demonstrated a role for Th-2 derived cytokines, including IL-13, IL-4, and eotaxin-1, and macrophages, in airway inflammation and lung fibrosis (40-42). Our pilot data of inflammatory responses in OVA/RV treated animals, which was obtained from C57BL/6 strain (data not shown), also revealed eosinophil infiltration and airway hyperresponsiveness to methacholine 4 days post infection compared to all other groups, although the extent of inflammation was lower in comparison to the Balb/c strain. Further, IL-4, IL-5, IL-13 and eotaxin have been detected in bronchial biopsy specimens from human asthmatics (43-46). RV infection of asthmatic subjects increases BAL eosinophils and airway hyperreactivity (15). In the clinical studies performed in our laboratory, nasal aspirates from asthmatic children showed a 6-fold increase in eotaxin-1 protein following respiratory infection (Toby Lewis, Marc Hershenson, unpublished data). Our *ex vivo* studies on BAL macrophages from asthmatic children also indicate

increased expression of IL-13 and human M2 markers stabilin (47), following RV infection compared to non-asthmatic RV stimulated cells (Dina Schneider, Marc Hershenson, unpublished data). Based on these results, we are confident that at least a subset of our observations from mice will be retained in human studies.

Finally while allergic asthma is characterized by the presence of Th-2 cytokines, a significant portion of asthma is not characterized by eosinophils but neutrophils, responds poorly to corticosteroids, and correlates with acute and sometimes even fatal asthma (48-51). We have not attempted to delineate the role of neutrophils in asthma and the precise mechanisms which may be involved. Furthermore, there is an emerging role of Th-17 cells which produce IL-17, and stimulate neutrophil infiltration, and exacerbate asthma (52).

OVA/RV-induced cytokine responses

We focused our experimental design on requirement of eosinophils, and the eosinophil-specific chemokine, eotaxin-1. However, there are other potential therapeutic targets. Any chemokine or cytokine implicated in the initial asthmatic response could play a role in viral-induced responses. Overexpression of IL-13 resulted in airway hyperreactivity (53). Therefore RV induced IL-13 may play a key role in bronchoconstriction and would hence be a therapeutic target in itself. In recent studies, IL-5 and eotaxin-2/ CCL24 have also been implicated in eosinophil accumulation and airway responses (54, 55). MIP-1α and IL-17 could also contribute to airway pathology (data not shown). RANTES, MIP-1α and MCP-1 neutralization in allergen sensitized and challenged animals show diminished airway hyperreactivity (56). When lung

homogenates from animals sensitized to schistosome egg allergens are preincubated with neutralizing antibodies to MIP-1α and RANTES *in vitro*, significant reductions in eosinophil chemotaxis are observed (57), thus suggesting that eosinophil accumulation and degranulation may be related to the actions of more CC chemokines than just eotaxins. IL-17 has been implicated in airway responses to methacholine in animals-sensitized and -challenged with OVA containing a low dose of LPS (58). Each of these cytokines could potentially provoke exacerbations in response to RV.

To address the contribution of other cytokines and chemokines to RV-induced airway inflammation and hyperresponsiveness, we have performed focused gene arrays on lungs from OVA/RV mice. Our preliminary studies indicate increases in MCP-1/CCL2, MCP-3/CCL7, MIP-3β/CCL19, GRO-α/CXCL1, MIP-3α/CCL20 and MIP-1β/CCL4 are increased in OVA/RV mice compared to OVA/sham mice. Future studies utilizing neutralizing antibodies or CCR antagonists could shed light on their role after allergen challenge and RV infection.

Macrophage profiles of naïve and OVA-treated mice, and potential contribution of dendritic cells

Along with the overall change in macrophage activation state observed after allergen sensitization and challenge, we also found that BAL macrophages from PBS- and OVA-sensitized mice underwent a phenotypic switch from CD11c ^{high}/CD11b ^{low} to a CD11c ^{low} /CD11b^{high} population, as detected via flow cytometric analysis. Consistent with this, asthmatic alveolar macrophages also have shown to express a high CD11b population compared to non-asthmatics (59, 60). On this basis, we could perform FACS

sorting of these CD11c ^{low} /CD11b^{high} cells from OVA mice and transfer them to naïve mice after RV infection and determine whether they are sufficient for IL-4, IL-13 and eotaxin-1 induction.

On the other hand it is conceivable that dendritic cell populations, key effector cells in asthma, also contribute to HRV responses in allergic mice. First, while clodronate treatment, a widely-used method for depleting macrophages (40, 61, 62), reduced eosinophilic inflammation and airways responsiveness, clodronate treatment may also affect certain dendritic cell populations (63). Additionally, though we identified our macrophage populations via CD68 labeling, CD68 is also expressed on certain subsets of dendritic cells (reviewed in (64)). Adoptive transfer experiments with macrophages (described above) using CD11b as a marker could lend confirmation to our findings, as dendritic cells express high levels of CD11c (Rudd BD, et al. Type I Interferon Regulates Respiratory Virus Infected Dendritic Cell Maturation and Cytokine Production. Viral Immunology 2007). Currently there is little information about dendritic cell involvement in response to RV infection. Eosinophils, when co-cultured with dendritic cells and lymphocytes, produced robost eosinophil peroxidase when infected with RV *in vitro* (65). Thymic stromal lymphopoetin (TSLP), a product of epithelial cells stimulates dendritic cells to induce Th-2 cytokine production and has been shown to be upregulated after RV 16 infection (66). On the other hand, HRV14 induces type-1 interferon production but fails to induces maturation of monocyte-derived dendritic cells (67). Thus there is a need to quantify and characterize dendritic cell populations after OVA/RV treatment in order to understand their relevance to RV induced asthma exacerbations.

Combined effects of viral infections and allergic inflammation: Effects of allergen sensitization on viral load, and potential deleterious effects of the interferon response on airway function

We have found that allergic inflammation qualitatively alters the response of lung macrophages to RV infection. But what about the effect of allergic inflammation on viral replication and clearance? We found a significant reduction in positive strand viral RNA in the allergen-sensitized and -challenged mice after RV infection, as well as increased interferon gamma production. Reduced viral load has been previously noted in parainfluenza-infected allergic guinea pigs {Adamko, 1999 #14326}. Further, macrophages from OVA mice expressed greater mRNA levels of IFN-γ after RV infection *ex vivo*. This seems to be in contrast to the current dogma of RV infection triggering inflammation and airway hyperreactivity possibly due to deficient interferon response patterns (15, 18, 19). Our results indicate that viral load and inflammatory mechanisms may not be directly coupled. In fact our data suggests that the presence of an allergic environment may confer an evolutionary advantage to the host which aids in alleviating viral binding or alternatively increases viral clearance possibly via increased interferon production. For example, eosinophils may have antiviral effects.

However, the production of interferons may also trigger the recruitment of other inflammatory cells to the airway which could reduce airflow. Pneumovirus-infected IFN-αβR null mice show fewer BAL leukocytes and prolonged survival despite increased virus titers {Garvey, 2005 #14974}. Dendritic cells from norovirus-infected MDA-5 null mice show reduced levels of IFN-α, IL-6, MCP-1 and TNF-α {McCartney, 2008 #14975}. Lungs from Sendai virus-infected MDA-5 null mice with reduced levels of

IFNs-α2, -β, -γ and λ3 also show reduced expression of TNF-α {Gitlin, 2010 #14977}. Together, these data suggest that the IFN response to viral infection is pro-, not anti-inflammatory. Indeed, our unpublished data from MDA5 -/- mice with reduced IFN levels show reduced airway neutrophils, C-X-C chemokines and airway responses to methacholine in response to RV infection (Q. Wang, M. Hershenson, unpublished data). Increased mucus production observed in the OVA mice and aggravated by RV infection (data not shown) may obstruct binding of virus to the ICAM or LDLR. Thus while the sustenance of the virus may be reduced in the allergic environment, the exacerbations of asthma may be more directly related to the inflammatory cascades in response to the virus rather than the viability of the virus in the lungs.

Physiological Relevance and Significance

RV accounts for the most virus induced asthma exacerbations (1), although the precise mechanisms are not well known. Much of the current understanding revolves around epithelial cell cytokine production after RV exposure (7-10). However, this paradigm fails to explain differences in the pathological role of RV in normal individuals and asthmatics (15). It is essential therefore to examine both the quantity as well as the quality of RV infection in naïve and asthmatic subjects in order to address potential therapeutic strategies. We have shown that RV infection of naïve mice causes CXCR2 mediated neutrophil infiltration and airway hyperresponsiveness. We have also shown that TNFR1 is required for RV induced airway neutrophils and responses to methacholine. Furthermore, we demonstrate that RV infection of allergen sensitized and challenged mice switches the immune reaction from a predominantly neutrophilic to a

predominantly Th-2 driven inflammatory response which is characterized by macrophage mediated production of eotaxin-1, recruitment of eosinophils, and airway hyperreactivity. In contrast to naïve mice, macrophages from OVA treated animals display a functionally polarized phenotype which is exacerbated by RV. Thus for the first time, we have shown that macrophage response to RV can be regulated by its cytokine milieu. Unlike normal individuals, a viral exposure may drive "Th-2 primed" macrophages from asthmatics to a pro-Th-2, pro-eosinophilic response which ultimately leads to bronchoconstriction. The above observations provide a novel paradigm to explain RV induced asthma and if held true in humans will in the future identify intervention techniques that could provide significant relief to asthmatics.

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