A MURINE MODEL OF GENETIC DETERMINANTS OF RSV DISEASE

Monica High

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Approved by:

David Peden, MD

Farhad Imani, PhD

Ilona Jaspers, PhD

Steven Kleeberger, PhD

Tim Wiltshire, PhD

ABSTRACT

Monica High
A murine model of genetic determinants of RSV disease
(Under the direction of Steven Kleeberger)

Respiratory Syncytial Virus (RSV) is the leading cause of lower respiratory tract infection (LRTI) in infants. Approximately 70% of infants are infected with RSV within the first year of life. Epidemiological studies show that individuals have varying severity of RSV disease, ranging from "cold-like" symptoms to death. Previous *in vivo* studies have suggested that RSV susceptibility is a polygenic trait; however, the specific genes regulating RSV disease have yet to be identified. The objective of this dissertation was to identify candidate genes that regulate differences in RSV disease severity between inbred mouse strains.

Thirty-six inbred mouse strains were infected with a single dose of 1×10^6 plaque forming units of RSV or control and sacrificed 1 and 5 days post-infection (pi). Inflammatory response, lung permeability, pathology, mucus cell metaplasia, and viral load were analyzed and compared among and within all inbred strains. Using *in silico* haplotype association mapping, the degree of correlation between the observed phenotypic differences and the genotype of the inbred strains was used to determine candidate QTLs.

RSV disease phenotypes were distributed continuously across inbred strains. Furthermore, the strain distribution patterns varied for each phenotype and time point, suggesting that multiple mechanisms influence RSV susceptibility. Correlation analysis of phenotype relatedness also suggests that the response to RSV at 1 and 5 days pi are independent.

Gene expression was minimally altered by RSV infection in "non-responsive" strains (e.g. C3H/HeJ); however, gene expression did vary significantly as a result of RSV infection in responsive inbred strains (e.g. BALB/cByJ). In responsive strains the most significant change in gene expression was noted 1 day following infection when a significant number of genes were either up-regulated or down-regulated. It was determined that genes involved in antigen presentation, infection mechanisms, and inflammatory response pathways were differentially activated between responsive and non-responsive strains. Based on the expression profile and biological plausibility a few of these genes (e.g. *Marco, Tlr4, Stat4, Mx1, Ccl12, Cx3cr1*) were identified as candidate genes for response to RSV infection. *In vivo* proof-of-principle investigations confirmed the role of *Cx3cr1* and *Marco* as candidate genes for RSV susceptibility and disease.

To my parents who always believed in me.

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LIST OF ABBREVIATIONS

BAL..Bronchoalveolar Lavage

HAM .... Haplotype Association Mapping

MCM.. Mucus Cell Metaplasia

PMN...Polymorphonuclear Leukocyte

RSV..Respiratory Syncytial Virus

SNP ....Single Nucleotide Polymorphism

CHAPTER 1

INTRODUCTION

Oxidative Stress and Lung Disease

Oxidative stress results from an oxidant/ antioxidant imbalance in favor of oxidants, which results in a process generating reactive oxygen species (ROS). ROS are highly reactive molecules with unpaired electrons, capable of causing oxidation (1). Common ROS include superoxide anion, hydrogen peroxide, and hydroxyl radicals. Biological systems are constantly challenged by endogenous ROS or ROS formed from a metabolic process (2). Due to their reactive nature ROS may cause damage to lipids, proteins, and DNA, in addition to inducing numerous cellular responses (3). Since the lung epithelium is exposed directly to the environment it is exposed to high levels of oxygen, oxidant gases, and particulate matter, it is especially vulnerable to ROS mediated injury. There is accumulating evidence that oxidative stress and ROS plays a role in the pathogenesis of asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), pulmonary fibrosis, lung cancer, acute lung injury (ALI) (4-6).

Oxidative Stress and Asthma

Asthma is a chronic inflammatory disease of the airways that is characterized by airway eosinophilia, goblet cell hyperplasia, and airway hyperresponsiveness (AHR) in response to inhaled allergens (7, 8). It is widely agreed that inflammation, which is characteristic of asthma results in increased oxidative stress and ROS production in the airways (9). Alveolar macrophages, eosinophils, and neutrophils of asthmatics have increased O_2 release and ROS production compared to healthy individuals (10, 11). This increase leads to increased hydrogen peroxide, 8-isoprostane and CO in breath condensates, increased glutathione peroxidase and superoxide dismutase in lung cells, and elevated levels of eosinophil peroxidase and myeloperoxidse in the peripheral blood, sputum and bronchoalveolar lavage fluid (BALF) in patients (12-14). These ROS contribute to airway smooth muscle contraction, β-adrenergic receptor dysfunction, loss of function of epithelial cilia, increased mucus production, an increase of inflammatory

cells and amplified vascular permeability (15). β-adrenergic receptor dysfunction leads to an increase in vagal tone and H_2O_2 production initiates airway smooth muscle contraction, both resulting in AHR in animal models (16, 17). Previous studies indicate that ROS also prompt mast cells to release histamine, and cause epithelial cells to secrete mucus (18). Continuous exposure to ROS will cause oxidative damage and epithelial cell sloughing (19). Once this barrier is damaged and unable to function properly the lung is vulnerable to permeability to fluid, macromolecules and inflammatory cells.

Oxidative Stress and Acute Lung Injury

Acute lung injury (ALI) is a disease characterized by diffuse inflammation of the lung parenchyma resulting in a sudden reduction in gas exchange, static compliance and non-hydrostatic pulmonary edema (15, 20). The pathogenesis of ALI is not completely understood; however, oxidant-mediated tissue injury is believed to be an important factor in the pathogenesis of ALI. ROS are believed to play an important role in pulmonary vascular endothelial damage, which is hypothesized to be responsible for the signs and symptoms of ALI. Patients with ALI have elevated levels of hydrogen peroxide in exhaled air and urine. In addition to enhanced ROS production, diminished antioxidant levels are also linked to ALI pathogenesis (21, 22). For example, decreased levels of GSH, vitamins C and E, endogenous scavenger of ROS, have been observed in detected in the alveolar fluid of patients with ALI (23). Exposure to ozone (O_3) is a widely accepted animal model for ALI. Under this circumstance ROS are generated as a result of neutrophil and macrophage activation (24).

In response to inflammatory stimuli, lung endothelial cells, alveolar cells, airway epithelial cells and activated macrophages can indirectly mediate production of ROSs such as NO and O_2^- (25). These compounds form peroxynitrite, which nitrates and oxidizes key amino acids in various lung proteins, resulting in modified proteins and inhibits their function. Modified proteins have been detected *ex vivo* in samples from

patients with ALI. Expression of inflammatory cytokines, chemokines and adhesion molecules are elevated during ALI, and may result in ROS formation. Studies have suggested that high concentrations of TNF-α and IL-1β are detected in the BALF of patients suffering from ALI (26).

Ozone (O₃)-Induced Oxidative Stress and Lung Injury

Ozone (O_3) is an allotropic form of O_2, which is more reactive, less stable and decomposes more readily than O_2. O_3 in ambient air is attributed to intrusion of stratospheric O_3 or through a photochemical reaction requiring reactive hydrocarbons, sunlight and nitrogen dioxide (NO_2). NO_2 is a major component of air pollution, produced mainly by motor vehicles. In the troposphere, NO_2 absorbs longer wavelength UV light, which leads to the following reactions:

$$NO_2 + UV\ photons \rightarrow O + NO$$

$$O + O_2 \rightarrow O_3$$

$$O_3 + NO \rightarrow NO_2 + O_2$$
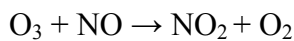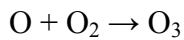
Clinical studies found that increases in ambient outdoor O_3 levels were associated with exacerbation of respiratory disease and excess mortality in some populations (27-29). Exposure to O_3 causes airway inflammation, airway hyperresponsiveness, and overall decreased lung function (30-32). In controlled human studies O_3 inhalation increases the airway levels of inflammatory mediators (e.g IL-8, GRO-α, ICAM-1), and depletes levels of protective antioxidants (e.g. ascorbic acid, glutathione peroxidase) in bronchoalveolar lavage fluid (BALF) (30, 33-35).

Even though studies have determined that certain inflammatory mediators regulate host response to O_3, the exact mechanism by which O_3 mediates these effects is mostly unknown, but may involve the generation of ROS such as H_2O_2, NO and hydroxyl radicals (18, 36-39). O_3 reacts with lipids in the epithelial lining fluid, producing aldehydes and hydroperoxides which initiate a cascade of events leading to the release of

chemokines from the epithelium that recruit inflammatory cells into the airways (40-42). The responding inflammatory cells release ROS which are believed to contribute to the lung injury normally found following O_3 exposure (43-45). Antioxidants such as ascorbic acid, are scavengers of O_3 and NO_2, and protect the lung from the damaging effects of O_3 (46).

Genetic aspect of O_3 susceptibility

While earlier studies suggest that O_3 mediates respiratory disease, later research found that these effects differ between individuals. Certain sub-sets of the population including the elderly, immunocompromised, and individuals with pre-existing respiratory disease are more susceptible to O_3 (47, 48). Besides these factors additional evidence suggests that the genetic background of the individual influences the disease response (24, 49). In humans, gene polymorphisms have been associated with susceptibility to many environmental agents such as pesticides and particulate matter. Currently there are many ongoing studies aimed at identifying genes believed to be associated with O_3 susceptibility.

Approaches to identifying candidate genes

Two approaches can be used to identify genes that regulate susceptibility (50-52). The first and most common method is the candidate gene approach, in which genes are chosen *a priori* as likely mechanisms that influence the phenotype of interest. Association is assessed between the phenotype of interest and polymorphisms within the candidate gene(s). This approach is effective at identifying genes that regulate the phenotype of interest. However, since it does not examine the entire genome it is unable to identify all the genes that affect the phenotype. Furthermore this approach fails to take gene-gene interaction into account (53). The second approach is a genome scan (positional cloning). Similar to the candidate gene approach this method associates gene

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expression and polymorphisms with the phenotype of interest. However, unlike the candidate gene approach a genome-wide screen is used to identify linkage to any chromosomal interval throughout the entire genome between two or more differentially responsive strains. This approach requires no prior knowledge of gene function and has the potential to discover novel disease genes.

CX3CR1 as a candidate gene

CX3CR1 is the only member of the CX3C family of chemokines receptors, and is the primary receptor for fractalkine (CX3CL1). Membrane-bound CX3CR1-fractalkine interaction regulates adhesion of monocytes, macrophages, natural killer (NK) cells and T lymphocytes (54). Soluble fractalkine exhibits efficient chemotactic activity for monocytes, macrophages, NK cells, and T lymphocytes expressing CX3CR1 (54-57). This ligand-receptor complex is very diverse, regulating both cell-cell interaction and cell migration. In the years since it was identified (1997), CX3CR1 expression and activation has been associated with inflammation, and the progression of many diseases. The role of *CX3CR1* in carotid cardiovascular disease is well characterized. Single nucleotide polymorphisms (SNPs) of *CX3CR1* are linked to attenuation of cardiovascular disease (58-60), and additional studies found that polymorphisms within *CX3CR1* are associated with resistance to HIV-1 infection (61-63). In spite of all that is known about the function *CX3CR1* and its role in disease progression, very little in known about how it regulates pulmonary inflammation and injury. A previous study investigated the role *CX3CR1* plays in lung injury. Investigators found that polymorphisms in *CX3CR1*associated with decreased incidence of CWP. This group concluded that the chemokine receptor *CX3CR1* plays a role in lung injury resulting from chronic exposure to coal dust (64). However, the role of *CX3CR1* in lung injury following subacute exposure is still unknown. While no evidence exists that *CX3CR1* promotes pulmonary responses to acute oxidant exposures, this gene is expressed in the lung and modulates

chronic inflammation (e.g. CWP, emphysema), and therefore might influence the initiation or development of inflammatory responses in that organ (64, 65). Based on what is known about *CX3CR1* it is a promising candidate gene for susceptibility to O_3-induced inflammation.

Objective of Chapter 2

The overall objective of the studies described in Chapter 2 was to test the hypothesis that *Cx3cr1* contributes to O_3-induced inflammation in the mouse, and to investigate the mechanisms through which *Cx3cr1* modulates the effect.

Virus-induced oxidative stress and lung injury

Respiratory Syncytial Virus

RSV is an enveloped ssRNA virus in the Paramyxovirus family. The viral genome of RSV encodes 8 structural and 2 non-structural proteins. Important structural proteins include the fusion (F) protein and the attachment (G) protein (66, 67). These two proteins are essential for viral penetration and attachment to host cells, and both are important in the development of immune responses and producing protective antibodies. The G protein regulates viral attachment, and the F protein mediates viral penetration and syncytium formation (68).

RSV replication follows specific events. The virus attaches to the host cell using the G protein, and the viral envelope fuses with the plasma membrane through the F protein. Following penetration, the viral nucleocapsid is released into the cellular cytoplasm. The viral RNA is a template for mRNA, which is a template for translation of viral proteins and cDNA is a template for transcription of virion RNA. This is a complex process that can only occur inside of the bronchopulmonary tree and viral infection is restricted to the respiratory mucosa.

Epidemiology and clinical impact

Respiratory syncytial virus (RSV) is the primary cause for hospitalization during the first year of life, and is the leading cause of bronchiolitis, pneumonia, mechanical ventilation and respiratory failure in infants worldwide (69, 70). RSV infection is also associated with the development of childhood asthma, and recurrent wheezing up to 7 years old (71-73). In the United States alone, more than 100,000 infants are hospitalized with RSV-induced bronchiolitis each year (74). Hospitalization rates are the highest among premature infants, and those with compromised immune systems. Throughout the world approximately 100% of children are infected with RSV by their second birthday,

and over half of these children have already experienced reinfection (75). RSV "season" usually correlates with flu season and cases in the United States peak in January or February (76). RSV is highly virulent and only a small percentage of infections are completely asymptomatic. While the majority of infected individuals present symptoms, the nature and severity of the symptoms vary among individuals. For some infants infection induces cold-like symptoms, others require hospitalization, and a few cases actually result in death. In most infants RSV causes an upper respiratory tract infect (URTI), which is followed by a mild lower respiratory tract infection (LRTI) in about 40% of cases. Viral LRTI are particularly serious in infants, because the lungs are still adapting to the extrauterine environment (77). In humans, signs of URTI include tachnypea, hyperinflation, crackles and expiratory wheezing (75). RSV infection usually subsides after a week. In general very few "healthy" suffer from life threatening RSV infection, and hospitalization and deaths are confined to infants who have preexisting cardiopulmonary disease or those who are immunocompromised (70, 78). Epidemiology studies found that the average age of onset is 13 months; the most severe cases are usually found in infants between 8 and 30 weeks old (70, 78, 79). The highest mortality risk for RSV is among infants younger than 6 months (80).

Children are not the only population at risk to RSV infection; RSV is also a significant cause of respiratory illness in immunocompromised adults and the elderly (81, 82). Among these populations RSV is responsible for 10.6% of pneumonia cases, 11.4% of chronic obstructive pulmonary disease (COPD) hospitalizations, 7.2% of hospitalizations related to asthma, and 5.4% of congestive heart failure cases (81, 83). The wide spread occurrence of RSV infection has fueled the urgency of research for prophylactic therapies. In the 1960's Chanock and Parrott developed a vaccine containing formalin-inactivated RSV (FI-RSV). This vaccine was administered to 141 children in the Washington, DC area. Nine months after vaccination approximately 60 of the 141 children presented symptoms of RSV infection. Not only did the vaccine fail to

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protect the children from infection, it also caused these children to experience an exacerbated form of the disease and was the cause of at least 4 infant deaths (68, 84, 85). The exact reason for enhanced disease is still not known. Despite the vast amount of research aimed at RSV infection, the complexities of the disease and its phenotypes cause the specific pathogenic mechanism to remain a mystery. Without some insight into the pathogenesis of primary infection effective vaccines and efficacious post-infection therapies are not currently available.

Respiratory Syncytial Virus and the immune response

Over the years nearly all components of the immune system have been implicated in the pathogenesis of RSV infection, including inflammatory cells and their products such as chemokines, cytokines, and soluble inflammatory mediators. Currently there is controversy surrounding the role all of these components play in the pathogenesis of RSV infection; specifically, whether RSV-specific antibodies produce disease enhancing effects (75, 85-89). Some studies show that infants with high titers of transplacentally acquired antibodies are less likely to develop severe bronchiolitis, and pre-treatment with RSV-intravenous immunoglobulin and monoclonal antibodies mitigates RSV infection, suggesting that RSV-specific antibodies may have a protective role against infection (90, 91). However, elevated levels of RSV-specific IgE antibodies were detected in individuals with severe RSV infection, compared to those with milder forms of the disease. These elevated levels suggest that the immune response to RSV infection predisposes the child to wheezing. Epidemiology studies found that asthma and allergic sensitization were both significantly higher in children previously infected with RSV (71, 72, 77, 92-94). However, there is still controversy on whether RSV is the actual cause of allergy or if genetic predisposition to allergy increases the child's susceptibility to RSV infection.

As mentioned previously, immunocompromised individuals are at greater risk for developing severe complications from RSV infection, suggesting that the cellular immune response is essential in terminating the infection. Several studies show depressed levels of CD8+ T cells in the peripheral blood children with RSV bronchiolitis, confirming that the defect in the immune system is regulated by CD8+ cells or redistribution of CD8+ cells to the lungs (95-98). In mice, investigators found extensive redistribution of CD8+ cells toward the lungs from peripheral blood during RSV infection. Response to RSV infection is widely believed to be mediated by a balance between Th1 and Th2 cells (99, 100). Th1 cells produce interferon gamma (IFNγ) and interleukin 2 (IL-2), and are generally observed in infectious disease processes involving microorganisms and delayed hypersensitivity. A Th2 response is associated with parasitic infections and allergic diseases such as asthma; these cells secrete IL-4, IL-5, IL-10 and IL-13, cytokines that induce specific immunoglobulin production by B cells. Recent studies have found associations with all the aforementioned cytokines and their receptors to either RSV susceptibility or immune response (88, 93, 101-108). IL-4 is known to have a chemotactic effect on eosinophils, and IL-5 stimulates eosinophil proliferation, which leads to eosinophilia in RSV infected individuals. In mice, distinct patterns of cytokine production are observed after RSV challenge following priming with either G or F RSV surface proteins. Mice treated with G-specific cells exhibited a severe form of RSV characterized by significant eosinophilia. Conversely, F-specific cells only exhibited only a slight increase on pulmonary inflammation, and eosinophilia was not observed. These finding suggest that RSV bronchiolitis results from Th2 response, and Th1 response is responsible for the non-wheezing illnesses associated with RSV. RSV reduces IFNγ production, and IL-4 production is reduced to a lesser degree, resulting in a significantly increased IL-4/ IFNγ ratio compared to controls (109). Because IFNγ levels were affected more than IL-4 it is believed that RSV infection favors a Th2 profile.

IFNγ, which is produced by natural killer cells, has antiviral activity and promotes cell-mediated immune responses to viruses. High levels of this cytokine can be found in mice in the early stages of viral infection (108). Elevated IFNγ is detected in BAL from BALB/c as early as 1 day post-infection, and peak levels are detected once viral clearance is near completion, and lung function abnormalities are at their maximum (roughly 6 days after infection) (108). While RSV induces IFNγ secretion in mice it is not a potent inducer in humans. However, treatment with anti- IFNγ reduced airway hyperresponsiveness, but has no effect on eosinophilia (109). Receptors for IFNγ are found on eosinophils and activation of IFNγ is believed to initiate eosinophilia associated with RSV infection.

Respiratory Syncytial Virus and Oxidative Stress

Previous studies suggest that RSV infection leads to oxidative stress, which contributes to alveolar epithelial cell injury (110-112). As mentioned previously IFNγ is believed to contribute the RSV-induced immune response. As part of the Th1 response IFNγ causes lymphocyte migration and nitric oxide (NO) synthase induction, resulting in ROS formation and epithelial cell damage (108, 113).

Previous studies indicate that the transcription factor NF-E2 like 2 (Nrf2) has a protective effect against oxidative stress, and ultimately the disease phenotypes (114). Nrf2 was originally identified in a screen for proteins that interact with the control region of β-globin gene. Nrf2 is found in almost all tissues in the body, but is present in high concentrations in tissues where detoxification occurs regularly, such as the lung (110). Cho *et al.* found that *Nrf2*$^{-/-}$ mice were at significantly greater risk for RSV disease phenotypes, including pulmonary inflammation, oxidative stress, and mucus cell metaplasia, compared to *Nrf2*$^{+/+}$ mice (110). Furthermore, antioxidant and other protective enzymes normally upregulated by RSV were inhibited in *Nrf2*$^{-/-}$ mice relative

to $Nrf2^{+/+}$ mice. These studies were consistent with a role for Nrf2-mediated defense against RSV-induced oxidative stress and disease.

Respiratory Syncytial Virus and Genetic Susceptibility

As previously mentioned the severity and nature of RSV infection varies between individuals. Effects can range from asymptomatic to death, with the majority of cases presenting mild upper respiratory tract infection (URTI) with cold-like symptoms. Viral strain differences play a minor role in this variation (115-119); and studies indicate that environmental factors, age and gender may contribute to susceptibility of RSV infection (94, 120-122). However, genetic variation of the host is believed to be the greatest influence on response and susceptibility to RSV. The genetics of RSV susceptibility and immune response has been heavily investigated using the candidate gene approach. Using this technique many genes were found to be associated with RSV infection, including *CCR5* (C-C chemokines receptor type 5), *Il8* (Interleukin-8), *IL4* (Interleukin-4), *IL9* (Interleukin-9), *IL10* (Interleukin-10), *TNFα* (Tumor necrosis factor-α) and *TLR4* (Toll-like recptor 4) (93, 103, 123-128). One gene that is well characterized in respects to RSV infection is *Tlr4*. In various studies C3H/HeJ mice, which have a *Tlr4* mutation, were found to be resistant and relatively non-responsive to RSV infection (129, 130); C3H/HeOuJ mice have a fully functional *Tlr4* gene and present severe pulmonary inflammation after infection. Because the only difference between these two strains is *Tlr4*, it is believed that *Tlr4* is responsible for RSV disease phenotypes in mice. A number of studies have found that polymorphisms in *TLR4* are associated with RSV disease severity in humans, though the mechanism(s) though which this effect occurs is not understood (127, 131-133).

Studies using inbred mouse strains have confirmed that susceptibility to infection is influenced by genetic background of the individual. Furthermore, *in vivo* and epidemiology studies indicate that susceptibility to RSV is multigenic (129, 130, 134,

135). Further *in vivo* studies using either recombinant inbred strains or F1 progeny found that susceptibility to RSV infection is a heritable trait (130, 135). Recently the positional cloning approach has linked a variety of genes to RSV susceptibility (135). This approach revealed that initial response to RSV primarily involves early antiviral defense changes, including genes associated with T-cell differentiation, antigen presentation, innate immune response, and early response to virus. For example, essential transcription factors responsible for virus-induced transcription such as *Stat1* (Signal Transducers and Activator of Transcription 1)*, Stat2* ((Signal Transducers and Activator of Transcription 2), and chemokines *Cxcl10* (C-X-C motif ligand 10)*, Ccl1* (C-C ligand 1)*, Ccl4* (C-C ligand 4) were associated with RSV susceptibility. Associations were also found with antiviral genes including *Adar* (adenosine deaminase, RNA-specific)*, Mx1* (myxovirus resistance 1)*, Mx2* (myxovirus resistance 2)*, Oas1a* (2'-5' oligoadenylate synthetase 1A)*, and *Tyki* (thymidylate kinase) (135). This study only investigated associations between genetic markers and susceptibility to RSV, which was determined by viral titers. Due to its complexity the investigators did not attempt to determine the genes that regulate response to RSV, and to date no study has used positional cloning to identify the genes responsible for RSV-induced disease phenotypes. Despite the amount of research dedicated to identifying the gene(s) responsible for RSV susceptibility and response, the exact cascade of events remains unknown.

Objective of Chapters 3 - 6

Considering that RSV bronchiolitis is the leading cause of infant hospitalizations worldwide, and 100% of the population will be infected with RSV at some point in their life, it is important to identify the genetic profiles that cause some individuals to be more susceptible than others. If the exact genetic profiles were known, specific and more effective prophylactic treatments could be developed. Therefore, the overall objective of Chapters 3 and 4 were to, first, characterize RSV disease phenotypes across multiple

inbred strains of mice to model variation in human disease and, second, use positional cloning to identifying the genes that regulate response to RSV infection.

Overall Objective of the Dissertation

Oxidant stress and inflammation are common characteristics of pulmonary responses to air pollutant and viral exposures. The overall objective of this project was two-fold. The first objective was to utilize a multi-disciplinary approach to investigate the role of a candidate susceptibility gene (*Cx3cr1*) in response to ozone and, subsequently, to RSV. The second objective was to utilize a positional cloning approach to identify genes that contribute to RSV disease phenotypes. It was hypothesized that these studies would identify mechanisms related to oxidative stress and inflammation may be common to ozone and RSV disease.

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CHAPTER 2

Cx3cr1 CONTRIBUTES TO PULMONARY INFLAMMATION IN THE MOUSE

Abstract

Background. Exposure to ozone (O_3) causes airway inflammation, lung hyperpermeability, and epithelial cell injury. Enhanced expression of the chemokine receptor *Cx3cr1* increases leukocyte adhesion, and interleukin-17 (IL-17) is linked to neutrophilic mobilization, potentially via CX3CR1. This study tested the hypothesis that *Cx3cr1* contributes to O_3-induced pulmonary inflammation, and that IL-17 is an effector mechanism.

Methods: $Cx3cr1^{+/-}$, $Cx3cr1^{-/-}$, $Il17ra^{+/+}$ and $Il17ra^{-/-}$ mice were exposed to air or 0.3 ppm O_3 for 24, 48 or 72 hours. Bronchoalveolar lavage (BAL) fluid analyzed for total protein (a marker of lung permeability) and cell differentials were used to evaluate inflammation. Pulmonary PCNA levels were measured by western blot analysis, and immunohistochemistry. Cytokine IL-17A expression in lung homogenate was analyzed by ELISA.

Results: Significantly greater BAL protein content and numbers of inflammatory cells were found in O_3-exposed $Cx3cr1^{+/-}$ mice compared to $Cx3cr1^{-/-}$ mice. O_3-induced IL-17R mRNA expression was significantly elevated in $Cx3cr1^{+/-}$ mice compared to $Cx3cr1^{-/-}$. Further, significantly greater numbers of neutrophils were found in $Il17ra^{+/+}$ mice compared to $Il17ra^{-/-}$ mice after O_3.

Conclusions: Results of this study are consistent with the hypothesis that *Cx3cr1* and *IL17ra* contribute to the pulmonary inflammatory response to O_3. Moreover, results suggest that *Cx3cr1* may have a regulatory role in IL-17 expression. The findings of this investigation provide novel insight to mechanisms of pulmonary inflammation induced by environmental oxidants.

Introduction

Air pollution is a heterogeneous mixture of particles such as aldehydes, peroxyacylnitrites, alkyl nitrites, and photochemical by-products including ozone (O_3). Ozone causes respiratory neutrophilic inflammation, airway hyperactivity, epithelial cell injury, and microvascular permeability. Individuals with pre-existing inflammatory lung disease such as asthma are particularly vulnerable to O_3 and are at risk for exacerbations (1). However the precise molecular events that cause the inflammatory response seen after O_3 exposure are not completely understood.

A number of studies have focused on the possible roles of chemokines/cytokines such as macrophage inflammatory protein-2 (MIP-2), interleukin-8 (IL-8), and RANTES, in the pathogenesis of O_3-induced lung inflammation and injury (2-6). *Cx3cr1* is known to mediate both leukocyte migration and adhesion (7). Recent studies suggest that *Cx3cr1* has a role in atherosclerosis, rheumatoid arthritis, coal workers' pneumoconiosis (CWP), and microglial neurotoxicity (8-10). Polymorphisms within this gene have also been linked to rapid progression of AIDS in HIV-positive individuals (11).

A previous study investigated the role *Cx3cr1* plays in lung injury. Investigators found that polymorphisms in *Cx3cr1* associated with decreased incidence of CWP. This group concluded that the chemokine receptor *Cx3cr1* plays a role in lung injury resulting from chronic exposure to coal dust (9). However, the role of *Cx3cr1* in lung injury following subacute exposure is still unknown. While no evidence exists that *Cx3cr1* promotes pulmonary responses to acute oxidant exposures, this gene is expressed in the lung and modulates chronic inflammation (e.g. CWP, emphysema), and therefore might influence the initiation or development of inflammatory responses in that organ (9, 12).

Increased expression of MIP-2, IL-1β, TNF-α and neutrophilia are markers of O_3-induced inflammation (5, 13, 14). Regulation of these events in other disease models has been linked to the chemotractant interleukin-17A (IL-17A) (15-17). IL-17A is secreted

by activated memory T cells and binds specifically to homodimeric receptor IL-17R. Recent studies found that IL-17R-deficient mice are unable to recruit neutrophils to the lung following insult (17). Earlier studies found that expression of CX3CR1 and IL-17R was associated with pulmonary inflammation and injury, however it is not known if CX3CR1 and IL-17R are co-dependent during pulmonary inflammation (15, 18, 19). In the present study we tested the hypothesis that *Cx3cr1* contributes to O_3-induced pulmonary inflammation and injury, and does so through upregulation of IL-17R.

Material and Methods

Animal and Inhalation Exposure

Male C57BL/6J$^{Cx3cr1+/-}$ and C57BL/6J$^{Cx3cr1-/-}$ mice (6-9 wk) were obtained from European Mouse Mutant Archive (EMMA) (Munich, Germany). Due to availability C57BL/6J$^{Cx3cr1+/-}$ mice were used as genotype controls instead of C57BL/6J$^{Cx3cr1+/+}$ mice, preliminary data suggest that both genotypes had a similar pulmonary inflammatory response to O_3. In a separate study male C57BL/6J$^{Il17ra+/+}$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and C57BL/6J$^{Il17ra+/-}$ (12-14 wk) mice were obtained from Taconic Farms Inc (Germantown, NY, USA) by permission from Amgen Inc (Thousands Oaks, CA, USA). Inhalation exposures were done in a barrier facility at Alion Science and Technology, Inc, Research Triangle Park, NC. Mice were placed in individual stainless-steel wire cages within a Hazelton 1000 chamber (Lab Products, Maywood, NJ) equipped with a charcoal and high-efficiency particulate air–filtered air supply. Mice were exposed to 0.3-ppm O_3 or filtered air for 24, 48 or 72 hr (23.5 hr/day) as described previously (20). All animals use was approved by the National Institute of Environmental Health Sciences Animal Care and Use Committee.

Lung Bronchoalveolar Lavage Fluid (BALF) and Cell Preparation

Immediately after 24, 48 or 72 hr exposure to O_3 or air, mice were euthanized with sodium pentobarbital (104 mg/kg), BAL was performed. BAL cell differentials and total protein concentration (a marker of lung permeability) were determined as previously described (20), The three time points chosen for evaluation of inflammation and injury were based on previous investigations that characterized the kinetics of inflammation and injury responses to continuous exposure to 0.3 ppm O_3.

Lung Tissue Preparation for Histopathology and Immunohistochemistry

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Left lung lobes were fixed with 10% neutral buffered zinc formalin for 24 hrs. Proximal and distal intrapulmonary axial tissues were excised and embedded in paraffin and sectioned (5 μm). Tissue sections were histochemically stained with hemotoxylin and eosin (H&E) for comparison of pathology and injury between genotypes. The terminal bronchioles and alveoli were the primary focus of study because after 48 hrs of exposure to 0.3 ppm O_3 causes inflammation and epithelial cell sloughing in these regions of the mouse lung.

Western Blot Analysis

PCNA expression was detected in nuclear protein (20 μg) using an anti-PCNA antibody (sc-56) in the right lungs from mice exposed to O_3 or air was assessed as previously described (20).

Cytokine Enzyme-linked Immunosorbent (ELISA) Measurement

Immunoreactive macrophage inflammatory protein (MIP-2), interleukin-1β (IL-1β), and interleukin-17A (IL-17A) were quantified in aliquots of BALF (50 μl) using commercially available ELISA kits (R&D Systems), according to the manufacturer's instructions. Each cytokine quantity was calculated from absorbance at 450 nm using a standard curve.

Quantitative Polymerase Chain Reaction (qPCR)

Total RNA was isolated from non-lavaged left lung pieces RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's procedure. One μg of total RNA was reverse transcribed into cDNA. For quantitative PCR, an aliquot (1-2.5 μl) of cDNA was amplified in a 25 μl reaction containing 12.5 μl 2X PowerSYBR Green Master Mix (Applied Biosystems, Foster City, CA) and 250 nM of each gene-specific forward and reverse primer by 10 minute hold at 95°C and 45 cycles at 95°C (15 seconds) -60°C (1

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minute) using the Applied Biosystems StepOne Plus Real-Time PCR Systems. Quantification of gene expression was determined by ΔC_T values obtained by subtracting fluorescence detected number of cycles to threshold (C_T) of 18s mRNA from those of target gene RNA in the same sample.

Statistics

Data were expressed as the mean ± SEM. Two-way analysis of variance (ANOVA) was used to evaluate the effects of exposure and genotype in all experiments. All statistical analyses were performed using Sigma-Stat software program (SPSS, INC. Chicago, IL).

Results

Cx3cr1 effect on O_3-induced pulmonary inflammation and injury

The role of *Cx3cr1* on O_3-induced pulmonary inflammation was examined by exposing continuously *Cx3cr1*$^{+/-}$ and *Cx3cr1*$^{-/-}$ mice to air or O_3 for 24, 48 and 72 hr. Significant differences in BAL protein concentration and cell numbers were found between genotypes following air exposure (Figure 2.1A). Relative to respective air controls, 48 and 72 hr O_3 caused significant increases in mean numbers of BAL macrophages, polymorphonuclear leukocytes (PMNs), epithelial cells, and lymphocytes in *Cx3cr1*$^{+/-}$ and *Cx3cr1*$^{-/-}$ mice. However, numbers of all cell types were significantly greater in *Cx3cr1*$^{+/-}$ mice compared to *Cx3cr1*$^{-/-}$ (Figure 2.1). O_3 caused significant increases in total BAL protein in *Cx3cr1*$^{+/-}$ (24, 48, and 72 hr) and *Cx3cr1*$^{-/-}$ (48 and 72 hr) mice (Figure 2.1B), but no significant differences in mean total protein were found between genotypes at any time points following O_3.

H&E stained lung sections from *Cx3cr1*$^{+/-}$ and *Cx3cr1*$^{-/-}$ air controls were normal, and no histological differences were found between them. Significant peribronchiolar inflammation, cellular proliferation, and epithelial hyperplasia were found in terminal bronchioles of *Cx3cr1*$^{+/-}$ and *Cx3cr1*$^{-/-}$ mice after 48 hr O_3 (Figure 2.2). However, O_3-induced injury in the terminal bronchioles was markedly attenuated in *Cx3cr1*$^{-/-}$ mice compared to *Cx3cr1*$^{+/-}$. Furthermore, O_3 enhanced nuclear PCNA expression (an indicator of cellular proliferation) in lung homogenates of both genotypes in an exposure-dependent manner after 48 and 72 hr (Figure 2.3A). However, PCNA levels were significantly attenuated in *Cx3cr1*$^{-/-}$ mice compared with *Cx3cr1*$^{+/-}$ mice after O_3 (Figure 2.3A). Immunostaining for PCNA also indicated that PCNA expression was enhanced in *Cx3cr1*$^{+/-}$ mice relative to exposure-matched *Cx3cr1*$^{-/-}$ mice (Figure 2.3B).

Influence of *Cx3cr1* on O_3-induced chemokine expression

To investigate the possible mechanisms through which *Cx3cr1* modulates pulmonary PMN inflammation, we measured BAL levels of MIP-2 and IL-1β, chemokines known to be involved in PMN recruitment. No significant difference in MIP-2 or IL-1β concentration was found between $Cx3cr1^{+/-}$ and $Cx3cr1^{-/-}$ mice after exposure to air (Figure 2.4A). Following O_3 exposure, the concentration of MIP-2 was increased and was greatest after 48 hrs in both genotypes, but was significantly greater in $Cx3cr1^{+/-}$ mice compared to $Cx3cr1^{-/-}$ mice. IL-1β concentration was significantly elevated in $Cx3cr1^{+/-}$ mice following O_3, and concentration peaked after 72 hrs O_3; however, O_3 had no effect on IL-1β concentration in $Cx3cr1^{-/-}$ mice (Figure 2.4B). Consistent with BAL data and pathology the concentrations of MIP-2 and IL-1β were greater in $Cx3cr1^{+/-}$ mice than in $Cx3cr1^{-/-}$ (Figure 2.4).

Effect of *Cx3cr1* on expression of IL-17R mRNA

IL-17R is critical for the recruitment of neutrophils, and regulates chemokine expression in the lung after microbial or particulate challenge. IL-17R mRNA expression was measured to determine if *Cx3cr1* modulates O_3-induced inflammatory cell migration by upregulating IL-17R. Relative to air controls, IL-17R mRNA expression was enhanced in $Cx3cr1^{+/-}$ mice following O_3 exposure (Figure 2.5). Expression of IL-17R mRNA in $Cx3cr1^{+/-}$ mice increased significantly after 48 hr O_3, and then expression was greatly diminished after 72 hr O_3, and not significantly different from air controls. IL-17R expression also increased significantly in $Cx3cr1^{-/-}$ mice after 48 hr O_3 relative to air control mice, but expression level was significantly less than that found in $Cx3cr1^{+/-}$ mice.

IL-17R effect on O_3-induced pulmonary inflammation and injury

To further investigate the role of IL-17R in O_3-induced pulmonary inflammation, we assessed BAL response phenotypes in $Il17ra^{+/+}$ and $Il17ra^{-/-}$ mice. Relative to respective air controls O_3 significantly increased mean numbers of BAL macrophages (72

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hr), PMNs (48 and 72 hr), epithelial cells (72 hr), and lymphocytes (72 hr) in $Il17ra^{+/+}$ mice (Figure 2.6A). O_3 also significantly increased BAL macrophages (48 and 72 hr) and PMNs (72 hr), but not epithelial cells or lymphocytes in $Il17ra^{-/-}$ mice. However, the number of O_3-induced BAL macrophages (72 hr), PMNs (48 and 72 hr), epithelial cells (72 hr) and lymphocytes (72) were significantly greater in $Il17ra^{+/+}$ mice compared to $Il17ra^{-/-}$ mice.

No significant differences in mean total BAL protein concentration were found between $Il17ra^{+/+}$ and $Il17ra^{-/-}$ following air, or 48 hr O_3 exposure (Figure 2.6B). After 72 hr O_3, total BAL protein concentration was significantly attenuated in $Il17ra^{-/-}$ mice compared to $Il17ra^{+/+}$ mice.

Cx3cr1 and IL-17R deficiency on differential recruitment of cytokine IL-17A

Because IL-17R mRNA levels were elevated in $Cx3cr1^{+/-}$ mice following 48hr O_3 exposure, BAL IL-17A concentration was measured to determine if O_3 exposure influences release of this proinflammatory cytokine. Relative to air-exposed controls, mean concentration of IL-17A increased significantly after 24 and 48 hr O_3 and returned toward basal levels after 72 hr. Interestingly, IL-17A was not detected in $Cx3cr1^{+/-}$ mice at any time point. Relative to air controls, 72 hr O_3 also caused a significant increase in BAL IL-17A in $Il17ra^{-/-}$ mice (Figure 2.7B). However, O_3 had no significant effects on mean BAL IL-17A in $Il17ra^{+/+}$ mice relative to genotype matched air controls (Figure 2.7B).

Discussion

        The functional importance of CX3CR1 as a mediator of lung toxicity has been documented in previous human epidemiological studies. Loss of function polymorphisms in *CX3CR1* associated with reduced risk of respiratory ailments following chronic exposure to coal dust suggesting that CX3CR1 contributes to the disease pathogenesis (7). The role of *CX3CR1* in pulmonary injury resulting from shorter term, sub-acute exposures to environmental pollutants has not been investigated. The present study was designed to investigate the role of *Cx3cr1* in inflammation and injury induced by subacute exposure to O_3, and the role of IL17 in this effect. Deletion of either *Cx3cr1* or IL-17R significantly diminished pulmonary inflammation and injury induced by O_3 relative to wild type mice. Furthermore, data from this study suggest that of the effect of CX3CR1 may be mediated through regulation of IL-17R.

        Previous studies have identified PMN infiltration into the airways as one of the primary indicators of O_3-induced toxicity (21). Following O_3, an exposure-dependent increase of lavageable PMNs was found in both genotypes, but numbers of PMNs were significantly reduced in $Cx3cr1^{-/-}$ mice compared to $Cx3cr1^{+/-}$ mice. Furthermore, BAL lymphocytes and macrophages (other cellular inflammatory phenotypes) were also increased by O_3 and were significantly reduced in $Cx3cr1^{-/-}$ mice compared to $Cx3cr1^{+/-}$ mice. It was also noted that there was a significant difference in pulmonary inflammation between genotypes following air exposure; suggesting that $Cx3cr1^{+/-}$ may be responding to air, and in the future all BAL phenotypes should be normalized to genotype-matched air controls in order to determine the effect of *Cx3cr1* on O_3-induced pulmonary inflammation. Consistent with BAL inflammation, peribronchiolar inflammation, cellular proliferation and epithelial hyperplasia were also more prominent in $Cx3cr1^{+/-}$ mice compared to $Cx3cr1^{-/-}$ mice following O_3 exposure. This is the first study to identify the functional importance of *Cx3cr1* in O_3-induced lung disease. Previous studies found that expression of CX3CR1 was associated with increased pulmonary

inflammation and pathological signs of disease in other disease models (12, 19, 22). For example, significantly greater pulmonary inflammation was found in mice with BAL inflammatory cells expressing CX3CR1 following exposure to cigarette smoke; in particular, the macrophage inflammatory response was associated with CX3CR1 function (12). Data reported previously suggests that deficiency *CX3CR1* mitigates BAL protein concentration and immune cell migration to the lung following tuberculosis infection in humans (19). Zhang *et al.* also found that CX3CR1 inactivation reduces RSV-induced pulmonary inflammation and pathology (22). Findings from these investigations are consistent with BAL cell differentials and protein concentration in this study.

Previous investigations suggest that expression of a number of proinflammatory cytokines (eg. MIP-2, IL-1β) are enhanced following a pulmonary insult, and are linked to PMN and macrophage recruitment (5, 6, 13, 23). To determine whether deficiency of *Cx3cr1* influences proinflammatory cytokine activity, and thus a potential mechanism through which Cx3cr1 modifies inflammation, MIP-2 and IL-1β expression was measured in lungs of O_3-exposed $Cx3cr1^{+/-}$ and $Cx3cr1^{-/-}$ mice. Interestingly MIP-2 expression was greater in $Cx3cr1^{+/-}$ mice than $Cx3cr1^{-/-}$ mice after air exposure, suggesting that *Cx3cr1* deficiency alters MIP-2 independent of O_3 exposure. O_3 exposure also induced MIP-2, as well as IL-1β, expression in both genotypes, but the increases found in $Cx3cr1^{+/-}$ mice were significantly greater than expression in $Cx3cr1^{-/-}$ mice. These results suggest that *Cx3cr1* may modulate pulmonary inflammation through activation of MIP-2 and IL-1β. While these findings are informative, further investigation is needed to determine the functional relationships between cytokines, *Cx3cr1* and pulmonary inflammation.

Because IL-17R is essential for lung neutrophil recruitment and lung expression of MIP-2 (16, 17), we hypothesized that *Cx3cr1* regulates pulmonary inflammation and injury in response to O_3 through upregulation of IL-17R (15, 17, 18, 24). To initially test this hypothesis, we measured IL-17R expression in lung after O_3 exposure in $Cx3cr1^{+/-}$

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and *Cx3cr1*$^{-/-}$ mice. IL-17R expression correlated with the observed inflammatory response, and was elevated in *Cx3cr1*$^{+/-}$ mice after O_3 exposure. Moreover, IL-17R expression was significantly depressed in *Cx3cr1*$^{-/-}$ mice relative to wild-type mice. These results are consistent with a role for Cx3cr1 in regulation of the expression of IL-17R in the lung.

To further investigate the role of IL-17R in this model, we asked whether genetic deletion of *Il17ra* would attenuate the inflammatory response induced by O_3. Similar to our findings with *Cx3cr1*$^{-/-}$ mice, deficiency of *Il17ra* caused a significant reduction in the inflammatory response (BAL PMNs, macrophages, and lymphocytes) following O_3 relative to wild type mice, suggesting that IL-17R is involved in modulating the inflammatory cell recruitment into the lung. This is consistent with findings of other studies that used *Il17ra*$^{-/-}$ mice to investigate mechanisms of pulmonary inflammation. A recent study found that IL-17R was required for OVA allergen and viral-induced neutrophilia and general inflammation (18, 25, 26). Wilson *et al.* also found that neutrophilia seen in the airways was associated with increased production of the chemokine CXCL5, which was found to be dependent on IL-17RA (18). CXCL5 and CX3CL1 (fractalkine), the ligand for CX3CR1 are members of the same cytokine family, thus findings from previous studies (18, 26, 27) are consistent with the hypothesis that CX3CR1 initiates pulmonary inflammation via IL-17R activation.

Interestingly IL-17A (cytokine) expression did not correlate with IL-17R activation. Negligible levels of IL-17A were found in *Cx3cr1*$^{+/-}$ mice throughout the study, and IL-17A expression increased significantly in *Cx3cr1*$^{-/-}$ mice following O_3 exposure, and peaked following 48 hrs of O_3 exposure. Previous studies similarly found elevated IL-17A expression in IL-17R deficient mice (16). Smith *et al* (16) suggested that there is a negative feedback loop where bound IL-17A prevents expansion of IL-17A -producing T-cells, therefore mice with functioning IL-17R will produce significantly less IL-17A than mice deficient for the receptor. Real time PCR analysis suggested

activation of IL-17R is linked to *Cx3cr1* function and this synergistic relationship may influence the inflammatory and injury response observed after O_3.

In summary our study found that, relative to wild-type mice, targeted deletion of *Cx3cr1* enhanced the pulmonary inflammation and injury, proinflammatory mediator release, and IL-17R expression induced by O_3. Furthermore, *Il17ra*^{-/-} mice were also protected against O_3-induced inflammation and injury relative to wild-type mice. Results therefore indicate that *Cx3cr1* and *Il17ra* are important determinants of the pulmonary inflammatory response to O_3 exposure. Results also suggest a potential interaction between *Cx3cr1* and *Il17r*, but further investigation is required to understand the mechanisms of this effect. These novel findings provide insight to the mechanisms of pulmonary responses to environmental oxidant stimuli, and may lead to new intervention modalities.

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chemokine expression in non-small cell lung cancer: role in angiogenesis and prognosis. *Clin Cancer Res* 9(2):853-60.

Figure Legends

Figure 2.1. BAL inflammatory parameters in *Cx3cr1*^{+/-} v. *Cx3cr1*^{-/-} mice exposed to 0.3 ppm O$_3$. Data are presented as means ± SEM (n=6-12 per group). +, significantly different from strain-matched air controls (p<0.05) *, significantly different from exposure-matched *Cx3cr1*^{+/-}.

Figure 2.2. *Cx3cr1* deficiency effects on O$_3$-induced pulmonary pathology. Paraffin-embedded left-lung tissue sections were stained with H&E. Representative light micrographs show less severe cellular proliferation in perivascular and peribronchiolar lesions and terminal bronchioles and neutrophilic infiltration from blood vessels to lung parenchyma in *Cx3cr1*^{-/-} mice relative to *Cx3cr1*^{+/-} mice exposed to O$_3$. av, alveoli; tb, terminal bronchioles; bv, blood vessels; br, bronchi. Arrows indicate neutrophils infiltrating blood vessels. Bars=100 μm.

Figure 2.3. Lung nuclear PCNA levels in *Cx3cr1*^{+/-} and *Cx3cr1*^{-/-} mice after O$_3$. PCNA is a marker of lung injury and proliferation. (A) Representative western blots of PCNA expression. (B) Immunostaining for PCNA in lung sections.

Figure 2.4. **Affect of O$_3$ exposure on chemokine expression in *Cx3cr1*^{+/-} and *Cx3cr1*^{-/-} mice.** (A) BAL level of MIP-2. (B) Level of IL-1β found in BAL. Data are presented as means ± SEM (n=6-12 per group). +, significantly different from strain-matched air controls (p<0.05) *, significantly different from exposure-matched *Cx3cr1*^{+/-}.

Figure 2.5. Expression of IL-17R in *Cx3cr1*^{+/-} and *Cx3cr1*^{-/-} mice after O$_3$ exposure. Fold change of IL-17R mRNA expression normalized to *Cx3cr1*^{+/-} air control. Expression determined by qPCR.

Figure 2.6. BAL inflammatory parameters in *Il17ra*^{+/+} v. *Il17ra*^{-/-} mice exposed to 0.3 ppm O₃. Data are presented as means $\pm$ SEM (n=6-12 per group). +, significantly different from strain-matched air controls ($p<0.05$). *, significantly different from exposure-matched *Il17ra*^{+/+}

Figure 2.7. Recruitment of cytokine IL-17A *Cx3cr1*^{+/-} v. *Cx3cr1*^{-/-} mice and *Il17ra*^{+/+} v. *Il17ra*^{-/-} mice determined by ELISA. Data are presented as means $\pm$ SEM (n=6-12 per group). +, significantly different from strain-matched air controls ($p<0.05$). *, significantly different from exposure-matched *Cx3cr1*^{+/-} or *Il17ra*^{+/+}

Figure 2.1.

Figure 2.2.

Figure 2.3.

A

Cx3cr1

Air | 24 | 48 | 72
Ozone (hr)

+/- -/- | +/- -/- | +/- -/- | +/- -/-

Nuclear PCNA

B

Air | 48h O₃

Cx3cr1+/-

Cx3cr1-/-

Figure 2.4.

Figure 2.5.

IL-17R expression/ 18s expression

Cx3cr1^{+/-}
Cx3cr1^{-/-}

Air   Ozone (hr)
24   48   72
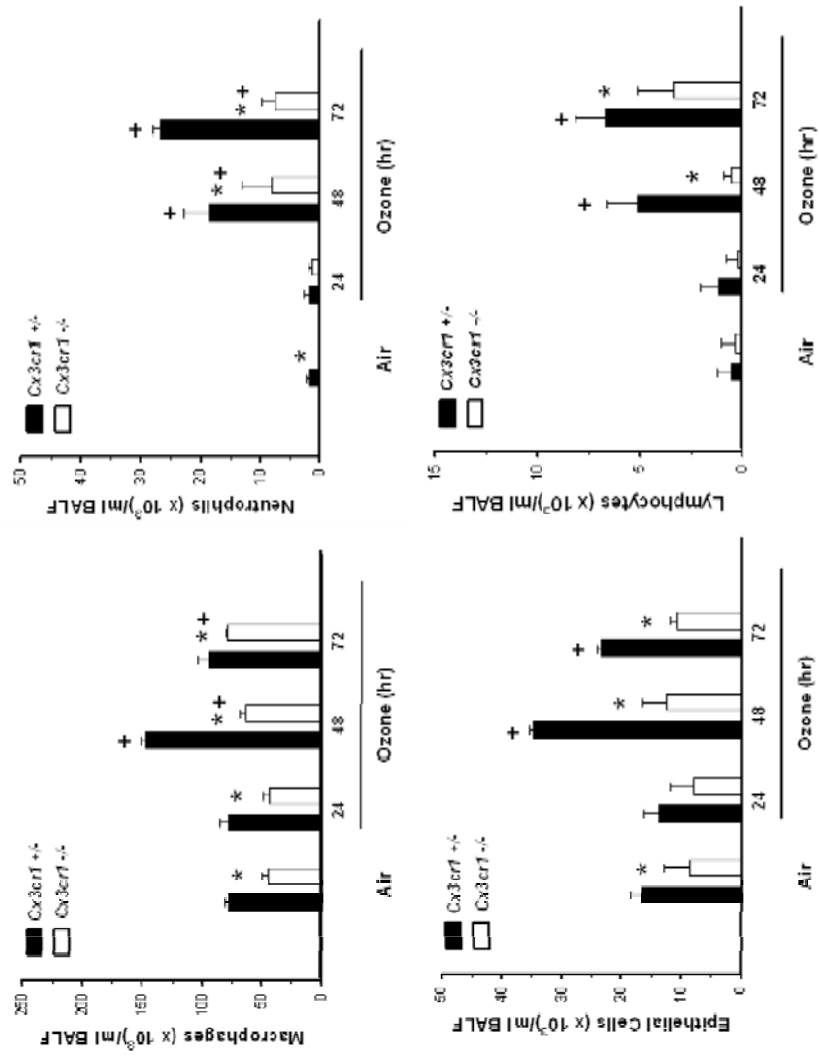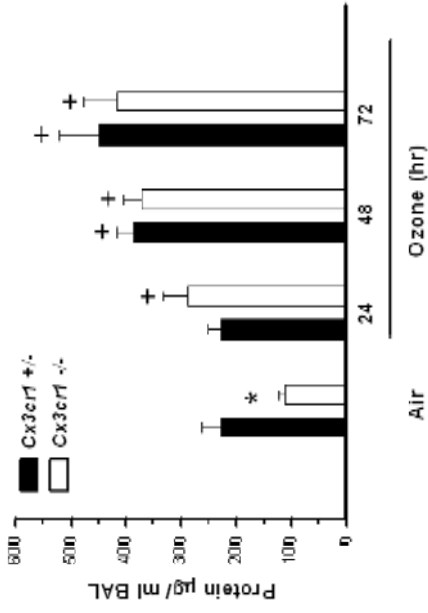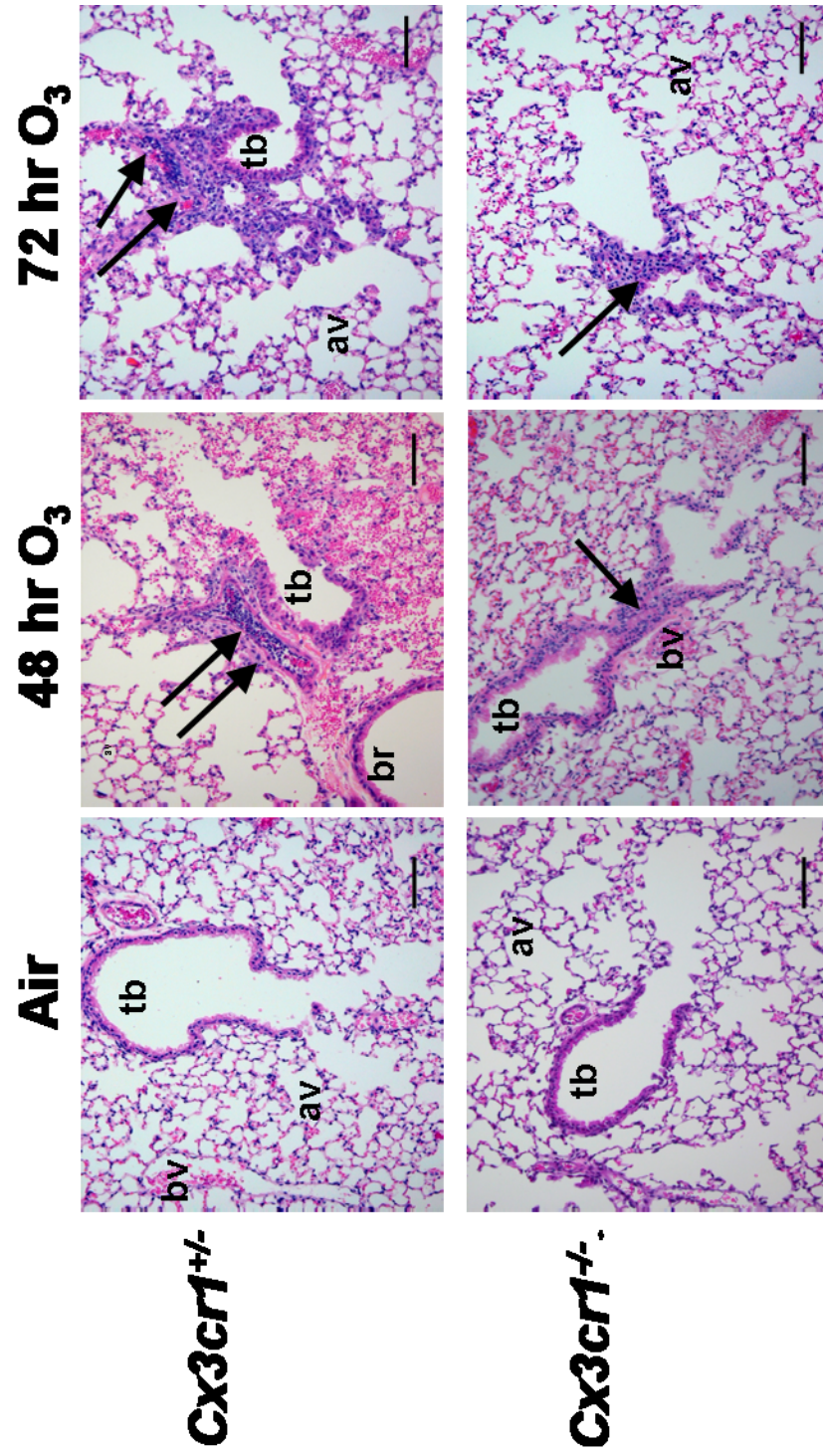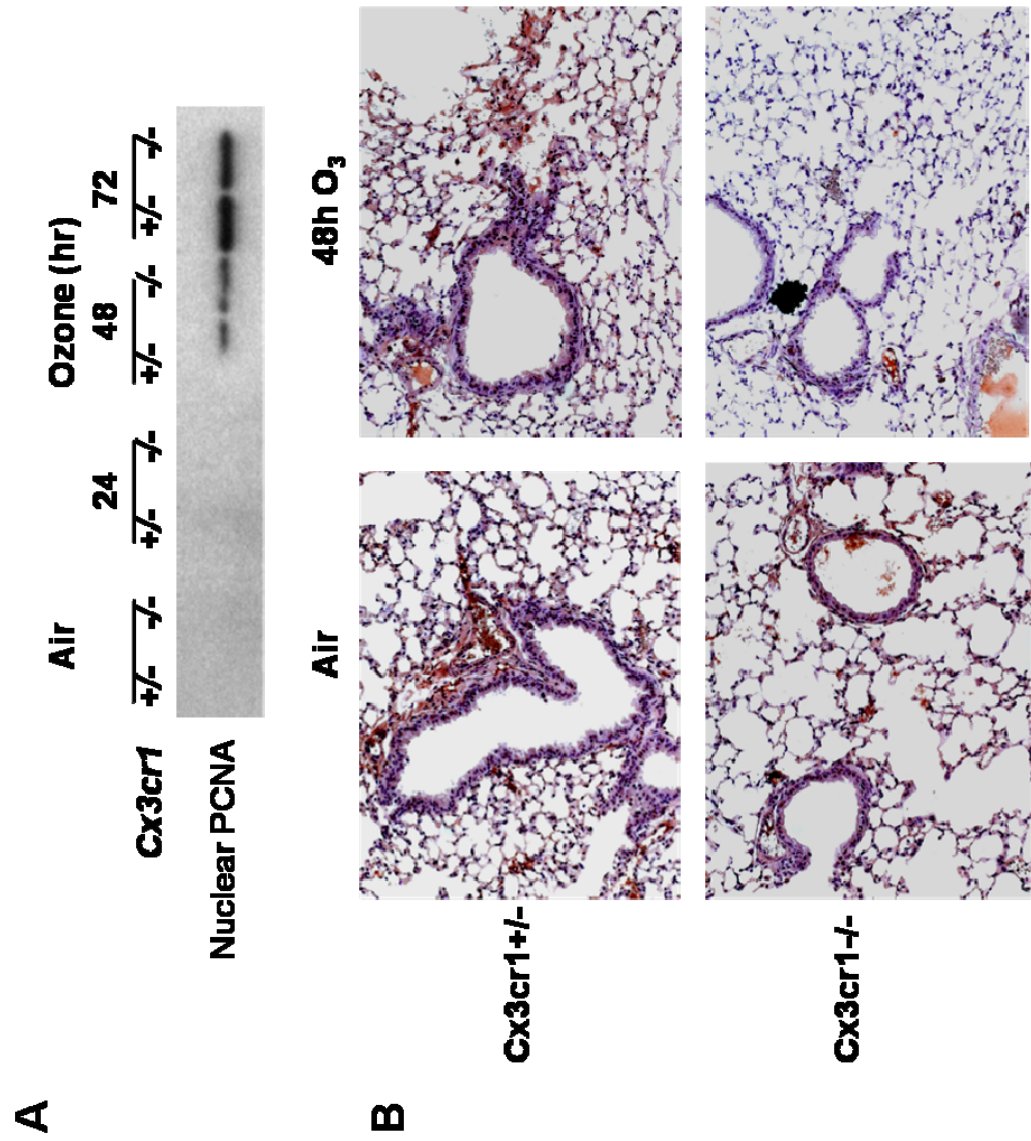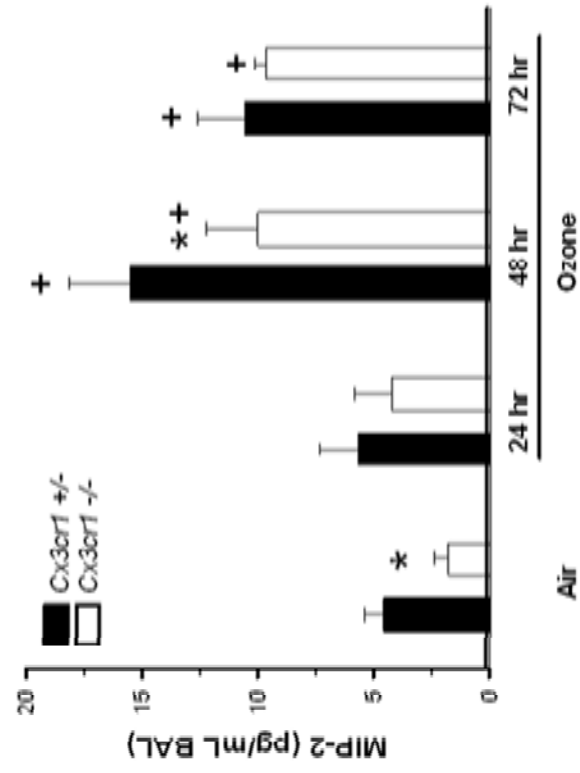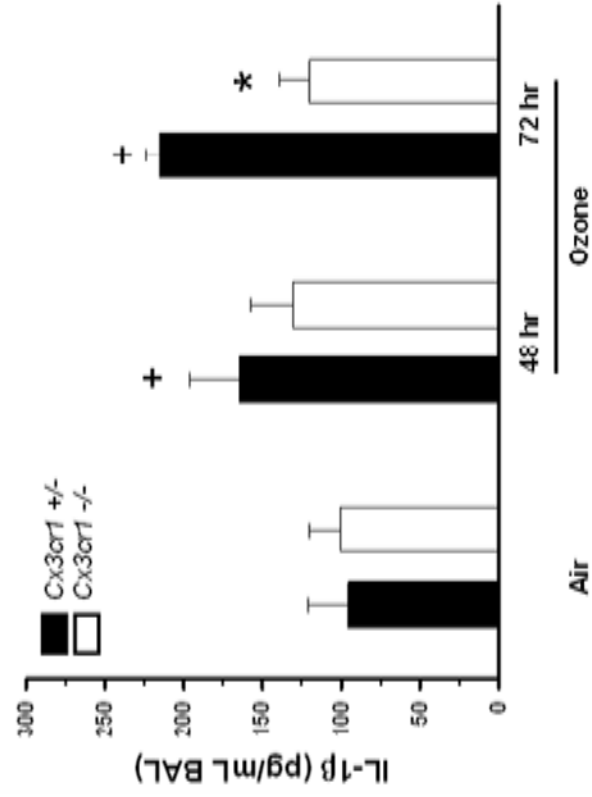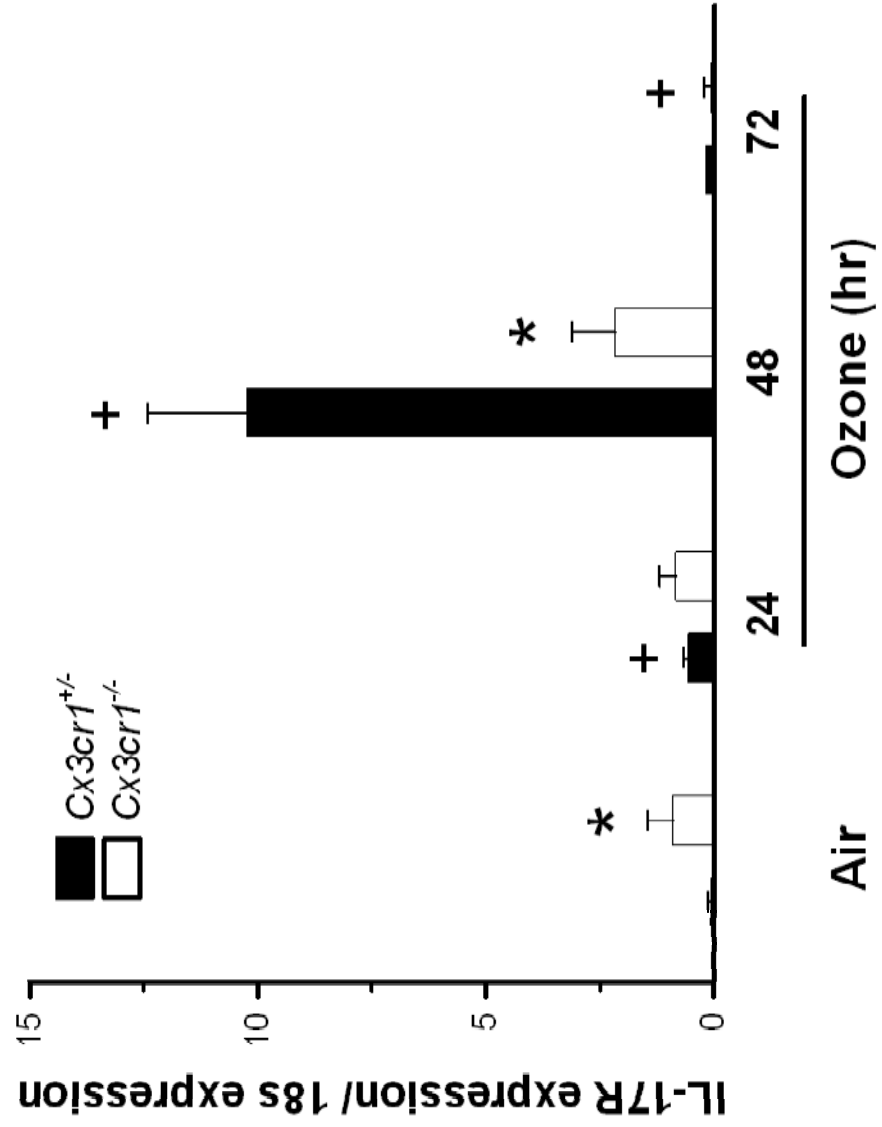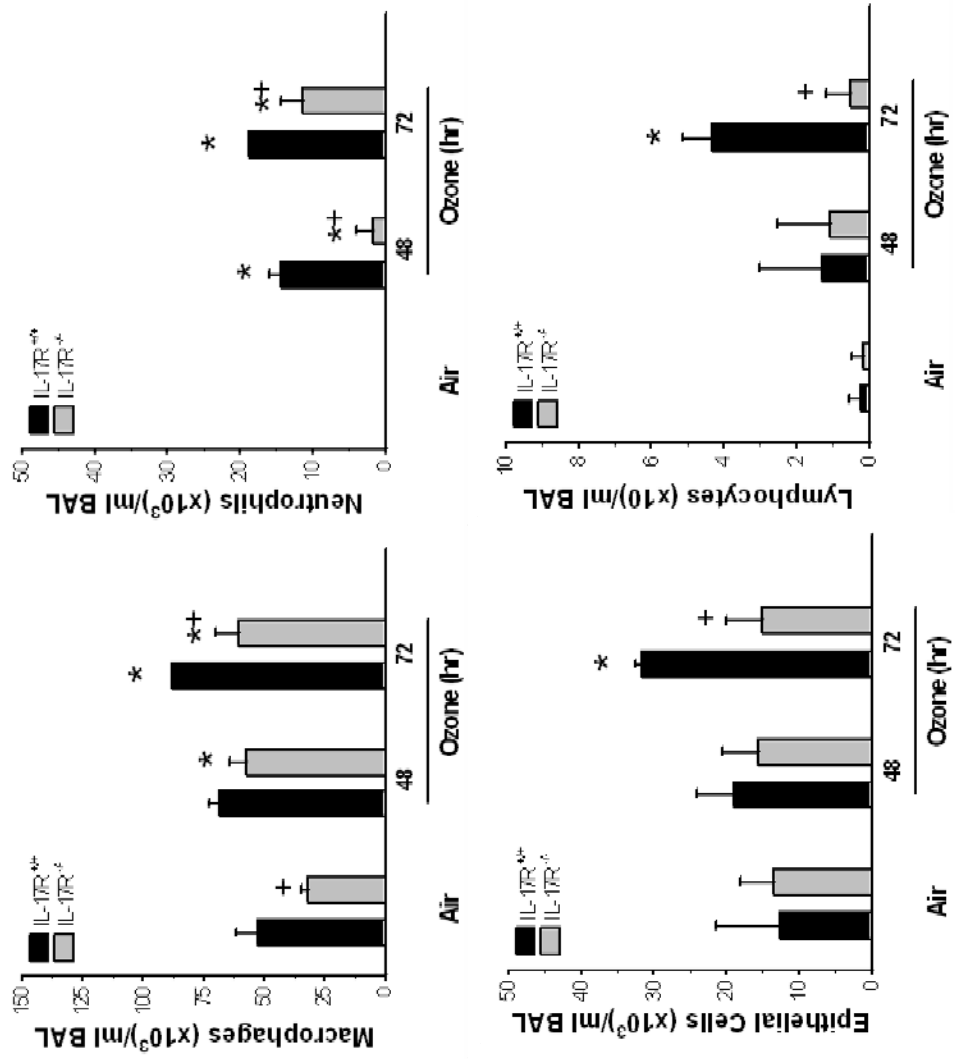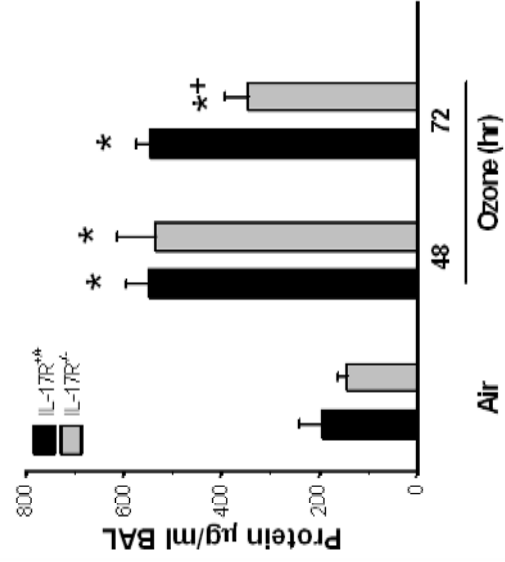
Figure 2.6.

Figure 2.7.
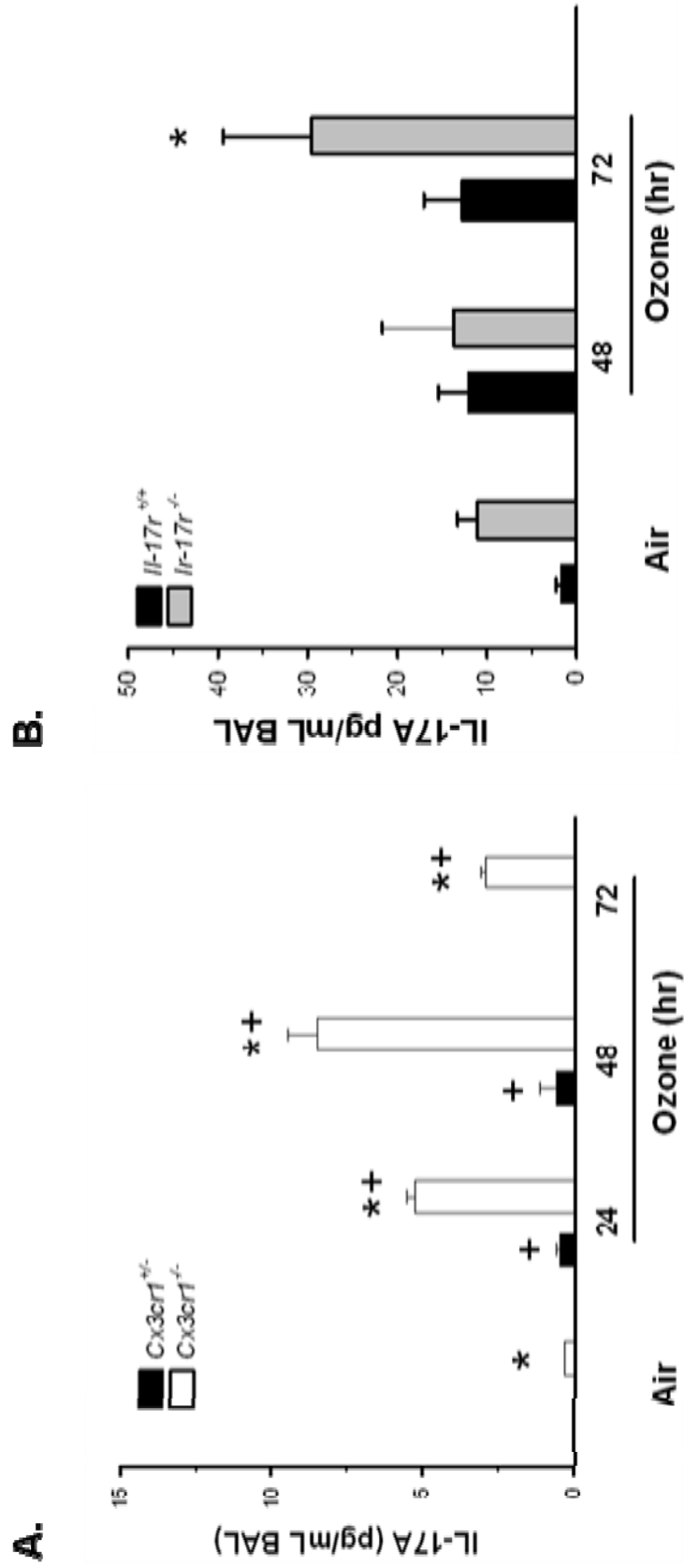
CHAPTER 3

Cx3cr1 REGULATES PULMONARY INFLAMMATION FOLLOWING RSV INFECTION

Introduction

A previous study has determined that *Cx3cr1* contributes to pulmonary inflammation and injury in the mouse following sub-acute O_3 exposure (Chapter 2). In humans, CX3CR1 has been linked to asthma, bronchiolitis and lung injury (1, 2). Clinical studies found that CX3CR1 activity and/ or expression was directly linked to RSV-induced bronchiolitis and inflammation (3-7). Because *Cx3cr1* contributes to pulmonary inflammation following exposure to the oxidant O_3 (Chapter 2), and oxidant stress has been implicated as an important component to RSV infection and disease, it was hypothesized that *Cx3cr1* is also important in the pulmonary response to RSV infection and RSV-induced pulmonary inflammation. To test this hypothesis, wild type controls and mice with targeted deletion of *Cx3cr1* (*Cx3cr1$^{-/-}$*) were infected with RSV and vehicle controls. Infection and RSV disease phenotypes were compared between the two groups. Significantly reduced numbers of inflammatory polymorphonuclear leukocytes (PMNs) and macrophages in *Cx3cr1$^{-/-}$* mice compared to wild type mice after RSV infection was consistent with a role for Cx3cr1 as an important determinant of susceptibility to RSV disease in mice.

Material and Methods

Animal and Inhalation Exposure

Age-matched (5-8 week), specific pathogen-free, inbred strain male C57BL/6J$^{Cx3cr1+/+}$ and C57BL/6J$^{Cx3cr1-/-}$ mice were obtained from European Mouse Mutant Archive (EMMA) (Munich, Germany). One group of mice from each strain was infected with 1×10^6 plaque forming units (pfu) of RSV (RSV-19 provided by Dr. Fernando Polack) and sacrificed 1 day after infection, another group was sacrificed 5 days following infection, and vehicle control group was given Hep 2 cells in 50µL of HBSS and sacrificed after 5 days. Previous work from the laboratory has indicated that a minimum of 6 animals/ sample group were required for statistical evaluation. A sample size of 6-8 was chosen to account for unplanned deaths during the study. Mice were anesthetized with 100 mg/kg Nembutal (pentobarbital sodium), weighed, and the chest opened. Lungs were lavaged in situ four times with Hanks' balanced salt solution (HBSS; 35 ml/kg, pH 7.2-7.4). Recovered BAL fluid was immediately cooled to 4°C and centrifuged. The supernatant from the first lavage return was assayed for total protein (a marker of lung permeability) with the Bradford assay. The four cell pellets were resuspended and pooled in 1 ml of HBSS, and the cells were counted with a hemacytometer. An aliquot (10 µl) of BAL cell suspension was cytocentrifuged and stained with Wright-Giemsa stain (Diff-Quik, Baxter Scientific Products, McGaw Park, IL) for differential cell analysis. Differential counts for epithelial cells (a marker of epithelial damage), neutrophils, lymphocytes, and macrophages (markers of inflammation) were done by identifying 300 cells according to standard cytological technique.

Results and Discussion

BAL PMN, macrophage, and lymphocyte concentrations were greatest 1 day after RSV infection and were significantly higher in $Cx3cr1^{+/+}$ mice compared to $Cx3cr1^{-/-}$ mice. In both genotypes BAL monocyte concentration was the greatest 5 days after RSV infection; however; no significant differences were found between genotypes. These results were consistent with previous work that directly linked neutrophilia and lymphocyte recruitment to RSV infection (8-10). Overall, BAL inflammatory cell concentration increased significantly following RSV infection in both genotypes, but was significantly greater in $Cx3cr1^{+/+}$ mice compared to $Cx3cr1^{-/-}$ mice. These findings suggest that $Cx3cr1$ contributes to pulmonary inflammation following RSV infection. However, the presence of inflammation in $Cx3cr1^{-/-}$ mice indicates that $Cx3cr1$ is not the sole regulator of the host's response to RSV and other genes contribute to the observed response to RSV infection.

The inflammatory response in both genotypes following RSV infection was similar to the response found in these mice after O_3 exposure. This may suggest that RSV and O_3 induce pulmonary inflammation through similar mechanisms. Previous results (Chapter 2) suggest that Cx3cr1 recruits IL-17R+ cells in the inflammatory process induced by O_3. Additional experiments are necessary to determine if the same mechanism(s) regulates response to RSV infection.

Recent studies demonstrate that reactive oxygen species (ROS) production modulates RSV disease and O_3–induced pulmonary injury, and genes that regulate ROS production may be responsible for O_3 toxicity and RSV disease severity. For example, $Nrf2$ (nuclear erythroid 2 p45-related factor 2) is a redox sensitive transcription factor that is essential in protection against oxidative lung injury resulting from O_3 exposure (11) and RSV infection (12, 13) Studies using $Nrf2$ wild-type and deficient mice found that inflammation was significantly attenuated in wild-type mice relative to $Nrf2$ deficient mice following O_3 exposure or RSV infection, suggesting that the same mechanism is

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responsible for both responses. Similarly, data from this dissertation identifies *Cx3cr1* as a candidate gene for both O_3-induced and RSV-induced immune response. Additional genes that regulate RSV-induced inflammation can be identified by either using this candidate gene approach, or through genome-wide haplotype association studies.

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Figure Legend

Figure 3.1. BAL inflammatory parameters in *Cx3cr1*$^{+/+}$ v. *Cx3cr1*$^{-/-}$ mice infected with RSV. Data are presented as means ± SEM (n=6 per group). *, significantly different from strain matched air controls ($p<0.05$) +, significantly different from exposure-matched *Cx3cr1*$^{+/+}$.

Figure 3.1.
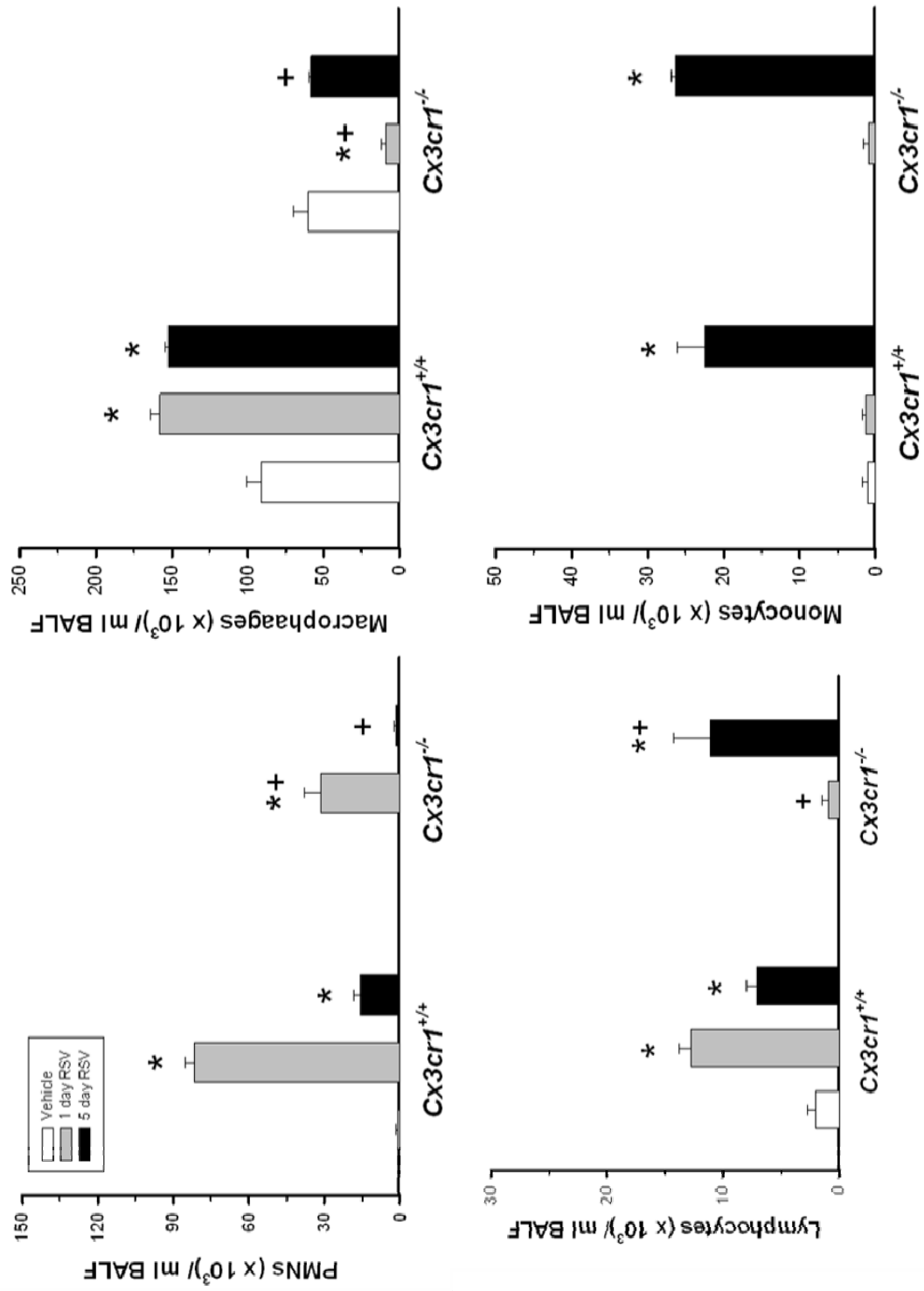
CHAPTER 4

PHENOTYPIC CHARACTERIZATION OF PULMONARY RESPONSES TO RSV INFECTION ACROSS MULTIPLE INBRED STRAINS OF MICE

Abstract

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection (LRTI) in infants. Approximately 70% of infants are infected with RSV within the first year of life. Epidemiological studies show that individuals have varying responses to RSV infection, ranging from "cold-like" symptoms to death.

Purpose: The purpose of this research was to characterize the phenotypes of various inbred mouse strains following RSV infection, identify inbred strains that are differentially responsive to RSV infection, and evaluate heritability of RSV disease phenotypes.

Methods: Thirty-six inbred mouse strains (5-8 wk) were infected with a single dose of $1x10^6$ plaque forming units (pfu) of RSV (RSV-19) or control and sacrificed after 1 and 5 days post-infection (pi). To limit the number of confounding factors, only male mice were used in this study. Pulmonary inflammation, changes in body weight, lung pathology, mucus cell metaplasia (MCM), and viral mRNA expression were analyzed and compared between and within all inbred strains of mice.

Results: No significant effects on inflammatory or other disease phenotypes were detected among strains treated with vehicle (control). Statistically significant strain distribution patterns (SDPs) for protein, cellular, and MCM phenotypes in response to RSV infection were found among the inbred strains. C3H/HeJ was the most resistant strain, and BALB/cByJ was among the most susceptible strains. SDPs for the inflammatory phenotypes were largely concordant within 1 and 5 day post-infection sample times, but not between times. The magnitude of RSV expression (viral load) did not correlate with disease severity across the strains. Heritability estimates indicated that a significant portion of the variance in the RSV disease phenotype was attributed to genetic background.

Conclusions: Data from this study indicate differential strain-specific responses to RSV infection across multiple inbred strains. Correlation analyses suggest that response to

RSV infection is regulated by different mechanisms 1 and 5 day(s) following infection. The data also indicate that viral expression was not related to RSV disease response. Furthermore, heritability estimates suggest that a significant portion of the phenotypic variation found between strains is due to genetic background, and provide the basis for future investigations to identify specific genes responsible for RSV disease phenotypes.

Introduction

Respiratory syncytial virus (RSV) is the primary cause of hospitalization during the first year of life, and is the leading cause of bronchiolitis, pneumonia, mechanical ventilation and respiratory failure in infants worldwide (1). RSV infection is also associated with the development of childhood asthma, and recurrent wheezing up to 7 years old. In the United States alone, more than 100,000 infants are hospitalized with RSV-induced bronchiolitis each year (2). RSV is highly virulent and only a small percentage of infections are completely asymptomatic. While the majority of infected individuals present symptoms, the nature and severity of the symptoms vary among individuals. For some infants infection induces cold-like symptoms, others require hospitalization and a small percentage of cases result in death. The highest morbidity risk for RSV is among infants younger than 6 months (3, 4). However, infants are not the only sub-population at risk to the effects of RSV. The elderly and some adults are also at high risk for adverse effects of RSV disease (5). The varying nature and severity of disease within a population suggest that host genotype regulates the phenotype (6-8).

Inbred strains have been used successfully to identify genetic factors that regulate susceptibility and response to pulmonary pathogens, inhaled particulates, and other environmental stressors (9-12). A number of studies have reported mouse RSV phenotypes that resemble those found in humans (11). Furthermore, studies using inbred mouse strains have found that response and susceptibility to RSV infection is influenced by genetic background (13, 14). Prince *et al* (12) conducted an inbred strain screen on 20 inbred mouse strains using viral titer as a marker of response to RSV infection. The inbred strains formed a continuous strain distribution with P/N, C57L/N, and DBA/2N among the most susceptible and CBA/CaHN among the most resistant (13). Stark *et al* (13) conducted a similar experiment and they found that C57/BL6J and A/J were the least susceptible strains and AKR/J was the most susceptible. F1 progeny between C57/BL6J and AKR/J were relatively resistant to RSV infection suggesting that resistance to RSV

infection is a dominant trait. However, when these mice were backcrossed to AKR/J the resulting progeny were unexpectedly highly susceptible to infection (14). These findings suggest that susceptibility to RSV infection is a polygenic trait, but do not provide any insight into the genetics of RSV disease phenotypes and the host immune response. The present investigation was designed to carefully characterize disease phenotypes of response to RSV infection across a panel of genetically diverse inbred strains of mice in order to: 1) model resistant and susceptible human disease phenotypes; 2) evaluate relatedness of disease phenotypes; and 3) estimate the genetic heritability of RSV disease phenotypes. This study will also provide well-characterized phenotypes for haplotype association mapping of RSV disease quantitative trait loci (Chapter 5).

Material and Methods

Animals and Infection

Age-matched (5-8 week), specific pathogen-free, inbred strain male mice (Jackson Laboratory) were divided randomly into three groups per strain (Table 4.1). One group of mice from each strain were infected with $1x10^6$ plaque forming units (pfu) of RSV (RSV-19 provided by Dr. Fernando Polack) and sacrificed 1 day after infection, another group was sacrificed 5 days following infection, and vehicle control group was given Hep 2 cells in 50µL of HBSS and sacrificed after 5 days. Previous work from the laboratory has indicated that a minimum of 6 animals/sample group were required for statistical evaluation. A sample size of 6-8 was chosen to account for unplanned deaths during the study. Mice were anesthetized with 100 mg/kg Nembutal (pentobarbital sodium), weighed, and the chest opened. Lungs were lavaged in situ four times with Hanks' balanced salt solution (HBSS; 35 ml/kg, pH 7.2-7.4). Recovered BAL fluid was immediately cooled to 4°C and centrifuged. The supernatant from the first lavage return was assayed for total protein (a marker of lung permeability) with the Bradford assay. The four cell pellets were resuspended and pooled in 1 ml of HBSS, and the cells were counted with a hemacytometer. An aliquot (10 µl) of BAL cell suspension was cytocentrifuged and stained with Wright-Giemsa stain (Diff-Quik, Baxter Scientific Products, McGaw Park, IL) for differential cell analysis. Differential counts for epithelial cells (a marker of epithelial damage), neutrophils, lymphocytes, and macrophages (markers of inflammation) were done by identifying 300 cells according to standard cytological technique.

Lung Histopathology

Left lung lobes were fixed with 10% neutral buffered zinc formalin for 24 hrs. Proximal and distal intrapulmonary axial tissues were excised and embedded in paraffin and sectioned (5 µm). Tissue sections were histochemically stained with hemotoxylin and

eosin (H&E) for comparison of pathology and injury between inbred strains. The terminal bronchioles and alveoli were the primary focus of study because RSV infection causes inflammation and epithelial cell sloughing in these regions of the mouse lung. Tissue sections were stained via alcian blue-periodic acid shift (AB/PAS) double staining method for comparison of mucus cell metaplasia (MCM) between inbred strains.

Mucus Cell Metaplasia

Images of the histological sections were captured using a Axioskop 40 microscope, camera and the Axiovision software. Black and white images, taken with a 10X objective lens, were analyzed with Scion image analysis software. The mucus-containing area and the perimeter of the basal lamina from large airways, visually judged to be third generation bronchus, were used for the analysis. The volume of mucus per airway was calculated using the following formula (15):

$$SV = 4 / \Pi(BA)$$
$$VS = VV / SV$$

VV is the sum area of stained goblet cells, calculated during the Scion Image analysis. BA is the length of the basal lamina. SV is surface area per unit volume and VS is volume per unit surface area of basal lamina (nanoliters/mm^2). VS values were calculated for each lung section and averaged for vehicle control and RSV infected groups within each strain. Values for large airways were averaged for each treatment group, vehicle control or RSV infected within a strain. Values are reported as the mean ± SEM for each strain where appropriate (n=3/infection/strain).

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from non-lavaged left lung pieces using an RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's procedure. One μg of total RNA was reverse transcribed into cDNA. For quantitative PCR, an aliquot (1-2.5 μl) of cDNA was amplified in a 25 μl reaction containing 12.5 μl 2X PowerSYBR Green Master Mix (Applied Biosystems, Foster City, CA) and 250 nM of each gene-specific forward and reverse primer by 10 minute hold at 95°C and 45 cycles at 95°C (15 seconds) -60°C (1 minute) using the Applied Biosystems StepOne Plus Real-Time PCR Systems. Quantification of gene expression was determined by ΔC_T values obtained by subtracting fluorescence detected number of cycles to threshold (C_T) of 18s mRNA from those of target gene RNA in the same sample.

Statistical Analysis

Analyses of lavage and tissue parameters were analyzed by 3-way ANOVA (factors: exposure, time, strain) and comparisons of means followed Student-Newman- Keuls (SNK) a posteriori procedures. It was previously determined that these parameters were distributed normally, but data were analyzed for homoscedasiticity before ANOVA analyses and transformed accordingly. Particular attention was paid to compare duration of RSV infection to indicators of lung injury and inflammation. Since percentages are not normally distributed, they were transformed (arcsine) and the transformed data for each variable was analyzed by two-way ANOVA (factors: time, strain). Comparisons of the means followed SNK procedures. The statistical significance in these and all other comparisons was accepted at $p < 0.05$. Relatedness of RSV phenotypes was determined using Pearson and Spearman correlation coefficients (www.genenetwork.org).

Heritability, or the fraction of the phenotype variability that is due to gene effects, was estimated by calculating the intraclass correlation and the coefficient of genetic determination, following the methods outlined by Festing (16). Intraclass correlation, the

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proportion of total phenotype variation due to between-strain differences or heritability in the broad sense, was calculated by the following formula:

$$r_I = (MS_B - MS_W) / [MS_B + (n - 1)MS_W]$$

where r_I is the intraclass correlation estimate, MS_B is the mean square of the between-strain comparison, MS_W is the mean square of the within-strain comparison, and n is the number of animals tested per strain with corrections for differences in numbers of mice per strain.

Results

<u>Strain distribution patterns of RSV induced injury and airway inflammation</u>

Differences in BAL protein concentration were found between inbred strains among the vehicle control groups (Table 4.2). Among the inbred strains studied 129s1/SvlmJ and A/J were the most responsive (based on BAL phenotypes) to vehicle. Following RSV infection, lung protein concentration was the greatest in most inbred strains 5 days after RSV infection (Table 4.2), and the greatest percent increase in lung protein concentration (compared to respective vehicle controls) 5 days after infection was found in CAST/EiJ and WSB/WiJ mice (Table 4.2).

Following infection with vehicle control the BAL inflammatory cell recruitment was minimal, but significant differences were found between inbred strains (Table 4.3). The most striking difference was noted for macrophage recruitment following vehicle control treatment. Within this exposure group A/J and 129s1/SvlmJ were the most responsive. Interestingly analysis of total cells in BALF after 1 day identified a continuous strain distribution pattern (Figure 4.1A). This response was greatest in all strains 1 day after infection; and 5 days after infection the mean number of inflammatory cells in BAL was still significantly higher than strain-matched vehicle controls (Figure 4.1B). Mean BAL cell differentials 1 day after RSV infection ranged from 152.5 ± 4.4 (WSB/WiJ) to 806.5 ± 53.7 (BALB/cJ). Five days following infection cell differentials were significantly lower and ranged from 99.2 ± 2.7 to 482.7 ± 6.1. The BALB/cByJ strain was one of the most responsive and C3H/HeJ was among the least responsive strains at both time points.

Mean numbers of BAL PMNs were greatest 1 day after RSV in most strains ranging from 31.0 ± 0.3 (FVB/NJ) to 631.4 ± 5.4 (BALB/cByJ) (Figure 4.2A). Mean numbers of PMNs 1 day post RSV in SM/J, C3H/HeOuJ, and MA/MyJ mice were significantly different from all the other strains. BAL PMN numbers were significantly

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lower after 5 days compared to 1 day and ranged from 0 (KK/HlJ) to 202.9 ± 1.9 (PWD/PhJ). Five days after infection mean PMN numbers from PWD/PhJ and SWR/J mice were significantly greater than all other strains. Unlike PMNs, mean numbers of lymphocytes, macrophages and monocytes were highest 5 days after infection (Figure 4.3 and Table 4.3). After 5 days of infection, the greatest number of BAL lymphocytes was found in BTBRT mice (154.5 ± 4.4) and the fewest were found in SJL/J mice (0 ± 0). Lymphocyte numbers found in BAL of WSB/WiJ and BALB/cByJ mice were significantly different from other inbred strains.

BAL epithelial cells were greatest 1 day after RSV infection, and BAL epithelial cell concentration was significantly higher in all strains 1 day after infection compared to vehicle controls and 5 day RSV infected mice (Table 4.3). A continuous strain distribution pattern for mean numbers of BAL epithelial cells was found at 1 and 5 days post RSV infection, in which BALB/cByJ mice were consistently one of the highest responders and C3H/HeJ mice were one of the lowest responders.

Pulmonary pathology

No pulmonary histopathologic changes were noted in any strains of the vehicle exposed groups. Qualitative histopathologic inspection of H&E stained lung sections indicated that lung pathology was most severe 1 day after infection in all inbred strains. In susceptible strains (e.g. A/J) the airway epithelium lining of the first daughter branch was partially exfoliated and severe inflammatory cell infiltration was marked in alveoli and proximal airways. In resistant strains (e.g. C3H/HeJ) airway epithelium exfoliation was not found, however localized inflammatory cell infiltration was observed (Figure 4.4). After 5 days of infection, all lung pathology cleared in C3H/HeJ mice; however epithelial cell hyperplasia was still found in the airways of A/J mice (Figure 4.4).

Mucus cell metaplasia (MCM) following RSV infection

Intraepithelial mucus was not detected in vehicle controls (data not shown). Among all strains examined after RSV infection, mean mucus concentration was greatest after 1 day of infection (Figure 4.5A) and declined 5 days after infection (Figure 4.5B). Quantitative analysis determined that one day following RSV infection, the greatest amount of intraepithelial mucus was found in SJL/J mice, and the lowest was found in BTBRT mice (Figure 4.5A and 4.5B). Representative histology images indicated that intraepithelial mucus concentration is most prominent in the third generation bronchus of the airway (Figure 4.5C). After 5 days of infection the highest bronchial mucus concentration was found in A/J and BALB/cByJ mice (Figure 4.5B).

Viral expression

An estimate of RSV infection in control and RSV-infected mice was obtained by measuring mRNA expression of the RSV-N gene. RSV m-RNA expression directly reflects the amount of virus found in the lung (17). RSV-N mRNA was not detected in any of the control groups. Among the majority of infected strains, lung RSV viral mRNA expression was greatest 1 day following RSV infection, and 5 days after infection viral expression had significantly diminished, but the virus was still present (Table 4.4). One day post RSV infection, the greatest amount of RSV-N mRNA expression was found in AKR/J mice and the lowest mRNA expression was found in C57BL/6J mice (Table 4.4). In some strains, including DBA/2J and 129S1/SvlmJ, viral expression 5 days after infection was significantly higher than viral expression measured 1 day after RSV infection. While viral expression in these few strains were the greatest 5 days post infection, detected viral expression was still significantly lower than those detected in AKR/J mice 1 day after RSV infection.

Relatedness of RSV-induced phenotypes

BAL and histopathology data suggest that response to RSV infection was greatest 1 day following infection compared to 5 days after infection. Pearson and Spearman correlation analyses were performed to determine if the observed phenotypic responses were related to one another. Rank analyses found that the phenotypic responses to RSV 1 day after were almost completely independent of the observed responses 5 days following infection (Figure 4.6). Interestingly, change in body weight 1 day after infection was significantly correlated with inflammatory responses found 5 days following infection. A clinically important phenotype, MCM, was not correlated with other phenotypes (Figure 4.6). It was determined that viral mRNA expression 1 and 5 day(s) following infection were significantly correlated among the inbred strains of mice; however, viral mRNA expression was not related to any of the RSV disease phenotypes examined in this study (Figure 4.6).

Heritability

Heritability estimates were calculated for all the phenotypes to determine how much of the variance in the observed phenotypes is directly related to the genotype (between-strain variation) compared to environmental variance (with-strain variation). Heritability estimates for all the phenotypes in this study were greater than 35%, which is considered significant (18) (Table 4.5). The highest degree of heritability was found for intraepithelial mucus concentration 1 and 5 days after infection, with estimates of 95.7% and 92.1% respectively (Table 4.5). The lowest heritability was found for BAL epithelial cells 1 day following infection (44%).

Discussion

One of the overall objectives of this study was to characterize RSV disease phenotypes in multiple inbred strains of mice in order to model resistant and susceptible human-like pathologies. Multiple inflammatory and injury RSV disease phenotypes were characterized across 36 inbred strains, and significant inter-strain variation was found for each phenotype, and resembled differential disease severity found in humans (11).

Pulmonary hyperpermeability was estimated by the total BAL protein concentration, and wild derived inbred strains such as CAST/EiJ and WSB/WiJ mice had significantly more BAL protein than any other inbred strains. Interestingly, other markers of lung injury (e.g. histopatholgy and numbers of BAL epithelial cells) do not correlate with BAL protein in theses strains, and may suggest that different mechanisms are controlling these phenotypes in these strains.

Inflammatory disease phenotypes across the strains suggest that C3H/HeJ mice are the least responsive to RSV infection and BALB/cByJ mice are among the most highly responsive to the virus. BAL analysis determined that the inflammatory response found 1 day after infection was primarily driven by PMN infiltration. This result was consistent with previous work that directly linked neutrophilia to RSV infection (19-22). However, the strain distribution patterns for some of the key inflammatory cell types (e.g. PMNs, lymphocytes) were not concordant at 1 and 5 days after infection. This may suggest that inflammation does not peak at 1 day in all strains, and that the mechanisms regulating the kinetics of cellular inflammatory response to RSV differ between cell types. Although the strain distribution pattern was not identical for all phenotypes measured some strains were consistently more responsive, including BALB/cByJ, C3H/HeOuJ, and A/J. C3H/HeJ, C57BL/6J and DBA/2J were among the least responsive to RSV infection. Interestingly, previous strain screens identified BALB/cByJ and C57BL/6J mice as highly susceptible and mildly susceptible, respectively, to RSV infection based on viral titers 4 days following infection (14, 23).

Of particular interest in the current study was the differential response of C3H/HeJ mice and C3H/HeOuJ mice. C3H/HeJ and C3H/HeOuJ are genetically identical with the exception of a *Tlr4* dominant negative mutation in C3H/HeJ (24). Results are therefore consistent with the hypothesis that the differential response to RSV observed in these two strains is in part due to a mutation in *Tlr4* (24) A recent study found that *TLR4* contributes to RSV disease severity and *TLR4* polymorphisms result in milder severity in humans (25). The observed differential response between C3H/HeJ and C3H/HeOuJ mice in this study is consistent with other studies, and further SNP analysis found that the same *Tlr4* polymorphisms found in C3H/HeJ mice were also present in C57BL/6J, AKR/J, DBA/2J and 129s1/SvlmJ mice, all of which were determined to be among the least responsive strains (Chapter 6).

An unexpected observation in the inflammatory response was the complete absence of eosinophils across all strains. Previous reports have emphasized the importance of eosinophilia in the murine inflammatory response to RSV (26, 27). The absence of any eosinophils may be due to the strain of RSV (RSV-19 versus RSV-A2) used in this study, because different strains of RSV are known to elicit varying degrees of inflammation (28-32).

Stark *et al.* reported no variation in pulmonary pathology between inbred strains following RSV infection and Anh *et al.* reported that PMV (murine form of RSV) infection caused only subtle qualitative pathological effects (14, 33). Both studies concluded that pathological responses resulting from RSV infection did not correlate with other measured phenotypes. Results from the present study did not support those findings. Analysis of histopathology sections revealed that all inbred strains exhibited some degree of pathological response (mild – severe) following infection, and the peak of this response occurred 1 day after infection. Qualitative analysis of lung sections revealed pathological differences between inbred strains, and identified C3H/HeJ and C57BL/6J as the least responsive and A/J was noted to be the most responsive in terms of

pathology. Interestingly Anh *et al*. found C3H/HeN and C57BL/6N to be two of the most responsive strains. This discrepancy could be due to the source of the mice (mice used in this study were obtained from Jackson Laboratories, and mice used by Anh *et al*. were from Charles River Laboratories), or it may be due to the different phenotypes used to determine responsiveness; Anh *et al*. used viral titer, and this study used immune response to determine responsiveness (33). However, the viral titers reported by Anh *et al*. correlated well with the viral mRNA findings in this study. In both studies 129S1/SvlmJ (129S1/SvlmN) were determined to be highly susceptible to infection, and SJL/J (SJL/N) were relatively resistant to RSV infection.

Qualitative analysis of histopathology sections correlated with BAL inflammatory data, and future quantitative analysis of lung histopathology will be performed to determine the strength of this correlation. While the histopathology findings in this study differed from those of Anh *et al.* the results were highly reproducible and little intrastrain variation was detected. The main reason our histopathology conclusions varied from previous studies is likely due to the sample time for RSV-induced histpathological changes. Earlier studies examined mice for pathological signs of pulmonary inflammation 4 and 5 days following infection respectively (14, 33); in the present study we noted that pathology severity peaked 1 day following infection and was largely cleared 5 days after infection. It is possible that the lung pathology in the two previous studies was evident 1 day after infection and either cleared or was in clearing at the timepoint these groups examined the pathological effects of RSV infection.

In humans significant mucus cell metaplasia (MCM) is found in the airways of individuals suffering from RSV infection, is believed to be the main source of discomfort, and is thought to have a direct relationship to disease severity (34-36). In the present investigation, MCM was measured in the epithelium of the main bronchus, and Pearson and Spearman rank coefficients indicated that MCM was not correlated with viral susceptibility or responsiveness to RSV. Previously MCM in the mouse has been

associated with RSV infection and the resulting immune response using specific knock-out mice, but no such correlation has been found across multiple inbred strains (37-40). The disparate responses found between human and mouse may occur because RSV is not native to mice, which is a criticism of this model (33, 41). While no correlation with immune response or susceptibility was detected, significant inter-strain difference in the MCM phenotype were found. Of the strains examined in this study SJL/J mice had the greatest mucus response, and BTBRT were the least responsive. The response of SJL/J mice 1 day following RSV infection was significantly greater compared to the response of the other strains analyzed. Based on the BAL cell data SJL/J mice do not have a profound inflammatory response to RSV. This may suggest that MCM actually had a protective effect in SJL/J mice (42). This same protective effect is not noted in any of the other inbred strains, which may indicate that this phenomenon is specific to the genotype of SJL/J mice. Similar to other phenotypes examined in this study MCM had diminished significantly after 5 days, but still greater than MCM found in vehicle control mice.

Analysis of the relationship between all measured RSV disease phenotypes found that response 1 day following infection was generally unrelated to the response found 5 days following infection. This observation suggests that different mechanisms regulate response to RSV at each timepoint. BAL cell differentials indicate that the response found 1 day after infection is primarily driven by neutrophils. We also found that BAL cell types associated with adaptive immunity were greatest 5 days after infection. Interestingly Pearson correlation coefficients indicated that changes in body weight 1 day following infection was directly linked to the inflammatory response 5 days after infection. Loss of body weight is an indicator of clinical illness, and a change in body weight 1 day after infection may be a predictor of the response 5 days following infection. Data from a recent study suggest that some of the same genes that regulate RSV-induced pulmonary inflammation also influence the loss of body weight associated with RSV infection (43). It is hypothesized that recruitment of chemokines and

inflammatory mediators following infection is responsible for the observed change in body weight.

Viral mRNA expression analyses confirmed that all inbred strains were successfully infected with RSV, and suggested that no strain was completely resistant to infection. Although viral load was not definitively related to disease in this study, RSV viral load has been linked to disease severity noted mainly by weight loss and bronchiolitis in humans. In particular, when RSV viral replication was reduced the inflammatory response and clinical signs of disease were significantly dampened (44-47). Contrary to results of previous studies, correlation analyses in the present investigation found that viral mRNA expression was not related to other disease phenotypes. For example, based on all the phenotype data in this study BALB/cByJ were determined to be the most responsive strains to RSV infection, however measured viral load of this strain were one of the lowest. This finding is in contrast to previous reports which found that BALB/cByJ mice are highly susceptible to infection and had the highest viral loads, but does support findings reported by Jafri and colleagues (13, 14, 48). In humans, severity of RSV disease is measured by decrease lung function which usually results from pulmonary inflammation, and airway MCM. Since viral load did not correlate with any disease phenotypes in the present study, identification of the genes that regulate viral load and replication will likely not be useful predictors RSV severity. On the other hand, because BAL phenotypes were found to be relevant markers of disease, greater understanding of the genetics of RSV disease may provide great insight to the differential responses found in this study.

The results of this study indicate that susceptibility and response to RSV infection are complex traits. Heritability calculations suggest that the observed variability of phenotypes between strains is largely attributed to the genetic differences between inbred strains, although environmental factors also contribute to the phenotypic variance. Furthermore, the variation of response to RSV infection across inbred strains

approximates the wide variation in RSV disease found in humans, suggesting that identification of genetic determinants of RSV phenotypes in this model may be important for understanding human disease. Results of this study provide the basis for future investigations to identify specific genes responsible for RSV disease phenotypes (Chapters 5 and 6).

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Figure Legends

Figure 4.1. Total inflammatory cells measured in BAL following RSV infection.
Total number of inflammatory cells in BAL from inbred strains of mice 1 day following RSV infection (*A*) and 5 days following infection (*B*). Data are presented as mean ± SEM (n = 6-12 mice/group). *Significantly different compared with lowest responder. +Significantly different compared with highest responder. #Significantly different compared with all other strains, ($P < 0.05$).

Figure 4.2. Total PMNs measured in BAL following RSV infection. Total number of PMNs found in BAL from inbred strains of mice 1 day following RSV infection (*A*) and 5 days following infection (*B*). Data are presented as mean ± SEM (n = 6-12 mice/group). *Significantly different compared with lowest responder. +Significantly different compared with highest responder. #Significantly different compared with all other strains, ($P < 0.05$).

Figure 4.3. Total lymphocytes measured in BAL following RSV infection. Total number of lymphocytes found in BAL from inbred strains of mice 1 day following RSV infection (*A*) and 5 days following infection (*B*). Data are presented as mean ± SEM (n = 6-12 mice/group). *Significantly different compared with lowest responder. +Significantly different compared with highest responder. #Significantly different compared with all other strains, ($P < 0.05$).

Figure 4.4. Pulmonary pathology following RSV infection in inbred strains of mice.
Representative images of lung sections from RSV-resistant C3H/HeH and RSV-susceptible A/J inbred strains following vehicle and RSV infection. Arrows indicate

areas of increased airway inflammation and bronchial epithelial proliferation (hyperplasia). AV = Alveoli, BR = bronchus or bronchiole, BV = blood vessel. Bar = 100mm.

Figure 4.5. Pulmonary mucus cell metaplasia (MCM) in inbred strains of mice following RSV infection. Quantitative analysis of intraepithelial MCM across inbred mouse strains 1 day following infection (*A*) and 5 days following infection (*B*). Data are presented as mean ± SEM (n = 3 mice/group). *Significantly different compared with lowest responder. +Significantly different compared with highest responder. #Significantly different compared with all other strains, (*P* < 0.05). Representative images visualize AB/PAS-stained lung sections for intraepithelial mucous from two differentially responsive mouse inbred strains following vehicle and RSV infection (*C*). *Arrows* indicate intraepithelial mucous, which are stained blue. AV = Alveoli, BR = bronchus or bronchiole, BV = blood vessel. Bar = 100mm.

Figure 4.6. Node and edge representation of the relatedness of RSV response phenotypes in inbred strains of mice. Pearson correlation coefficients noted on edges indicate the association between phenotypes.

Figure 4.1.

Figure 4.2.

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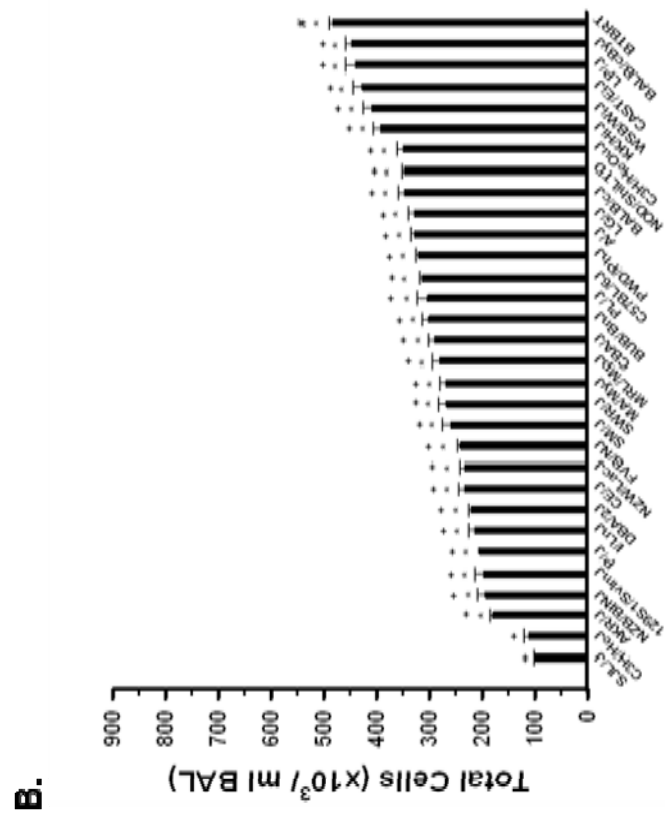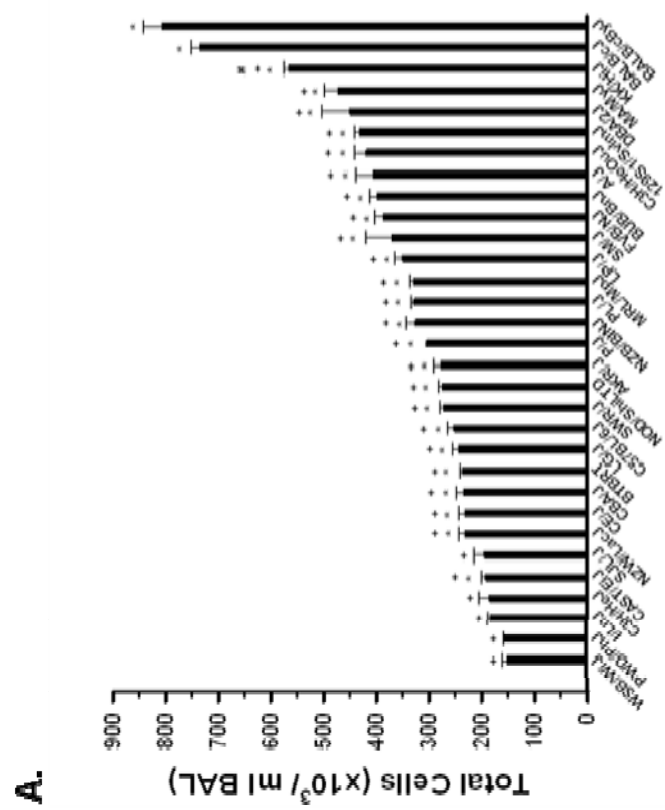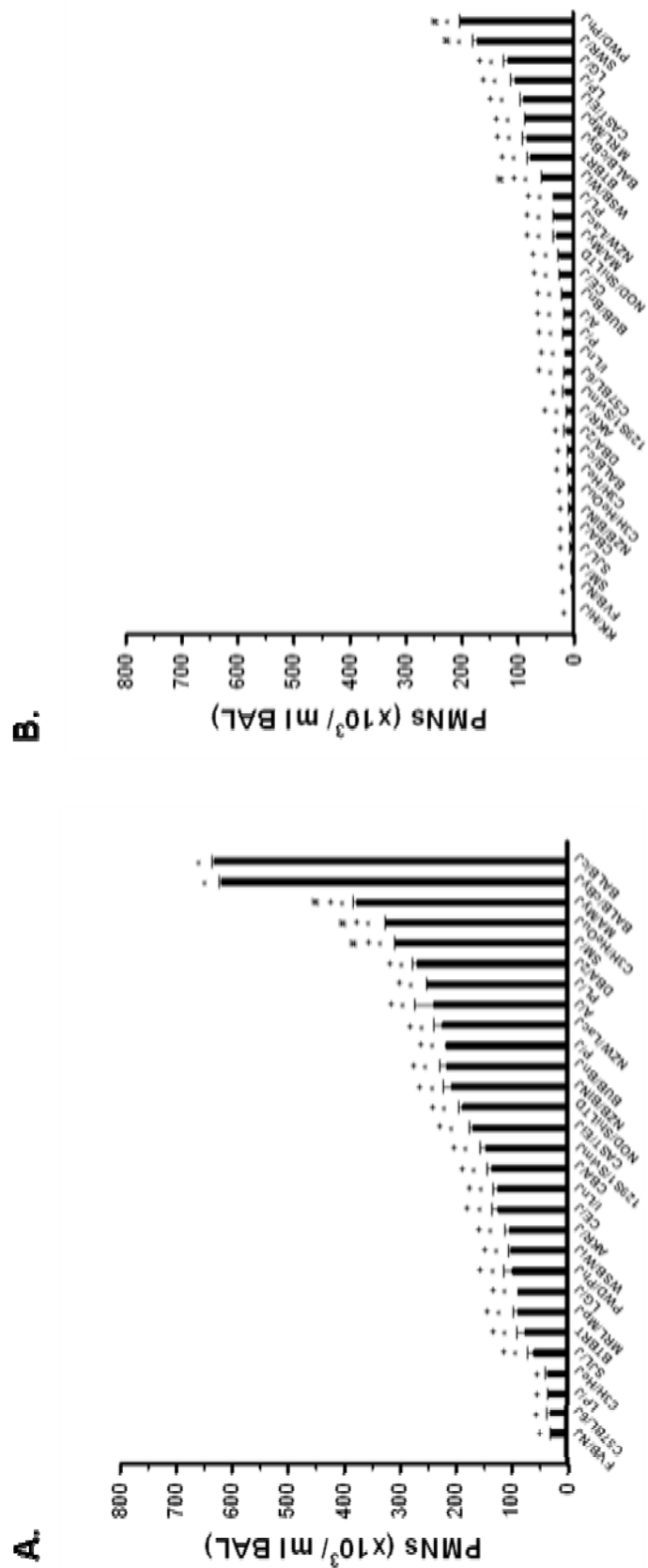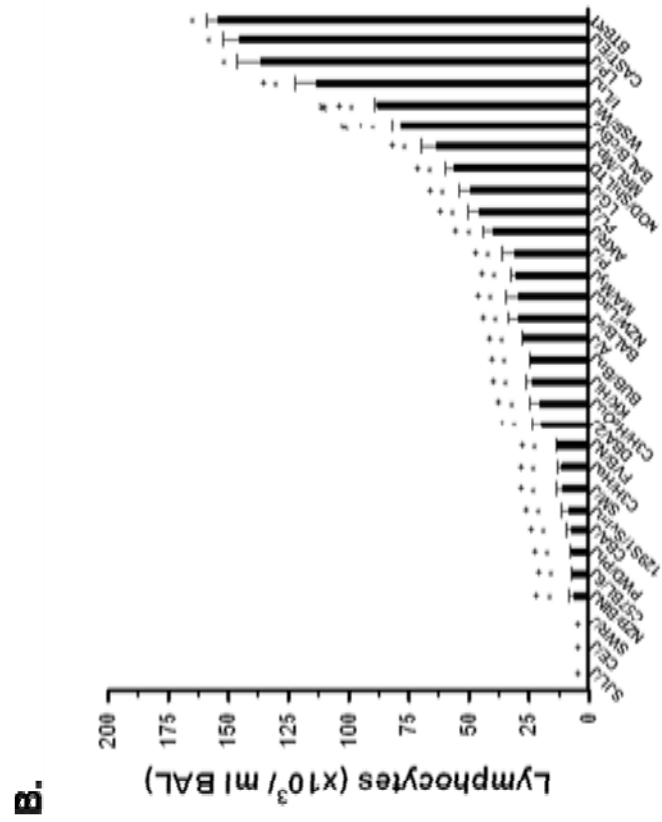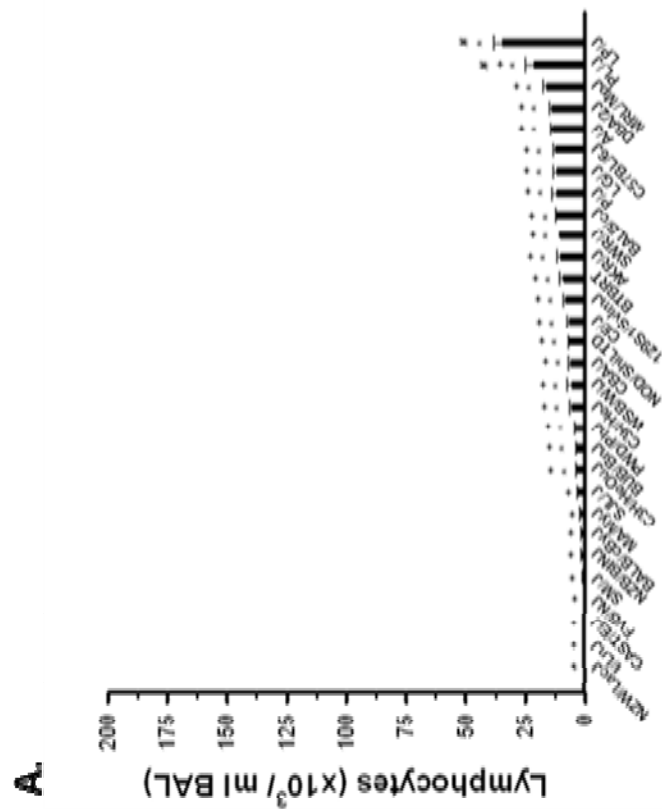
Figure 4.3.

Figure 4.4.

Figure 4.5.

Figure 4.6.

Pearson correlations
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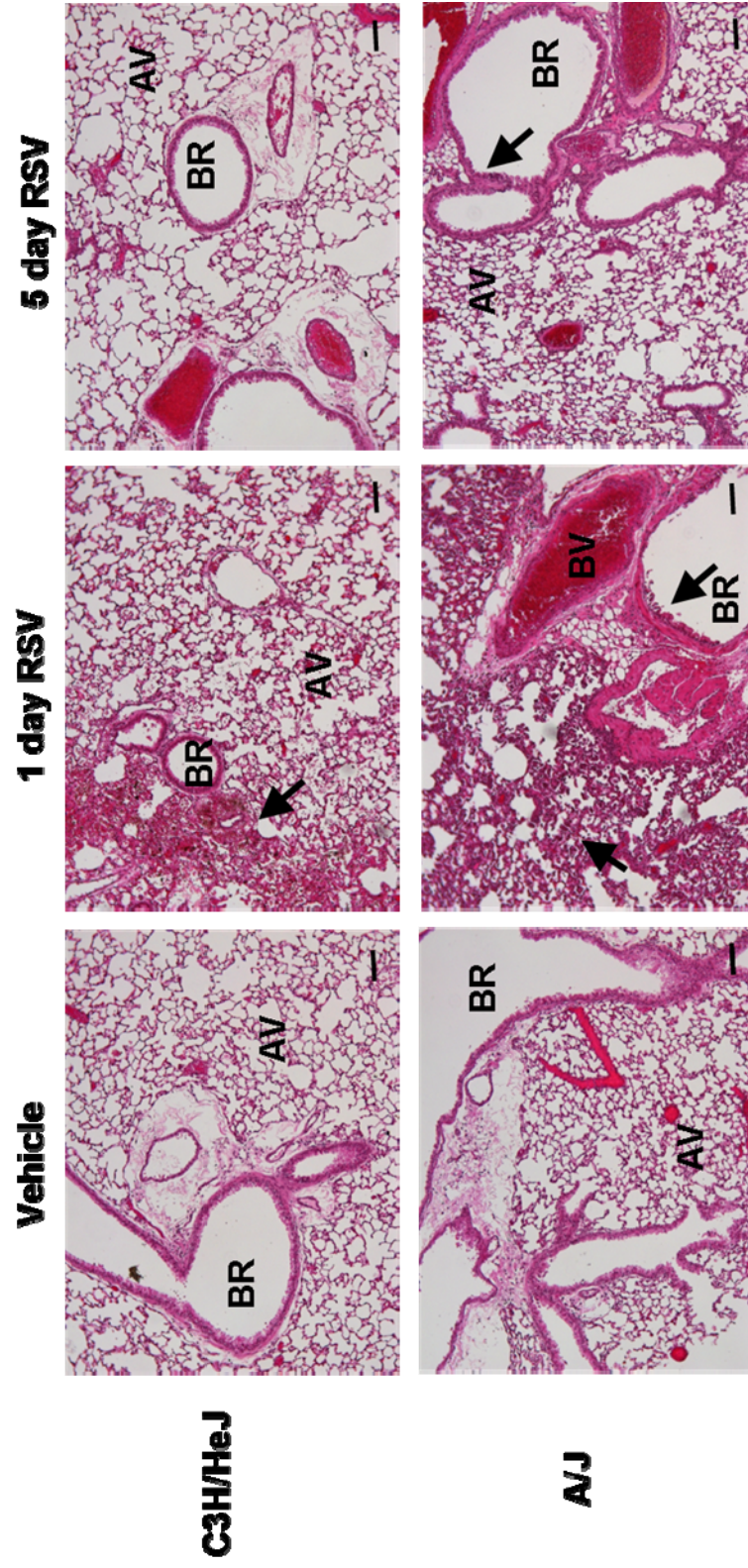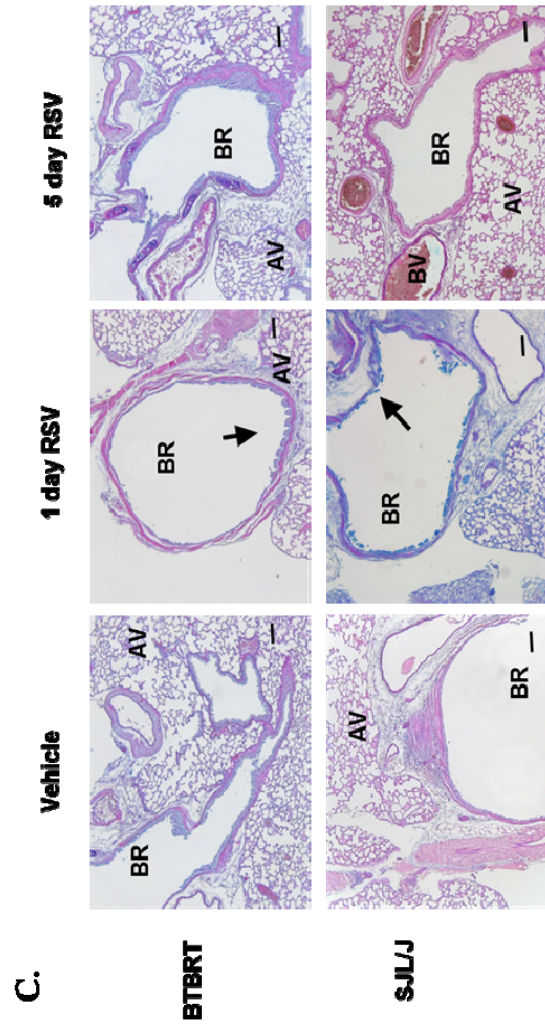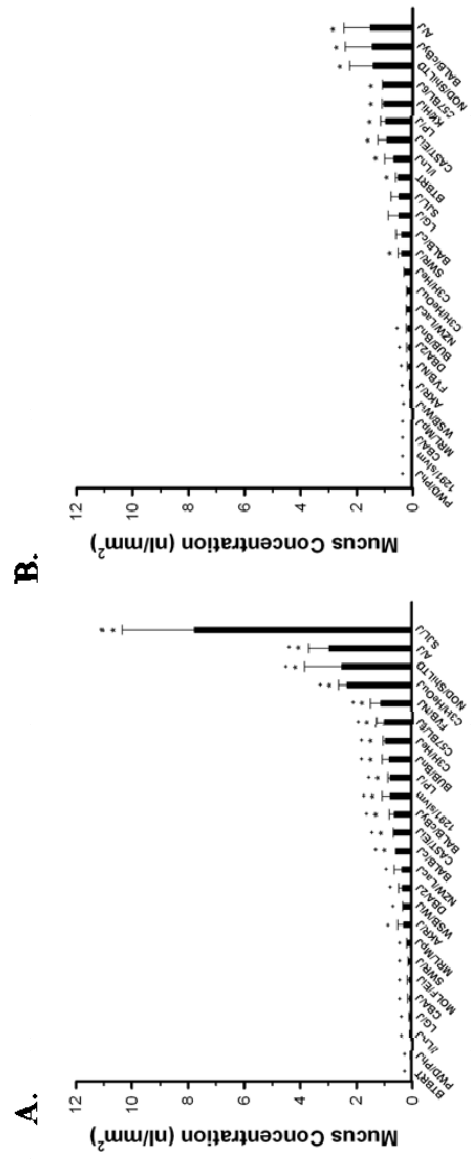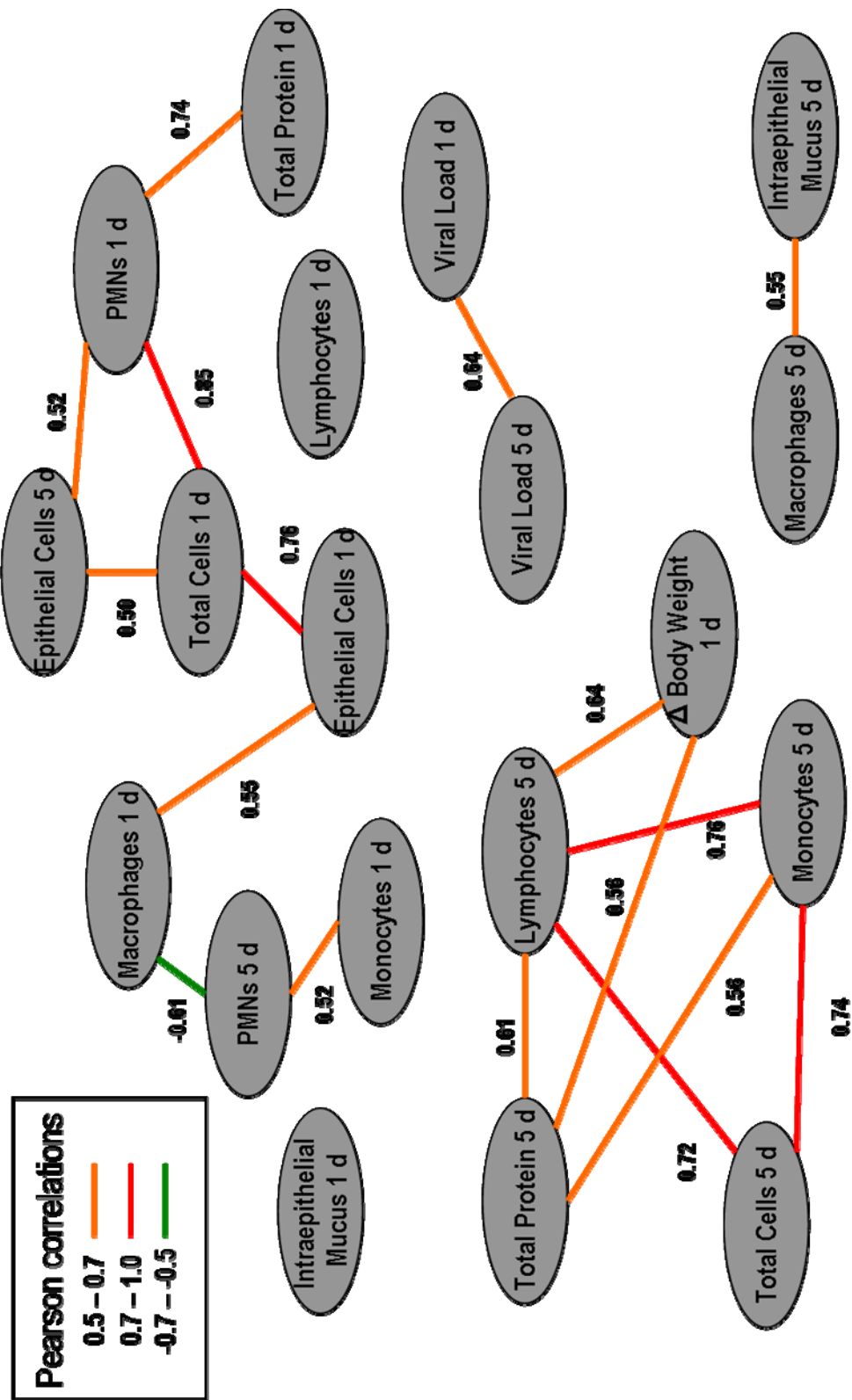
Table 4.1. Weight and age of inbred mouse strains.

Inbred strains	Approximate age (weeks)	Weight (g) ± SEM
A/J	7	22.3 ± 2.1
AKR/J	6	24.8 ± 2.4
BALB/cByJ	7	22.4 ± 1.9
BALB/cJ	7	21.5 ± 1.4
BTBRT	6	27.8 ± 3.2
BUB/BnJ	7 - 8	26.2 ± 1.8
CAST/EiJ	7 - 10	14.5 ± 1.6
CBA/J	8	23.5 ± 2.9
CE/J	8	21.5 ± 1.6
C3H/HeJ	6	23.1 ± 2.5
C3H/HeOuJ	6 – 8	21.8 ± 1.5
C57BL/6J	6	21.3 ± 1.8
DBA/2J	7 – 8	22.4 ± 2.1
FVB/NJ	7	20.6 ± 2.5
I/LnJ	6 – 7	23.6 ± 1.8
KK/HlJ	6 – 8	31.8 ± 2.7
LG/J	8	36.5 ± 2.2
LP/J	6	15.8 ± 2.2
MA/MyJ	6	24.6 ± 2.8
MOLF/EiJ	7 - 9	12.2 ± 0.2
MRL/MpJ	6	35.5 ± 3.3
NOD/LtJ	6 – 7	21.5 ± 1.3
NZB/BlNJ	7	25.2 ± 2.4
NZW/LacJ	7	30.2 ± 2.7
PL/J	6	24.2 ± 2.4
P/J	6	23.5 ± 2.1
PWD/PhJ	6 – 9	16.3 ± 2.3
SJL/J	7	20.9 ± 1.1
SM/J	6	24.6 ± 2.9
SWR/J	6	19.4 ± 1.7
WSB/WiJ	7 – 9	15.1 ± 2.1
129s1/svlmJ	7	18.2 ± 1.8

Table 4.2. **Total protein concentration in BAL** (μg/ml) after vehicle (5 days) or RSV infection (1 and 5 days) in 31 inbred strains of mice. Data are presented as mean ± SEM (n = 6-12 mice/group).

	Total BAL Protein		
Inbred Strains	**Vehicle**	**1 day RSV**	**5 day RSV**
A/J	120.9 ± 41.6	243.0 ± 49.8	231.2 ± 61.0
AKR/J	289.8 ± 93.1	263.6 ± 49.2	392.5 ± 114.4
BALB/cByJ	233.7 ± 17.7	359.3 ± 40.8	240.2 ± 19.2
BALB/cJ	196.9 ± 21.5	425.1 ± 42.3	425.1 ± 59.8
BTBRT	145.4 ± 8.3	258.0 ± 14.8	431.6 ± 59.8
BUB/BnJ	301.5 ± 13.1	343.9 ± 50.4	364.8 ± 34.4
CAST/EiJ	90.7 ± 15.1	212.4 ± 32.4	608.4 ± 100.4
CBA/J	216.9 ± 34.0	233.7 ± 14.1	209.9 ± 19.0
CE/J	54.5 ± 11.0	263.4 ± 73.2	189.1 ± 21.8
C3H/HeJ	239.7 ± 20.3	150.7 ± 13.1	176.4 ± 12.1
C3H/HeOuJ	223.6 ± 15.4	280.9 ± 95.4	319.8 ± 36.4
C57BL/6J	362.2 ± 35.3	227.5 ± 23.9	227.9 ± 48.9
DBA/2J	225.9 ± 45.4	313.4 ± 56.9	313.4 ± 80.5
FVB/NJ	153.0 ± 7.0	320.0 ± 57.0	306.1 ± 65.8
I/LnJ	242.4 ± 8.5	300.1 ± 26.1	307.5 ± 36.9
KK/HlJ	204.3 ± 36.4	195.9 ± 28.9	200.8 ± 37.3
LG/J	132.4 ± 19.5	125.6 ± 12.3	182.6 ± 23.1
LP/J	110.1 ± 14.5	327.7 ± 45.6	277.2 ± 28.7
MA/MyJ	127.0 ± 5.3	440.8 ± 57.7	245.5 ± 20.8
MRL/MpJ	130.1 ± 6.8	223.6 ± 34.3	229.1 ± 22.9
NOD/LtJ	173.2 ± 11.2	469.2 ± 27.8	168.5 ± 21.5
NZB/BlNJ	160.5 ± 18.2	190.5 ± 28.9	165.5 ± 14.7
NZW/LacJ	263.5 ± 21.6	214.1 ± 24.4	298.5 ± 32.3
PL/J	146.5 ± 14.1	314.4 ± 38.8	348.4 ± 33.7
P/J	56.3 ± 8.9	221.4 ± 28.9	181.6 ± 29.8
PWD/PhJ	83.7 ± 18.3	321.2 ± 65.8	189.1 ± 29.6
SJL/J	85.1 ± 12.2	141.4 ± 26.2	113.3 ± 22.2
SM/J	117.8 ± 20.9	329.7 ± 47.1	133.3 ± 4.8
SWR/J	80.0 ± 5.0	126.7 ± 21.6	108.6 ± 13.2
WSB/WiJ	176.6 ± 17.5	220.4 ± 20.8	629.5 ± 49.5
129s1/svlmJ	245.4 ± 33.2	258.6 ± 12.6	269.1 ± 30.2

Table 4.3. BAL phenotypes following RSV infection. Inflammatory cells recovered in bronchoalveolar lavage fluid ($x10^3$/ml) after vehicle (5 days) or RSV infection (1 and 5 days) in 31 inbred strains of mice. Data are presented as mean ± SEM (n = 6-12 mice/group).

BAL Cell Differentials								
Macrophages			**Monocytes**			**Epithelial Cells**		
Inbred Strains	**Vehicle**	**1 day**	**5 day**	**Vehicle**	**1 day**	**5 day**	**Vehicle**	**1 day**	**5 day**
A/J	192 ± 33.4	86.9 ± 8.4	205.7 ± 4.5	1.0 ± 0.3	24.8 ± 2.2	18.9 ± 1.5	23.5 ± 4.5	39.8 ± 2.2	30.5 ± 2.4
AKR/J	48.6 ± 1.7	111.2 ± 4.7	44.2 ± 8.5	0.7 ± 0.2	7.2 ± 0.7	5.4 ± 2.0	8.0 ± 3.1	43.7 ± 3.3	32.1 ± 3.4
BALB/cByJ	117.3 ± 4.8	57.0 ± 3.0	184.4 ± 3.6	2.1 ± 0.5	7.4 ± 0.7	26.8 ± 2.0	44.0 ± 4.8	57.0 ± 0.7	67.1 ± 1.6
BALB/cJ	64.5 ± 3.7	99.8 ± 3.3	141.8 ± 5.7	2.6 ± 1.5	5.0 ± 0.3	53.0 ± 2.7	6.7 ± 2.5	56.0 ± 2.6	80.7 ± 4.7
BTBRT	104.0 ±5.2	62.9 ± 6.1	153.2 ± 4.5	2.3 ± 0.4	5.9 ± 0.3	60.3 ± 3.2	13.3 ± 2.2	15.4 ± 0.3	36.2 ± 1.5
BUB/BnJ	121.9 ± 2.4	79.0 ± 4.9	209.5 ± 1.7	1.2 ± 0.3	23.9 ± 2.9	18.8 ± 1.0	15.2 ± 1.3	73.4 ± 6.1	24.8 ± 0.5
CAST/EiJ	78.8 ± 5.4	11.6 ± 1.5	111.1 ± 6.5	4.5 ± 1.0	3.9 ± 0.2	55.6 ± 5.7	15.7 ± 3.5	7.7 ± 1.9	12.8 ± 2.6
CBA/J	78.5 ± 5.9	58.8 ± 7.1	208.8 ± 6.2	2.3 ± 1.5	11.7 ± 2.4	22.6 ± 2.9	30.8 ± 4.9	19.3 ± 1.1	35.9 ± 3.1
CE/J	69.9 ± 2.9	79.2 ± 5.2	163.1 ± 4.2	0	0	0	16.8 ± 1.1	20.9 ± 1.4	44.2 ± 3.6
C3H/HeJ	129.5 ± 2.5	103.2 ± 0.8	59.6 ± 6.9	0.6 ± 0.3	3.4 ± 0.7	7.1 ± 2.2	30.5 ± 2.1	35.2 ± 5.5	37.7 ± 4.2
C3H/HeOuJ	103.8 ± 7.8	45.7 ± 0.3	169.6 ± 1.5	0.3 ± 0.2	1.8 ± 0.2	51.9 ± 1.6	25.2 ± 8.7	41.4 ± 0.8	76.9 ± 3.2
C57BL/6J	45.6 ± 0.9	157.9 ± 6.2	76.1 ± 2.0	0.6 ± 0.7	1.3 ± 0.5	22.5 ± 3.6	7.6 ± 0.8	48.9 ± 3.5	39.3 ±3.6
DBA/2J	136.5 ± 3.1	95.2 ± 6.1	61.8 ± 2.6	0.7 ± 0.3	8.9 ± 0.5	15.2 ± 1.3	25.8 ±2.9	59.1 ± 2.7	17.8 ± 1.5
FVB/NJ	98.5 ± 1.9	68.8 ± 4.1	193.3 ± 1.3	0	7.7 ± 0.5	4.8 ± 0.6	9.4 ± 1.8	31.0 ± 0.3	28.9 ± 3.5
I/LnJ	36.1 ± 0.4	29.8 ± 2.7	32.1 ± 8.7	3.4 ± 1.3	0.7 ± 0.1	3.2 ± 0.9	37.2 ± 0.4	17.9 ± 2.3	47.1 ± 2.6
KK/HiJ	60.7 ± 3.1	100.7 ± 8.9	282.8 ± 2.5	0	2.5 ± 0.3	15.7 ± 2.4	46.4 ± 1.8	66.4 ± 4.5	70.7 ± 5.6
LG/J	61.3 ± 2.5	122.7 ± 10	124.8 ± 15	0	0.9 ± 0.2	6.1 ± 1.0	10.3 ± 1.4	15.9 ± 4.2	60.1 ± 9.0
LP/J	83.8 ± 1.2	57.5 ± 1.5	103.3 ± 4.3	3.7 ± 1.7	2.3 ± 0.5	13.2 ± 1.7	36.9 ± 5.2	25.3 ± 1.5	106 ± 4.6
MRL/MpJ	90.5 ± 2.6	59.3 ± 2.7	69.7 ± 5.3	0	0.8 ± 0.2	7.5 ± 0.9	22.8 ± 2.0	21.8 ± 0.9	51.4 ± 1.6
NOD/LtJ	122.8 ± 6.1	55.2 ± 6.3	171.1 ± 4.9	3.4 ± 0.9	8.9 ± 2.1	18.0 ± 2.2	23.7 ± 3.7	21.4 ± 1.4	65.1 ± 3.6
NZW/LacJ	142.5 ± 1.4	84.5 ± 5.8	106.1 ± 1.5	0.4 ± 0.2	0.5 ± 0.1	19.7 ± 2.8	19.6 ± 1.4	22.7 ± 1.7	54.2 ± 2.5
PL/J	24.6 ± 0.2	24.6 ± 0.2	101.6 ± 2.0	0	0	25.4 ± 2.0	27.1 ± 3.4	21.9 ± 2.3	33.4 ± 0.2
PWD/PhJ	94.7 ± 8.7	10.9 ± 2.9	69.3 ± 1.3	0.6 ± 0.2	35.9 ± 3.5	19.2 ± 1.4	15.5 ± 1.4	4.6 ± 0.1	20.5 ± 1.1
SJL/J	95.0 ± 0.7	86.4 ± 12.1	66.8 ± 3.5	0.2 ± 0.1	0.8 ± 0.1	10.2 ± 1.3	11.1 ± 0.5	28.1 ± 2.7	14.6 ± 2.1
SWR/J	89.4 ± 1.7	135.6 ± 16	132.0 ± 8.4	0	8.2 ± 0.5	9.8 ± 0.8	20.9 ± 2.0	24.7 ± 2.9	17.8 ± 1.6
WSB/WiJ	80.1 ± 1.4	25.9 ± 2.7	126.8 ± 1.1	15 ± 0.7	3.0 ± 0.8	71.6 ± 0.8	37.0 ± 0.5	10.6 ± 0.2	63.4 ± 1.4
129s1/svlmJ	270.3 ± 2.0	158.0 ± 2.5	54.4 ± 5.6	3.1 ± 0.5	8.6 ± 1.1	5.2 ± 1.9	28.5 ± 2.1	41.1 ± 2.6	25.9 ± 6.1

Table 4.4. RSV-N mRNA expression following infection. RSV-N mRNA expression normalized to 18S mRNA expression in 32 inbred strains of mice. Data are presented as mean ± SEM (n = 6-12 mice/group).

RSV-N mRNA Expression			
Inbred Strains	**Vehicle**	**1 day RSV**	**5 day RSV**
A/J	0	1.0 ± 0.3	0.9 ± 0.2
AKR/J	0	5.8 ± 2.1	6.1 ± 0.7
BALB/cByJ	0	6.0 ± 1.1	4 ± 0.6
BALB/cJ	0	6.1 ± 1.1	4.5 ± 0.6
BTBRT	0	4.2 ± 1.0	4.3 ± 0.8
BUB/BnJ	0	1.4 ± 0.6	1.1 ± 0.2
CAST/EiJ	0	0.9 ± 0.2	0.8 ± 0.4
CBA/J	0	4.2 ± 1.0	2.5 ± 0.8
CE/J	0	1.1 ± 0.5	2.1 ± 0.9
C3H/HeJ	0	1.3 ± 0.6	1.1 ± 0.5
C3H/HeOuJ	0	5.4 ± 1.2	3.3 ± 0.9
C57BL/6J	0	2.7 ± 0.8	2.5 ± 0.9
DBA/2J	0	4.3 ± 1.1	6.2 ± 1.2
FVB/NJ	0	6.1 ± 2.0	2.2 ± 0.7
I/LnJ	0	2.6 ± 1.3	1.4 ± 0.7
KK/HlJ	0	5.5 ± 1.9	4.1 ± 2.3
LG/J	0	3.3 ± 1.4	2.9 ± 1.1
LP/J	0	0.9 ± 0.3	1.3 ± 0.2
MA/MyJ	0	3.9 ± 1.0	3.2 ± 0.6
MOLF/EiJ	0	2.2 ± 0.4	1.7 ± 0.2
MRL/MpJ	0	1.5 ± 0.4	1.2 ± 0.3
NOD/LtJ	0	7.1 ± 1.4	5.1 ± 0.2
NZB/BlNJ	0	5.1 ± 1.5	4.7 ± 2.1
NZW/LacJ	0	5.5 ± 2.5	4.3 ± 0.7
PL/J	0	2.6 ± 0.3	1.9 ± 0.5
P/J	0	3.3 ± 1.1	3.9 ± 0.8
PWD/PhJ	0	2.3 ± 0.9	2.6 ± 0.7
SJL/J	0	7.5 ± 2.6	2.6 ± 1.0
SM/J	0	4.3 ± 1.1	2.1 ± 0.6
SWR/J	0	5.3 ± 1.4	1.1 ± 0.3
WSB/WiJ	0	2.5 ± 0.9	2.6 ± 0.7
129s1/svlmJ	0	3.0 ± 1.6	7.9 ± 1.1

Table 4.5. **Heritability of phenotypes.** Estimated heritability of RSV phenotypes among 33 inbred strains of mice. Inflammatory cells differentials were measured in BAL, Intraepithelial mucus was determined from AB/PAS stained light micrographs and viral load was measured using right lung mRNA.

Heritability of Phenotypes		
	1 day after infection	**5 days after infection**
Lung Hyperpermeability	47.6%	55.8%
Total cells in BALF	72.1%	74.8%
Epithelial Cells	44.0%	64.6%
Lymphocytes	63.1%	68.0%
Macrophages	55.8%	68.0%
Monocytes	63.1%	61.7%
Neutrophils	65.0%	89.8%
Intraepithelial Mucus	95.7%	92.1%
Viral load	78.5%	72.6%

CHAPTER 5

GENETIC CONTRIBUTION TO RSV RESPONSE AND SUSCEPTIBILITY

Abstract

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection (LRTI) in infants. Approximately 70% of infants are infected with RSV within the first year of life. Previous *in vivo* studies have suggested that RSV susceptibility is a polygenic trait; however, the specific genes regulating this phenotype have yet to be identified.

Purpose: The overall objective of this research was to utilize haplotype association approaches to identify quantitative trait loci (QTL) that contain genes that contribute to differential disease phenotypes in response to RSV infection among multiple inbred strains of mice.

Methods: BAL and pathology phenotypes following vehicle and RSV infection were measured across 36 inbred mouse strains. Using haplotype association mapping, the degree of correlation between the observed phenotypic differences and the genotype of 36 inbred strains was used to determine candidate quantitative trait loci (QTLs). Candidate genes were identified within the most prominent QTLs based on biological plausibility (i.e. potential role in the phenotype based on published literature). Statistical analysis was performed to determine the relationship between genetic SNPs and RSV disease. SNPs with $p<0.05$ were considered important to RSV-induced immune response.

Results: A previous study (Chapter 4) found a continuous strain distribution pattern of phenotypes in response to RSV infection across inbred mouse strains, and indentified highly responsive (e.g. BALB/cByJ, C3H/HeOuJ) and low responsive (C57BL/6J, C3H/HeJ) mouse strains. Data from this same study suggest that response to RSV infection is a polygenic trait. In the present study, haplotype association analyses identified eleven QTLs that were associated with RSV inflammation, and 9 candidate genes were identified within these QTLs. SNPs within *St18, Marco, Stat4, Kif27,*

Slc28a3 and *Mpg* were significantly associated with RSV disease phenotypes, suggesting a potential role in regulating the immune response to RSV infection.

Conclusions: Haplotype association mapping identified a number of QTLs that partially accounted for genetic variance in important RSV disease phenotypes. Further, candidate genes were identified that have biological plausibility and functional polymorphisms correlated with RSV responses among the inbred strains. These findings add to our understanding of RSV-induced immune responses, and may lead to novel intervention strategies to prevent disease progression.

Introduction

Respiratory syncytial virus (RSV) is the primary cause for hospitalization during the first year of life, and is the leading cause of bronchiolitis, pneumonia, mechanical ventilation and respiratory failure in infants worldwide (1). RSV is also a significant cause of respiratory illness in immunocompromised adults and the elderly (2). While the majority of infected individuals present symptoms, the nature and severity of the symptoms vary among individuals (3, 4). For some, infection induces cold-like symptoms, others require hospitalization, and a small percentage of cases result in death. The wide variation in response to RSV infection suggests that susceptibility and response to RSV is influenced by the genotype of the host (1, 5, 6).

Previous *in vivo* studies have found that susceptibility and response to RSV infection is a multigenic trait, but did not determine the genes responsible for susceptibility and response (5, 6). Other studies found that transcription factors including NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and c/EBP (CCAAT-enhancer-binding proteins) regulate susceptibility to infection, and inflammatory and viral response mediators such as *TLR4* (toll-like receptor 4)*, CX3CR1* (chemokine (C-X3-C motif) receptor 1)*, MX1* (myxovirus resistance 1) and *STAT* (signal transducer and activator of transcription) may modulate the response to infection (7-10). Nearly all components of the immune system have been implicated in the pathogenesis of RSV infection, including inflammatory cells and their products such as chemokines, cytokines, and soluble inflammatory mediators (11-15). Epidemiology studies in children suggest that polymorphisms in surfactant association proteins and several cytokines are associated with the immune response to RSV (16-24). Currently there is controversy surrounding the role all of these components play in the pathogenesis of RSV infection. A number of animal and epidemiological studies have sought to identify the genes that regulate RSV disease; however, the exact genes responsible for susceptibility and response to RSV infection have yet to be identified.

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Severity of RSV infection is a complex, multigenic trait, which is characterized by many phenotypes. Several genes have previously been linked to certain RSV phenotypes using a candidate gene approach; however, the genetic profile responsible for the disease as a whole is still unknown. In the present study, haplotype association analyses were used to identify QTLs and candidate genes that account for genetic variations in RSV disease severity among inbred strains of mice.

Material and Methods

Animals and Treatment

Thirty-six male inbred strains of mice were studied (Table 3-1). All mice were approximately 6-10 weeks of age, and purchased from Jackson Laboratory (Bar Harbor, ME). *Marco* deficient mice (*Marco^-/-^)* were developed using homologous recombination and were backcrossed for at least 8 generations to the BALB/cByJ background (provided by T. Kodama, University of Tokyo, Tokyo, Japan). All mice were individually housed and handled as described in Howden *et. al* (25). Mice were infected with 10^6 plaque-forming units (PFU) of human RSV-19 strain in 50 μL Hank's balanced salt solution (HBSS) by intranasal instillation. HBSS containing Hep-2 cell lysates were intranasally instilled into mice for vehicle controls. Animals were killed 1 or 5 days after intranasal instillation (n = 6 per group). On designated post-instillation days, mice were killed by sodium pentobarbital overdose (104 mg/kg body weight). All animal use was approved by the National Institute of Environmental Health Sciences and Animal Care Use Committee.

Haplotype Association Mapping

Haplotype association mapping was performed as previously described (26, 27). Haplotype associations were calculated using a modified F-statistic based on genotype-phenotype pairings at each 3-SNP window across a 650,000 SNP data set. SpotFire (SpotFire, Inc.) was used to plot Log*P* values across the mouse genome. Genes within the most prominent QTLs (those with Log*P* values >4) were identified using SpotFire and their functions were assessed using BioGPS (www.biogps.gnf.org). SNPs of candidate genes were identified and analyzed using The Jackson Laboratory Mouse Phenome Database (www.phenome.jax.org).

Lung Bronchoalveolar Lavage Fluid (BALF) and Cell Preparation

Mice were euthanized with sodium pentobarbital (104 mg/kg) 1 or 5 days following vehicle or RSV infection, and BAL was performed and cell differentials were determined as previously described (28). The time points chosen for evaluation of inflammation and injury were based on previous investigations that characterized the kinetics of inflammation and injury responses to RSV infection (see Chapter 4).

Lung Histopathology.

Left lung lobes were fixed with 10% neutral buffered zinc formalin for 24 hrs. Proximal and distal intrapulmonary axial tissues were excised and embedded in paraffin and sectioned (5 µm). Tissue sections were histochemically stained with hemotoxylin and eosin (H&E) for comparison of pathology and injury between inbred strains. The terminal bronchioles and alveoli were the primary focus of study because RSV infection causes inflammation in epithelial cell sloughing in these regions of the mouse lung. Tissue sections were stained via alcian blue-periodic acid shift (AB/PAS) double staining method for comparison of mucus cell metaplasia (MCM) between inbred strains.

Statistical Analysis

The statistical association between genotype and phenotype within mouse strains was calculated using the two-tailed Student's t-test. The statistical significance in these comparisons was accepted at $p < 0.05$. Only the significance of nonsynonymous coding SNPs were used in this study, if more than one nonsynonymous coding SNP was found within a gene multiple comparisons were performed.

Results

Phenotypic characterization of RSV disease across inbred mouse strains

Thirty-six inbred mouse strains were previously infected with vehicle control or RSV and BAL and pathology phenotypes were measured 1 and 5 day(s) following infection (Chapter 4). Data from this study indicated that the total cell and PMN responses to RSV infection are greatest 1 day after infection, and the lymphocyte and monocyte responses were greatest 5 days after infection (Figures 4.1). A continuous strain distribution pattern was found for all of the phenotypes analyzed and certain strains were determined to be highly responsive to RSV infection, and others were found to be relatively non-responsive to infection. For example BALB/cByJ was one of the most responsive strains and C3H/HeJ was among the least responsive strains (Table 4.3). The observed continuous strains distribution pattern (Figures 4.1, 4.2, 4.3), limited intrastrain variation (Table 4.3) and high heritability estimates (Table 4.5) indicate that response to RSV infection is a multigenic trait.

Identification of candidate genes for susceptibility and response to RSV infection

Haplotype association was utilized to perform genome-wide searches for QTLs for four BAL phenotypes (total inflammatory cells and PMNs 1 day RSV infection, lymphocytes and monocytes 5 days following infection) using the entire inbred strain data set (Table 4.3). Haplotype association mapping identified QTLs that exceeded Log$P$ = 4 for the total cellular inflammatory response located on chromosomes 1, 10, and 12 (Figure 5.1A). QTLs on chromosomes 10 and 13 were linked to the PMN response (Figure 5.1B). Haplotype association mapping for monocyte response identified QTLs on chromosomes 1, 14 and 15 (Figure 5.1C). Linkage analysis for BAL lymphocyte responses identified QTLs located on chromosomes 1, 4, 11 and 13 (Figure 5.1D). Further examination of these QTLs identified genes that have a strong association with particular RSV phenotype (Figure 5.2).

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Identification of candidate susceptibility genes

The aforementioned QTLs contained many genes; however several of these genes that could be reasonably linked to RSV-induced inflammation and lung injury were identified and considered to be candidate genes (i.e. they have biological plausibility based on published studies about their effects). Included among these gene candidates are *St18, Tbk1, Marco, Fndc3a, Stat4, Stat1, Spnb2, Il9r, Slc28a3, Mpg* and *Kif27* (Tables 4.1 and 4.2). Nonsynonymous coding SNPs of these candidate genes were identified using The Jackson Laboratory Mouse Phenome Database across inbred mouse strains used in this study and statistical analysis revealed that several of the SNPs identified were significantly associated with the immune response to RSV infection (Table 5.2). Figure 5.3 illustrates the effect of these significant SNPs on RSV disease phenotypes. For example, *St18* contains a Thr961Ser mutation that was associated with RSV-induced increase of BAL total inflammatory cells across all of the strains ($p<0.0001$) (Table 4.1). An Asp392Val mutation in *Marco* on chromosome 1 significantly correlated with BAL monocyte concentration 5 days after infection ($p<0.0001$). *Stat4, Slc28a3, Mpg,* and *Kif27* contain SNPs that resulted in amino acid mutations (Table 4.3) and were significantly associated with BAL lymphocytes after 5 days RSV infection ($p<0.05$). Statistical analysis was not performed using *Stat1* and *Il9r* because no nonsynonymous coding SNPs were found within these genes.

Genetic deletion of *Marco* modifies RSV-induced pulmonary inflammatory response and injury

The role of *Marco* on RSV-induced pulmonary inflammation was further examined by infecting *Marco*$^{+/+}$ and *Marco*$^{-/-}$ mice with vehicle control or RSV for 1 or 5 days. Genotype specific differences were found in vehicle control groups, in which a significantly greater BAL protein concentration was found in *Marco*$^{+/+}$ mice compared to

Marco^{-/-} mice (Figure 5.4). Relative to respective vehicle controls RSV infection caused a significant increase of BAL protein concentration in *Marco*$^{-/-}$ mice; however infection did not affect BAL protein concentration in *Marco*$^{+/+}$ mice. No significant differences in BAL monocytes, lymphocytes, and macrophages were found between genotypes following vehicle treatment; however, *Marco*$^{+/+}$ mice had significantly greater BAL polymorphonuclear leukocytes (PMNs) after vehicle treatment compared to *Marco*$^{-/-}$ mice. Relative to respective vehicle treated mice, 1 and 5 days of RSV infection caused significant increases mean numbers of all BAL inflammatory cells. Numbers of all cell types were significantly higher in *Marco*$^{-/-}$ mice compared to *Marco*$^{+/+}$ mice (Figure 5.5).

H&E stained lung section from *Marco*$^{+/+}$ and *Marco*$^{-/-}$ vehicle treated mice were normal and showed no signs of disease. Indication of neither pulmonary injury nor inflammatory cell response was found in either genotype after 1 day of RSV infection. However, significant peribronchiolar inflammation, cellular proliferation, and epithelial hyperplasia were found in the terminal bronchioles of both genotypes following 5 days of RSV infection. Peribronchiolar inflammation observed in *Marco*$^{-/-}$ mice was greater than the response found in *Marco*$^{+/+}$ mice; which supported BAL protein and inflammatory cell data following RSV infection. Yet, cellular proliferation and epithelial hyperplasia was greater in *Marco*$^{+/+-}$ mice (Figure 5.6).

Discussion

Considerable evidence has suggested that genetic background is an important determinant of RSV disease severity, but the identity of susceptibility genes remains unclear. To determine the within-strain variation in RSV disease phenotypes, we phenotyped 36 inbred mouse strains following infection with vehicle and RSV (Chapter 4). Results of that study suggested that response to infection is a multigenic trait. To dissect the specific chromosomal regions responsible for the phenotypic variation between inbred strains, genomewide haplotype association mapping analysis was performed for BAL inflammatory cell phenotypes, and numerous QTLs were found for all of these phenotypes. QTL identification was based solely on haplotype association mapping, but candidate genes were selected according to prior knowledge of the function of a gene. Several well characterized genes known to be essential for viral response, pulmonary inflammation, and cytokine trafficking were found within the identified QTLs. Some of the genes strongly associated with the measured phenotypes are poorly characterized and little is known about their function. Statistical analysis found that SNPs within these genes significantly enhanced or reduced the response to infection among inbred strains, and suggested they are important targets for future investigation of their roles in RSV disease progression.

Haplotype association mapping was performed for BAL total inflammatory cells and PMNs (1 day after RSV infection), and lymphocytes and monocytes (5 days following infection) to identify QTLs within the mouse genome associated with these phenotypes. Haplotype association mapping of total lavageable cells identified 3 QTLs (Log$P$ values greater than 4) on chromosomes 1, 10 and 12. Several QTLs with Log$P$

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values between 3.5 and 4 (chromosomes 1, 3 and 5) were also identified. Query of the QTLs on chromosomes 1, 10, and 12 identified a number of candidate genes based on biological plausibility. Within the chromosome 10 QTL is *St18*, a transcription factor that regulates expression of proinflammatory genes such as *IL-6, IL-1α, and TNF-α* in human fibroblast (29). Possible functional SNPs within this candidate gene were identified using The Jackson Laboratory Mouse Genome Informatics (MGI) website (www.informatics.jax.org). Interestingly, inbred strains of mice that are homozygous for functional mutations in *St18* (Thr961Ser) had significantly greater numbers of BAL lymphocytes after RSV infection compared to inbred strains that were wild type for the mutations. These results are consistent with a functional role of *St18* in RSV disease, and understanding the mechanisms of *St18* activity in response to RSV should provide novel insight to susceptibility.

The QTL associated with BAL monocytes on chromosome 1 contained *Marco*. Previous studies suggest that *Marco* has protective effects in the lung following inhalation of particulate matter, and bacterial infection (30-33). Infection with RSV leads to oxidative stress, which contributes to alveolar epithelial cell injury, and pulmonary inflammation (34-36). Oxidative stress phenotypes are regulated by various genes, one of which is *Marco* (37, 38). Recent studies found that *Marco* expression reduces pulmonary injury and inflammation (31). Because *Marco* modulates oxidative stress-induced lung injury, and haplotype association mapping linked *Marco* genotype to BAL monocyte migration, it was hypothesized that *Marco* provides protection against RSV-induced pulmonary inflammation, especially monocyte response. Following RSV infection, an infection-dependent increase in lavageable monocytes was found in both

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genotypes, but numbers of monocytes were significantly reduced in $Marco^{+/+}$ mice compared to $Marco^{-/-}$ mice. Consistent with BAL data, peribronchiolar inflammation, and cellular inflammation were more prominent in $Marco^{-/-}$ mice relative to $Marco^{+/+}$ mice following RSV infection. The role of $Marco$ in reducing neutrophilia is well documented (30, 31, 37), and is supported by this study. However, little is known about how $Marco$ regulates monocyte migration, but the data from this investigation suggest that $Marco$ may be an important regulatory component of this RSV disease phenotype.

$Stat1$ and $Stat4$ on chromosome 1 are candidate genes that were identified through haplotype association mapping for BAL lymphocytes 5 days after RSV. RSV vaccine-enhanced disease is dependent on $Stat4$ expression, and $Stat4$ is believed to modulate RSV-induced inflammation through activation of IL-12, and other studies have found that RSV activates STAT signaling in human epithelial cells (10, 39). Computational analyses identified a functional mutation (Thr709Met), and strains of mice with this mutation had significantly enhanced BAL lymphocyte recruitment relative to strains that have the wildtype $Stat4$ genotype. Results suggest that this polymorphism is a gain of function mutation, but further investigation is necessary to gain molecular insight. $Stat1$ has very different functions from those of $Stat4$; however, SNP data for $Stat1$ were unavailable for analysis.

Another lymphocyte QTL was found on chromosome 11, and $Slc28a3$ was identified as a candidate gene within this QTL. Genetic variants of $Slc28a3$ are associated with the immune response in chronic lymphocytic leukemia (CLL) patients. The immune response in patients with polymorphisms of this gene was significantly reduced compare to individuals without these polymorphisms, suggesting that mutations

to *Slc28a3* result in loss of function (40). Similarly, in the present study a reduced lymphocyte response to RSV was found in strains with the *Slc28a3* Asn697Asp mutation compared to strains without the mutation.

Kif27 is highly expressed in ciliated cells and extremely important to cilia function. Without full function of *Kif27* lung cilia cannot clear mucus and other foreign particles from the lung (41). Cilia dysfunction such as ciliostasis, clumping, or loss of cilia is responsible for some phenotypes of RSV disease (42). Two nonsynonymous coding SNPs within *Kif27* were identified, and lymphocyte migration was significantly higher in inbred strains of mice with either of the SNPs relative to strains with wild type *Kif27*. Because of the previous association of Kif27 with RSV disease in humans (43), this candidate gene is particularly interesting for additional investigation into its functional role in differential response to RSV in the mouse model.

Data for nonsynonymous coding SNPs were not available for *Il9r*. However, it may be hypothesized to play a role in regulating inflammation following RSV infection. Numerous studies have found that *Il9r* is responsible for asthma and general allergic airway inflammation (31, 43-45). Due to the many similar phenotypes between the two, it is believed that response to RSV and asthma inflammation may have common regulatory mechanisms (47, 48). Furthermore, *IL-9* mRNA is elevated in the lungs of infants with RSV bronchiolitis, and is believed to regulate MCM (46). Analysis of the mouse genome found that MOLF/EiJ, PWD/PhJ and CAST/EiJ contain SNPs within the promoter region for *Il9r*. Very little is known about *Mpg*, however its location within the QTL of chromosome 11 and significant *p* value may suggest that it is important to RSV response.

The large number of QTLs identified in the genome scans that exceeded Log*P* values of 4 for the RSV disease phenotypes reconfirms that response to RSV is a complex (i.e. multigenic) trait. However, the genome-wide significance of these QTLs is unknown, and while extremely promising and informative the significance of some of the identified QTLs is questionable. Furthermore, it is likely that because of multiple gene-environment (phenotype) and gene-gene interactions the haplotype association mapping approach was unable to identify all of the SNPs that modulate the observed responses, and important SNPs may be overlooked. For example, data from previous studies suggest that *IL-6* and *IL-12* contribute to the RSV-induced inflammatory response in mice (39, 49-51). Neither of these genes were found within the QTLs of this study, suggesting that these genes may not be polymorphic between strains; however expression of these genes is associated with *St18* and *Stat4* activation respectively (29, 52, 53), both of which were identified in this study. Similar limitations are noted in other association mapping studies investigating complex phenotypes (25, 54). While limitations to the approach are acknowledged, results from this method should be viewed as a starting point and follow-up mechanistic investigations of the identified candidate genes should provide substantial insight to RSV disease susceptibility. It should be mentioned that haplotype association mapping was only performed on 4 of the 16 previously measured phenotypes. Pearson correlation coefficients indicated that the 4 phenotypes used for haplotype association mapping were the most relevant RSV phenotypes. Haplotype association mapping for some of the less relevant phenotypes may also identify associations with genes important to RSV disease.

Collectively this study details a clear genetic influence on RSV disease phenotypes using inbred strains of mice. Six of the candidate genes in this study were found to have influence on the host response to RSV infection. Previous knowledge along with data from this study suggest that *Marco, Slc28a3,* and *Kif27* protect the host from RSV-induced inflammation; while *St18, Stat4, Stat1, Mpg* and *Il9r* contribute to the inflammatory response following infection.

Using a similar approach a recent study linked several of the genes (e.g *Stat4, Il9r*) identified in the present study to RSV susceptibility (55). In contrast to disease response phenotypes used in our investigation, Stark *et al.* used viral load as the RSV response phenotype and did not evaluate inflammatory or other immunological response phenotypes (6, 55). While informative for mechanisms of infection, data presented earlier (Chapter 4) suggests that susceptibility to infection does not influence disease severity. Therefore identifying genes that regulate susceptibility does not help elucidate the genetic mechanisms that regulate RSV disease severity. In the current study identification of QTLs and candidate genes was based solely on disease phenotypes, suggesting that these genes are responsible for disease severity.

Throughout the years many genes have been linked to RSV-induced pulmonary inflammation and injury, but the complexity of the disease phenotype makes it difficult to determine which genes are absolutely essential for response to RSV infection (9, 10, 56-58). The haplotype association analysis of clinically relevant RSV disease phenotypes across 35 inbred strains of mice identified a number of potentially important candidate susceptibility genes. Association of nonsynonymous coding SNPs with RSV response phenotypes across the inbred strains suggests that further analyses of mechanistic roles of

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the genes in RSV response should lead to novel insight about genetic susceptibility. Future investigations may evaluate the role of functional SNPs in homologous human genes.

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Figure Legends

Figure 5.1. Genome-wide haplotype association mapping. Manhattan plot of haplotype association mapping of RSV disease phenotypes, concentration of BAL total cells (A), PMNs (B), monocytes (C) and lymphocytes (D). X-axis represents the genomic location. Y-axis indicates the strength of the genotype-phenotype correlation. Each color block represents a different chromosome of the mouse genome.

Figure 5.2. Detailed view of representative significant QTLs associated with RSV disease phenotype and corresponding candidate genes. Chromosome 10 association with total BAL cells 1 day post infection (pi) (A). Chromosome 10 association with BAL PMNs 1 day pi (B). Chromosome 1 association with monocytes (C) and lymphocytes 1 day pi (D).

Figure 5.3. Effect of candidate gene SNPs on BAL (total cell, monocyte, and lymphocyte) **phenotypes** among inbred strains of mice following RSV infection. Number above columns indicates the number of strains with the genotype. Group means ± SD are presented. * Significantly different from wild-type genotype, p < 0.05.

Figure 5.4. Effect of *Marco* on pulmonary hypermeability. *Marco* deficiency enhanced RSV-induced pulmonary hyperpermeability, determined by BAL protein concentration. Data presented as group mean ± SEM (n=3-5/group). * Significantly different from genotype-matched vehicle control. + Significantly different from infection-matched *Marco*$^{+/+}$, *p*<0.05.

Figure 5.5. Effect of *Marco* on pulmonary inflammation. *Marco* deficiency enhanced RSV-induced pulmonary inflammation. Numbers of PMNs, monocytes, lymphocytes, and macrophages were determined by BAL fluid analysis in *Marco*$^{+/+}$ and *Marco*$^{-/-}$ mice following vehicle or RSV infection. Data presented as group mean $\pm$ SEM (n=3-5/group). * Significantly different from genotype-matched vehicle control. + Significantly different from infection-matched *Marco*$^{+/+}$, *p*<0.05.

Figure 5.6. Pulmonary pathology following RSV infection in *Marco*$^{+/+}$ and *Marco*$^{-/-}$ mice. Arrows indicate areas of increased airway inflammation and bronchial epithelial proliferation (hyperplasia). AV = Alveoli, BR = bronchus or bronchiole, BV = blood vessel. Bar = 100mm.
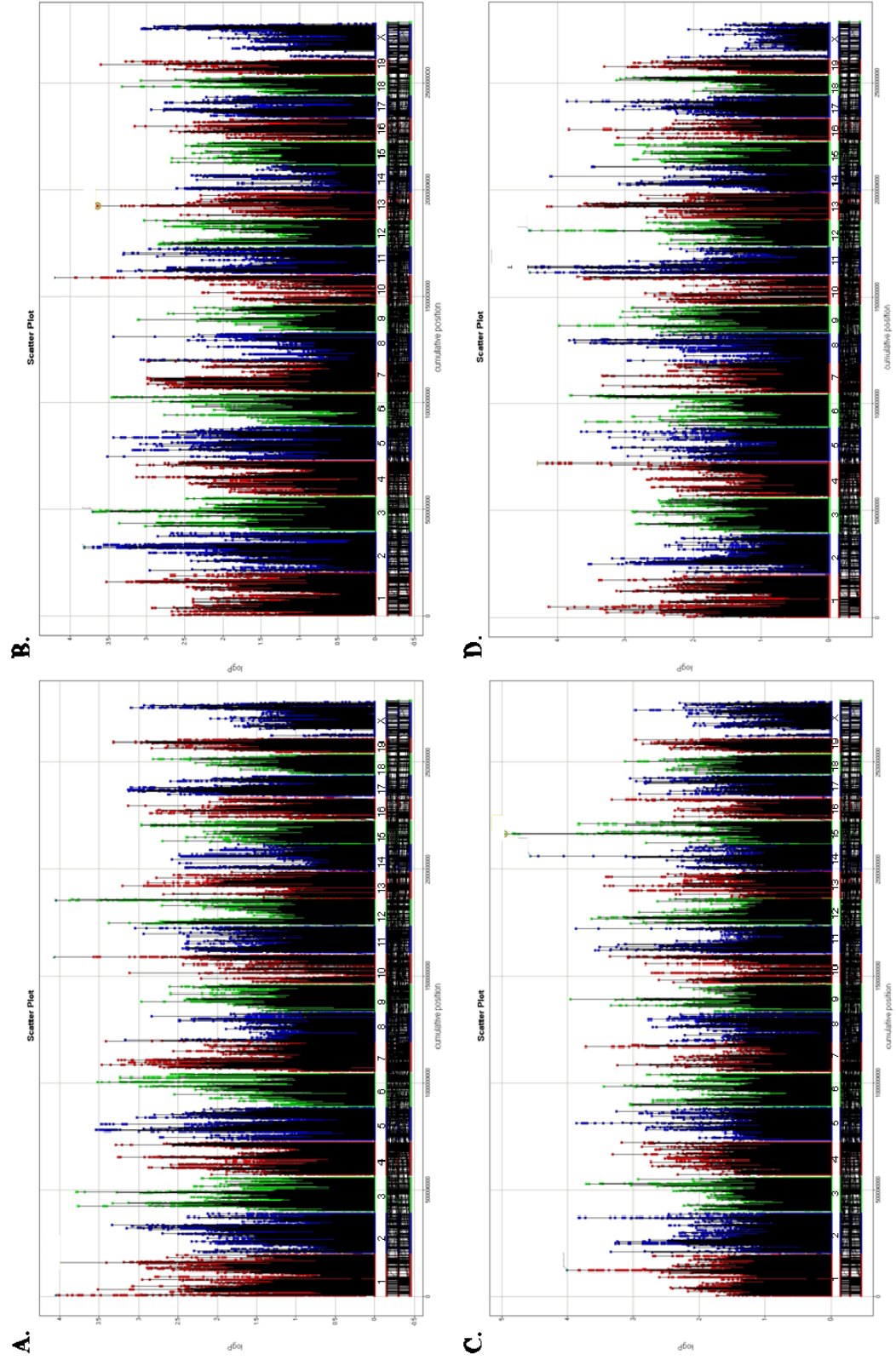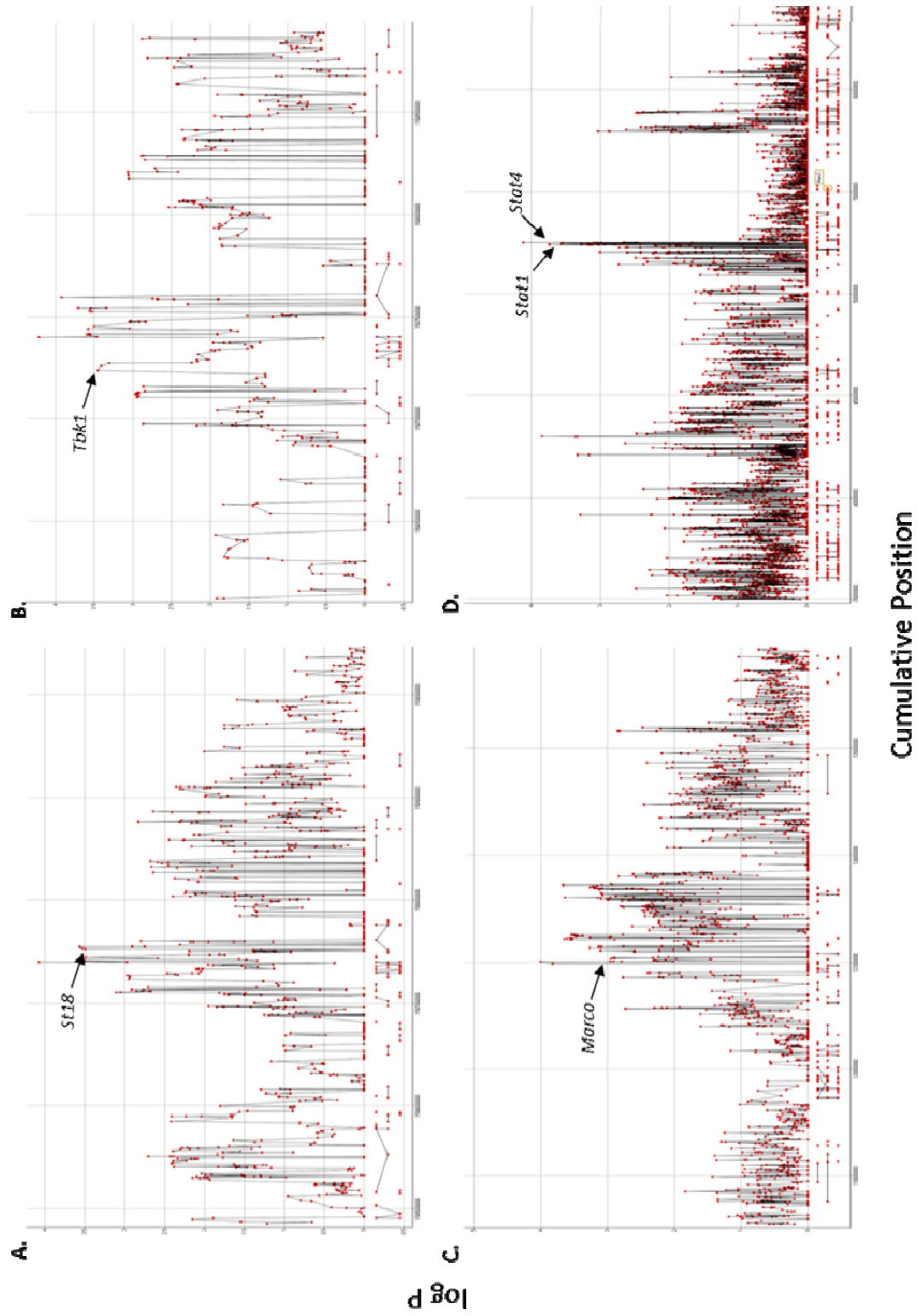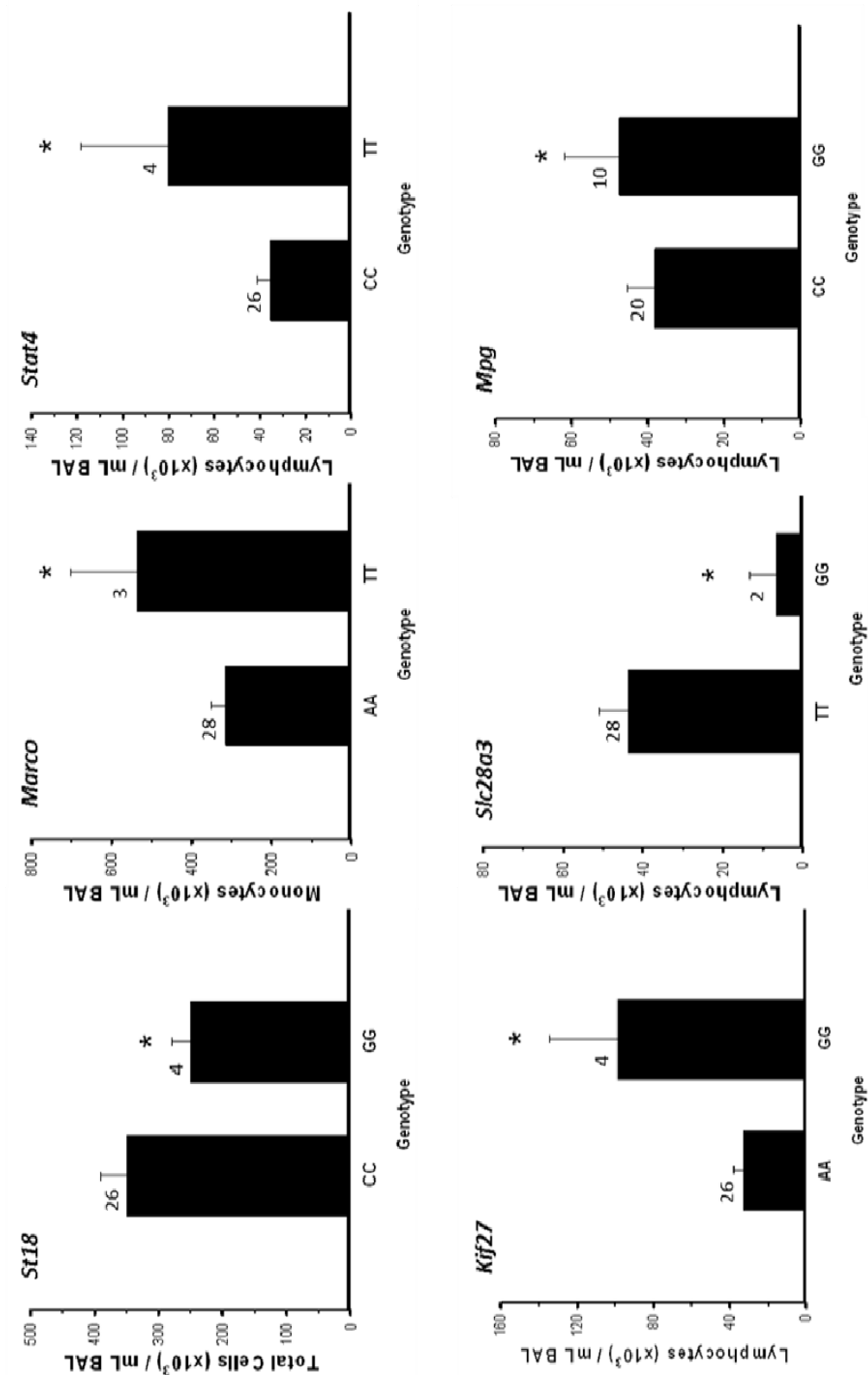
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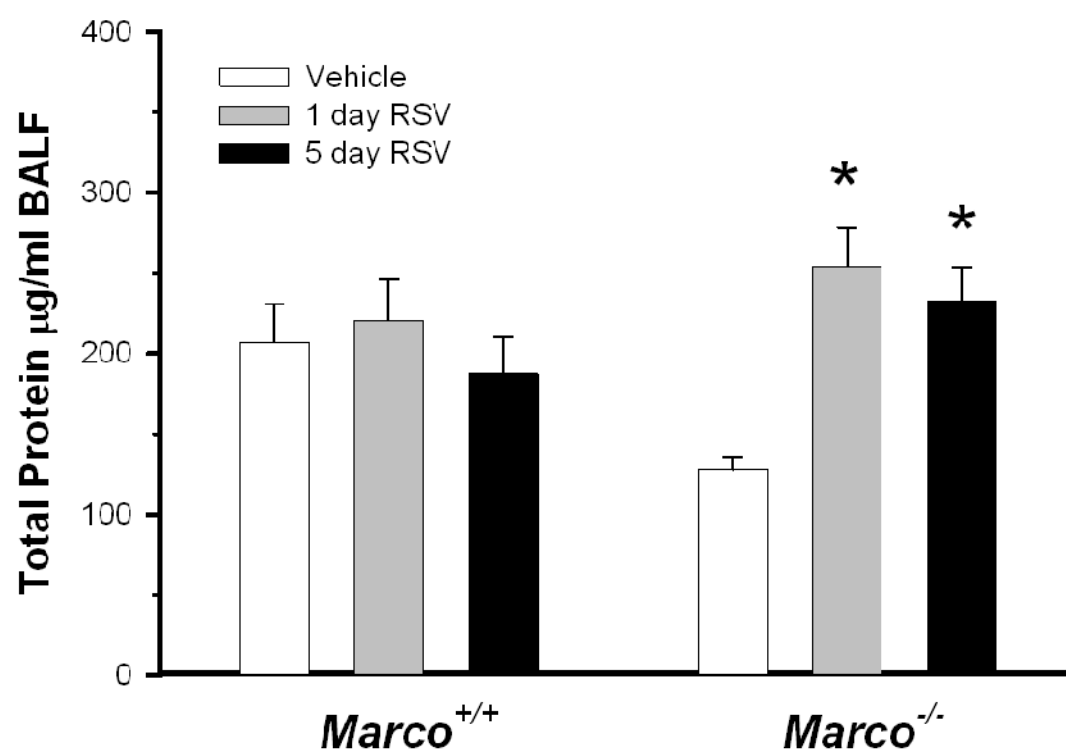
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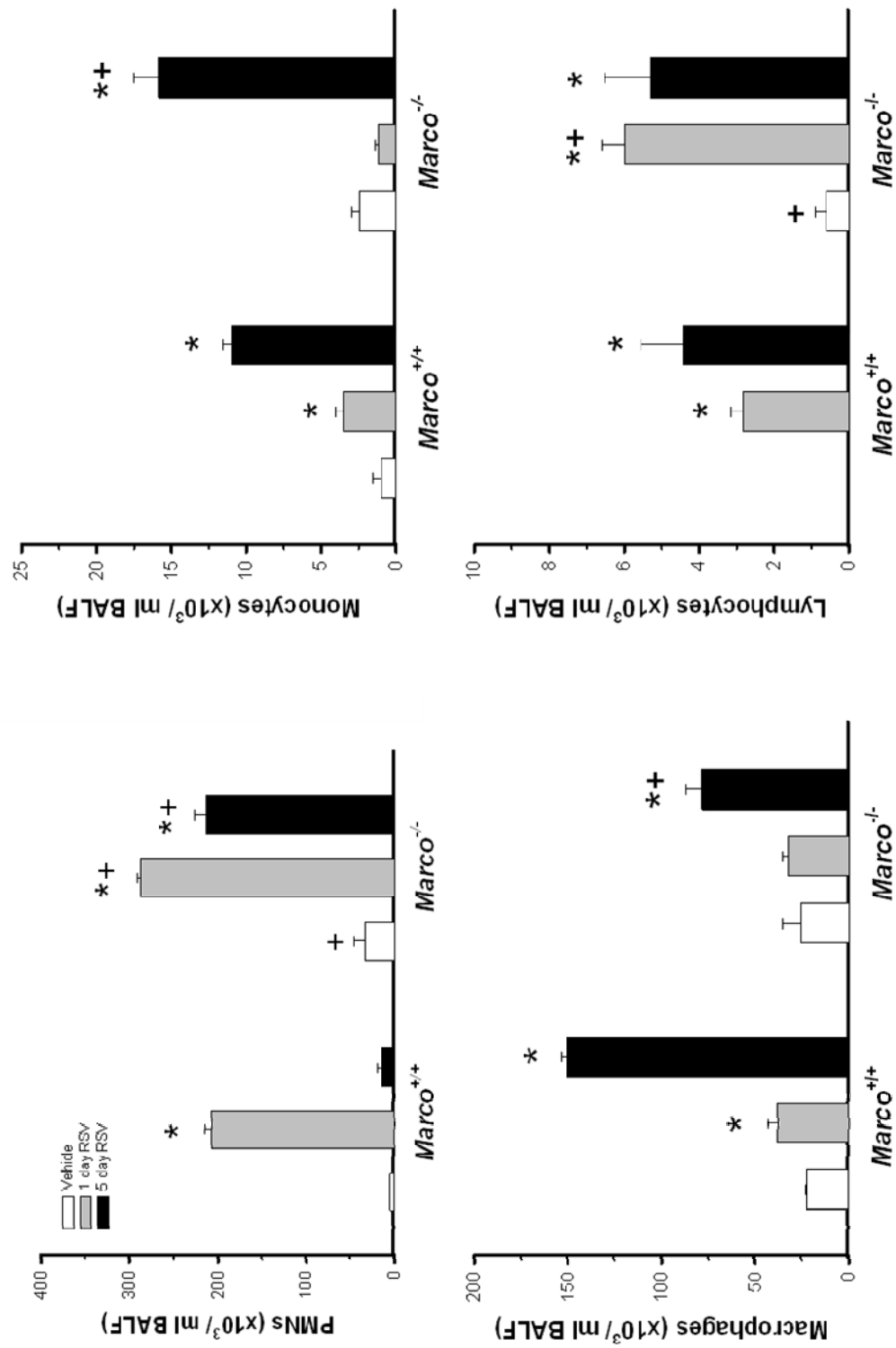
Figure 5.3.

Figure 5.4.

Figure 5.5.

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Figure 5.6.
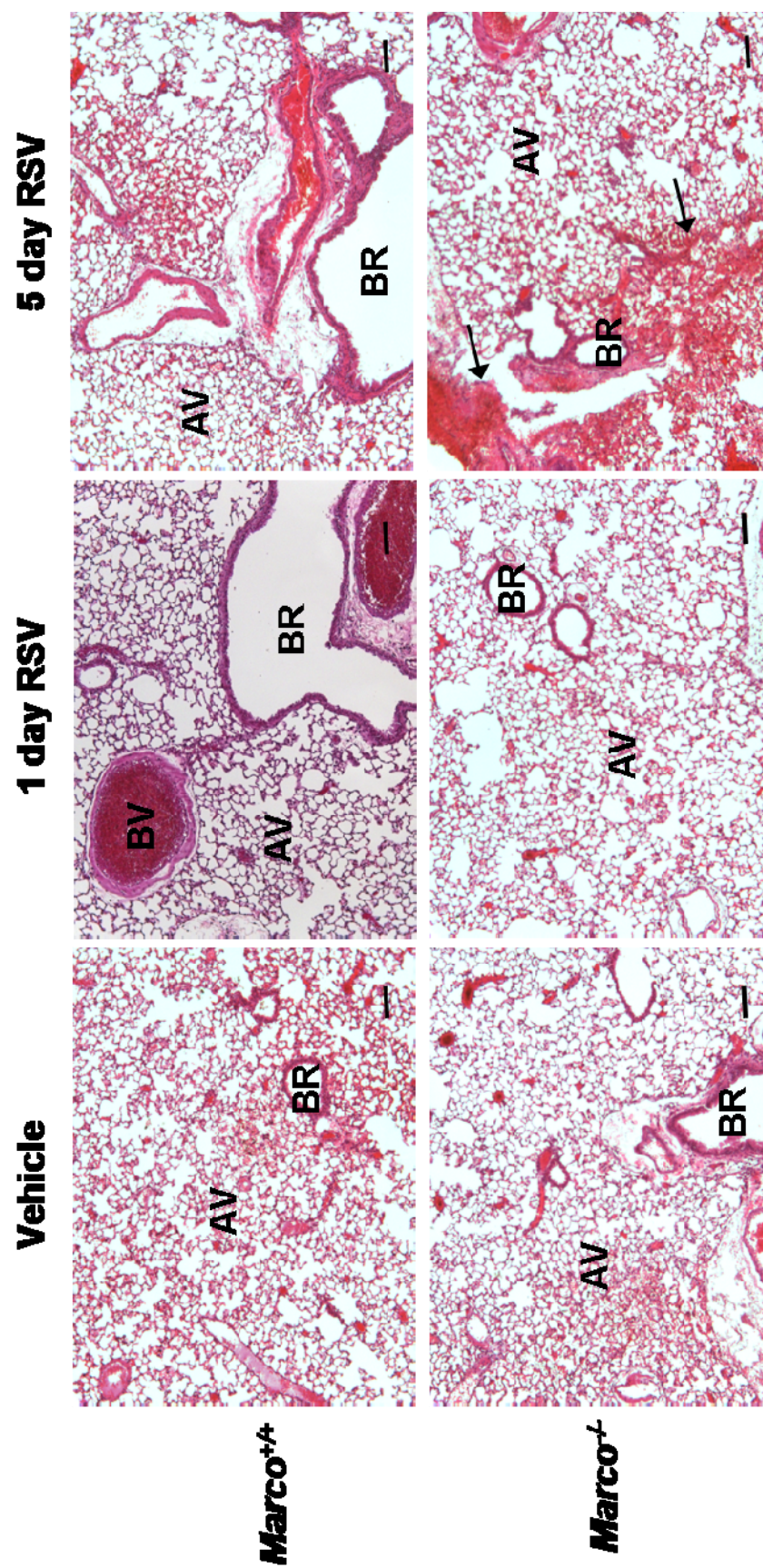
Table 5.1. QTLs associated with RSV disease severity identified by HAM. Genes of interest were found within these QTLs, genes with a priori biological relevance to response to RSV infection are in bold. Gene identifications are listed in Table 4.2.

Phenotype	QTL	Genome position (Mb)	Genes in region
PMN (1 day pi)	1	Chr 10: 120.75-121.50	*Gns, Rassf3,* **Tbk1***, Xpot, Srgap, Tmem5*
Total Cells (1 day pi)	1	Chr 1: 159.20-160.9	*Astn1*
	2	Chr 10: 5.91-6.76	*Npbwr1, Rb1cc1,* **St18**
	3	Chr 12: 114.00-117.26	*Adam6, Ankrd12, Ptpn2*
Monocytes (5 day pi)	1	Chr 1: 121.74-122.30	*Tmem177, Sctr, Dbi, Steap3,* **Marco**
	2	Chr 14: 70.41-71.51	**Fndc3a**
	3	Chr 15: 48.52-48.89	*Csmd3*
Lymphocytes (5 day pi)	1	Chr 1: 51.82-52.47	*Myo1b,* **Stat4**, **Stat1***, Gls, Nab1, Gm553*
	2	Chr 4: 153.48-153.55	*Prdm16, Actrt2, Mmel1, Tnfsf14, Hes5, Pank4, Plcl4*
	3	Chr 11: 30.00-33.12	**Spnb2***, Acyp2, Psme4,* **Il9r***, Cpeb4,* **Mpg***, Mare, Tlx3, Rhbdtf1*
	4	Chr 13: 57.91-58.57	*Spock1, Klh13, Hnrpa0, Ubqln1, Gkap1,* **Kif27**, **Slc28a3***, Hnrpk*

Table 5.2. Complete list of genes of interest identified through HAM. Genes with *a priori* biological relevance based on the literature are in bold.

Gene Symbol	Offical Gene Name
PMNs	
Chromosome 10	
Gns	Glucosamine (N-acetyl)-6-sulfatase
Rassf3	Ras association (RalGDS/AF-6) domain family member 3
Tbk1	**TANK-binding kinase 1**
Xpot	Exportin, tRNA
Srgap	SLIT-ROBO Rho GTPase activating protein
Tmem5	Transmembrane protein 5
Total Cells	
Chromosome 1	
Astn1	Astrotactin 1
chromosome 10	
Npbwr1	Neuropeptides B/W receptor 1
Rb1cc1	RB1-inducible coiled-coil 1
St18	**Suppression of tumorigenicity 18**
chromosome 12	
Adam6	A disintegrin and metallopeptidase domain 6A
Ankrd12	Ankyrin repeat domain 12
Ptpn2	Protein tyrosine phosphatase, non-receptor type 2
Monocytes	

	Chromosome 1	
Tmem177	Transmembrane protein 177	
Sctr	Secretin receptor	
Dbi	Diazepam binding inhibitor	
Steap3	STEAP family member 3	
Marco	**Macrophage receptor with collagenous structure**	

	Chromosome 14	
Fndc3a	**Fibronectin type III domain containing 3A**	

	Chromosome 15	
Csmd3	CUB and Sushi multiple domains 3	

Lymphocytes

	Chromosome 1	
Myo1b	Myosin IB	
Stat4	**Signal transducer and activator of transcription 4**	
Stat1	**Signal transducer and activator of transcription 1**	
Gls	Glutaminase	
Nab1	Ngfi-A binding protein 1	
Gm553	Predicted gene 553	

	Chromosome 4	
Prdm16	PR domain containing 16	
Actrt2	Actin-related protein T2	
Mmel1	Membrane metallo-endopeptidase-like 1	
Tnfsf14	Tumor necrosis factor ligand superfamily member 14	
Hes5	Hairy and enhancer of split 5	
Pank4	Pantothenate kinase 4	

Plcl4	Phospholipase C-like 4

Chromosome 11	
Spnb2	**Spectrin beta 2**
Acyp2	Acylphosphatase 2
Psme4	Proteasome activator subunit 4
Il9r	**Interleukin 9 receptor**
Cpeb4	Cytoplasmic polyadenylation element binding protein 4
Mpg	**N-methylpurine-DNA glycosylase**
Mare	Alpha globin regulatory element containing gene
Tlx3	T-cell leukemia, homeobox 3
Rhbdf1	Rhomboid family member 1

Chromosome 13	
Spock1	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan 1
Klh13	Kelch-like protein 13
Hnrpa0	Heterogeneous nuclear ribonucleoprotein A0
Ubqln1	Ubiquilin 1
Gkap1	G kinase anchoring protein 1
Kif27	**Kinesin family member 27**
Slc28a3	**Solute carrier family 28 member 3**
Hnrpk	Heterogeneous nuclear ribonucleoprotein K

Table 5.3. Responses to RSV associated with SNPs in candidate genes identified by HAM. Genes of interest containing SNPs with significant phenotypic associations were classified as candidate genes and are listed in bold.

Gene	Mutation Genomic Location (Mb)	Geno-type	Amino Acid	No. of Strains	Phenotype, day pi	Phenotype (mean ± SD)	*p*-value
Tbk1	121.000992	T	Ser	28	PMNs, 1 day	187.9 ± 26	0.5072
		C	Gly	2		203.9 ± 103	
St18	**6.847409**	**C**	**Thr**	**26**	Total cells, 1 day	**349.4 ± 40**	**<0.0001**
		G	**Ser**	**4**		**249.8 ± 29**	
Marco	122.371364	A	Thr	24	Monocytes, 5 days	332.8 ± 37	0.4692
		T	Ser	6		352.2 ± 110	
	122.374458	**A**	**Asp**	**28**	Monocytes, 5 days	**315.1 ± 34**	**<0.0001**
		T	**Val**	**3**		**536.8 ± 164**	
Fndc3a	72.961494	T	Thr	25	Monocytes, 5 days	342.3 ± 40	0.1035
		C	Ala	5		306.7 ± 55	
	72.974433	A	Phe	26	Monocytes, 5 days	336.8 ± 39	0.9346
		G	Leu	4		334.9 ± 61	
Stat4	**52.162526**	**C**	**Thr**	**26**	Lymphocytes, 5 days	**35.4 ± 5.9**	**<0.0001**
		T	**Met**	**4**		**80.3 ± 37.8**	
Spnb2	30.004580	A	Ala	24	Lymphocytes, 5 days	39.8 ± 7.1	0.1760
		T	Ala	7		45.9 ± 21.3	
Kif27	**58.389425**	**A**	**Glu**	**26**	Lymphocytes, 5 days	**32.7 ± 5.5**	**<0.0001**
		G	**Gly**	**4**		**98.7 ± 36.7**	
	58.445828	**C**	**Ala**	**28**	Lymphocytes, 5 days	**39.9 ± 7.0**	**0.0022**
		T	**Val**	**2**		**59.1 ± 19.0**	
Slc28a3	58.657074	T	Asn	26	Lymphocytes, 5 days	41.2 ± 6.8	0.9999
		C	Asp	4		41.4 ± 34.7	
	58.657166	**T**	**Lys**	**28**	Lymphocytes, 5	**43.6 ± 7.1**	**<0.0001**

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					days		
		C	**Arg**	**2**		**6.6 ± 6.6**	
Mpg	**32.129868**	**C**	**Ala**	**20**	Lymphocytes, 5 days	**38.2 ± 7.1**	**0.0239**
		G	**Gly**	**10**		**47.5 ± 14.3**	
	32.127831	C	Ser	21	Lymphocytes, 5 days	43.0 ± 8.2	0.0835
		T	Leu	9		36.6 ± 10.5	

CHAPTER 6

GENE EXPRESSION PROFILES OF INBRED MOUSE STRAINS FOLLOWING RSV INFECTION

Introduction

Data from inbred strain screens indicate that susceptibility to RSV infection is a polygenic trait (1, 2). Using a candidate gene approach and genome-wide haplotype association mapping, several genes linked to RSV susceptibility and immune response have been identified (3-6).

Previous studies have identified statistically significant inter-strain variation in inflammation and lung pathology phenotypes among inbred mice (Chapter 5). Moreover, haplotype association mapping has identified quantitative trait loci for a number of these RSV response phenotypes and candidate genes within them. In the present study, a genomics approach was initiated to provide further insight to the mechanisms of differential susceptibility to RSV disease among inbred strains. Global gene expression profiles were determined for lung homogenates obtained from RSV-susceptible BALB/cByJ and RSV-resistant C3H/HeJ mice. In C3H/HeJ mice, gene expression was minimally altered by RSV infection. However, gene expression did vary significantly as a result of RSV infection in responsive BALB/cByJ mice. In the BALB/cByJ mice, a number of "biologically interesting" expression patterns were identified, but the most significant change in gene expression was noted 1 day following infection when a significant number of genes were either up-regulated or down-regulated. Altered gene transcript expression approached baseline 5 days after RSV infection.

Material and Methods

Microarray Data Analyses

RNA was extracted from whole lung homogenates from each mouse within a given group and pooled (n = 3) to provide a single RNA sample for each experimental group. Two arrays per group were hybridized. Total RNA was used only after it passed quality testing performed using a 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). Gene expression analysis was conducted using the Affymetrix Mouse Genome 430 2.0 experimental platform. The log(10) of the Affymetrix processed signal intensity was visualized using Spotfire k-means clustering. Visual inspection of clusters identified 9 biologically interesting patterns. Probes were collected for each pattern, and entered the probe lists (GenBank IDs from the chip) for each pattern into GeneSpringGX10 (Agilent Technologies, Inc., Santa Clara, CA) to analyze gene expression profiles.

Results and Discussion

Microarray analysis indicated that RSV infection significantly altered gene expression in both strains; however, changes in gene expression are greater in BALB/cByJ mice compared to C3H/HeJ mice (Figure 6.1). One day following RSV infection over 850 genes were significantly upregulated or downregulated in both genotypes relative to vehicle controls; however, the observed gene expression was greater in BALB/cByJ mice than C3H/HeJ. The Gene Ontology feature of GeneSpringGX10 identified many of the differentially regulated genes; some of which have biological relevance and plausibility to RSV susceptibility and disease severity. For example, initial analyses determined that expression of *Mx1* (myxovirus 1), *Mx2* (myxovirus 2), *Oas1* (2',5'-oligoadenylate synthetase 1), *Oas2* (2',5'-oligoadenylate synthetase 2), *Stat1* (signal transducer and activator of transcription 1) and *Stat2* (signal transducer and activator of transcription 2) was significantly upregulated 1 day after RSV infection (Figure 6.2; clusters 6, 7, 10, 12, 13, 15). Previous studies have found that *Mx1* and *Mx2* are responsible for specific antiviral activity against influenza virus infection (7). A recent published study and preliminary data from this laboratory suggests that *Mx1* and *Oas1* regulate susceptibility to RSV infection (1, 7, 8).

Relative to vehicle control BALB/cByJ mice, expression was significantly lower for approximately 100 gene transcripts in mice after RSV infection (Figure 6.2; clusters 3, 5, 11). Among these genes were *Marco* (macrophage receptor with collagenous structure), *Xpot* (exportin, tRNA), and *Mpg* (N-methylpurine-DNA glycosylase). Interestingly, previous studies associated these genes with pulmonary inflammation and injury (9-11), and haplotype association mapping analysis reported in Chapter 5 also linked QTLs that contained these genes to RSV-induced pulmonary inflammation.

Gene expression profiles found in clusters 11 and 14 (Figure 6.2) indicated that there are significant differences in gene expression between C3H/HeJ mice and BALB/cByJ mice at baseline. This finding suggests that further investigation of this gene

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set may identify genes or gene networks that predispose to differences in host susceptibility to RSV infection and disease. Cluster 11 identifies genes for chemokines such as *Ccl12* (Chemokine (C-C motif) ligand 12), and *Cxcl3* (Chemokine (C-X-C motif) ligand 3) as well as *Tlr4* (toll-like receptor 4). Interestingly all of these genes are associated with inflammation (12-14). Expression of these genes was greater in BALB/cByJ mice than in C3H/HeJ mice, which suggests these genes contribute to susceptibility to infection. Cluster 14 identifies genes with greater expression in C3H/HeJ mice, these genes include *Cyr61* (cysteine-rich, angiogenic inducer 61) and *Fos* (FBJ murine osteosarcoma viral oncogene homologue) both of which promote apoptosis (15-17). Cluster 14 gene expression profiles along with phenotype data presented in chapter 5 suggest that these genes contribute to resistance to infection.

Initial microarray analysis of C3H/HeJ and BALB/cByJ mice has identified several interesting candidate genes, some of which are consistent with HAM analyses (Chapter 5). Future microarray analysis on additional responsive and non-responsive strains will determine if the expression profiles identified in C3H/HeJ and BALB/cByJ mice are predictive of RSV disease severity in other strains of mice. Programs such as IPA (Ingenuity Pathway Analysis) and DAVID (Database for Annotation, Visualization and Integrated Discovery) will be used to identify pathways responsible for RSV susceptibility and severity. Subsequent studies using knock-out mice and cell lines will be used to confirm the role of these candidate genes in RSV disease.

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Figure Legends

Figure 6.1. Effects of RSV on gene expression profiles. Gene expression patterns for lung tissues from C3H/HeJ and BALB/cByJ mice infected with vehicle or RSV. Expression patterns of 854 genes were significantly affected by RSV (p<0.01). (n=3 per group)

Figure 6.2. K-means cluster analysis of gene expression profiles. Gene expression patterns for lung tissues from C3H/HeJ and BALB/cByJ mice infected with vehicle or RSV. Expression patterns of 854 genes were significantly affected by RSV (p<0.01). K-means cluster analysis was performed on the Affymetrix genome array probes, and 16 "biologically interesting" clusters were found among the 854 genes used in this analysis. (n=3 per group)
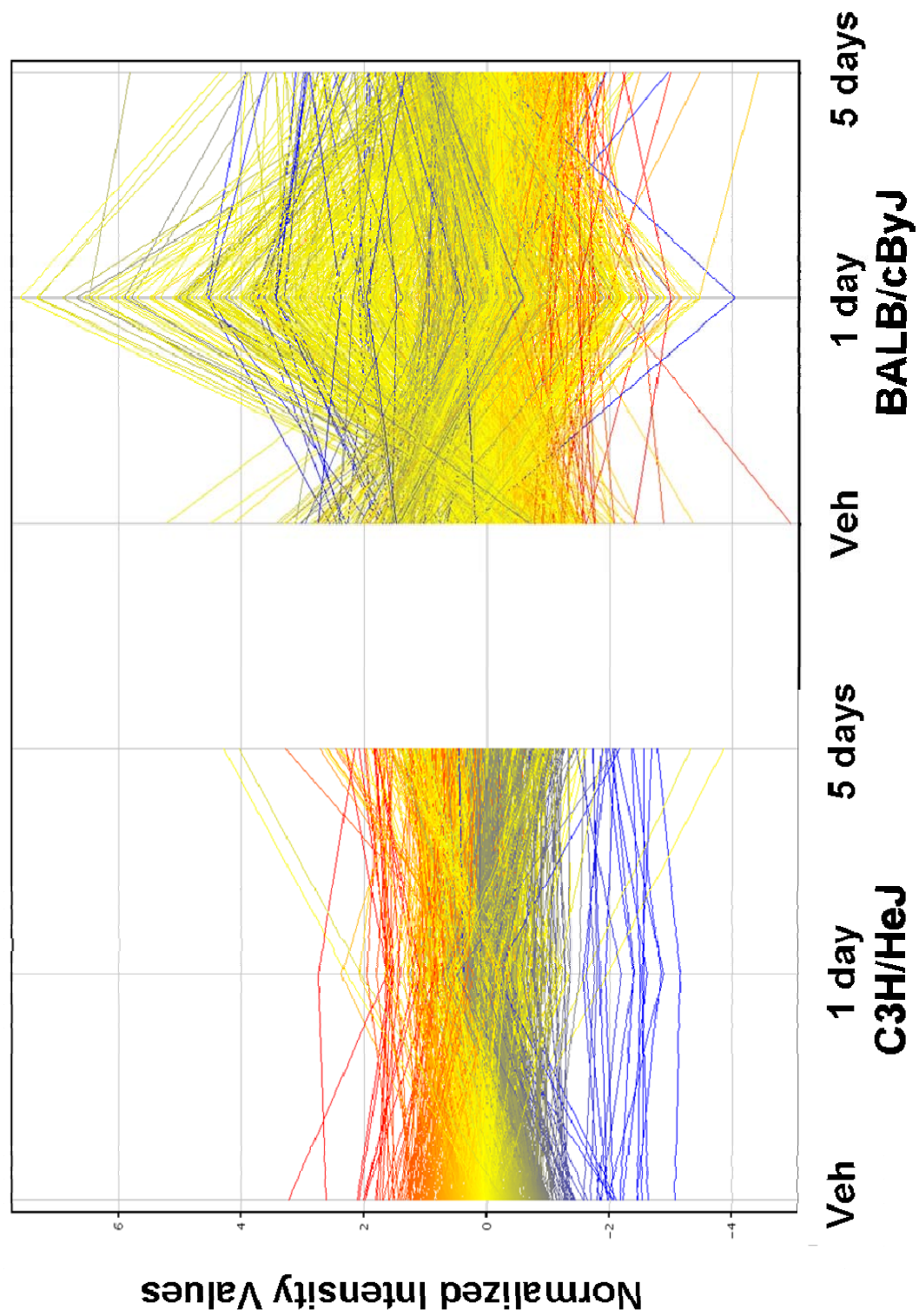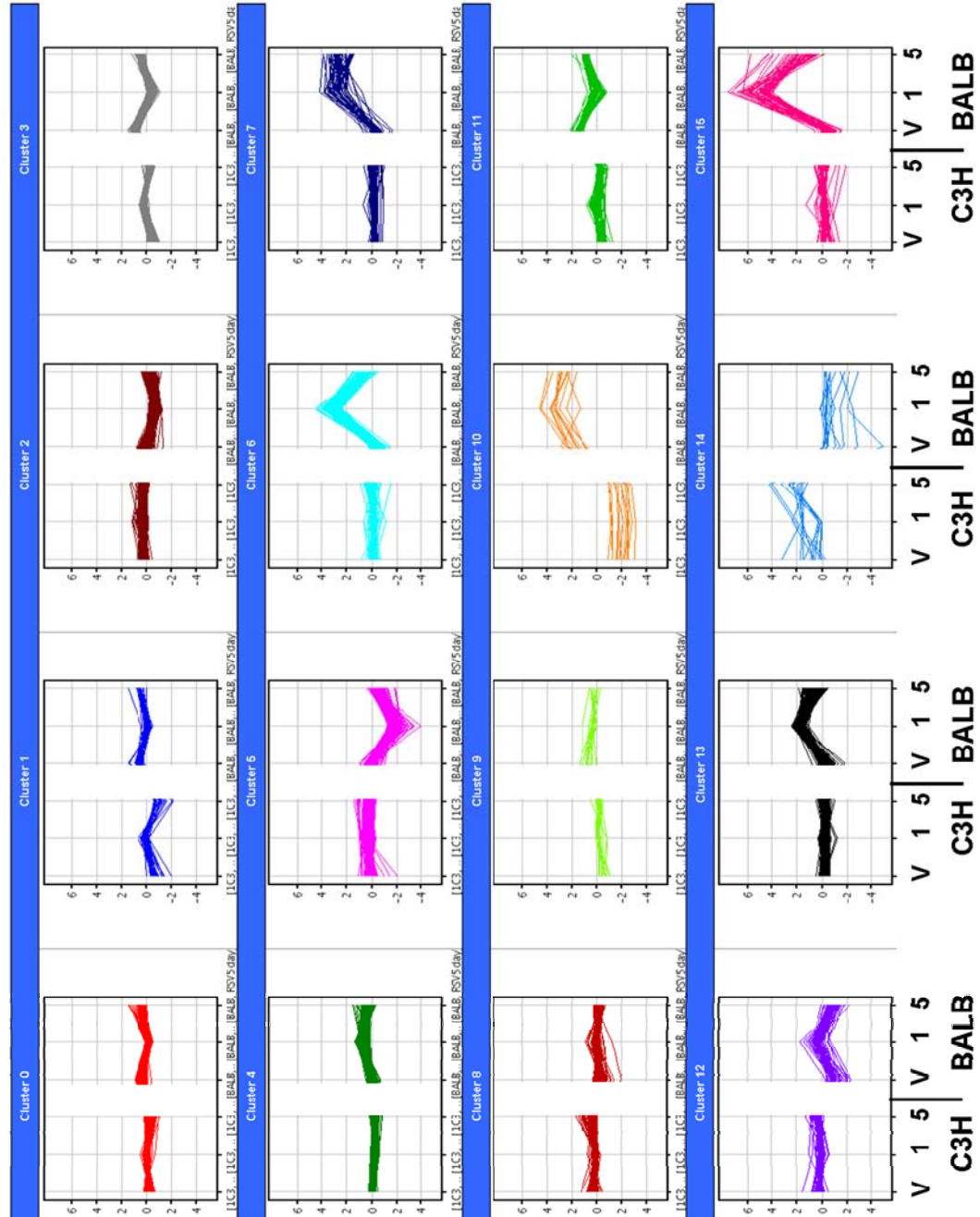
Figure 6.1.

Figure 6.2.

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CHAPTER 7

SUMMARY AND CONCLUSIONS

The overall objective of this project was two-fold. The first objective was to utilize a multi-disciplinary approach to investigate the role of a candidate susceptibility gene (*Cx3cr1*) in response to O_3 and, subsequently, to RSV. The second objective was to utilize a positional cloning approach to identify genes that contribute to RSV disease phenotypes. It was hypothesized that these studies would identify mechanisms related to oxidative stress and inflammation that may be common to ozone and RSV disease.

Previous studies have characterized the role of *Cx3cr1* in pulmonary inflammation following chronic exposure in humans. Pulmonary inflammation and injury were greatly diminished in individuals with *CX3CR1* polymorphisms following chronic exposure to coal dust compared to individual with wild-type *CX3CR1* (1). Results of the present study demonstrated that sub-acute O_3 exposure (0.3 ppm) induced pulmonary inflammation, and *Cx3cr1* contributes significantly to the inflammatory response. That is, when compared to wild type mice, targeted deletion of *Cx3cr1* significantly reduced the inflammatory response to O_3 exposure, thus implicating *Cx3cr1* as an important determinant of host response to this environmental oxidant. To further investigate the mechanism through which *Cx3cr1* regulates O_3-induced pulmonary inflammation, the role of IL-17 was examined in relation to *Cx3cr1* activity. Targeted deletion of the IL-17 receptor (*Il17ra*) significantly reduced the inflammatory response induced by O_3 exposure thus suggesting that, like *Cx3cr1*, *Il17ra* contributes to the O_3 effects. Moreover, *Il17ra* expression correlated with *Cx3cr1* expression, suggesting that these receptors work together to modulate pulmonary inflammation and injury. Data from this dissertation and earlier studies suggest that *Cx3cr1* may contribute to oxidative stress response following exposure to inhaled pollutants.

Oxidant stress has been found to be a contributing factor to the pathogenesis of RSV-induced lung injury (2). Further, fractalkine (Cx3cl1), the main endogenous ligand for CX3CR1, is a chemoattractant for T cells and monocytes and also promotes strong adhesion of leukocytes to activated endothelial cells where it is primarily produced and

expressed (3, 4). Recent studies have also found that the G protein of respiratory syncytial virus is able to bind to *Cx3cr1* (5). These observations led to the hypothesis that *Cx3cr1* is an important component of the inflammatory response induced by viral infection. To test this hypothesis, *Cx3cr1*^{+/-} and *Cx3cr1*^{-/-} mice were infected with RSV and BAL was analyzed for inflammatory cell infiltration and total protein. Results indicated that lack of *Cx3cr1* reduced pulmonary inflammation and injury following RSV infection. Other *in vivo* and *in vitro* studies are consistent with these observations (5-7).

Two approaches can be used to identify genes that regulate susceptibility (8-10). The first and most common method is the candidate gene approach, in which genes are chosen *a priori* as likely mechanisms that influence the phenotype of interest. Association is assessed between the phenotype of interest and polymorphisms within the candidate gene(s). In this dissertation the candidate gene approach was used to demonstrate that *Cx3cr1* and *Marco* are important modulators of RSV susceptibility and inflammation. This approach is effective for identifying genes that regulate the phenotype of interest. However, since it does not examine the entire genome it is unable to identify all the genes that affect the phenotype. Furthermore this approach fails to take gene-gene interaction into account (11). The second approach to identification of susceptibility genes is to perform a genome scan (positional cloning). Similar to the candidate gene approach this method associates gene expression and polymorphisms with the phenotype of interest. However, unlike the candidate gene approach a genome-wide screen is used to identify linkage to any chromosomal interval throughout the entire genome between two or more differentially responsive strains. This approach requires no prior knowledge of gene function and has the potential to discover novel disease genes. The second part of this dissertation was dedicated to identifying the genes responsible for susceptibility to RSV infection as well as the genes that regulate the response to infection.

An important characteristic of the inbred mouse is the availability of the complete genome sequence, and the many similarities between the human and mouse genomes (12). The continuous strain distribution patterns found for all the phenotypes measured in this study demonstrate that similar to humans, RSV infections elicits a wide range of disease severity that can be measured and is highly heritable.

Previous studies have shown that susceptibility and response to RSV infection is a multigenic trait, but only a few genes including *Cx3cr1* have previously been associated with RSV disease (13-15). RSV viral titer is often used as a marker of susceptibility to RSV infection; using only this phenotype previous studies found that susceptibility is multigenic, and AKR/J are the most susceptible to infection, and C57BL/6J are the most resistant to infection. These findings are consistent with viral load data reported in this dissertation. However, these findings are inconsistent with data for RSV disease phenotypes (BAL measurements, changes in body weight, histopathology), and Pearson ranked correlation coefficients indicate that RSV disease severity is not influenced by viral load in the lung.

In the present study, haplotype association mapping (HAM) was used to identify QTLs and candidate genes that determine susceptibility to inflammatory and other pulmonary responses to RSV infection. A similar approach has been used by other investigators to identify genetic mechanisms responsible for susceptibility to RSV infection (14). While determining the genes that regulate susceptibility to infection is informative, it does not explain the differential disease responses found between inbred mouse strains. Furthermore, current literature and data presented in this dissertation (Chapter 4) suggest that the signs and symptoms of RSV disease are directly related to the host's response to infection, and not the viral concentration (16). Deciphering the mechanisms responsible for this differential response is important in order to understand why certain individuals only present cold-like symptoms after RSV infection, while other

individuals require hospitalization. Using haplotype association mapping *St18, Marco, Stat4, Kif27* and *Slc28a3* were identified as candidate genes for regulating inflammation and injury in response to RSV infection. All of the identified candidate genes are known regulators of inflammation (especially bronchiolitis) and cell cycle (17-21).

Polymorphisms within these genes were significantly associated with RSV-induced pulmonary inflammation. Polymorphisms of *Kif27* result in cilia dysfunction, which is associated with bronchiolitis and MCM (21, 22). Interestingly, inbred strains such as CAST/EiJ and KK/HlJ which have a nonsynonomous coding SNP (Glu1335Gly) in *Kif27* were among those inbred strains with the greatest amount of intraepithelial mucus concentration 5 days following RSV infection. These results suggest that *Kif27* may have a role in determining response to RSV and warrant additional investigation to the potential mechanism(s) through which this gene influences host susceptibility.

Little is known about the function of *Slc28a3* other than it is a membrane transport protein. Data from this study suggests that *Slc28a3* contributed to pulmonary inflammation and polymorphisms of this gene significantly attenuated the response. *St18* is a transcription factor that regulates expression of proinflammatory genes such as *IL-6, IL-1α, and TNF-α* in human fibroblast (18). Statistical analysis suggests that polymorphisms of this gene results in a decreased inflammatory response. *Stat4* is also an inflammatory mediator and operates downstream of *IL-12* (23). Specifically, studies have shown that *Stat4* contributes to pulmonary inflammation (19, 24-26) and *Stat4* deficiency is associated with protection against ischemic injury (27). However, in this study polymorphisms of this gene elevated inflammation in the lung. However, no *Stat4* coding SNPs were found in C3H/HeJ mice and other inbred strains with minimum lung injury following RSV infection. The role of *Stat4* in the immune response following RSV infection cannot be determined based on these data, investigation into activation and expression of *IL-12* may provide insight into the role *Stat4* plays in response to RSV.

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The role of *Marco* in relation to RSV disease in mice was further investigated. Targeted deletion of *Marco* significantly enhanced RSV-induced injury and inflammation, especially the lymphocyte response. These findings were consistent with the HAM analysis which identified a QTL on chromosome 1 that encompassed *Marco*. Interestingly, similar to RSV, *Marco* also was found to have an important role in the inflammatory response to O_3 (28), suggesting another potential common response mechanism for these two environmental agents. Similar "proof of concept" investigations of other candidate genes identified in the present study should provide unique, important insight to roles of these genes in susceptibility to RSV disease.

HAM identified *Il9r* as a gene of interest. *Il9r* regulates inflammation and lung hyper-reactivity associated with allergic asthma, both of which are prominent human RSV phenotypes. Recent studies have also linked *Il9r* expression to MCM and bronchiolitis. SNP analysis found that MOLF/EiJ, PWD/PhJ, CAST/EiJ mice have *Il9r* coding SNPs; and these same inbred strains presented the minimum amounts of MCM following RSV infection. The association of these SNPs with reduced MCM suggests that *Il9r* may regulate RSV-induced bronchiolitis, which is associated with severe RSV infection.

Genome sequence analysis revealed that wild-derived inbred strains (CAST/EiJ, PWD/PhJ, MOLF/EiJ, WSB/ EiJ) contain coding SNPs of *Myd88, Tlr4, Mx1, Mx2* and *Il9r*, which may explain why these strains were relatively nonresponsive to RSV infection.

This HAM approach provides an important platform for indentifying gene(s) that are responsible for specific, well-characterized phenotypes with high heritability. Microarray analyses were performed to identify gene expression patterns that change throughout the course of disease and may be predictive of RSV disease phenotypes. microarray analyses identified numerous genes with greater expression following RSV infection compared to vehicle controls. Most of the genes with enhanced expression after

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infection were associated with inflammation, specifically chemokines such as fractalkine; the ligand for *Cx3cr1*. This was not surprising since a number of the RSV disease phenotypes (e.g. pulmonary edema, emphysema, pulmonary epithelial damage) are linked to inflammation. Expression of inflammatory mediators 1 day after infection was consistent with BAL and pathology data of this study (i.e. the greatest changes in gene expression were found 1 day after infection). Changes in gene expression profiles of the RSV-non responsive C3H/HeJ inbred mouse were markedly reduced compared to expression profiles of the RSV-responsive BALB/cByJ strain. Using microarray analysis (genomics) together with HAM (genetics) is a powerful approach to identify genetic polymorphisms and expression, as well as their interaction (genetical genomics) to provide insight into the genetic mechanisms of RSV disease.

While preliminary, initial integration of the candidate genes and gene networks identified in this study via HAM and microarray analysis have identified potentially interesting targets for future investigation. For example, initial query of the array data via cluster analyses found genes that regulate virus attachment and replication such as *Mx1* and *Mx2* are highly expressed in responsive strains compared to low responsive strains. A recent study by Stark *et al.* (13) found similar results using F_2 mice; furthermore, unpublished *in vitro* data from this laboratory suggest that functional polymorphisms of *Mx1* significantly decrease susceptibility to RSV infection (Ciencewicki, pers. comm.). Another example of a potentially important gene identified by these analyses is *Myd88*, a critical signaling molecule in *Tlr4*-mediated innate immunity. *Myd88* is of particular interest because both HAM and microarray suggested *Myd88* was associated with RSV disease susceptibility and response. Past studies found that *Myd88* was essential to inflammation in RSV disease (29, 30), and suggests further validation of *Myd88* as a candidate gene for further validation in the model used in this study.

Tlr4 is another gene of great interest in this model. A number of studies have suggested that *Tlr4* is necessary for RSV disease and polymorphisms of *Tlr4* are linked to

mild RSV disease in animal models and humans (29, 31). In the present study, microarray analysis determined that *Tlr4* was differentially expressed between C3H/HeJ and BALB/cByJ mice following vehicle, suggesting that *Tlr4* may regulate susceptibility to RSV infection. Furthermore, RSV disease phenotypes were significantly greater in C3H/HeOuJ (*Tlr4* normal) compared to C3H/HeJ (*Tlr4* mutant) mice (see Chapter 4), which indicates that *Trl4* may not only regulate susceptibility to RSV infection, but it may contribute to disease severity as well. These results are therefore consistent with a role for this gene in RSV disease, and further investigation into the mechanism(s) through which *Tlr4* modulates disease could provide insight to intervention strategies to reduce disease severity. For example, other disease/ injury models indicate that *Tlr4* activates *Myd88* to induced pulmonary injury and inflammation. Additional *in vitro* and *in vivo* studies could determine if *Tlr4* uses this same mechanism to regulate RSV disease.

Supporting earlier findings in this dissertation, microarray analysis found *Cx3cr1* was differentially expressed between high and low responders. *Cx3cr1* along with other genes coding for chemokines (e.g. *Ccl12, Cxcl1*) were significantly upregulated following RSV infection relative to vehicle controls. However, RSV infection resulted in a significantly greater increased gene expression in high responders (BALB/cByJ mice) relative to low responders (C3H/HeJ mice). No significant difference in *Cx3cr1* expression was found between high and low responders in vehicle control groups, indicating that *Cx3cr1* contributes to RSV disease severity, but may not influence disease susceptibility.

As expected microarray analysis found that numerous genes were upregulated following RSV infection; however, it was determined that several genes were significantly downregulated in high responders following RSV infection. These include genes coding for Arrdc3 (arrestin domain containing 3) and Cyr61 (cysteine rich protein 61). However the same genes were upregulated in non-responsive strains 1 day after

infection, suggesting that these genes impede RSV-induced inflammation and injury and provide protection against RSV infection.

It is important to note that the murine model of RSV disease has some limitations. Probably the greatest criticism is that pathogenesis of RSV disease in mice fails to mimic what is observed in humans. Specifically RSV infection does not result in a measurable degree of morbidity, eosinophilia, nor acute respiratory distress syndrome, and RSV viral loads in mice are typically low (32, 33). For these reasons some investigators suggest that pneumonia virus of mice (PVM) is a more accurate model of human RSV infection (15). Unlike RSV, PVM infection results in a broader range of phenotypes including death. In this study, infection with 1×10^6 pfu of RSV-19 resulted in wide range of disease phenotypes, but no deaths resulted from infection. Other investigators have attempted to use higher doses of RSV-19 in order to mimic human disease; however, this resulted in mass mortality within 2 days, and 1×10^6 pfu of RSV-19 is believed to be the dose that most accurately reflects human RSV disease.

While some criticisms of this model are valid, they largely stem from the fact that most previous investigations of this model use a single inbred strain of mouse. The approach used in the present studies characterized important human disease phenotypes across many inbred strains and identified strains of mice with a wide spectrum of RSV disease that approximates the human experience. This multiple strain approach leveraged with the extensive genetic information available for multiple strains to perform HAM can provide gene candidates for testing in human populations.

In summary the inbred strain screen identified high and low responders to RSV infection. HAM analysis of these strains identified several genes that were linked to disease progression, inflammation and injury following RSV infection. Microarray analysis also indicated that many genes were differentially expressed between high and low responder strains prior to and after RSV infection. Identification and validation of the genes and gene patterns will provide insight into what causes differential disease

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severity in individuals. It is also possible that the gene expression profiles can be used to predict disease severity in additional mouse strains. These gene profiles may be used to predict disease severity in humans. If informative, they could significantly advance current novel therapies for RSV infection. At the present time treatment options for RSV are still limited. Palivizumab and RSV immune globulin intravenous (RSV-IGIV) have been approved for prophylactic use (34). Effective treatment requires multiple doses of either drug, which can be very costly, and not very cost efficient considering most individuals only experience a mild form of the disease (35). By indentifying the genes that regulate RSV infection and thus the individuals prone to severe cases of RSV, current treatments can be targeted specifically to these individuals,

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