REGULATION OF MONOCYTIC CELL IMMUNE RESPONSES TO RHINOVIRUS

By

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ABSTRACT

One of the main causes of asthma and wheezing in both children and adults is respiratory infections by the common cold virus, rhinovirus (RV). RV is the most commonly found virus in the respiratory tract and has been detected in over 50% of asthma exacerbations. In addition to RV infections, allergies have long been recognized as another major risk factor for asthma. Recent studies have also shown that the lower airways are not sterile and that asthmatics have increased bacterial presence, as well as different microbiome composition compared to healthy individuals. Therefore, the overall aim of this thesis is to elucidate mechanisms by which these different environmental factors interact to influence airway immune responses and contribute to asthma exacerbations. While there are many immune cells in the airways, macrophages and cells of monocytic lineage are the most prevalent leukocyte in the lower airways and act as first responders during infections. Therefore, the focal point of this thesis is understanding how bacterial products and allergies alter the monocytic cell responsiveness to rhinovirus infections, specifically, in the secretion of inflammatory mediators.

Chapter Two explores the effect of bacterial product, lipopolysaccharide (LPS), on RV-induced signaling mechanisms in primary human monocytes and airway macrophages. The data presented in this chapter demonstrates that LPS can significantly diminish (~90%) RV-induced CXCL10 and CXCL11 secretion, while maintaining or promoting CCL2 and CCL8 secretion. Additionally, LPS can upregulate parts of the interferon α signaling pathway while diminishing its downstream effectors. Chapter Three tests the hypothesis that allergen exposure alters macrophage responsiveness to RV exposure. This chapter demonstrates that in vivo allergen challenge significantly alters primary human airway macrophage responsiveness to RV by diminishing CXCL10 and CXCL11 chemokine secretion, while maintaining CCL2 and CCL8
secretion. These findings also demonstrate that the effect of allergen is consistent among the
different RV types and is independent of viral target receptor surface expression. Together, the
data presented in this thesis demonstrate novel mechanisms by which environmental exposures,
such as bacteria and allergies, may alter RV-induced airway immune responses that could
thereby influence severity of illness and the risk of asthma exacerbations.
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CHAPTER ONE

Literature review of the role of rhinovirus, monocytic cells, bacteria, and allergic inflammation in asthma
Overview

Asthma is a chronic inflammatory disease of the airways and is a source of morbidity and mortality for both children and adults. The pathogenesis of asthma is multifactorial, and a variety of factors including viral infections and allergen exposure have been shown to exacerbate asthma. By investigating how individual risk factors influence asthma, mechanisms by which asthma is initiated may be elucidated. Additionally, understanding the underlying mechanisms of asthma may aid in the design of future drug targets and therapeutics. Asthma symptoms stem from airway inflammation, involving mononuclear cells, eosinophils, and neutrophils. The most prevalent immune cells in the lower airways are of monocytic lineage, and therefore the major focus of this work is to further examine the role of monocytic cells in asthma through the effects of environmental factors on monocytic cell immune responses to viral infections.

This introductory chapter begins with a description of rhinovirus (RV)-induced signaling in cells. Next, I will briefly describe the role of monocytes and macrophages in immune responses to stimuli and in asthma. This will be followed by a depiction of how monocyte-derived chemokines, specifically CXCL10, CXCL11, CCL2, and CCL8 contribute to airway inflammation. Then I will describe how environmental factors, such as bacteria and allergen exposure, influence airway inflammation and asthma.

Rhinoviruses

Rhinoviruses (RV) are the primary viral cause of the common cold. RV belongs to Picornaviridae and represents a group of small non-enveloped positive-strand RNA viruses and includes over 150 different genotypes.¹ There are currently three species (A, B, and C) into which all the RV types fit phylogenetically according to their RNA genome sequence. Viruses
from the RV-A and -B species can be differentially classified into two different groups (major and minor) based on the cellular host receptor to which they bind. The major group rhinoviruses (88 types) bind intercellular adhesion molecule 1 (ICAM-1), while the minor group (11 types) bind members of the low-density lipoprotein receptor (LDLR) family, which include LDLR, LDLR related protein-1 (LRP-1), megalin, apoprotein E receptor 2 (apoER2), and very low density lipoprotein receptor (VLDLR).\textsuperscript{2, 3} RV-C have only recently been discovered and the receptor target has yet to be identified. While there are many cytokines and chemokines that are induced by RV, the precise mechanisms by which RV induces inflammatory responses is not fully defined.\textsuperscript{4} The sections below describe several of the mechanisms by which RV can induce the transcription of key inflammatory mediators.

**Rhinovirus-induced toll-like receptor signaling**

Binding of RV to its respective receptor on the cell surface initiates internalization of the virus-receptor complex into the endosome. Once in the endosome, acidification of the endosome occurs, allowing for dissociation of the viral protein VP4 from the capsid and release of viral RNA into the cell. The viral RNA is then translated, replicated, and progeny are released through lysis of the cell.\textsuperscript{5-7} Respiratory symptoms arise from both the direct destruction of normal airway epithelium and the host pro-inflammatory response to the virus.

Once RV RNA is released in the endosome, viral RNA is detected by toll-like receptors (TLR).\textsuperscript{6} TLRs play an important role in the innate immune responses as they recognize specific molecular patterns found in bacteria and viruses and initiate inflammatory and immune responses.\textsuperscript{8} TLRs 7/8 and 3 in particular recognize single stranded RNA and double stranded RNA, respectively. TLR 7/8 and 3 can therefore recognize rhinovirus RNA in the endosome and
activate transcription factors, such as activating protein-1 (AP-1) and nuclear factor κB (NF-κB), for the induction of inflammatory cytokines and chemokines. The mechanisms by which the TLRs activate transcription factors differ slightly and are illustrated in Figure 1-1. TLR 7/8 signal through the central adapter protein myeloid differentiation primary response protein 88 (MyD88)-dependent pathway, which activates transforming growth factor-β-activated protein kinase 1 (TAK1) via tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6). TAK1 is a member of the mitogen-activated protein kinase family (MAPK) and in turn activates downstream MAPKs to initiate transcription through AP-1. TAK1 also promotes translocation of NF-κB to the nucleus through activation of inhibitor of NF-κB kinase (IKK) complex. In addition, TLRs can activate interferon regulatory factors (IRFs), specifically IRFs 3 and 7, that combined with NF-κB promote the transcription of type I interferons (IFN), such as IFNα and IFNβ. TLR7/8 activates IRF7 through the association of IRF7 with MyD88, interleukin-1 receptor-associated kinase 1 (IRAK1), and TRAF6, which results in IRF7 phosphorylation by IRAK1. On the other hand, TLR3 initiates a MyD88-independent pathway through TRIF (TIR-domain-containing adapter-inducing interferon-β), which activates TAK1 through TRAF 6 and subsequently AP-1 and NF-κB. In addition, the TRIF-dependent pathway initiates, through TRAF3, TBK1 (TANK-binding protein 1) and IKKi phosphorylation of IRF3. Additionally, association of the virus with TLR3 can upregulate intracellular RNA helicases, such as (retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated protein 5 (MDA-5), which are pattern recognition receptors. These receptors can then recognize viral RNA in the cytoplasm and stimulate RV-induced IFN and cytokine production. Thus, through the recognition of viral RNA in the endosome by TLRs and the activation of subsequent signaling cascades, the host cell initiates anti-viral responses to the virus.
FIGURE 1-1

Lipid raft
DNA

TLR 3
Endosome
22000
RV/RNA

TLR 7/8
MyD88

TRIF
TRIF
TRAM

TRAF3
TRAF6

IRF3
NF-κB
MAPKs

IRF7

Nucleus

Type I IFNs
Inflammatory cytokines & chemokines
FIGURE 1-1.

RV- and LPS-induced signaling through TLRs in monocytic cells. RV viral RNA is detected in the endosome by TLR 7/8 and 3. TLR 7/8, via the MyD88 adaptor protein, and TLR 3 via the adaptor protein TRIF, lead to subsequent activation of TRAF6. TRAF6 can then activate both the MAPK and NF-κB pathways leading to the transcription of a variety of inflammatory cytokines and chemokines. TLR7/8 also activate IRF7, while TLR3 activates IRF3 via TRAF3, and these IRFs, along with NF-κB, are important transcription factors for type I IFNs. LPS is detected by LBP and anchored to the cell membrane by CD14, which allows for LPS to interact with the TLR4-MD-2 complex. Similar to RV signaling, LPS can activate NF-κB and MAPK pathways via both the MyD88-TRAF6 and the TRAM-TRIF-TRAF6 dependent pathway. LPS can also induce activation of IRF3 through the TRAM-TRIF-TRAF3 pathway. Through these pathways LPS can induce the transcription of inflammatory mediators, including type I IFNs. Details of LPS signaling are described below (page 17). IFN, interferon; IRF, interferon regulatory factor; LBP, LPS-binding protein; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation primary response protein 88; NF-κB, nuclear factor κB; RV, rhinovirus; TLR, toll-like receptor; TRAF, tumor necrosis factor receptor (TNFR)-associated factor; TRAM, TRIF-related adaptor molecule; TRIF, TIR-domain-containing adapter-inducing interferon-β.
Rhinovirus-induced signaling and induction of interferon

While rhinovirus activation of TLRs and helicases can directly promote the transcription of inflammatory cytokines and type I IFNs, the autocrine effect of type I IFN secretion on the cell can induce additional inflammatory cytokine and chemokine release.\textsuperscript{11, 12} IFNs are classified into three subgroups, type I, II, and III, based on differences in sequence, receptors and producing cells, yet all IFNs can induce similar downstream effectors.\textsuperscript{11} A recent study showed that IFNα is the primary interferon produced by peripheral blood mononuclear cells in response to RV, followed by IFNβ and IFNλ, respectively.\textsuperscript{13}

Type I IFNs, which include IFNα and IFNβ, are key effectors in host immunity to viral infections through their ability to interfere with viral replication and protect surrounding uninfected cells.\textsuperscript{14, 15} The type I IFN receptor is made up of two subunits, IFNAR1 and IFNAR2, and is commonly found on the cell surface of multiple cell types, such as monocytes, macrophages, dendritic cells, epithelial cells, natural killer (NK) cells, and B lymphocytes.\textsuperscript{14, 16} Each of the subunits associate with the members of the Janus activated kinase (JAK) family: JAK1 and tyrosine kinase 2 (TYK2).\textsuperscript{17} Through the binding of IFNs to the receptors and dimerization of the subunits, the JAKs are activated through phosphorylation and in turn phosphorylate signal transducer and activator of transcription (STAT) proteins. This signaling cascade occurs rapidly and results in the translocation of activated STAT complexes to the nucleus that bind IFN-stimulated response elements (ISREs) or IFNγ-activated site (GAS) elements to initiate transcription. IFNs can induce the transcription of a variety of genes involved in anti-viral/anti-bacterial, pro-inflammatory, and apoptotic responses.\textsuperscript{15} Specifically, our lab has found that RV A016-induced secretion of the chemokine CXCL10 from primary blood
monocytes is significantly impaired after neutralization of IFNAR, which further demonstrates a role for type I IFNs in RV-induced chemokine secretion \textit{ex vivo}.^{18}

\paragraph*{Rhinoviruses and asthma}

Viral infections are strongly associated with asthma and wheezing in both adults and children.\textsuperscript{4} RV infections specifically are the major (> 50 \%) cause of exacerbations of asthma in both children and adults.\textsuperscript{2,19} In addition, RV is the most commonly isolated virus in the respiratory tract.\textsuperscript{4,20-22} Thus, RV infections play a major role in asthma pathogenesis and exacerbation.\textsuperscript{20,23,24} It is thought that recurrent lower respiratory infections during early childhood/infancy can promote asthma through changes in airway structure, as viral infections can induce factors that modulate airway development, repair, and growth [\textit{e.g.}, interleukin 6 (IL-6), and transforming growth factor \(\beta\) (TGF\(\beta\)]\textsuperscript{3,25}. In addition, it has been shown through birth cohort studies that development of asthma at age 6 years is strongly associated with RV-induced wheezing illnesses during the first three years of life.\textsuperscript{25} Asthmatics do not tend to have increased numbers of RV infections, yet studies have shown that they demonstrate increased duration and severity of the RV-induced symptoms in the lower airways.\textsuperscript{26,27} Interestingly, these differences are not found in the upper airways, implicating the lower airways in asthma-related differences during RV infections. Recently, studies have shown that the rhinoviruses differ in virulence and illness severity depending on the strain. Specifically, it has been shown that RV-C and RV-A are more virulent than RV-B,\textsuperscript{28} demonstrating that viral factors contribute to the severity of RV infections. Further investigation of airway immune responses to RV infections will help elucidate the mechanism by which asthma is exacerbated by RV.
Monocytic cells

Monocytic cells include a number of cell types, as circulating blood monocytes are precursor cells that can differentiate into different cell types depending on the environment and tissue.\textsuperscript{29} In particular, monocytes circulate in the blood and once they enter the airways can mature into alveolar macrophages. Other tissue monocytic cells include microglial cells (brain), Langerhans cells (skin and mucosa), osteoclasts (bone), and Kupffer cells (liver).\textsuperscript{29-31} The goal of the studies in the following chapters is to further understand the role of monocytes and alveolar macrophages in airway immunity during RV exposure. These cell types have an important role in the host defense against pathogens and surveillance, as they express a large variety of pathogen-recognition receptors and scavenger receptors.\textsuperscript{29, 31} They are considered the first responders to the site of infections and are responsible for phagocytosis and digestion of debris and infectious agents, wound healing, and remodeling.\textsuperscript{30, 32, 33} In addition, monocytic cells are important in the secretion of inflammatory mediators, such as cytokines and chemokines, that play an important role in the recruitment and activation of other immune cells, as well as additional monocytic cells, to the site of tissue damage.\textsuperscript{32, 33} Circulating monocytes comprise approximately 10\% of the leukocytes in the blood. In contrast, alveolar macrophages are the primary immune cell in the lower airways and comprise over 90\% of the immune cells in the resting airway,\textsuperscript{34, 35} thus making them a major player during innate airway anti-viral responses and airway inflammation.

\textit{Monocytic cell involvement in asthma}

There is an increasing appreciation for the role of monocytes and macrophages in regulation of airway inflammation in asthma.\textsuperscript{32, 36-38} Several differences have been observed
between healthy and asthmatic alveolar macrophage function, including lowered phagocytic
function and greater antigen presentation activity in asthmatic macrophages. A study
comparing gene expression profiles of asthmatic alveolar macrophages to healthy macrophages
demonstrated that 38% of the differentially expressed genes are part of the immune and stress
responses, indicating an inherit underlying component to altered asthmatic macrophage
function. In addition, it has been shown that asthmatic macrophages secrete less interleukin-10
(IL-10), which has an inhibitory role in inflammation, but increased inflammatory cytokines,
such as IL-8 and IL-6, thus suggesting a reduced inhibitory function and increased inflammatory
function in asthmatic responses.

Interestingly, altered macrophage function can affect the activity of other cell types.
Specifically, there is evidence that changes in macrophage subpopulations due to inflammation
may influence T cell responses during inflammation. Additionally, supernatants from
peripheral blood mononuclear cells from atopic individuals, compared to nonatopic individuals,
increased eosinophil viability in vitro, a main characteristic of asthma. Altogether, these
studies highlight the importance of macrophage dysfunction in asthma and in the regulation of
overall airway immune and inflammatory responses, and indicate that additional information is
needed to understand how the airway environment and stimuli influence macrophage immune
responses.

Models for studying rhinovirus effects on asthma

While the development of small-animal models of asthma and RV infections is important
and useful in understanding the pathogenesis of asthma and viral infections, several limitations
exist in the use of murine models for RV-induced asthma exacerbations. The first limitation is
that major group RV does not bind mouse ICAM-1, which renders mice insusceptible to major
group RV infections. This limitation has been partially overcome by either infecting mice with
minor group RV, which represents less than 10% the total RV types known, or by creating
transgenic mice that express either human ICAM-1 or a mouse-human ICAM-1 chimera.\textsuperscript{47,48}
Another limitation is that mice lack the development of key asthma characteristics that are
commonly observed in humans.\textsuperscript{47,49} In addition, mouse models often inadequately compare to
humans in acute inflammatory genomic responses.\textsuperscript{50} However, in attempt to closely mimic
human allergic inflammation, ovalbumin is commonly used to sensitize mouse airways.\textsuperscript{6,49} Due
to these limitations and the inherit differences between murine and human models, there is a vital
role for human-based studies in RV-induced asthma to validate findings from murine models.

While utilizing human cell lines can be useful, transformed cell lines can often lose some
differentiated functions, as in the case when comparing THP-1 cells (human monocytic cell line)
with primary monocytes and macrophages.\textsuperscript{51} This inability of even human cell lines to fully
mimic primary monocytic cell behavior highlights the value in utilizing primary human cells.
Besides the apparent advantages to utilizing primary human airway cells, an important advantage
is the ability to conduct both \textit{in vivo} and \textit{ex vivo} studies using RV inoculation and allergen
challenge. Acquiring primary human airway macrophages requires a process known as a
bronchoalveolar lavage (BAL). This, medical procedure, performed by a physician, requires
careful regulation, as it involves human subjects, and is moderately invasive and expensive.
Therefore, BAL cells are quite precious and are highly limited. Using precursor cells for airway
macrophages, such as primary human blood monocytic cells, is highly relevant, feasible, and less
expensive and limited than using BAL cells.
Despite potential inherit differences in responses to stimuli between blood monocytes and airway macrophages, primary human blood monocytes serve as an appropriate model system to study changes in airway macrophage immune responses to viral infections. This has been demonstrated previously by our lab, as well as by several other groups.\textsuperscript{18, 52, 53} In addition, monocytes have previously been considered precursors for other tissue macrophages, yet as the research in the field moves forward we see that monocytes have specific functions during inflammation as well.\textsuperscript{29} Therefore, the following studies in the subsequent chapters were all performed on primary human peripheral blood monocytes and/or primary human BAL macrophages.

*Monocytic cell interactions with rhinovirus*

Monocytic cells respond to rhinoviruses during the early stages of infection. Both monocytic cells and epithelial cells are able to respond to major and minor group RV, indicating that the respective receptors are present on the surface of these cell types.\textsuperscript{4, 54} RV replicates primarily in epithelial cells, and little to no RV replication occurs in monocytic cells. Production of inflammatory mediators has been studied extensively in epithelial cells, but less is known about effects of RV on monocyte activation and function.\textsuperscript{52}

Our lab has previously shown that monocytic cells not only respond to RV exposure but that they can direct epithelial cell responses to RV.\textsuperscript{18, 55, 56} Specifically, we have shown that when examining epithelial-monocytic cell co-cultures, monocytic cells account for 84% of the variance in RV-induced CXCL10 secretion.\textsuperscript{56} This is in agreement with another study indicating that the monocytic cell source can influence epithelial cell secretion of cytokines and chemokines in response to RV.\textsuperscript{57} Additionally, we have demonstrated that the transfer of media
from RV-stimulated monocytic cells to epithelial cells results in a significantly robust secretion of CXCL10, suggesting that monocytic cells can secrete factors that influence epithelial cell responses.\textsuperscript{56} More recently, a study by Bentley and colleagues demonstrated that staining of lower airway biopsies from human subjects experimentally infected with RV confirm colocalization of the airway monocytes/macrophages with RV.\textsuperscript{58} These findings provide evidence that the airway monocytic cells interact with RV and contribute to anti-viral responses \textit{in vivo}, and suggest that altered monocytic cell responses to RV may influence general airway anti-viral responses leading to asthma exacerbations.

**Chemokines and asthma**

Asthma is a disease of chronic airway inflammation. The immune response during viral infections is regulated by the secretion of chemokines, which play an integral role in intercellular communication and in the migration of other immune cells. Therefore understanding how different airway environments cause differences in chemokine expression is essential to understanding asthma exacerbations. The increasing appreciation for a role of chemokines in asthma pathogenesis has prompted the present study of four specific chemokines that represent both the CXCL and CCL families of chemokines.\textsuperscript{59-62}

**Chemokine characterization**

Chemokines are small 8-10 kD proteins that are secreted from cells in order to attract leukocytes from the blood stream to the tissues. Chemokines are subdivided into groups based on the positioning of the first two cysteine residues in the mature protein. Of the four subfamilies known, the two most well characterized are the $\alpha$-chemokines, in which the cysteine residues are
separated by a single amino acid (CXC), and the β-chemokines, in which the cysteine residues are adjacent to one another (CC). These secreted molecules act as chemoattractants via binding to specific G-protein coupled receptors on the surface of the leukocytes. These receptors are also characterized into subfamilies according to the subfamily of chemokines in which they interact. Therefore, the CXC ligands (CXCL) bind to the family of CXC receptors (CXCR), while the CC ligands (CCL) bind the CC receptors (CCR). 63

The binding of the chemokine to its receptor leads to a series of signaling cascades, involving specifically the Rho proteins, which play a role in actin-dependent processes resulting in cell motility. 64 Specificity of chemotaxis is therefore influenced by the particular chemokine released and the expression of the respective receptor present on the surface of the immune cells. This provides directional cues locally and systemically for leukocytes in normal development and homeostasis, as well as in inflammation.

**CXCL10, CXCL11, CCL2, and CCL8**

This thesis focuses on four specific chemokines that represent both the CXCL and CCL families of chemokines. Among the CXCL family of chemokines are two closely related proteins CXCL10, also known as IFN-γ-inducible protein 10 (IP-10), and CXCL11, also known as IFN-inducible T cell α-chemoattractant (ITAC). Both of these chemokines primarily bind the CXCR3 receptor. 65 CXCR3 is highly expressed on IL-12 activated T cells, but is not expressed on resting B and T cells, monocytes, nor is it expressed on granulocytes. 63 Therefore CXCR3 expression is suggested to be important in the selective recruitment of T cells. CXCL10 acts as a chemokine for T cells and natural killer cells, but is of particular interest due to its strong association with
asthma and viral-induced exacerbation of asthma.$^{62,66}$ Finally, CXCL11 is suggested to be a T cell and dendritic cell chemotactant.$^{67,68}$

The larger group of chemokines, the CCL family, contains a subgroup of structurally related proteins known as the monocyte chemoattractant proteins (MCP) which play an important role in allergic inflammation.$^{64}$ This group of chemokines regulate the chemotaxis of monocytes through binding the CCR2 receptor and a subset of MCPs induce the chemotaxis of eosinophils through CCR3 binding.$^{65}$ The main stimuli for the secretion of these chemokines are pro-inflammatory cytokines (e.g. TNF-α), bacterial products (e.g. LPS), and viral infections.$^{69}$ CCL2, also known as MCP-1, binds only CCR2 and therefore is mainly a chemokine for monocytes, but can also attract dendritic cells and activated T cells. CCL2 has been found to be elevated in asthmatic airways and is thought to contribute to the regulation of airway inflammation and the pathogenesis of asthma.$^{55,70-72}$ Specifically, RV-induced CCL2 from BAL macrophages of asthmatic children is significantly increased compared with non-asthmatic children, which suggests a role for CCL2 in viral-induced asthma exacerbations.$^{73}$ CCL8 (MCP-2) binds CCR1, CCR2, CCR3, and CCR5, and recruits several cell types, including monocytes, eosinophils, basophils and activated T cells.$^{67,74}$ While these cells are all involved in allergic inflammation, this suggests a role for CCL8 in eosinophil airway infiltration in asthma.$^{61}$

Several studies have shown that CCL2, CCL8, and CXCL10 levels are increased in BAL samples of asthmatic patients, yet the cellular source of these chemokines remains unclear.$^{61,75-78}$ RV-elicited chemokine production has been extensively studied in epithelial cells, but there is less information about RV-induced chemokine responses in monocytes and macrophages. Epithelial cells have been shown to induce the secretion of CXCL10, CXCL11, and CCL2 in response to RV infections,$^{4}$ however whether RV induces CCL8 in epithelial cells remains
controversial. On the other hand, RV-induced CXCL11 and CCL8 expression and secretion from monocytic cells had yet to be determined prior to this thesis. Subsequent chapters demonstrate that both blood monocytes and airway macrophages secrete CXCL11 and CCL8 in response to RV exposure, which suggests that monocytic cells may contribute to the CCL8 levels in the airways.

The specific roles for the aforementioned chemokines during virus-induced exacerbations of asthma have yet to be identified. During natural colds, nasal secretions contain increased CCL2 and CXCL10 levels and a natural cold study in children found that CCL2, but not CXCL10, levels were positively related to symptom scores. The authors suggested that chemokines such as CCL2 contribute to acute viral-induced asthmatic response, while CXCL10 may set the stage for protective future immune responses. These findings highlight the potential role for CXCL10 and CXCL11 in viral immunity and for CCL2 and CCL8 in viral-induced airway hyperresponsiveness.

**Bacterial colonization and asthma**

The lower airways are not sterile, and it has been shown that the composition of the airway microbiome is important for maintaining health, but can also contribute to different disease states, such as asthma, cystic fibrosis, and chronic obstructive pulmonary disease (COPD). The majority (> 70%) of bacterial species in/on the body cannot be cultured using standard laboratory techniques. Sequencing of bacterial 16S ribosomal RNA (rRNA) genes has led to increased understanding of the microflora that populate tissues, and has provided insights into how microbiome composition may contribute to different disease states.
There is evidence that airway bacteria affect airway inflammation and function. Bacteria can activate innate immune responses through their detection by pathogen-recognition receptors on the surface of immune cells, such as monocytes and macrophages.\textsuperscript{85} There is growing evidence that the presence of atypical bacteria is associated with increased airway inflammation and potentially increased asthma severity.\textsuperscript{85} Recent studies have identified several atypical bacterial species that may either be actively contributing to asthma pathogenesis or may be prognostic of asthma.\textsuperscript{81}

**Lipopolysaccharide structure and signaling**

One of the major immunogenic components of Gram-negative bacteria is lipopolysaccharide (LPS), which is a component of their cell walls, and is ubiquitously found in the environment.\textsuperscript{86} In early life, exposure to endotoxin may be associated with decreased risk for atopic asthma.\textsuperscript{87} One the other hand, high-level exposure to endotoxin once asthma is established can increase airway inflammation, provoke symptoms of asthma,\textsuperscript{86} and adversely affect pulmonary function.\textsuperscript{88, 89} Effects of endotoxin on asthma may be dependent on the timing and dose of exposure, and host genetics.\textsuperscript{82, 86, 90}

LPS is made up of a specific O-chain, a core oligosaccharide, and a lipid component.\textsuperscript{91} Most of the adaptive immune response to LPS is due to the O-chains, which are structurally heterogeneous across bacterial species, however the lipid component is sufficient in activating the innate immune response and also varies within bacterial species.\textsuperscript{53, 92} LPS binds to the plasma protein LPS-binding protein (LBP), which increases LPS sensitivity and aids in the binding of LPS to CD14, which anchors LPS to the cell membrane. CD14 is commonly expressed on monocytes and macrophages in the membrane bound form, known as mCD14, but can also be
found in the a free soluble form in the plasma, known as sCD14. mCD14 lacks a transmembrane domain and therefore binding of LPS to CD14 does not induce an intracellular signaling cascade. Instead, LPS once anchored to the membrane can interact with toll-like receptor 4 (TLR4) and the accessory protein MD-2, which induces the activation of monocytes and macrophages, and the TLR4-MD-2 complex serves as the receptor for LPS signaling. As mentioned above, TLRs utilize similar downstream effectors. Therefore, LPS and RV signaling via TLRs share many commonalities (Figure 1-1).

LPS signaling has been extensively studied, and LPS can induce several intracellular signaling cascades, namely MAPK and NF-κB pathways, which lead to the transcription of many LPS-induced genes, including cytokines, chemokines, receptors, and other transcription factors in both monocytes and macrophages. Specifically, LPS signaling can occur through a MyD88-dependent or a MyD88-independent pathway, which utilizes both the adaptor proteins TRIF and TRIF-related adaptor molecule (TRAM). Through the MyD88-dependent pathway LPS activates p38, c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and NF-κB. Alternatively, through the MyD88-independent pathway LPS can also activate MAPK and NF-κB pathways, and can also activate IRF 3 and lead to the transcription IRF-dependent genes, namely type I IFNs (e.g., IFNβ). In addition, LPS can induce a variety of secondary indirect transcriptional responses through the secretion of cytokines, such as IFNβ and IL-10, in monocytes and macrophages. Therefore, the intracellular signaling events that occur after detection of LPS by monocytic cells encompass a vast number of cellular processes.

Clinical support for the association of airway microbiome with asthma

It has long been recognized that infections with Mycoplasma pneumoniae and
Chlamydophila pneumoniae can affect asthma. More recently, in a longitudinal study following infants to 5 years of age, Bisgaard and colleagues found that colonization of asymptomatic neonate airways with Streptococcus pneumoniae, Haemophilus influenzae, and/or Moraxella catarrhalis, but not Staphylococcus aureus, at one month of age is associated with increased risk of first wheezy episode, persistent wheeze, and hospitalization due to wheeze. In addition, they demonstrated that colonization of the airways by these atypical bacteria is associated with early asthma biomarkers such as increased blood eosinophil counts and total IgE, increased reversibility of airway resistance, and development of asthma by 5 years of age.

A study by Hilty and colleagues examined differences in microbiome composition between children and adult asthmatics and healthy controls. This study found that the major bacterial colonies in healthy airways are anaerobes from the phylum Bacteroidetes, particularly Prevotella and Veillonella, which have been suggested to play a role in the direct inhibition of other bacterial growth and yet their prevalence is diminished in asthmatics. On the other hand, they observed in asthmatics an increase in prevalence of the phylum Proteobacteria, which contains important potential pathogens, such as Haemophilus, Moraxella, and Neisseria. These studies indicate that asthmatic airways and healthy airways differ in airway bacterial colonization and that asthmatics may have increased bacterial pathogens.

Other studies have expanded upon these observations. Huang and colleagues demonstrated that asthmatics have greater bacterial burden and diversity in the airways compared to healthy individuals. In agreement with other studies, they showed an increase in Proteobacteria in asthmatic airways, and that the asthmatic microbiome composition correlated with bacterial burden and the degree of airway hyperresponsiveness. Zhang and colleagues showed that the bacterial colonization of the airways of severe asthmatics is linked to increased
duration of asthma and greater number of exacerbations in the previous year.\textsuperscript{100} These findings indicate that the presence of specific bacterial species, and bacterial community composition as a whole, are important in asthma.

\textit{Bacteria-rhinovirus interactions}

One mechanism by which bacteria may influence airway immune responses is by modulating airway responses to viral infections, including infections with rhinovirus. Bacterial products, particularly endotoxin, can increase epithelial cell expression of ICAM-1, which may increase susceptibility of the epithelial cells to major group rhinovirus infections.\textsuperscript{86} Despite these findings, there is relatively little information about how bacteria and viruses interact with regards to cellular signaling in asthma. One study by Oliver and colleagues demonstrated that RV can alter LPS-induced cytokine secretion from BAL macrophages and impaired the ability of the macrophages to phagocytose the bacterial particles.\textsuperscript{101} In addition, Contoli and colleagues demonstrated that LPS induction of IFN\(\lambda\) from BAL macrophages isolated from asthmatics was diminished compared to healthy controls.\textsuperscript{102} When considered together, these findings provide evidence that the composition of the airway microbiome influences airway immune responses to rhinovirus infections and plays a significant role in asthma exacerbations.

\textbf{Allergic inflammation in asthma}

Allergen sensitization (atopy) is considered a primary risk factor for development of asthma in childhood.\textsuperscript{103} Therefore, it is not surprising that among children and adult asthmatics, approximately 60-80 \% are sensitized to at least one allergen.\textsuperscript{104} The allergic inflammatory response is characterized by an induction of the T\(_{\text{H}2}\)-cell pathway. This pathway is initiated by
the uptake and presentation of allergens by antigen presenting cells, which then leads to the activation of leukocytes and the secretion of inflammatory cytokines.\textsuperscript{105, 106} Particularly, IL-4 and IL-13 secretion play a role in the synthesis of IgE, and granulocyte-macrophage colony-stimulating factor (GM-CSF) family members are important in eosinophil maturation and survival.\textsuperscript{105} The activation of the leukocytes, combined with the recruitment of additional leukocytes such as eosinophils and mast cells, leads to the secretion of histamine, leukotrienes, and prostaglandins mediate allergic inflammation. Additionally, the release of these inflammatory mediators and other growth factors can lead to bronchoconstriction, mucous metaplasia and airway remodeling, which are all common features of asthma.\textsuperscript{105, 107}

\textit{Rhinovirus-allergen interactions}

Both RV infections and allergen sensitization in the first year of age independently increase the risk of asthma.\textsuperscript{25, 108} In addition, it has been shown that children sensitized to allergens in their first year of life are predisposed to RV-induced wheezing, suggesting a sequential relationship between atopy and viral-induced asthma.\textsuperscript{109} More notably, the combination of allergen sensitization and viral infections, particularly by RV, synergistically increase the risk of asthma, and worsen symptoms in patients with established asthma.\textsuperscript{110, 111} Olenec and colleagues demonstrated that rhinovirus infections induce more severe asthma symptoms in children, and children with allergic asthma experience longer duration of illness and increased severity of both cold and asthma symptoms.\textsuperscript{112} Additionally, the combination of allergen sensitization, allergen exposure, and viral infections is strongly associated with exacerbation of asthma leading to hospitalization in adults.\textsuperscript{113} In children, the combination of allergen exposure and viral infection substantially increases the risk of hospitalization due to
asthma. Together, these studies demonstrate a synergistic effect on asthma during allergen exposure and viral infections.

Despite the mounting support for allergen-rhinovirus interaction in asthma, the underlying mechanisms that contribute to this relationship still remain unclear. Allergic asthma is associated with decreased RV-induced IFN production, suggesting that allergic asthmatics have impaired innate immune responses. There is also evidence to support that atopy, regardless of asthma status, decreases airway epithelial anti-viral responses to RV infections and that circulating IgE levels are inversely correlated to IFN production, suggesting that atopy increases susceptibility to RV infections. Furthermore, asthma and atopy can be associated with damage to the bronchial epithelium. Since the bronchial epithelium acts as a barrier to prevent the entry of allergens, viruses, and other airborne particles into the airway tissue, the impairment of this barrier in asthma could increase susceptibility of the airways to viral infections and effects of allergen exposure. Therefore, allergic sensitization and rhinovirus infections can interact on multiple levels to alter airway inflammation. Further elucidation of these synergistic effects of atopy and RV infections on asthma will aid in the discovery of new therapeutic targets for asthma.

Summary of introduction, hypothesis and outline of dissertation

Rhinoviruses, which cause colds in normal individuals, are closely related to wheezing and exacerbations in patients with asthma. This suggests that host immune response to rhinovirus is altered in asthmatics. Allergic inflammation and viral infections promote asthma exacerbations. Recent observations suggest that there are differences in bacterial colonization between healthy and asthmatic individuals, and that bacteria and allergen exposure may alter
airway immune responses to rhinovirus infections in patients with asthma. Considering the integral role of monocytic cells in innate immune responses, there is reason to believe that these cells are important responders to both bacteria and allergen exposure. These findings suggest the two main hypotheses for this thesis: 1) bacterial products, specifically LPS, alter RV-induced signaling in monocytic cells; 2) allergic inflammation modifies BAL macrophage chemokine responses to RV exposure.

The experiments presented in Chapter Two address the first hypothesis by determining the effects of LPS exposure on RV-induced chemokine secretion from primary human airway macrophages and blood monocytes. LPS selectively attenuates RV-induced CXCL10 and CXCL11 secretion, while promoting or maintaining RV-induced CCL2 and CCL8 secretion from both blood monocytes and airway macrophages. Additionally, a possible mechanism is demonstrated for LPS attenuation of RV-induced chemokine secretion, which may be due in part to LPS inhibition of RV-induced JAK/STAT1 signaling downstream of IFNα release. Chapter Three addresses the second hypothesis by investigating the effects of allergen challenge on airway macrophage immune responses to RV stimulation. The findings in this chapter demonstrate that in vivo allergen challenge can robustly alter RV-induced chemokine secretion from primary human airway macrophages, independent of its effects on RV target receptor expression. Together, these interactions provide novel mechanisms by which increased airway bacteria or allergic sensitization in the airways may modify airway anti-viral responses and promote more severe lower airway illness in asthma.
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CHAPTER TWO

Lipopolysaccharide modulates rhinovirus-induced chemokine secretion in monocytes and macrophages

Adapted from

ABSTRACT

Recent studies suggest that both bacteria and rhinoviruses (RV) contribute to asthma exacerbations. We hypothesized that bacteria might alter antiviral responses early in the course of infection by modifying monocyte-lineage chemokine responses to RV infection. To test this hypothesis, human blood monocytes or bronchoalveolar lavage (BAL) macrophages were treated with RV types A016, B014, A001, and/or A002 in the presence or absence of lipopolysaccharide (LPS), and secretion of chemokines (CXCL10, CXCL11, CCL2, and CCL8) and IFNα was measured by ELISA. Treatment with RV alone induced blood monocytes and BAL macrophages to secrete CXCL10, CXCL11, CCL2, and CCL8. Pretreatment with LPS treatment significantly attenuated RV-induced CXCL10, CXCL11, CCL8 secretion by 68-99.9 % on average (p < 0.0001, p < 0.004, and p < 0.002, respectively), but did not inhibit RV-induced CCL2 from blood monocytes. Similarly, LPS inhibited RV-induced CXCL10 and CXCL11 secretion by > 88 % on average from BAL macrophages (p < 0.002 and p < 0.0001, respectively). Furthermore, LPS inhibited RV-induced STAT1 phosphorylation (p < 0.05) as determined by immunoblotting, yet augmented RV-induced IFNα secretion (p < 0.05) and did not diminish expression of RV target receptors as measured by flow cytometry. In summary, major and minor group RV strongly induce chemokine expression and IFNα from monocytic cells. The bacterial product, LPS, specifically inhibits monocyte and macrophage secretion of RV-induced CXCL10 and CXCL11, but not other highly-induced chemokines or IFNα. These effects suggest that airway bacteria could modulate the pattern of virus-induced cell recruitment and inflammation in the airways.
CLINICAL IMPACT

Viral infections and more recently bacterial colonization of the airways have been shown to play a role in asthma exacerbations. We show that bacterial product, LPS, alters rhinovirus-induced secretion of chemokines from monocytic cells. The presence of bacterial products in the airway could therefore alter host immune responses to respiratory viruses such as rhinovirus.

ABBREVIATIONS

BAL: bronchoalveolar lavage
ICAM-1: intercellular adhesion molecule-1
IFNα: interferon α
LDLR: low density lipoprotein receptor
LPS: lipopolysaccharide
MAPK: mitogen activated protein kinase
MOI: multiplicity of infection
RV: rhinovirus
STAT1: signal transducer and activator of transcription 1
INTRODUCTION

Asthma prevalence has steadily increased globally, making acute asthma exacerbations a major cause of morbidity that entail substantial healthcare costs worldwide.\(^1\),\(^2\) Resident airway cells of the lower respiratory tract are consistently in contact with the external environment and therefore it is not surprising that the airways are not sterile.\(^3\) Interestingly, recent studies have detected asthma-related differences in microbiome composition of the lungs, raising the possibility that they may alter airway function.\(^4\),\(^5\) For example, there is an association between Proteobacteria (Gram-negative bacteria) in the airways and the degree of bronchial hyperresponsiveness in subjects with asthma.\(^4\) In addition, a recent study has shown that viral and bacterial co-infections resulted in an increased risk of hospital readmission after hospitalization for acute asthma exacerbations.\(^6\) In young children, detection of respiratory viruses or common bacterial pathogens (\textit{S. pneumoniae}, \textit{H. influenza}, \textit{M. catarrhalis}) are associated with acute wheezing episodes.\(^7\)-\(^9\)

Asthma is often exacerbated by respiratory infections.\(^10\),\(^11\) Viral respiratory infections, most commonly caused by rhinoviruses (RVs), contribute to 50-85 \% of asthma exacerbations.\(^10\),\(^12\) RV consists of over 150 different types, classified into three species (A, B, and C) according to their RNA genome sequence.\(^13\),\(^14\) RV from the A and B species can be separated into two different groups (major and minor) based on the cellular host receptor to which they bind. Major group RV bind intercellular adhesion molecule 1 (ICAM-1), while minor group RV bind members of the low-density lipoprotein receptor (LDLR) family.\(^13\) RV-C types have only recently been discovered and the receptor target has yet to be identified.\(^15\)-\(^17\)

While there are many immune cells in the lower airway, 80-90 \% are resident alveolar macrophages.\(^18\) Monocytic cells play an important role in immunological surveillance as they
express many cytoplasmic and cell surface innate immune sensors for viral and bacterial pathogens. These findings suggest that monocytes and macrophages are likely to play an important role in initiating the immune response to RV infection during the early stages of infection. Although little to no RV replication has been detected in monocytic cells, these cells can respond to RV stimulation through the secretion of a variety of chemokines. During RV infections, chemokine responses such as CCL5, CXCL8, and CXCL10 correlate with severity of respiratory symptoms, and CXCL10 is increased during viral-induced asthma exacerbation. In addition, two closely related chemokines, CCL2 and CCL8, have been suggested to contribute to the airway hypersensitivity in asthmatics, and while both have been shown to be induced in response to RV in epithelial cells, only CCL2 has previously been shown to be induced in monocytic cells. Despite the growing appreciation for the role of monocytic cells during RV infections and asthma, there are many aspects yet to be elucidated.

When considered together, these findings raise the possibility that airway bacteria might alter the monocytic antiviral response to RV infections. Therefore, we conducted a series of experiments to analyze the chemokine response to RV, and then tested the hypothesis that bacterial products, specifically LPS, modulate monocytic cell chemokine responses to RV infections. A portion of these results have been presented in abstract form.
MATERIALS AND METHODS

Cell sources

Human BAL macrophages and peripheral blood were obtained from human atopic men and women (ages 18-50 years) with or without mild asthma, under informed consent. The study protocol was approved by the University of Wisconsin-Madison, Human Subjects Committee. Exclusion criteria included corticosteroid use within one month of screening, smoking, pregnant/lactating women, unstable asthma, and other major health problems.

Purification and isolation of cells

Human peripheral blood monocytes and BAL macrophages were purified as previously described.28 Briefly PBMCs, enriched from heparinized whole blood via centrifugation with a Percoll monolayer (1.090 g/mL Percoll, GE Healthcare, Piscataway, NJ, USA), underwent washes with 2% heat-inactivated calf serum/HBSS (Mediatech, Herndon, VA, USA) to remove platelets. Monocytes were enriched by negative antibody selection (RosetteSep, Stemcell Technologies, Vancouver, BC, CA) and centrifugation over lymphocyte separation media (Mediatech). The cells were resuspended in monocyte complete medium (MCM): RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, 2 mM sodium pyruvate, 2 mM L-glutamine. Monocytes, 85-90% pure as determined by flow cytometry 29, were plated and allowed to adhere (2 h, 37 °C), then washed twice with HBSS to enhance purity.

BAL macrophages were recovered from BAL fluid after washing with 2% newborn calf serum/HBSS. Average BAL populations contain 94% macrophages based on morphological examination. Cells were resuspended and plated in MCM supplemented with 0.25 µg/mL amphotericin B (Mediatech), incubated (2 h, 37 °C), and washed twice with HBSS to enhance purity.
Rhinovirus production and purification

RV A016, B014, A001, and A002 were grown in HeLa cells and purified as previously described. The infectivity of the virions in HeLa cell monolayers was determined in tissue culture [plaque forming units (pfu)].

Microarray analysis

RNA preparation, sample labeling, hybridization, and data analysis was performed similarly as previously described. Briefly, human peripheral blood was acquired from 5 subjects and monocytes were prepared as previously described. Each subject's cells were treated (4 h, 37 °C) with RV A016 (MOI = 10) or mock infected. Cells were lysed in TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA). RNA was isolated as described in manufacturer's protocol and the total RNAs were pooled from the five donors. Biotin-labeled complementary RNA was synthesized using ENZO BioArray High Yield RNA Transcript Kit (ENZO Diagnostics, Farmingdale, NY, USA) from reverse transcribed RNA to cDNA and hybridized to Affymetrix HuGeneFL arrays (Affymetrix, Santa Clara, CA, USA). Microarray data analysis initially was done by analysis of the scanned microarray images and normalization to total signal intensity of chip by GeneChip software (Affymetrix). Quantification of abundance was performed as previously described, and fold increase was determined by dividing A016-treated RNA transcript quantification by the mock-treated transcript quantification for each transcript.

Treatment conditions for chemokine detection

Blood monocytes were plated (5x10^5 cells/well) in 24-well CoStar plates (Corning Inc.). Monocytes were either (1) co-treated with LPS (Escherichia coli, serotype 0111:B4, 0.01-100 ng/mL, Sigma) or vehicle (40 nM HEPES/H_2O, Sigma) and either mock infected (0.00025%
HSA/ HBSS, Irvine Scientific, Santa Ana, CA, USA) or RV treated (MOI = 10) for 8-48 h (34.5 °C); or (2) pretreated (16 h at 37 °C) with +/- LPS (100 ng/mL), followed by 24 h (34.5 °C) +/- RV. All treatments were diluted in MCM. BAL macrophages were plated and treated similarly to the monocytes, but in 12-well plates.

**Detection of CXCL10, CXCL11, CCL2, and CCL8 secretion**

Chemokine quantification was done via ELISA, as previously described. Capture and biotinylated antibodies and recombinant protein for CXCL10/IP-10 and CCL8/MCP-2 were purchased from R&D Systems (Minneapolis, MN, USA); CXCL11/I-TAC from PeproTech Inc. (Rocky Hill, NJ, USA); and CCL2/MCP-1 from BD Biosciences (San Jose, CA, USA). The assay sensitivity level was 3.1 pg/mL for CXCL10, CXCL11, and CCL2, and 7.8 pg/mL for CCL8. All determinations were performed in duplicate.

**Cell viability assay**

Human peripheral blood monocytes were plated (1x10^5 cells/0.1 mL media) in 96-well plates and incubated overnight (16 h, 37 °C). Cells were co-treated (24 h, 34.5 °C) with +/- LPS (100 ng/mL) and +/- RV A016, B014, and A001 (MOI = 10). After treatment the cells were treated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and the electron-coupling reagent phenazine methosulfate (PMS) according to manufacturer's protocol (Promega, Madison, WI, USA). The mixtures were incubated at 37 °C for 2 h and formazan production was quantified by absorbance at 490 nm.

**Flow cytometry**

Monocytes (1x10^6/0.5 mL) co-treated with +/- RV (MOI = 10), and +/- 100 ng/mL LPS were lifted by incubation with Cell Dissociation Solution (Sigma). Cells, suspended in 1 % FBS/RPMI, were immunostained (30 min, 4 °C) with 5 μl of V450-conjugated anti-CD14 (Clone
MΦPg, BD Biosciences), PE-conjugated anti-CD54 (ICAM-1,Clone HA58, BD Biosciences), or APC-conjugated anti-LDLR (Clone 472413, R&D Systems). Cells were washed with 1% FBS/RPMI and suspended PBS. Propidium iodide (3 µg/mL) staining was used exclude dead cells, and 10,000 events were assessed on an LSRII flow cytometer (Becton–Dickinson, Bedford, MA, USA). After gating for CD14+ cells, geometric mean fluorescence intensity (gMFI) was determined for each receptor using FlowJo software (TreeStar, Ashaland, OR, USA).

**Immunoblots**

Human peripheral blood monocytes were plated (1x10^6 cells/well) and incubated overnight (16-20 h, 37 °C). Cells were co-treated (2-4 h, 34.5 °C) with +/- LPS (100 ng/mL) and +/- RV (MOI = 10). After treatment, cells were lysed with SDS sample buffer (20 mM Tris, pH 6.8, 2 mM EDTA, 1 mM Na_3VO_4, 2 mM DTT, 2 % SDS, and 20 % glycerol). After SDS-PAGE and transfer, membranes were probed for phosphoSTAT1 (pSTAT1), total STAT1 (as a control for overall changes in STAT1 between treatments) and actin (as a loading control between the wells). Immunoblotting was performed as specifically described in the respective antibody manufacturer's protocol and as previously described. Antibodies were purchased from the following companies: anti-STAT1, anti-rabbit IgG, and anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-actin (BD Biosciences), and anti-phosphoSTAT1 (pY701; Cell Signaling Technologies Inc., Danvers, MA, USA). The immunoreactive bands were visualized using Epichemi II darkroom (UVP, Upland, CA, USA) and images were processed and analyzed using ImageJ 1.46r (NIH).

**Detection of IFNα secretion**

Blood monocytes were plated (5x10^5 cells/well) in 24-well plates. Monocytes were co-treated for 8-24 h (34.5 °C) with +/- LPS (100 ng/mL) and +/- RV A016 (MOI = 10). After
incubation, supernatants were collected and centrifuged (15800 x g, 1 min, 4 °C). Supernatants were stored at -80 °C until IFNα quantification via Human IFN-α Multi-Subtype ELISA Kit (#41105, PBL Interferon Source, Piscataway, NJ, USA). ELISA was performed as specifically described in the manufacturer's protocol. The assay detects 14 of the 15 identified IFNα subtypes with a sensitivity level of 12.5 pg/mL. All determinations were performed in duplicate.

Statistical analysis

Measurements of chemokine and IFNα secretion were log transformed to approximate normal distribution. Student's two-tailed paired t tests were used to calculate statistical differences between treatment effects. One-way repeated measures ANOVA (Friedman test) was used to evaluate treatment effects in Figure 4 (C & D). Analysis was performed with Prism 5 version 5.01 (GraphPad Software, Inc., La Holla, CA, USA) and a 2-sided p-value of 0.05 was considered statistically significant.
RESULTS

Monocytes secrete CXCL and CCL chemokines in response to RV exposure

To identify chemokines highly induced by RV, blood monocytes were incubated with RV A016 (MOI = 10) and mRNA expression was determined by a microarray. Among the most highly induced mRNA were multiple chemokines (Table 2-1), and we selected the four most highly expressed chemokines (CXCL10, CXCL11, CCL2, and CCL8; fold increase of 116, 49, 34, and 26, respectively) for further study. To determine effects of RV-induced chemokine secretion, monocytes purified from human peripheral blood were treated with RV types representing both the major (i.e., A016, B014) and minor groups (i.e., A001, A002), as well as representing the A and B species. All four RV types tested caused a significant increase in chemokine (CXCL10, CXCL11, CCL2, and CCL8) secretion after 24 h treatment (Figure 2-1). The upregulation of CXCL10 secretion was the highest [A016-induced geometric mean (GM) 74880 pg/mL], followed by CCL2 (A016-induced GM 6651 pg/mL), CCL8 (A016-induced GM 6155 pg/mL), and CXCL11 (A016-induced GM 677 pg/mL), respectively. These data suggest that major and minor group RV, which utilize different receptors to infect cells, induce qualitatively similar monocyte chemokine responses.

LPS effects on RV-induced chemokine secretion in monocytic cells

Peripheral blood monocytes were inoculated with major and minor group RV for 24 h in the presence or absence of LPS and the secretion of CXCL10, CXCL11, CCL2, and CCL8 was assessed (Figure 2-2). LPS co-treatment alone induced secretion of CCL2 and CCL8 and LPS pretreatment alone induced secretion of CCL2, but not the other chemokines. When added as co-stimulus with A016, B014, or A001, LPS significantly attenuated RV-induced secretion of CXCL10 (Figure 2-2 A, p ≤ 0.0001) and CXCL11 (Figure 2-2 B, p < 0.008). This effect was
TABLE 2-1. Expression levels of the top 50 mRNA transcripts most highly induced by A016 in purified human monocytes.

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* Fold increase describes the abundance of the given transcript in A016-treated versus mock infected preparation, pooled from five donors.

# Bolded font indicates chemokines selected for further study.
FIGURE 2-1

A

CXCL10 Release (log pg/mL)

Mock A016 B014 A001 A002

B

CXCL11 Release (log pg/mL)

Mock A016 B014 A001 A002

C

CCL2 Release (log pg/mL)

Mock A016 B014 A001 A002

D

CCL8 Release (log pg/mL)

Mock A016 B014 A001 A002
FIGURE 2-1.

Multiple RV types significantly induce chemokine (A, CXCL10; B, CXCL11; C, CCL2; and D, CCL8) secretion from primary human monocytes. Data are log transformed for normalcy and depicted as individual points representing three different donors and the geometric mean represented as a line. (*) p < 0.05, (**) p < 0.01, (***) p < 0.001 compared to mock infected cells by Student’s paired t-test.
FIGURE 2-2

Co-treatment

A  CXCL10 Release (log pg/mL)

LPS: Mock A016 B014 A001

B  CXCL11 Release (log pg/mL)

LPS: Mock A016 B014 A001

C  CCL2 Release (log pg/mL)

LPS: Mock A016 B014 A001

D  CCL8 Release (log pg/mL)

LPS: Mock A016 B014 A001

Pretreatment

E  CXCL10 Release (log pg/mL)

LPS: Mock A016 B014 A002

F  CXCL11 Release (log pg/mL)

LPS: Mock A016 B014 A002

G  CCL2 Release (log pg/mL)

LPS: Mock A016 B014 A002

H  CCL8 Release (log pg/mL)

LPS: Mock A016 B014 A002
FIGURE 2-2.

**LPS modifies RV-induced chemokine secretion in primary human blood monocytes.**

Peripheral blood monocytes were co-treated +/- LPS (100 ng/mL) and +/- RV (MOI = 10) for 24 h at 34.5 °C. Supernatants were analyzed for the presence of (A) CXCL10, (B) CXCL11, (C) CCL2, and (D) CCL8 by ELISA. Monocytes were pretreated +/- LPS (100 ng/mL, 16 h at 37 °C), followed by mock infection or RV (MOI = 10, 24 h at 34.5 °C). Supernatants were analyzed for the presence of (E) CXCL10, (F) CXCL11, (G) CCL2, and (H) CCL8 by ELISA. (*) p < 0.05, (**) p < 0.01, (***) p < 0.001 by Student’s paired t-test. The data are log transformed for normalcy and are summarized as boxplots (whiskers: 10-90 percentile), each representing cells from ten different donors.
specific to CXCL10 and CXCL11, as LPS co-treatment significantly (p < 0.05) increased A016- and B014-induced CCL2 secretion and did not attenuate virus-induced secretion of CCL8 (Figure 2-2 C-D). Similarly, pretreatment with LPS 16 h prior to RV exposure also significantly inhibited RV-induced CXCL10 (Figure 2-2 E, p < 0.0001) and CXCL11 (Figure 2-2 F, p < 0.004) secretion by a larger magnitude compared to LPS co-treatment. While LPS pretreatment alone did significantly increase CCL2 secretion, it did not affect RV-induced CCL2 secretion (Figure 2-2 G). In addition, LPS pretreatment significantly decreased RV-induced CCL8 secretion from blood monocytes (Figure 2-2 H, p < 0.002). Overall, these findings indicate that the effects of LPS are similar whether the exposure is simultaneous with, or prior to, RV stimulation on RV-induced CXCL10, CXCL11, and CCL2 secretion. No significant differences in monocyte metabolic activity were observed between any of the treatment conditions used, demonstrating that cell viability is unaffected by LPS and RV co-treatment (Figure 2-3).

In order to test these relationships in airway cells, human BAL macrophages were either co-treated with LPS and RV or pretreated with LPS for 16 h and then incubated for 24 additional hours with RV (Figure 2-4). LPS co-treatment alone significantly increased CXCL10 and CXCL11 secretion from BAL macrophages in the absence of RV. Although LPS co-treatment of mock infected cells induced CXCL10 and CXCL11 secretion (p = 0.0002), LPS co-treatment significantly attenuated RV B014- and A002-induced CXCL10 (Figure 2-4 A, p < 0.03) and CXCL11 (Figure 2-4 B, p < 0.03). In addition, LPS co-treatment decreased A016-induced CXCL10 secretion (from GM 140494 pg/mL to GM 44848 pg/mL; p = 0.07). Interestingly, LPS pretreatment appeared to cause a more robust effect on RV-induced CXCL10 and CXCL11 secretion than LPS co-treatment (Figure 2-4 E-F). LPS pretreatment significantly attenuated RV A016-, B014-, and A002- induced CXCL10 (p < 0.002) and CXCL11 (p < 0.0001) secretion in
FIGURE 2-3.

LPS and RV co-treatment conditions do not significantly change human peripheral blood monocyte metabolic activity. Summary of 2 h MTS viability assay, displayed as percent metabolic activity. All values are means ± SEM using cells from five different donors.
FIGURE 2-4

Co-treatment

CXCL10 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CXCL11 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CXCL10 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CXCL11 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CXCL2 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CXCL10 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CXCL11 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CXCL2 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL8 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL2 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL8 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL2 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL8 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL2 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL8 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL2 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL8 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL2 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL8 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL2 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL8 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL2 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL8 Release (log pg/mL)

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CCL2 Release (log pg/mL)

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Mock A016 B014 A002

CCL2 Release (log pg/mL)

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Mock A016 B014 A002

CCL8 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL2 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL8 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL2 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL8 Release (log pg/mL)

LPS: - + - + - + - +

Mock A016 B014 A002

CCL2 Release (log pg/mL)
**FIGURE 2-4.**

LPS modifies RV-induced chemokine secretion in primary human BAL macrophages. BAL macrophages were co-treated +/- LPS (100 ng/mL) and +/- RV (MOI = 10) for 24 h at 34.5 °C. Supernatants were analyzed for the presence of (A) CXCL10, (B) CXCL11, (C) CCL2, and (D) CCL8 by ELISA (n = 9). BAL macrophages were pretreated +/- LPS (100 ng/mL, 16 h at 37 °C), followed by mock infection or RV treatment (MOI = 10, 24 h at 34.5 °C). Supernatants were analyzed for the presence of (E) CXCL10, (F) CXCL11, (G) CCL2, and (H) CCL8 by ELISA (n = 8). (*) p < 0.05, (**) p < 0.01, (***) p < 0.001 by Student’s paired t-test. The data are log transformed for normalcy and are summarized as boxplots (whiskers: 10-90 percentile).
BAL macrophages, which is similar to peripheral blood monocytes. In the absence of RV, BAL macrophages alone secreted large amounts of CCL2 (GM 4200 pg/mL) and minor amounts of CCL8 (GM 10 pg/mL), and LPS co-treatment and LPS pretreatment alone enhanced both CCL2 and CCL8 secretion (Figure 2-4 C-D and G-H, p < 0.04). Although LPS co-treatment slightly reduced A002-induced CCL2 secretion, both LPS co-treatment and pretreatment generally maintained RV-induced CCL2 or CCL8 secretion from BAL macrophages (Figure 2-4 C-D and G-H).

**Effects of treatment time and LPS dose on RV-induced chemokine secretion**

We next evaluated effects of timing and dose on interactions between simultaneous LPS and RV infection with respect to chemokine secretion. A016 treatment for 8-48 h stimulated CXCL10 secretion (p < 0.01, Figure 2-5 A). LPS co-treatment significantly attenuated A016-induced CXCL10 at both 24 and 48 h. A different pattern was observed for CCL2. Either LPS or A016 alone induced increasing amounts of CCL2 over time, while LPS and A016 co-treatment induced a significantly (p < 0.05) more robust secretion of CCL2 compared to A016 alone at both 24 and 48 h (Figure 2-5 B).

To determine if the effect of LPS on RV-induced chemokine secretion was dose-dependent, CXCL10 and CCL2 secretion from monocytes was measured in the presence or absence of A016 and increasing doses of LPS (0-100 ng/mL). A016-induced CXCL10 secretion (Figure 2-5 C) was inversely proportional to concentrations of LPS (p = 0.007), while LPS alone increased modest amounts of CXCL10 secretion (p = 0.02). On the other hand, LPS and A016 had additive effects on CCL2 secretion (Figure 2-5 D, p = 0.01).

**Major and minor group RV receptor expression in response to stimuli in monocytes**

Cell surface receptors for minor and major group RV are LDLR and ICAM-1,
FIGURE 2-5

A

CXCL10 Release (pg/mL)

100000
10000
1000
100
10
1
0.1

12 24 48

Time (h)

Mock LPS A016 LPS+A016

B

CCL2 Release (pg/mL)

100000
10000
1000
100
10
1
0.1

12 24 48

Time (h)

Mock LPS A016 LPS+A016

C

CXCL10 Release (pg/mL)

100000
10000
1000
100
10
1
0.1

0.01 1 100

LPS (ng/mL)

Mock A016

p = 0.007

p = 0.02

D

CCL2 Release (pg/mL)

100000
10000
1000
100
10
1
0.1

0.01 1 100

LPS (ng/mL)

Mock A016

p = 0.006

p <0.0001
FIGURE 2-5.

LPS modification of RV-induced chemokine release from primary human monocytes is dose dependent. *Open circles*, mock infected; *open triangles*, LPS treated; *solid circles*, A016 treated; *solid triangles*, LPS and A016 co-treated. Supernatants were analyzed for the presence of chemokine secretion by ELISA. All values are means ± SEM using cells from six different donors. (A) CXCL10 and (B) CCL2 time-dependent release, (*) p < 0.05, (**) p < 0.01 A016 compared to LPS+A016 by Student’s paired t-test. LPS concentrations were significantly related to (C) CXCL10 and (D) CCL2 secretion, p values by one-way repeated measures ANOVA (Friedman test).
respectively. To test whether LPS effects on CXCL10 and CXCL11 were due to downregulation of target receptors for RV, we cultured primary human monocytes with LPS, A016, and/or A001 for 24 h and analyzed ICAM-1 and LDLR expression. After gating for live, CD14+ cells, geometric mean fluorescence intensity (gMFI) was determined for each receptor. Rather than diminishing expression, LPS stimulation enhanced the expression of ICAM-1, irrespective of RV co-treatment (Figure 2-6 A). Treatment with A001 alone also enhanced the expression of ICAM-1, whereas A016 alone did not have a significant effect. Similarly treatment with RV and/or LPS enhanced LDLR expression (Figure 2-6 B). These data indicate that treatment of monocytes with major and minor group RV or LPS does not diminish the expression of RV target receptors, ICAM-1 and LDLR.

**Phosphorylation of STAT1 in monocytes in response to LPS and RV A016**

One of the pathways involved in RV A016-induced CXCL10 secretion is the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. To determine whether LPS inhibition of RV-induced CXCL secretion was mediated through the JAK/STAT pathway, monocytes were co-treated with RV with or without LPS, and RV-induced phosphorylated STAT1 (pSTAT1) was determined 2 or 4 h later (Figure 2-7 A). At 2 h (Figure 2-7 B), LPS significantly induced pSTAT1, whether alone or in combination with A016, whereas RV A016 treatment alone did not significantly induce detectable phosphorylation of STAT1 above mock infected cells. At 4 h (Figure 2-7 C), RV A016 induced more robust pSTAT1 compared to LPS alone. Interestingly, co-treatment of monocytes with LPS and A016 at 4 h significantly diminished RV-induced pSTAT1. These data indicate that pSTAT1 induction occurs more rapidly with LPS treatment versus RV treatment. In addition, LPS co-treatment with A016 at 4 h significantly attenuates RV-induced pSTAT1 signaling in monocytes, without
FIGURE 2-6

A
ICAM-1 gMFI (CD14+ cells)

Mock  LPS  Δ016  LPS+Δ016  Δ001  LPS+Δ001

B
LDLR gMFI (CD14+ cells)

Mock  LPS  Δ016  LPS+Δ016  Δ001  LPS+Δ001
FIGURE 2-6.

Effects of LPS and RV co-treatment on major and minor group RV receptor expression on monocytes. CD14+ monocyte expression of (A) ICAM-1 and (B) LDLR is reported as geometric mean fluorescence intensity (gMFI). (*) p < 0.05, (**) p < 0.01, (***) p < 0.001 compared to mock infected cells by Student’s paired t-test. The data are summarized as boxplots (whiskers: 10-90 percentile) using cells from 5-6 different donors.
FIGURE 2-7

A

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<tr>
<td>A016:</td>
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B

C

Normalized pSTAT1 (fold change)

LPS:          | -   | +   | -   | +   |
| A016:        | -   | -   | +   | +   |
FIGURE 2-7.

LPS reduces RV-induced pSTAT1 in primary human peripheral blood monocytes. (A) Representative immunoblot images for pSTAT1, total STAT1 and actin. (B) Summary of 2 h co-treatment (n = 10). (C) Summary of 4 h co-treatment (n = 8). Data were normalized to total actin levels, and fold changes in pSTAT1 were in respect to mock infected cells. All values are means ± SEM using cells from different donors. (*) p < 0.05, (**) p < 0.01, (***) p < 0.001 compared to A016; (#) p < 0.05, (# #) p < 0.01, (# # #) p < 0.001 compared to mock infected cells by Student’s paired t-test.
altering the overall STAT1 available in the cells.

**LPS increases A016-induced IFNα secretion from monocytes**

RV induces IFNα secretion, and autocrine binding of IFNα to the type I IFN receptor leads to activation of the JAK/STAT pathway. In addition, treatment of monocytes with IFNα induces CXCL10 secretion. We therefore tested for effects of LPS on RV-induced IFNα secretion. Monocytes that were co-treated with LPS and A016 showed little to no secretion of IFNα at 8 and 24 h (Figure 2-8). A016 alone induced IFNα secretion after 24 hours (p = 0.02). In contrast, LPS and A016 co-treatment induced significant IFNα secretion earlier (by 8 hours), and LPS significantly augmented A016-induced IFNα secretion at both time points (Figure 2-8 A and B, p = 0.01 and 0.03). These findings suggest that while LPS diminishes RV-induced pSTAT1 at 4 h, this effect unlikely due to a reduction in RV-induced IFNα secretion.
FIGURE 2-8

A

IFN-α Release at 8 h (log pg/mL)

Mock  LPS  A016  LPS+A016

B

IFN-α Release at 24 h (log pg/mL)

Mock  LPS  A016  LPS+A016

*  **  **  **

*  *  *  *

**  **  **  **
FIGURE 2-8.

**LPS augments RV-induced IFNα secretion in primary human monocytes.** Peripheral blood monocytes were co-treated with LPS (100 ng/mL) and A016 (MOI = 10) or the respective vehicle controls for either (A) 8 h or (B) 24 h at 34.5 °C. Supernatants were analyzed for the presence of IFNα by ELISA. (*) p < 0.05, (**) p < 0.01, (***) p < 0.001 by Student’s paired t-test. The data are log transformed for normalcy and are summarized as boxplots (whiskers: 10-90 percentile) using cells from six different donors.
DISCUSSION

Our results demonstrate that primary blood monocytes and BAL macrophages secrete CXCL10, CXCL11, CCL2, and CCL8 in response to all the RV types tested. Furthermore, LPS modifies monocyctic cell response to RV exposure, in a chemokine-specific manner. LPS co-treatment inhibits RV-mediated CXCL10 and CXCL11, but either has no effect or else potentiates RV-mediated CCL2 and CCL8. These findings were demonstrated in human peripheral blood monocytes as well as in human BAL macrophages. Interestingly, LPS augments RV-induced IFNα secretion, yet diminishes RV-induced pSTAT1 in the blood monocytes. These findings suggest that the quantity of Gram-negative bacteria in the airway could influence the immunological response to RV infections. Because asthma is associated with increased bacterial colonization, our findings suggest that monocytic chemokine responses to RV could be distinct.

Recent findings have demonstrated that after in vivo RV infections in humans, RV colocalizes with macrophages in the tissues, suggesting a direct role for macrophages in antiviral responses. In addition, Rajan and colleagues have shown that the source of monocytic cells influence RV-induced cytokine and chemokine secretions from epithelial cells. This is in agreement of our previous study demonstrating that monocytic cells can regulate epithelial cell responses to RV infections. We have demonstrated that monocyte-epithelial cell co-cultures have more robust RV-induced CXCL10 secretion compared to the mono-cultures. Interestingly, conditioned medium from RV-treated monocytic cultures enhanced epithelial cell CXCL10 responses to RV. When considered together, these findings provide evidence that RV interacts with monocytic cells in vivo, which in turn secrete chemokines and modify epithelial cell responses to RV exposure.
If these effects also occur in vivo, how would LPS affect the course of an RV infection? CXCL10 and CXCL11 are ligands for the receptor CXCR3, which is commonly found on activated T cells and natural killer T cells. Thus, these chemokines are thought to promote viral clearance. CCL2 primarily binds CCR2, found on the surface of a variety of myeloid cells including monocytes, macrophages, dendritic cells, eosinophils, and neutrophils. CCL8 can bind to several receptors, CCR-1, -2, -3,-5. CCR3 is highly expressed on eosinophils and its expression in the airways is increased in asthma. CCL2 and CCL8 levels are elevated in asthmatic airways and likely contribute to inflammatory responses that promote exacerbations of asthma. These findings suggest that LPS attenuation of RV-mediated CXCL10 and CXCL11 secretion could impair viral clearance. In contrast, LPS does not inhibit RV-induced CCL2 and CCL8 secretion, and these two chemokines have been linked to airway obstruction and exacerbations of asthma. Since LPS did not diminish RV-induced IFNα secretion, these findings imply that LPS could impair RV-induced antiviral responses downstream of IFNα secretion, while maintaining or augmenting pro-inflammatory effects in the airway.

A study by Oliver and colleagues demonstrated that infectious RV reduces BAL macrophage responsiveness to LPS. Specifically, they found that RV pretreatment impaired LPS-induced secretion of TNFα and IL-8, and impaired the ability of macrophages to phagocytose E. coli particles. Our findings, when considered together with those of the Oliver study, suggest the presence of RV and bacterial products in the airway leads to altered BAL macrophage responsiveness to either stimuli.

The effect of LPS on RV-induced CXCL10, CXCL11, and CCL2 secretion were consistent regardless of whether LPS exposure was prior to RV stimulation or whether LPS was
co-stimulated with RV in both the monocytes and the BAL macrophages. Whereas this was also true for RV-induced CCL8 secretion from BAL macrophages, LPS timing-dependent differences were observed in blood monocytes. RV-induced CCL8 secretion was unaffected by LPS co-treatment, but was inhibited by LPS pretreatment in blood monocytes (Figure 2-2 D and H). Interestingly, prior exposure of the monocytic cells to LPS appeared to exaggerate LPS-induced attenuation of RV-induced CXCL10 and CXCL11 secretion compared to LPS and RV co-treatment. While BAL macrophages are differentiated cells, blood monocytes can differentiate depending on their environment. LPS is commonly used in vitro to differentiate blood monocytes into various types of macrophages. Therefore, it is reasonable that the differences observed between LPS pretreatment and co-treatment are due to LPS-induced low levels of monocyte differentiation.

The similar induction of chemokine secretion by all RV types tested (major and minor), suggests that the overall pattern of chemokine responses is independent of RV type and RV receptor utilization. Furthermore, RV and/or LPS treatment increased the expression of RV target receptors, ICAM-1 and LDLR. Together these data suggest that attenuation of RV-induced CXCL10 and CXCL11 secretion is not explained by LPS effects on cell surface receptors, and implies that LPS instead modifies RV-induced intracellular signaling.

LPS-mediated attenuation of RV-induced CXCL10 and CXCL11 occurs within hours and is maintained, suggesting LPS alters one or more of the early RV-induced signaling pathways. RV induces the secretion of IFNα, which acts in an autocrine manner through the type I IFN receptor to activate the JAK/STAT1 pathway, leading to the secretion of CXCL10. Here we demonstrate that although LPS diminishes RV-induced pSTAT1, it increases RV-induced IFNα. Therefore, the mechanism by which LPS reduces RV-induced pSTAT1 and CXCL10/CXCL11
is likely to be downstream of IFNα secretion. One possible mechanism by which LPS could reduce the phosphorylation of STAT1 may relate to the ability of LPS to rapidly phosphorylate STAT1. LPS stimulation of macrophages can induce the secretion of IFNβ, which activates the type I IFN receptor and promotes phosphorylation of STAT1.44,45 By inducing phosphorylated STAT1, LPS co-stimulation with RV could either divert activated STAT1, or lead to its downregulation, and thereby diminish the pool of activated STAT1 available for RV-induced signaling. This mechanism could therefore contribute to LPS attenuation of RV-induced genes that are STAT1-dependent, such as CXCL10 and CXCL11. Furthermore, LPS can activate multiple mitogen-activated protein kinase (MAPK) signaling pathways in human monocytes that induce secretion of CCL2.46,47 Thus, LPS effects on signaling pathways are likely to direct the selective effects on RV-induced chemokine secretion. Alternatively, suppressor of cytokine signaling (SOCS) family proteins directly inhibit the phosphorylation of STATs by inhibiting JAK catalytic activity and by marking it for proteasomal degradation, and both LPS and IFN can induce SOCS-1 and -3 under some experimental conditions.48 It is possible that the combination of LPS and RV increases SOCS levels which would diminish STAT1 phosphorylation.

In this study we use multiple purified RV types, representing both the major and minor groups as well as RV A and B species, allowing for characterization of RV-induced signaling in a general context. Recent studies have shown differences in virulence and illness severity across RV species.14,49 Thus, it is advantageous to utilize representative RV types in experimental models. In addition, we used human monocytes and BAL macrophages, which demonstrate that LPS has similar immunomodulating effects on blood and airway cells. One limitation of this study is the use of monocytic cells from donors with atopy or atopic asthma. We selected these donors because of our interest in mechanisms of RV-induced inflammation, however, it is
possible that effects of LPS on virus-induced chemokine secretion in non-atopic individuals could have distinct features. Another limitation to consider is that while LPS is a single component, bacteria are complex organisms, and more work is needed to determine the effects of whole bacteria on RV-induced airway inflammation.

Several clinical studies provide evidence that bacteria may contribute to asthma. Martin and colleagues have shown that lower airway colonization or infection by atypical bacteria is more common in asthmatic adults compared to healthy controls.\textsuperscript{50} Furthermore, studies by Hilty and Huang and colleagues have shown that asthmatics have characteristic airway microbial flora compared to healthy individuals and that these differences strongly associate with airway disease.\textsuperscript{4,8} In addition, a recent study by Wark and colleagues demonstrated that bacterial and RV co-infection is an independent predictor of more severe acute asthma exacerbations and that co-infections increase the likelihood for hospital readmission.\textsuperscript{51} Thus, providing clinical support for the immunomodulating effects of bacteria in RV-induced asthma.

In summary, we demonstrate a mechanism by which endotoxin exposure can alter the mononuclear cell immune response to RV infections. RV infections are a substantial cause of asthma exacerbation, and with increasing appreciation that airway bacteria are also related to asthma, it is critical to understand how microbial products can alter airway immune responses. Additional studies to determine how LPS affects RV-induced signaling \textit{ex vivo} correspond to physiologic responses \textit{in vivo} could aid in the development of future therapeutics.
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CHAPTER THREE

Allergen challenge *in vivo* alters rhinovirus-induced chemokine secretion from human airway macrophages

Adapted from


I co-wrote this manuscript and provided Figure 3-1.
CAPSULE SUMMARY

Allergen challenge modifies RV-induced chemokine secretion and RV target receptor expression on airway macrophages. Allergy sensitization in the airway could therefore alter macrophage immune responses to RV.

ABBREVIATIONS

BAL: bronchoalveolar lavage
ICAM-1: intercellular adhesion molecule-1
LDLR: low density lipoprotein receptor
LRP-1: low density lipoprotein receptor-related protein 1
RV: rhinovirus
To the Editor:

Allergy and viral infection are the two main risk factors for the inception of persistent wheezing and asthma in early childhood and for acute exacerbations of established asthma. The underlying mechanisms of how these two distinct sources of inflammation contribute to asthma inception and exacerbation are incompletely understood.

Macrophages are the most numerous leukocyte (>90%) in the lower airways. Although rhinovirus (RV) does not replicate in macrophages, it directly interacts with macrophages in airway tissues. Once activated by RV, macrophages can secrete a variety of inflammatory chemokines, depending on the differentiation state and phenotype of the cells. For example, macrophages can secrete CXCL10 and CXCL11, which recruit adaptive immune cells that direct viral clearance, and/or CCL2 and CCL8, which can recruit myeloid cells such as monocytes and eosinophils. In mouse models of RV infection, macrophage responses contribute to airway inflammation and hyperresponsiveness. Furthermore, RV activation of monocytes and macrophages can potentiate antiviral responses of airway epithelial cells. These findings provide evidence that macrophages are important immunoregulatory cells during RV infections.

A recent study demonstrated reduced anti-viral responses to RV stimulation in allergic asthmatic children, corresponding with increased susceptibility to RV-induced exacerbations. Therefore, we hypothesized that allergen exposure modifies RV-induced chemokine responses of airway macrophages to impair the anti-viral response and promote inflammation. Together, these effects could increase the severity of viral respiratory infections and lead to lower respiratory symptoms in patients with asthma.
To test this hypothesis, in accordance with IRB-approved protocols from the Human Subjects Committee at University of Wisconsin-Madison, bronchoalveolar lavage (BAL) was performed to obtain airway mononuclear cells (MNCs) from 10 donors with a history of mild atopic asthma (Table 3-1). BAL was performed before (D0) and 48 h after (D2) segmental bronchoprovocation with allergen as previously described. Contaminating granulocytes were removed from D2 cells by Percoll density gradient centrifugation (see Methods section), and cell populations were comparable between the two isolations (Figure 3-1). Macrophages were isolated from D0 cell and D2 MNC populations by adherence to plastic (2 h).

To determine the effect of allergen challenge on RV-induced macrophage responses, D0 and D2 BAL macrophages were incubated (24 h) with RV A016, B014, and A002 (see Methods section). The supernatants were then analyzed for CXCL10, CXCL11, CCL2, and CCL8 by ELISA (Figure 3-2). In the absence of virus, D0 macrophages secreted low levels of CXCL10 and CCL2 [geometric mean (GM) 95 and 3,739 pg/mL, respectively], but not CXCL11 and CCL8, and incubation with RV A016, B014, and A002 significantly induced the secretion of all four of the chemokines tested (p < 0.001).

After allergen challenge, RV stimulation still significantly induced the secretion of CXCL10, CXCL11, CCL2, and CCL8 (p < 0.001 compared to D2 vehicle treated cells), but there were several differences noted. Compared to D0 macrophages, D2 cells incubated in vehicle alone secreted less CXCL10 (GM 26 vs. 95 pg/mL, p < 0.05), and more CCL2 (GM 11,392 vs. 3,739 pg/mL, p < 0.001, Figure 3-2 A and C). After incubation with RV A016, D2 vs. D0 macrophages secreted significantly less CXCL10 (GM 17,271 vs. 114,973 pg/mL, p < 0.001, Figure 3-2 A) and CXCL11 (GM 117 vs. 453 pg/mL, p < 0.05, Figure 3-2 B) and more CCL2 (GM 27,264 vs. 10,245 pg/mL, p < 0.01, Figure 3-2 C). In addition, these effects were similar for
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<sup>a</sup> Methacholine concentration that resulted in a 20% reduction in forced expiratory volume in 1 s (FEV<sub>1</sub>).  
<sup>b</sup> Percentage of predicted value.  
<sup>c</sup> Provocative dose of allergen that resulted in a 20% decrease in lung function, measured in cumulative breath units.  
<sup>d</sup> Subjects requested to hold medications (NSAIDs, corticosteroids, and antihistamines) for a week prior to BAL.  
<sup>e</sup> Cat dander.  
<sup>f</sup> House dust mite.  
<sup>g</sup> Information not reported.  
<sup>h</sup> Median (25 and 75 percentiles).
FIGURE 3-1

- **Macs**: D0 BAL cells
- PMNs: D0 BAL MNCs
- Lymph: D0 BAL MNCs
- Eos: D0 BAL MNCs

% Cells in population
FIGURE 3-1.

Cell differentials in BAL cell population on D0 and D2. *Macs*, macrophages; *PMNs*, polymorphonuclear leukocytes; *Lymph*, lymphocytes; *Eos*, eosinophils. The values are means ± SEM from nine individual subjects. (*) p<0.05 by two-way repeated measures ANOVA (Bonferroni post-tests).
**FIGURE 3-2**

A. CXCL10 Release (log pg/mL)

B. CXCL11 Release (log pg/mL)

C. CCL2 Release (log pg/mL)

D. CCL8 Release (log pg/mL)
FIGURE 3-2.

Allergen challenge alters RV-induced chemokine secretion in primary human BAL macrophages. CXCL10 (A), CXCL11 (B), CCL2 (C) and CCL8 (D). NS, not significant; (*) p<0.05, (**) p<0.01, (***) p<0.001 by two-way repeated measures ANOVA (Bonferroni post-tests). The data are log transformed for normalcy and summarized as boxplots (whiskers: 10-90 percentile) from nine individual subjects.
RV A016, B014, and A002 (p < 0.05, Figure 3-2). Allergen challenge had no significant effect on RV-induced CCL8 secretion (Figure 3-2 D). Preliminary experiments demonstrated that Percoll treatment did not alter the overall effect of allergen challenge on RV-induced macrophage chemokine response (Figure 3-3). These findings suggest that allergen exposure modifies RV-induced induction of specific chemokine responses from airway macrophages.

One mechanism by which allergen could alter anti-viral responses is through altered expression of cellular receptors for RVs. Allergen challenge can induce expression of intercellular adhesion molecule-1 (ICAM-1), the major group RV receptor, but effects on minor group receptors (low-density lipoprotein receptor [LDLR] and LDLR-related protein-1 [LRP-1]) are unknown. Because allergen challenge had similar effects on RV-induced chemokine secretion induced by both the major (A016 and B014) and minor group (A002) RVs, we hypothesized that the differences in chemokine secretion were independent of effects on RV target receptors. To test if allergen challenge affects RV receptor expression on airway macrophages, D0 BAL macrophages and D2 MNCs were stained for ICAM-1, LDLR, and LRP-1, and surface expression was measured by flow cytometry (Figure 3-4). As expected, allergen challenge significantly increased ICAM-1 expression on D2 compared to D0 macrophages (Figure 3-4 A). In contrast, allergen challenge decreased both LDLR and LRP-1 surface expression on D2 BAL macrophages (Figure 3-4 B and C, respectively). Allergen challenge did not affect ICAM-1, LDLR, and LRP-1 surface expression on peripheral blood monocytes from the same patients, suggesting that D2 allergen-induced changes in receptor expression are localized to the airways. These findings indicate that allergen-induced effects on the RV-induced chemokines are independent of RV receptor utilization, and raise the possibility that allergen challenge affects RV-induced intracellular signaling.
FIGURE 3-3

A

Normalized CXCL10 Release (pg/mL/100K macrophages)

B

Normalized CXCL11 Release (pg/mL/100K macrophages)

C

Normalized CCL2 Release (pg/mL/100K macrophages)

D

Normalized CCL8 Release (pg/mL/100K macrophages)

○ D0 BAL cells
○ D2 BAL MNCs (separation by Percoll)
● D2 BAL cells (unprocessed)
FIGURE 3-3.

Comparison of macrophage responsiveness to RV before and after Percoll separation. CXCL10 (A), CXCL11 (B), CCL2 (C) and CCL8 (D). White, D0 BAL cells; gray, D2 BAL MNCs (after separation by Percoll); black, D2 BAL cells (without Percoll processing). The data are normalized to $1 \times 10^5$ macrophages plated per treatment condition and displayed as three individual subjects and the mean. (*) $p<0.05$, (**) $p<0.01$, (***) $p<0.001$ compared to D0 BAL cells and (#) $p<0.05$, (##) $p<0.01$ D2 BAL cells compared to D2 BAL MNCs; all comparisons tested by two-way repeated measures ANOVA with Bonferroni post-tests.
FIGURE 3-4

A

ICAM-1 gMFI

BAL macs blood monocytes

D0 D2 D0 D2

B

LDLR gMFI

BAL macs blood monocytes

D0 D2 D0 D2

C

LRP-1 gMFI

BAL macs blood monocytes

D0 D2 D0 D2
Allergen challenge differentially affects major and minor RV target receptor expression.

Live (propidium iodide negative), CD14 positive BAL macrophages and blood monocytes. Open circles, D0 BAL macrophages; solid circles, D2 BAL macrophages; open triangles, D0 blood monocytes; solid triangles, D2 blood monocytes. ICAM-1 (A), LDLR (B), and LRP-1 (C) surface expression measured by flow cytometry, and represented as geometric mean fluorescent intensity (gMFI) from three individual subjects. (*) p < 0.05, (**) p < 0.001 by one-way ANOVA (Bonferroni post-tests).
In summary, allergen exposure modifies the quality and quantity of RV-induced chemokine secretion independent of allergen effects on RV target receptors. These findings complement and extend earlier observations, which showed that allergen challenge during RV infection leads to enhanced airway eosinophil recruitment and CXCL8.\textsuperscript{8,9} Our data demonstrate that \textit{in vivo} allergen challenge decreases \textit{ex vivo} macrophage responses to RV by inhibiting CXCL10 and CXCL11 and enhancing CCL2 secretion. By decreasing RV-induced CXCL10 and CXCL11 secretion, allergen challenge may dampen the anti-viral response in the airways by decreasing the influx of active T cells, which direct the adaptive immune response to viral infections and viral clearance. On the other hand, by increasing CCL2 and maintaining CCL8 secretion, allergen exposure may further promote the recruitment of proinflammatory cells, such as eosinophils, neutrophils, and macrophages to the airways. If similar processes occur \textit{in vivo}, these allergen-induced changes may lead to greater airway inflammation and a less effective anti-viral response, and thereby promote more severe respiratory illness and exacerbations of asthma.

We thank Yury Bochkov and Wai-Ming Lee for preparation of virus stocks; and Sameer Mathur, Elizabeth Schwantes, and Lei Shi for support and technical assistance.
METHODS

Segmental bronchoprovocation with antigen and bronchoalveolar lavage

Bronchoalveolar lavage (BAL) and segmental bronchoprovocation were performed as previously described. In addition, mononuclear cell (MNC) population was enhanced from the BAL fluid on the second day (D2) by Percoll density centrifugation (1.085 g/mL Percoll; 700 x g, 20 min, 25 °C).

Rhinovirus production and purification

RV A016, B014, and A002 were grown in HeLa cells and purified by centrifugation through a 30 % sucrose cushion as previously described. Titers of virus were determined by measuring the infectivity of the virions in HeLa cell monolayers [plaque forming units (PFU)]. All experiments were done using the same viral stock preparation that was aliquoted and stored at -80 °C.

Chemokine detection

BAL macrophages were plated (5x10^5 cells/well, 12-well CoStar plates) in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, 2 mM sodium pyruvate, 2 mM L-glutamine, and 0.25 µg/mL amphotericin B (Mediatech). To enhance purity macrophages were allowed to adhere (2 h, 37 °C), then washed twice with HBSS. Following overnight incubation, macrophages were treated (24 h, 34.5 °C) with vehicle (0.00025% HSA/ HBSS) or RV [Multiplicity of infection (MOI) = 10]. After incubation, supernatants were collected, centrifuged (15800 x g, 1 min, 4 °C) and analyzed for the presence of CXCL10, CXCL11, CCL2, and CCL8 via ELISA, as previously described. Capture and biotinylated antibodies and recombinant protein for CXCL10/IFN-10 and CCL8/MCP-2 were purchased from R&D Systems (Minneapolis, MN); CXCL11/I-TAC from PeproTech Inc. (Rocky Hill, NJ); and CCL2/MCP-1
from BD Biosciences (San Jose, CA). The assay sensitivity level was 3.1 pg/mL for CXCL10, CXCL11, and CCL2, and 7.8 pg/mL for CCL8. All determinations were performed in technical duplicates.

**Flow cytometry**

D0 BAL cells and D2 BAL MNCs were suspended in 1 % FBS/RPMI and immunostained (30 min, 4 ºC) with V450-conjugated anti-CD14 (Clone MΦPg, BD Biosciences), PE-conjugated anti-CD54 (ICAM-1,Clone HA58, BD Biosciences), APC-conjugated anti-LDLR (Clone 472413, R&D Systems), or FITC-conjugated anti-CD91 (LRP-1, Clone A2MR-a2, BD Biosciences). Cells were washed with 1 % FBS/RPMI and suspended in PBS. Propidium iodide (3 µg/mL) staining was used exclude dead cells, and 10,000 events were assessed on an LSRII flow cytometer (Becton–Dickinson, Bedford, MA, USA). After gating for CD14⁺ cells, geometric mean fluorescence intensity (gMFI) was determined for each receptor using FlowJo software (TreeStar, Ashaland, OR).

**Statistical analysis**

Measurements of chemokine secretion were log transformed to approximate normal distribution. Two-way and one-way repeated measures ANOVA (Bonferroni post-tests) were used to evaluate treatment effects. Analysis was performed with Prism 5 version 5.01 (GraphPad Software, Inc., La Holla, CA) and a 2-sided p-value of 0.05 was considered statistically significant.
REFERENCES


CHAPTER FOUR

Summary, conclusions, and future directions
SUMMARY AND CONCLUSIONS

The data presented in this thesis contributes to the knowledge of how environmental factors may alter monocytic cell responses to rhinovirus (RV) infections. Specifically, I present evidence that bacterial products, specifically lipopolysaccharide (LPS), and allergen exposure modulate the immune response to RV infection. Each chapter focuses on a different risk factor for asthma exacerbations, and demonstrates novel mechanisms that illustrate the importance of RV-induced airway immune responses as part of multifaceted immune system that contribute to asthma exacerbations.

Chapter Two tests the hypothesis that bacterial colonization of the airway leads to altered monocytic cell immune responses to RV. A microarray performed on primary human blood monocytes treated with A016 demonstrated a robust induction of chemokine and cytokine mRNA in response to RV. The chemokines that showed the highest fold induction in their mRNA were CXCL10, CXCL11, CCL2, and CCL8, which were the chemokines selected for further study. The findings confirm that RV induces both mRNA and protein for these chemokines, and further demonstrate that RV from both the major group (A016 and B014) and the minor group (A001 and A002) strongly induce the secretion of CXCL10, CXCL11, CCL2, and CCL8 from both primary human monocytes and airway macrophages. I then demonstrate that the bacterial product, LPS, significantly attenuates RV-induced CXCL10 and CXCL11 secretion, yet maintains or promotes RV-induced CCL2 and CCL8 secretion.

The mechanism by which LPS alters RV-induced chemokine secretion from monocytic cells was shown to be independent of RV target receptor expression, as treatment conditions increased surface expression of intercellular adhesion molecule 1 (ICAM-1) and low-density lipoprotein receptor (LDLR). In addition, while LPS augments RV-induced interferon α (IFNα)
secretion, LPS significantly diminishes RV-induced phosphorylation of signal transducer and activator of transcription 1 (STAT1), which is downstream of IFNα signaling. A recent study revealed that CXCL10 transcription is dependent on STAT1 phosphorylation, but not on the transcription factor activating protein-1 (AP-1), which is downstream of mitogen-activated protein kinase (MAPK) signaling. In addition, the study showed that inhibition of STAT1 phosphorylation inhibits RV-induced CXCL10 production in epithelial cells. CXCL10 and CXCL11 belong to a group of closely related chemokines that, along with CXCL9, are transcriptionally regulated by IFN and STAT signaling and are likely to be inhibited by similar cell signaling pathways. When considered together with the data presented in Chapter Two, these findings suggest that LPS attenuates RV-induced CXCL10 and CXCL11 secretion on a transcriptional level through the inhibition of STAT1 phosphorylation. The data presented in Chapter Two are summarized in Figure 4-1, which depicts a simplified diagram of the proposed mechanism by which LPS alters RV-induced chemokine secretion from monocytic cells.

Chapter Three tested the hypothesis that allergen exposure in vivo alters ex vivo airway macrophage responsiveness to RV exposure. Allergic is a major risk factor for childhood asthma pathogenesis, as well as exacerbations of asthma in adults. Therefore, we investigated the effects of in vivo allergen exposure in allergic subjects on RV-induced chemokine secretion from primary human airway macrophages. The data presented in Chapter Three demonstrates that allergen challenge significantly attenuated both RV-induced CXCL10 and CXCL11 secretion, yet it increased RV-induced CCL2 secretion and did not alter RV-induced CCL8 secretion. These effects were independent of allergen-mediated effects on expression of cellular receptors used by RV, and in fact allergic sensitization increased surface expression of the major group RV target receptor (ICAM-1), but decreased minor group receptor expression [LDLR and LDLR-
Proposed mechanism for LPS modification of RV-induced signaling and chemokine secretion. Major and minor group RV bind intercellular adhesion molecule 1 (ICAM-1) and low-density lipoprotein receptor (LDLR) family members, respectively. RV signal through toll-like receptor (TLR) 7 and 3 to induce the transcription and secretion of CXCL10, CXCL11, CCL2, and CCL8. RV also induces the secretion of interferon α (IFNα) that works in an autocrine manner to bind the type I IFN receptor (IFNRI) and lead to subsequent Janus activated kinase 1 (JAK1) phosphorylation. Active JAK1 can then phosphorylate signal transducer and activator of transcription 1 (STAT1) that enters the nucleus and acts as a transcription factor for a variety of genes, including CXCL10 and CXCL11. Activation of the mitogen-activated protein kinase family (MAPK) signaling cascade plays a role in the transcription of multiple chemokines, including CCL2 and CCL8. Free LPS binds LPS binding protein (LBP) allowing LPS to bind CD14 and signal through the TLR4- myeloid differentiation protein-2 (MD 2) complex. Myeloid differentiation primary response protein 88 (MyD88) adaptor protein-dependent LPS signaling can also activate the MAPK signaling pathway and lead to CCL2 secretion, thus promoting or maintaining RV-induced CCL2 and CCL8 secretion. LPS also potentiates RV-induced IFNα secretion, yet diminishes RV-induced STAT1 phosphorylation, thus diminishing RV-induced CXCL10 and CXCL11 secretion. Solid lines, one or two steps indicated; and dotted lines, several steps indicated.
related protein 1 (LRP-1)) on airway macrophages. The effects of allergen challenge occurred in airway macrophages, but not blood monocytes from the same patients. Together, these data demonstrate that allergic sensitization alters macrophage responsiveness to RV exposure, suggesting that allergen exposure may have a local effect on airway anti-viral responses.

Altogether, my work demonstrates that both LPS exposure and allergic sensitization attenuate RV-induced CXCL10 and CXCL11 secretion, but maintain or increase RV-induced CCL2 and CCL8 secretion, independent of their respective effects on RV target receptors ICAM-1, LDLR, and LRP-1. As previously mentioned in Chapter One, CXCL10 and CXCL11 play an important role in the recruitment of lymphocytes to the airways and viral clearance, while CCL2 and CCL8 play a role in the recruitment of myeloid cells and inflammation. Thus, the data presented here suggests that LPS and allergen exposure may be skewing the airway immune response to RV towards increased inflammation and decreased viral clearance. While RV infections alone are an important risk factor for asthma exacerbations, bacterial colonization of the airway and allergic sensitization may alter airway immunological responses to RV, which may affect severity of illness and the risk of RV-induced asthmatic episodes. Considering the substantial effects of allergen exposure and LPS on airway immune responses, a better understanding of how these environmental factors modify airway responses could lead to the identification of new targets for asthma therapeutics.

FUTURE DIRECTIONS

It is an intriguing question how rhinovirus infections exacerbate asthma. My work focused on how two main risk factors for asthma, the airway microbiome and atopy, may act as airway immunomodulators. While the work presented here demonstrates that both these risk
factors modulate immune responses to rhinovirus infections in monocytic cells, several questions remain unanswered.

*Combined effects of lipopolysaccharide stimulation and in vivo allergen challenge on rhinovirus-induced chemokine secretion from primary airway macrophages.*

Chapters Two and Three discuss the individual immunomodulating effects of LPS exposure and *in vivo* allergen challenge on RV-induced chemokine secretion from airway macrophages. With the emerging importance of bacterial colonization in asthma and the established role of allergic sensitization in asthma pathogenesis, there is an increasing need to understand how different environmental factors, such as allergen exposure, bacterial prevalence, and viral infections, work in concert to exacerbate asthma. Therefore, the next step in understanding how asthmatic airways may respond differently to RV infection is to determine how these risk factors jointly affect airway immune responses. It has been previously shown that allergen exposure can enhance the secretion of LPS-binding protein (LBP) and soluble CD14 into the bronchoalveolar space, which can enhance sensitivity to LPS. In addition, it has been shown that asthmatics have increased bronchial obstruction and hyperresponsiveness to inhaled LPS. This suggests that allergic inflammation may potentiate airway responses to bacterial presence and vice versa. Due to this and based on the similarity of immunomodulating effects of LPS and allergen challenge reported in Chapters Two and Three, respectively, I hypothesized that together allergy sensitization and LPS have greater immunomodulating effects on viral-induced signaling in primary airway macrophages than the individual risk factors alone.

In order to determine the effects of *in vivo* allergen challenge in combination with LPS stimulation on RV-induced chemokine release from airway macrophages, mild atopic asthmatic
donors underwent bronchoalveolar lavages (BAL) to obtain airway macrophages. BAL was performed prior to (D0) and 48 h after segmental bronchoprovocation with allergen (D2) and macrophage purity was optimized, as described in Chapter Three. Both D0 and D2 macrophages were pretreated with LPS (100 ng/mL) or control vehicle for 16 h at 37 °C, followed by treatment with control vehicle or RV A016, B014, or A002 (MOI=10) at 34.5 °C for 24 h. The supernatants were collected and analyzed for the presence of the chemokines CXCL10, CXCL11, CCL2, and CCL8 by ELISA (Figure 4-2). Figure 4-2 represents pooled data, which parts of the graphs have been published elsewhere (D0 vs. D0+LPS⁹, and D0 vs. D2¹⁰). Any repeated data were included to serve as controls for the comparison of allergen sensitization combined with LPS treatment (Figure 4-2 purple boxes).

As demonstrated in Chapters Two and Three, the individual conditions of LPS treatment and allergen sensitization both attenuated RV-induced CXCL10 and CXCL11 secretion from airway macrophages, while maintaining both RV-induced CCL2 and CCL8 secretion. The combined effects of allergen sensitization and LPS pretreatment results in a significant attenuation in RV-induced CXCL10 and CXCL11 secretion (Figure 4-2 A and B) when compared to allergen challenged macrophages (D2; p < 0.001) and when compared to D0 macrophages without LPS (p < 0.01). When comparing D0 and D2 macrophages treated with LPS, there was a significant decrease (p < 0.05) in RV-induced CXCL10 and CXCL11 secretion. These effects were independent of viral type used, with the exception of A016-induced CXCL11, which is likely due to the small sample size (n=4), but still showed a trending decrease (p = 0.09). On the other hand, while there were small increases between allergen challenge alone or LPS treatment alone on RV-induced CCL2 and CCL8 secretion, here we demonstrate that together allergic sensitization and LPS stimulation significantly increase (p < 0.05) RV-induced
CCL2 and CCL8 secretion from LPS-treated D2 macrophages compared to untreated D0 macrophages (Figure 4-2 C and D). A002-induced CCL8 was only slightly elevated ($p = 0.09$) in the LPS-treated D2 macrophages, which may be due to donor variance and the larger range of CCL8 secretion observed.

Given recent studies demonstrating increased bacterial colonization (particularly by Proteobacteria) in asthmatic airways and the well-investigated role for allergic sensitization in both asthma pathogenesis and exacerbation, the work presented in this thesis aimed to further understand the role of these two factors on RV infections in the airway. Figure 4-2 demonstrates that the combination of allergen sensitization and LPS treatment have additive effects on RV-induced chemokine release from airway macrophages. CXCL10 and CXCL11 are associated with the anti-viral responses, while CCL2 and CCL8 are associated more with the recruitment of proinflammatory cells to the airway.\textsuperscript{11-13} These findings suggest that the combined effects of allergic sensitization and bacterial presence in the airway may further diminish the anti-viral responses in asthmatic airways, while increasing the proinflammatory response. Together, these data demonstrate a potential mechanism by which airway bacteria and allergens, commonplace in asthmatics, alter airway immune responses to RV infections leading to asthma exacerbations. This study portrays a more physiologically relevant understanding of what is occurring in the airways of asthmatics during RV infections and stresses the importance of furthering our understanding of the airways as multifactoral system.

\textit{Effects of bacterial stimulation on rhinovirus A016-induced chemokine secretion from primary human monocytes.}

Chapter Two demonstrates LPS attenuation of RV-induced CXCL10 and CXCL11
FIGURE 4-2

**CXCL10 Release (log pg/mL)**

- **A:**
  - D0
  - D2 (post-Ag)
  - D0 + LPS
  - D2 (post-Ag) + LPS

- **B:**
  - CXCL11 Release (log pg/mL)
  - p = 0.09

- **C:**
  - CCL2 Release (log pg/mL)
  - p = 0.09

- **D:**
  - CCL8 Release (log pg/mL)

Vehicle, A016, B014, A002
FIGURE 4-2.

Comparison of LPS effects on RV-induced chemokine secretion between D0 and D2 BAL macrophages. (A) CXCL10, (B) CXCL11, (C) CCL2, and (D) CCL8 secretion analyzed by ELISA. *D0*, pre-allergen challenged macrophages; *D2*, post-allergen challenged macrophages; *white*, D0 alone; *green*, D0 pretreated with LPS; *blue*, D2 alone (allergen challenged); *purple*, D2 (allergen challenged) pretreated with LPS. (*) p<0.05, (**) p<0.01, (***) p<0.001 by Student’s paired t-test. Data were log transformed to approximate a normal distribution and summarized as boxplots (whiskers: 10-90 percentile) from six independent experiments, except for D0 macrophages treated with LPS (*green*, n=4).
secretion from peripheral blood monocytes. On the other hand, LPS maintained or promoted RV-induced CCL2 and CCL8 secretion. LPS is a component of the cell wall of Gram negative bacteria; therefore, the next step in this study was to determine the effects of components from whole bacteria on RV-induced chemokine secretion. In order to expand upon the observations made in Chapter Two, I hypothesized that exposure to lysed bacterial components will alter RV-induced chemokine secretion in a similar manner as LPS.

*Acinetobacter lwoffii*, is a farm-associated Gram negative bacteria correlated with asthma protection characteristics in children, yet its effects during adulthood is not well studied. In order to test the hypothesis that bacterial exposure will alter monocytic cell responsiveness to RV A016 exposure in a similar fashion to *E. coli* LPS treatment, *A. lwoffii* lyophilized in PBS was sonicated in a water bath sonicator for 1 h, frozen (-80 °C) and thawed three times, heated at 95 °C for 10 m and then sonicated again for 1 h. This procedure was done in order to insure that the bacterial cells were no longer viable, as determined by lack of growth in liquid growth media. Nonviable cells were used to control for amount of bacterial products introduced into the monocyte culture and to avoid contamination by bacterial growth byproducts. Then primary human peripheral blood monocytes were treated with A016 (MOI=10) and increasing amounts of non-viable *A. lwoffii* (0-10⁶ CFU/mL) or *E. coli* LPS (100 ng/mL) and incubated at 34.5 °C for 24 h. The supernatants were collected and analyzed for the presence of CXCL10, CXCL11, CCL2, and CCL8 by ELISA (Figure 4-3). These preliminary data show a large induction of CXCL10 secretion from A016 treatment alone, while increasing amounts of *A. lwoffii* attenuated A016-induced CXCL10 release in a dose-dependent manner (Figure 4-3 A). In addition, *A. lwoffii* at a concentration of 10⁶ CFU/mL, which is within the physiologically relevant range of what airways can be exposed, shows similar effects on RV-induced CXCL10 release as *E. coli*
LPS. Due to the small sample size (n = 3), A016-induced CXCL11 and CCL2 secretion was not significantly increased above vehicle treated cells (Figure 4-3 B and C). However, the effects of *A. lwoffii* on RV-induced CXCL11 secretion are similar to its effects on RV-induced CXCL10 secretion, as *A. lwoffii* significantly diminished A016-induced CXCL11 secretion. On the other hand, *A. lwoffii* at $10^2$ CFU/mL significantly increased A016-induced CCL2 secretion compared, yet did not alter A016-induced CCL8 secretion at any of the concentrations of *A. lwoffii* tested (Figure 4-3 C and D).

Together, these observations demonstrate the effect of LPS on RV-induced chemokine secretion from monocytic cells coincides with the effect bacterial exposure has on monocytic cells. In addition, this suggests that exposure to either LPS or Gram-negative bacteria can alter monocytic cell responsiveness to RV infections. Additionally, these findings support the physiological relevance of understanding LPS as an airway immunomodulator. While these data are in agreement with the data presented in Chapter Two, these are preliminary results, which require further investigation. However, this study outlines a direction in which to proceed to further understand the effects of increased bacterial presence on RV-induced signaling in the airways.

*Further elucidation of underlying signaling mechanisms for LPS and allergy as immunomodulators.*

While the data presented in this thesis demonstrates that both bacterial products, specifically LPS, and allergic sensitization have similar effects on RV-induced chemokine secretion from primary human monocytic cells, the underlying mechanism that leads to altered chemokine secretion is not well understood. Chapter Two scratches at the surface of the
**FIGURE 4-3**

A. **CXCL10 Release (log pg/mL)**

- Vehicle
- A. lwoffii (CFU/mL): 10^2, 10^4, 10^6
- LPS 100 ng/mL

B. **CXCL11 Release (log pg/mL)**

- Vehicle
- A. lwoffii (CFU/mL): 10^2, 10^4, 10^6
- LPS 100 ng/mL

C. **CCL2 Release (log pg/mL)**

- Vehicle
- A. lwoffii (CFU/mL): 10^2, 10^4, 10^6
- LPS 100 ng/mL

D. **CCL8 Release (log pg/mL)**

- Vehicle
- A. lwoffii (CFU/mL): 10^2, 10^4, 10^6
- LPS 100 ng/mL

**Significance Levels**

- * p < 0.05
- ** p < 0.01
- *** p < 0.001
- # p < 0.05
- ## p < 0.01
- ### p < 0.001
- **p** 0.055

**Legend**

- □ Vehicle
- ■ A016
FIGURE 4-3.

Increasing doses of *A. lwoffii* modulate A016-induced chemokine secretion from primary human monocytes. Peripheral blood monocytes treated with A016 (MOI = 10) and either increasing amounts of *A. lwoffii* (0-10⁶ CFU/mL) or *E. coli* LPS (100 ng/mL) for 24 h and chemokine secretion was analyzed by ELISA. (A) CXCL10, (B) CXCL11, (C) CCL2, and (D) CCL8. *White bars*, RV vehicle treated; *black bars*, A016 treated. (#) p<0.05, (##) p<0.01, (###) p<0.001 A016 treated compared to respective *A. lwoffii/LPS* treatment alone by two-way repeated measures ANOVA (Bonferroni post-tests). (*) p<0.05, (**) p<0.01, (***) p<0.001 by Student’s paired t-test. Data displayed as bar graphs representing duplicate wells for each treatment and were log transformed to approximate a normal distribution. All values are means ± SEM from three independent experiments.
mechanism by which LPS alters RV-induced signaling, yet the potential for additional LPS modification of other RV signaling pathways is likely. While LPS potentiates RV-induced IFNα secretion, which is upstream of the JAK/STAT pathway, LPS diminishes RV-induced STAT1 phosphorylation. This suggests that LPS is either inhibiting RV-induced JAK phosphorylation or inhibiting the ability of JAK to phosphorylate STAT. Suppressor of cytokine signaling (SOCS) family of proteins directly inhibit the JAK/STAT pathway by either occupying the kinase site of JAK or by signaling JAK for proteasomal degradation. It has been shown that LPS can induce SOCS levels in macrophages and thus indirectly inhibit JAK/STAT signaling. Therefore, these data demonstrate a possible role for SOCS in LPS attenuation of RV-induced CXCL10 and CXCL11, as depicted in Figure 4-4.

In order to test the hypothesis that LPS increases RV-induced SOCS cellular levels, primary human peripheral blood monocytes were treated with A016 (MOI=10) and/or E. coli LPS (100 ng/mL) and incubated at 34.5 °C for 2-8 h. The cells were lysed, total RNA was extracted and SOCS1 and SOCS3 mRNA expression was measured by quantitative real-time PCR (Figure 4-5). Interestingly, this preliminary data demonstrates that LPS co-treatment with A016 increases A016-induced SOCS1 and SOCS3 mRNA expression levels above A016 treatment alone at all the time points tested (Figure 4-5). Therefore, this suggests that the exposure of cells to LPS and RV can increase cellular levels of SOCS proteins, which in return would diminish levels of phosphorylated STAT1. While LPS can induce a variety of signaling pathways initially, LPS can induce subsequent secondary pathways in human monocytes and macrophages (i.e., MAPK, NF-κB, IL-10, IFNβ, and TNFα), as previously elaborated in Chapter One. With this vast array of signaling cascades being initiated by LPS, it is important to further understand how they affect monocytic cell responsiveness to stimuli.
FIGURE 4-4.

Proposed mechanism for SOCS protein family in LPS attenuation of RV-induced CXCL10 and CXCL11 secretion. RV induces the secretion of interferon α (IFNα) that binds the type I IFN receptor (IFNRI) and leads to subsequent Janus activated kinase 1 (JAK1) and then signal transducer and activator of transcription 1 (STAT1) phosphorylation. Activated STAT1 is translocated into the nucleus and acts as a transcription factor for CXCL10 and CXCL11. LPS induces the transcription of suppressor of cytokine signaling (SOCS) family members, specifically SOCS1 and SOCS3, in a myeloid differentiation primary response protein 88 (MyD88) adaptor protein-dependent signaling mechanism. These SOCS proteins can directly interact with JAK1 and inhibit JAK1 ability to phosphorylate STAT1. By inhibiting the phosphorylation of STAT1, through the induction of SOCS proteins, LPS can inhibit RV-induced CXCL10 and CXCL11 transcription.
FIGURE 4-5

A

B

Vehicle

A016

LPS

LPS+A016
**FIGURE 4-5.**

LPS effects on A016-induced *SOCS1* and *SOCS3* mRNA in primary human monocytes.

Human peripheral blood monocytes co-treated with +/- A016 (MOI = 10) and +/- *E. coli* LPS (100 ng/mL) for 2-8 h and (A) *SOCS1* and (B) *SOCS3* mRNA induction were analyzed by qRT-PCR using SYBR Green Master Mix (SABiosciences). Data depicted as fold change relative to cells treated with the vehicle alone (2 h) using the comparative cycle threshold (ΔΔCT) method and values represent means ± SEM from three independent experiments. (*) p<0.05, (**) p<0.01, (*** p<0.001) LPS+A016 compared to A016 alone by Student’s paired t-test. Specific primers were designed using Primer Express 3.0 (Applied Biosystems); *SOCS1* (F-GCTGGCCCCTTTCTGTAGGAT, R-TGCTGTGGAGACTGCATTGTC) and *SOCS3* (F-GAGACTTTGATTCGGGACCAG, R- GAAACTTGCTGTGGGTTGACCA). Sequences were compared to the human genome to determine specificity using http://www.ncbi.nlm.nih.gov/tools/primer-blast. β-glucuronidase (*GUSB*; F-CAGGACCTGCGCACAAGAG, R-TCGCACAGCTGGGGTAAG) was used as a reference gene and to normalize the samples.
On the other hand, while allergen-virus interactions remain a focus point for several studies, not much is known about how allergic sensitization may alter RV-induced signaling in monocytic cells.\textsuperscript{19} A recent study by Durrani and colleagues demonstrated that children with allergic sensitization have more asthma exacerbations and lower respiratory tract illness.\textsuperscript{20} In addition, they found an inverse correlation between expression of a high-affinity IgE receptor on plasmacytoid dendritic cells, which are of monocytic cell lineage, and RV-induced IFNα and IFNλ1 secretion.\textsuperscript{20} This suggests a role for allergy sensitization in modulating innate immune responses important for viral clearance. One topic of debate in the field is whether asthmatics have impaired IFN responses in response to viral infections. Several studies suggest that allergic asthmatics have enhanced viral-induced IFN responses, yet others suggest that there is diminished IFN responses in atopic asthmatics. This controversy, along with gaps in understanding the interplay between allergens, bacteria, and viral infections, highlights the need for further investigation into the intra- and intercellular signaling cascades resulting from these types of exposures that may lead to asthma pathogenesis and exacerbation. Asthma therapeutics may be improved by expounding the fundamental mechanisms that control airway immunological responses to stimuli.

Understanding the role of CXCL10, CXCL11, CCL2, CCL8 in RV-induced asthma exacerbations.

Another key aspect to understanding the interplay of airway microbiome, allergen sensitization, and RV infections in airway immunology and asthma exacerbation is investigating how the differences in secretion levels of inflammatory mediators affect the overall immune response during viral-induced asthma. While the work demonstrated here describes LPS and
allergen challenge regulation of RV-induced chemokine levels, there is a gap in the knowledge of the field as to what effect this would have on inflammation, viral clearance, and asthma exacerbation. As previously described in Chapter One, CXCL10 and CXCL11 are chemokines for activated T cells and natural killer cells, while CCL2 and CCL8 attract more monocytic cells and eosinophils.\textsuperscript{3, 13} Although CXCL10 has been considered a strong candidate for a biomarker for RV-induced asthma exacerbations,\textsuperscript{21} there is little knowledge about what CXCL10 secretion indicates in regards to asthma and how altering its expression levels may play a role in asthma. On the other hand, CCL2 expression levels have been correlated to hyperresponsiveness and inflammation during RV infections\textsuperscript{22, 23}, it has been speculated that CXCL10 expression is linked to future immune responses to RV.\textsuperscript{23, 24} Currently the targets of these four chemokines and what cell type they may recruit have been delineated. However, it is unclear the effect of reduced or increased chemokine secretion levels on the recruitment and activation of airway immune cells and what this effect will have on asthma and viral clearance. Therefore, further understanding the airway milieu during RV, bacteria, and allergen exposure is crucial in understanding asthma exacerbations.
REFERENCES


