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Neonatal Respiratory Virus Infection Shapes Pulmonary Function in Adult Mice

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NEONATAL RESPIRATORY VIRUS INFECTION SHAPES PULMONARY FUNCTION IN ADULT MICE

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
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ABSTRACT

Respiratory syncytial virus (RSV) and influenza are the two most common causes of acute lower respiratory tract infections in infants. Epidemiological data suggest that both severe RSV and influenza infections during infancy are associated with long-term pulmonary function abnormalities.

Despite of the severe burdens of RSV and influenza infections in infants, no efficacious vaccines are available for RSV and the current vaccine strategy for influenza is inconvenient due to the fact that vaccines are made annually based on the prediction of circulating strains in the next season. New strategies in vaccine development have been slow to arrive partly due to the fact that pathogenesis of RSV and influenza, especially in infants, is not well understood; and this is further complicated by the fact that most researchers use adult animal models to study immunopathology observed in human infants.

To better model the infections of these two viruses in infants, we established neonatal mouse models for RSV and influenza infections. In our neonatal mouse model, both RSV and influenza infections led to long-term airway hyperreactivity associated with persistent pulmonary inflammation. An inchoate CD8⁺ T cell response was found to play a significant role in the pathogenesis of neonatal influenza infection; while a Th2 (IL-4-producing CD4⁺ T helper cell)-biased immune response was responsible for the pathogenesis of neonatal RSV infection and led to Th2-skewed secondary responses in adult mice originally infected as neonates.

In an effort to seek a better vaccine strategy for RSV infection, we used antisense oligonucleotides (ASO) against IL-4 receptor α (IL-4R α) to modulate the T cell responses at the time of primary RSV infection. Upon reinfection with RSV, IL-4R α ASO treated mice were completely protected from airway hyperreactivity. This finding suggested that there was a

delicate balance between Th1 (IFN- γ -producing CD4⁺ T helper cell) and Th2 cell responses and that only a slight push in one direction (i.e., Th1) had a tremendous impact on subsequent diseases. Our data indicate that modulation of the immune responses to RSV during infantile infection may be of significant benefit to infants and that IL-4R α may be part of a viable vaccine strategy.

CHAPTER ONE:
GENERAL INTRODUCTION

Infectious Diseases

Infectious diseases are a significant cause of morbidity and mortality worldwide and are responsible for nearly 15 million deaths per year, according to the Centers for Disease Control and Prevention (CDC; <http://www.cdc.gov/ncidod/EID/vol11no04/04-1167.htm>). Of all infectious diseases, respiratory infections are the number one cause of mortality, accounting for nearly 4 million annual deaths (Morens et al., 2004). Of all respiratory infections, including bacterial, viral, fungal, and protozoal infections (Lednicky and Rayner, 2006), viral infection, with variable rates of morbidity and mortality, is the most frequently diagnosed (Denny, 1995). Respiratory viral pathogens include rhinoviruses, influenza viruses, adenoviruses, parainfluenza viruses, respiratory syncytial viruses (RSV), human metapneumoviruses, and coronaviruses (Denny, 1995). Despite the fact that respiratory viruses usually lead to mild diseases in healthy children and adults, infants, elder adults, and immunocompromised individuals have a higher risk of developing severe lower respiratory tract infections (LRTIs) (Lee and Barton, 2007). RSV and influenza infections are the most common causes of severe respiratory distress and hospitalization of children less than five years of age (Carbonell-Estrany et al., 2004; Fiore et al., 2007).

Respiratory Syncytial Virus

The Virus

RSV is an enveloped virus with a negative-sense, single-stranded RNA genome of ten genes, which code for eleven proteins. There are two serotypes, A and B, which co-circulate in the human population; natural non-human reservoirs for RSV do not exist (Anderson et al., 1985). Serotype A is usually more virulent than serotype B (Walsh et al., 1997). Three glycoproteins — F (fusion), G (attachment), and SH (small hydrophobic) proteins — reside on

the lipid envelope. The M (matrix) protein consists of the matrix layer underneath the lipid envelop. The 15.2 kb RNA genome is associated with three proteins: the N (nucleocapsid), P (phosphoprotein), and L (large protein). These three proteins, together with two other proteins named M2-1 and M2-2, play essential roles in genome replication and transcription. Two nonstructural proteins (NS1 and NS2) are important in modulating host responses to the infection. Specifically, they are thought to inhibit interferon responses within the infected cells (Collins and Graham, 2008; Easton et al., 2004).

Pathogenesis

The RSV virus is usually transmitted by direct contact with secretions or aerosols produced by a sneeze or cough. The virus replicates in the nasopharynx with an incubation time of about four days before symptoms appear. Symptoms include cough, low-grade fever, rhinorrhea, and pharyngitis (Schwartz, 1995). RSV infects people of all ages. In most cases, only mild upper respiratory tract infection occurs, and this resolves in four to seven days (Kuzel and Clutter, 1993). Disease is more severe in infants, and about 25 to 40 % of infected infants develop LRTIs including bronchiolitis and/or pneumonia with 0.5 to 4.4 % requiring hospitalization (CDC; <http://www.cdc.gov/ncidod/dvrd/revb/respiratory/rsvfeat.htm>).

Once the virus enters the airway, it attaches via the G protein to ciliated and non-ciliated epithelial cells. The G protein contains heparin-binding domains, which interact with glycosaminoglycans (Feldman et al., 1999), and a CX3C motif, which binds to CX3CR1 (Tripp et al., 2001). The beating cilia on ciliated epithelial cells help spread RSV to the lower part of the respiratory tract, where type I pneumocytes become the primary target cells. The cytopathic effects from the virus itself or from inflammatory cells result in sloughing of epithelial cells in the airway. Inflammatory cells, such as neutrophils, monocytes, and lymphocytes, are recruited

to the airways, arterioles, and lung parenchyma. Some inflammatory cells also penetrate the smooth muscle and epithelial cells, reaching the airway lumen (Johnson et al., 2007). Necrotic epithelial cells, inflammatory cells, mucus, fibrin, and excessive liquid in the airways induce airway obstruction and increased airways resistance. Airway obstruction is a hallmark of RSV bronchiolitis (Peebles and Graham, 2005).

Prevention and Treatment

Despite intense studies of RSV, there are still no efficient antiviral drugs or vaccines. Two antibodies to RSV are currently approved for RSV prophylaxis: RSV-IGIV (RespiGam) and palivizumab (Synagis). The use of RSV-IGIV has been largely replaced by palivizumab since palivizumab's approval in 1998 (Barton et al., 2001); and palivizumab is only recommended for use in high-risk populations, including children less than two years old with chronic lung disease or congenital heart disease and preterm infants born at 35 weeks or less gestation (Feltes et al., 2003; Meissner and Long, 2003). Ribavirin is the only available antiviral drug for RSV infection. However, the efficacy of ribavirin in combating RSV infection is controversial (Randolph and Wang, 1996; Ventre and Randolph, 2007). In clinical practice, various supportive therapies are used, including such interventions as nasopharyngeal suctioning and ventilation or diverse reagents, such as oxygen, heliox (a combination of helium and oxygen), bronchodilators, epinephrine, corticosteroids, surfactants, nitric oxide, recombinant human DNase I, and antibiotics (Chidgey and Broadley, 2005). Each is prescribed to reduce the severity of the disease based on one or more aspects of the pathogenesis of RSV bronchiolitis; however, none of them cures RSV.

Vaccine development for RSV has been hampered largely by the tragedy which occurred in the 1960s with a formalin-inactivated viral vaccine. The vaccine was well tolerated and high

levels of neutralizing antibodies were found in the serum during the acute (active infection) phase of illness. However, RSV-vaccinated infants became seriously ill upon subsequent infection with community-acquired RSV, and 80% of the vaccinated infants required hospitalization for bronchiolitis and/or pneumonia. The severity of illness was inversely correlated with age, and histology examinations at lung autopsies showed extensive inflammation consisting of mononuclear cells and eosinophils within the peribronchiolar area (Kapikian et al., 1969; Kim et al., 1969).

Various types of new vaccines have been developed, including live attenuated viruses, attenuated temperature-sensitive strains, viral mutants with one or more genes deleted, purified RSV surface proteins (usually F proteins), synthetic viral peptides, and plasmid vaccines or vaccinia virus vectors expressing F or G proteins (Moore and Peebles, 2006; Venkatesh and Weisman, 2006). All of them have been tested in adult mouse models or chimpanzees, and some show a certain level of protection (Venkatesh and Weisman, 2006). However, to date, no vaccines have been approved for use in human beings.

Influenza

The Virus

Influenza is an enveloped virus with a segmented, negative-sense, and single-stranded RNA genome. There are three serotypes of influenza including type A, B, and C. Unlike type B and C, which are essentially restricted to humans, type A can infect a variety of hosts including humans, birds, pigs, seals, and horses (Brownlee, 2002). Influenza C usually causes mild upper respiratory tract infections, while influenza A and B are associated with severe respiratory diseases and seasonal epidemics (Matsuzaki et al., 2006). Influenza A also leads to pandemics

when major antigenic changes of its surface glycoproteins are introduced. The research presented in this dissertation focused on influenza A virus.

The RNA genome of influenza A contains eight linear segments encoding 10 proteins. Three surface proteins are localized on the host cell membrane-derived envelope: haemagglutinin (HA), neuraminidase (NA), and matrix (M2) proteins. Serological reactivity of HA and NA is used to subcategorize influenza A. In total, sixteen HA and nine NA subtypes exist; however, only three HA (H1, H2, and H3) and two NA (N1 and N2) subtypes are currently circulating in the human population (Fouchier et al., 2005; Obenauer et al., 2006; Webster et al., 1992). Beneath the lipid bilayer of the envelope is the matrix protein (M1). Inside the matrix, the eight RNA genomic segments are associated with nucleoproteins (NP) and with an RNA-dependent RNA polymerase complex consisting of three proteins, named polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), and polymerase acidic (PA) protein. Like RSV, influenza A also contains two nonstructural proteins: NS1 and NS2. NS1 enhances the translation of viral mRNA (de la Luna et al., 1995; Enami et al., 1994), inhibits nuclear export of viral and host mRNA (Qian et al., 1994; Qiu and Krug, 1994), and provides resistance to host anti-viral responses (Ludwig et al., 2002; Min and Krug, 2006; Talon et al., 2000; Wang et al., 2000). The NS2 protein mediates nuclear export of viral ribonucleoproteins (RNPs) and is also called nuclear export protein (NEP) for this reason (O'Neill et al., 1998).

Pathogenesis

The influenza virus is transmitted by aerosol or droplets and infects people of all ages. Once it enters the respiratory tract, influenza replicates rapidly, with a short incubation time of one to four days. Symptoms of infection in adults and adolescents include fever, chills, headache, and signs of upper respiratory tract infection such as dry cough, sore throat, and rhinitis (Cox and

Subbarao, 1999). Infections in mild cases usually resolve three to seven days after illness onset (CDC; <http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5408a1.htm>). However, as with infantile RSV infection, influenza infection in infants is more severe, consisting of pharyngitis, tracheobronchitis, and sometimes pneumonia (Taubenberger and Morens, 2008).

Influenza infects epithelial cells of both the upper and lower respiratory tract and alveolar macrophages (Fesq et al., 1994; Guarner et al., 2006; Guarner et al., 2000) via HA proteins binding to glycoproteins or glycolipids containing sialic acid on the cell surface. The binding triggers receptor-mediated endocytosis of the virus. HA further mediates fusion of the viral and cellular membrane releasing the viral RNPs into the cytosol. Multifocal necrosis and desquamation of infected epithelial cells in the luminal spaces of the airway are characteristically found in the trachea, bronchi, and bronchioles (Milton et al, 1920). Massive destruction of epithelial cells usually occurs, leaving only one layer of basal epithelial cells in larger airways and sometimes complete loss of epithelial layers in the bronchioli (Goodpasture, 1919; Milton et al, 1920). Edema and congestion of the submucosa and interstitium are often associated with a large amount of neutrophils and mononuclear cell infiltrates. In the resolution stage of infection, regeneration of epithelial cells, which can lead to metaplasia of epithelial cells and interstitial fibrosis, occurs in order to reconstruct the destroyed epithelial layer (Taubenberger and Morens, 2008).

Prevention and Treatment

Four antiviral drugs for influenza infection are licensed in the United States; all of them have to be administered within 48 hours of illness onset, reduce illness duration by only one to two days, and work best as prophylactics (Couch, 2000; Moscona, 2005). These include two M2 inhibitors, amantadine (Symmetrel) and rimantadine (Flumadine) (Dolin et al., 1982; Monto et

al., 1995), and two NA inhibitors, oseltamivir (Tamiflu) and zanamivir (Relenza) (Moscona, 2005). The M2 inhibitors are only effective against type A virus, and strains resistant to M2 inhibitors develop easily (Bright et al., 2006a; Bright et al., 2006b). In contrast, the NA inhibitors treat both type A and type B viruses, and resistant virus strains are rarely isolated (McKimm-Breschkin et al., 2003; Moscona, 2004). None of these antiviral drugs are approved for treatment of infants younger than one year of age (Munoz, 2003).

Despite the use of antiviral drugs to treat and prevent influenza infection, vaccination is still considered to be the most efficacious and safest way to prevent infection. Due to the constant and gradual antigenic change (antigen drift) for both influenza A and B, vaccines are developed annually based on the prediction of circulating strains for the next season (Fukuda and Kieny, 2006; Ohmit et al., 2006). There are currently two types of vaccines: live-attenuated and trivalent inactivated vaccines (Fukuda and Kieny, 2006; Ohmit et al., 2006). The CDC determines which populations are eligible for vaccination each year, a decision largely influenced by the availability of vaccines. In general, vaccination is recommended for individuals between six months and two years of age or older than 65 years of age and for those who are immune compromised (Pearson et al., 2006). No vaccine has been approved for infants younger than six months of age.

Contribution of Host Responses to Pathogenesis

Age as a Factor of Pathogenesis

Age has been identified as an independent risk factor for development of severe RSV disease (Boyce et al., 2000; Welliver, 2003). The rate of hospitalization associated with primary RSV infection declines as a function of age. About 4.4% of infants younger than six months of age require hospitalization for severe RSV bronchiolitis. This number decreases to 1.5% in

infants between six months and eleven months old and decreases further to 0.4% in the second year of life. Finally, by the third year of life, only 0.1% of infants require hospitalization due to RSV infection (Welliver, 2003).

As with RSV, influenza A infects humans at all ages; however, infants and the elderly have the highest hospitalization and mortality rates (Bhat et al., 2005; Brotherton et al., 2004). The hospitalization rate for children less than five years old is also comparable to that of the elderly (Thompson et al., 2004). As with RSV, the younger the child, the greater the risk for hospitalization, with the highest rate in infants younger than six months (Izurieta et al., 2000; Neuzil et al., 2000; O'Brien et al., 2004).

Neonatal Immunity

Although, under some circumstances, adultlike immune responses can be elicited in neonates (Forsthuber et al., 1996; Ridge et al., 1996; Sarzotti et al., 1996), generally speaking, neonatal immune responses do not completely recapitulate adult immune responses (Adkins et al., 2004).

Neonatal B Cell and Antibody Responses. Fewer B cells respond to the infection and those that do are inefficient at antibody production (Adkins et al., 2004). Specifically, in RSV infection, infants younger than six months have lower serum anti-RSV antibodies than older children (Welliver et al., 1980). More importantly, a poor correlation between neutralizing ability and the amount of RSV-specific IgA and IgG in nasal secretions and serum is observed in infants less than six months old (McIntosh et al., 1978; Murphy et al., 1986). Weak antibody responses are also observed in neonatal influenza infection. When stimulated *in vitro*, B cells from cord blood produce less anti-influenza antibodies than those from healthy adult blood (Yarchoan and Nelson, 1983).

Neonatal CD4+ T cell Responses. The number of neonatal CD4+ T cells responding to infections is smaller and the responses of these cells are Th2 biased (Adkins et al., 2004). CD4+ T cells are classified into different categories mainly based on the cytokines that they secrete: Th1 (IFN- γ and IL-12 producing), Th2 (IL-4, IL-5, and IL-13 producing), regulatory T (TGF- β and/or IL-10 producing), and Th17 cells (IL-17 producing) (Neurath et al., 2002). Th1 cells are usually thought to be protective against intracellular pathogens such as viruses, while Th2 cells allow for permissive responses. Th1 cells promote a cascade of inflammatory responses to clear the virus. Th2 cells stimulate IgE production and the activation and proliferation of eosinophils, which are responsible for clearing extracellular pathogens and are inefficient at clearing viruses. Particularly, Th2-skewed responses are reported in RSV infections in infants (Aberle et al., 1999; Renzi et al., 1999; Roman et al., 1997). A higher Th2-to-Th1 ratio of cytokine production is observed in peripheral blood mononuclear cells (PBMCs) from RSV-infected infants compared to non-infected controls (Roman et al., 1997).

Neonatal CD8+ T cell Responses. Responses of CD8+ T cells in infants are considerably weaker in quantity and quality than in adults (Adkins et al., 2004). Infants with severe influenza and RSV infections have fewer CD8+ T cells and a near absence of effector function (i.e., aberrant cytotoxic activity including decreased granzyme production) in the lung (Welliver et al., 2007).

Immunopathogenesis

A Th2-biased immune response to RSV infection has been proposed to explain pathogenesis. PBMCs from RSV-infected infants produce higher IL-4 and lower IFN- γ levels *in vitro* (i.e., a skewed Th2 response) compared to uninfected controls (Bendelja et al., 2000; Roman et al., 1997). Moreover, severe RSV infection in infancy is associated with development

of asthma and abnormal lung function into early adulthood (Korppi et al., 2004; Sigurs et al., 2000; Sigurs et al., 2005; Stein et al., 1999). Asthma is a Th2 inflammatory disease and allergic asthma involves the production of IgE and eosinophilia. Although severe RSV infection has been linked epidemiologically to asthma development, direct experimental data linking the two is scarce. The Th2-biased immune responses to RSV infection in infancy are thought to be the mechanism by which RSV predisposes to allergic asthma.

In contrast to human epidemiology data, mouse studies have shown that RSV infection before allergen sensitization alleviates pulmonary allergic responses, including airway hyperresponsiveness, IL-13 production, and eosinophilia (Peebles et al., 2001a; Peebles et al., 2001b). In addition, RSV infection alone in adult mice elicits a Th1-dominated response, characterized by a high ratio of Th1 to Th2 cells and large amount of IFN- γ in the lungs (Peebles et al., 2001b). Interestingly, all of these studies used adult mice. Three studies, including our own, investigated immune responses to RSV infection as a function of age (Becnel et al., 2005; Culley et al., 2002; Dakhama et al., 2005). In toto, these studies show that primary infection of neonatal (zero to seven days of age) mice followed by secondary infection in the same mice after they matured into adults (twelve weeks of age) resulted in Th2-biased immunity. In fact, mice originally infected as neonates recruited more IL-4+CD4+ T cells (Th2 cells), while mice originally infected as adults recruited more IFN- γ + CD4+ cells (Th1 cells) (Culley et al., 2002). These studies (Culley et al., 2002; Dakhama et al., 2005) and our own (Becnel et al., 2005; You et al., 2006) highlight the importance of using neonatal mouse models to understand disease pathogenesis in human infants.

Similarities and dissimilarities exist between RSV and influenza infections. Like RSV infections, severe influenza infections usually occur in the very young (less than six months old),

the elderly (more than 65 years old), and the immunocompromised indicating that the lack of immunity in these three populations is responsible for disease pathogenesis. Studies in mouse models demonstrate that immune responses play a dual role in the pathogenesis of influenza A infection. Knockout or depletion studies of various T cell subsets demonstrate that immune responses are beneficial for clearing the virus, but also correlate with massive inflammation and severity of lung tissue damage (La Gruta et al., 2007). Influenza infection initiates Th1 immune responses in adult mice, which are usually thought to be protective in viral infections. Adoptive transfer of Th1 cells decreases pulmonary inflammation, while the administration of Th2 cells delays viral clearance and enhances pulmonary pathology (Graham et al., 1994; La Gruta et al., 2007). CD8⁺ T cells eliminate the virus through either direct lysis of infected cells or secretion of cytokines that induce antiviral responses (Doherty et al., 1997; Topham et al., 1997). Absence of CD8⁺ T cells delays viral clearance and recovery from infection (Hou et al., 1992; Taylor and Askonas, 1986), and passive administration of influenza-specific CD8⁺ T cells is protective against subsequent challenge (Bender et al., 1992). Intriguingly, the same responses may cause extensive lung tissue damage and exacerbate respiratory distress (Moskophidis and Kioussis, 1998). All of these observations, again, were made in adult mice with few studies investigating infections in neonatal mice. In contrast to adult data, studies in human infants showed weakened CD4⁺ and CD8⁺ T cell responses to both influenza and RSV (Welliver et al., 2007). While the adult mouse is a good model for studying immunopathogenesis of highly virulent strains, such as the 1918 Spanish influenza, a neonatal mouse model (zero to seven days of age) is more relevant for understanding infections in human infants and for testing vaccines and therapies.

Summary

RSV and influenza infections are two of the primary causes of acute respiratory infections in infants. Both have been studied intensely in the past several decades. To date, no

efficacious anti-viral drugs or vaccines exist for combating RSV, and antigenic drift and shift in influenza A make vaccine development a challenge — “moving target”. This is partly because the mechanisms of pathogenesis are not well defined in either process, although immunopathology has recently been accepted as partly responsible for the pathogenesis of both RSV and influenza infections.

As discussed above, age is an important and independent factor, not only for disease severity but also for predicting the immune responses to initial and subsequent infections with respiratory viruses. Surprisingly, we are one of a small number of groups that study the pathogenesis of respiratory viruses in age-relevant animal models; seven-day-old mice are chosen, since the immunological status of mice at seven days of age is thought to be comparable to human infants (Adkins et al., 2004; Culley et al., 2002; Dakhama et al., 2005; You et al., 2006). **Our programmatic hypothesis is that the outcome of respiratory viral infections in neonates is determined by the interplay between the host (i.e., neonatal immunity) and viral factors (i.e., RSV versus influenza).** Using different viruses (i.e., RSV and influenza) and mice at different ages (i.e., neonates and adults), we will be able to identify these host and viral factors.

The majority of this dissertation research aimed to establish neonatal mouse models for RSV and influenza A infections, to demonstrate that the outcome of infection depends on age at initial infection; and to show that RSV and influenza infections cause pulmonary distress with similarities and dissimilarities via different mechanisms (Chapter 2 and 4, respectively). More importantly, the elucidation of the pathogenesis of viral infections should shed light on possible therapies or prevention strategies for disease. Hence, we started to explore a possible vaccine / therapeutic strategy for RSV infection in Chapter 3.

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CHAPTER TWO:

**EXPOSURE OF NEONATES TO RESPIRATORY
SYNCYTIAL VIRUS IS CRITICAL IN DETERMINING
SUBSEQUENT AIRWAY RESPONSES IN ADULTS***

*This is a revision of the article originally published in Respiratory Research

Introduction

Respiratory syncytial virus (RSV) is the most common cause of upper and subsequent lower respiratory tract infection in children and the elderly and is most severe in children between the ages of 8 and 30 weeks (Matthews et al., 2005). Severe RSV infection occurs in 1-2% of the cases and may result in acute bronchiolitis that requires mechanical ventilation (Leader and Kohlase, 2002).

Several studies have suggested that severe RSV lower respiratory tract infection in infancy may induce later development of asthma (McConnochie and Roghmann, 1984; Mok and Simpson, 1984; Murray et al., 1992; Noble et al., 1997; Pullan and Hey, 1982; Sims et al., 1978). In 2000, Sigur and colleagues reported that RSV infection in infancy, severe enough to require hospitalization, was associated with asthma and allergy in children up to age 7 (Sigurs et al., 2000). More recently, they reported that the relationship between severe RSV bronchiolitis in infancy and later development of asthma and allergy sensitization are still observed in children up to age 13 (Sigurs et al., 2005). In murine models, numerous studies have been carried out and controversial results have been reported. While some studies propose that previous RSV infection enhances allergen sensitization and exacerbates asthma, others believe that RSV infection in infancy protects against later allergic sensitization (Kalina and Gershwin, 2004). This controversy is now thought to arise from the age of initial RSV infection and the different protocols used for RSV infection and allergen sensitization and challenge. In two independent experiments, researchers suggested that the timing of primary RSV infection is significant for predicting disease outcome. Culley and colleagues reported that if neonatal mice (1 d or 7 d of age) were infected with RSV and rechallenged at 12 wks of age, inflammatory cell recruitment was enhanced (including Th2 cells and eosinophils) (Culley et al., 2002). Their data suggested

that neonatal exposure to RSV exacerbated subsequent disease upon rechallenge in the adult (Culley et al., 2002). More recently, Dakhama and colleagues infected 1-wk old or 3-wk old mice with RSV, and they found that both primary infections led to increased lung resistance, mucus hyperproduction and inflammation along with increased lymphocytes in the lung (Dakhama et al., 2005). Furthermore, they demonstrated that the bronchoalveolar lavage fluid (BALF) from mice infected with RSV at 1 wk of age had reduced levels of IFN- γ and elevated levels of IL-13 compared to mice initially infected at 3 wks of age. These data indicated that a Th2 polarized response occurred in mice infected with RSV at 1 wk of age, while a Th1-biased response occurred in the mice infected with RSV at 3 wks of age. When rechallenged with RSV 5 weeks later (i.e., infection at 1 wk and reinfection at 6 wks of age), these mice displayed exacerbated disease as indicated by enhanced pulmonary resistance, mucus hyperproduction, eosinophilia, and elevated levels of Th2 cytokines. In contrast, initial infection of mice at 3 wks of age resulted in protection upon RSV rechallenge as indicated by the abolished lung resistance and enhanced viral clearance at rechallenge (Dakhama et al., 2005). These experiments indicate that age at initial RSV infection influences the subsequent immune and physiologic responses upon re-exposure to RSV.

We hypothesized that neonatal exposure to RSV plays a critical role in the pathophysiological response to subsequent allergen exposure and the development of allergic asthma. To investigate whether neonatal exposure to RSV influences airway hyperreactivity (AHR), mice were infected with RSV at 1 wk of age. At 6 wks of age, these mice were then sensitized and challenged with ovalbumin (Ova). Pulmonary function, histopathology, and BALF cellularity and cytokine production were examined 24 hours after the last Ova challenge. The results were compared to sham infected and/or saline exposed mice and demonstrated that

neonatal exposure to RSV resulted in elevated and prolonged AHR, chronic pulmonary inflammation, and subepithelial fibrosis in adult mice. These pathophysiological endpoints were most severe when neonatal RSV infection was followed by allergen sensitization and challenge. These studies demonstrate the ability of neonatal exposures to chronically alter lung function and the importance of preventing RSV exposure during infancy.

Methods

The study protocol is outlined in Figure 2.1. Mice were divided into four groups. RSS mice were infected with RSV and mock sensitized and challenged. OVA mice were mock infected then Ova sensitized and challenged. ROO mice were infected with RSV and then Ova sensitized and challenged. As the control group, SAL mice were mock infected, mock sensitized and challenged. Data were collected on protocol days 0, 4, 69, and 96.

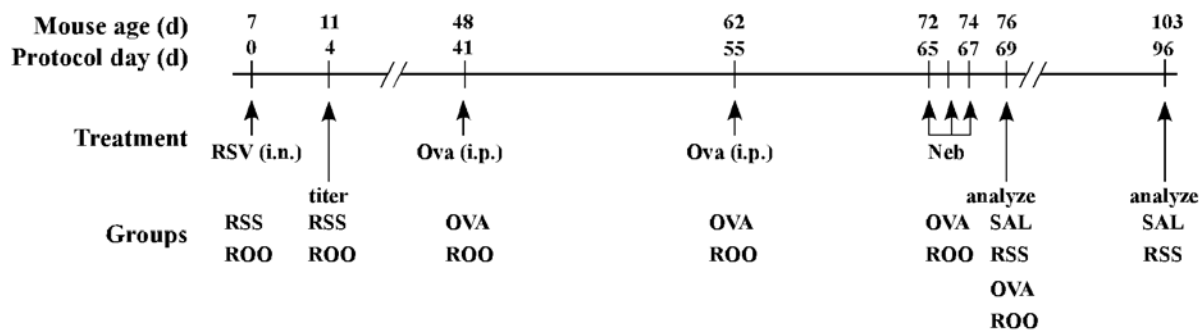


Figure 2.1 Schematic of study protocol and exposure groups. Neonatal mice (seven days of age) were infected with RSV (2×10^5 TCID₅₀/g body weight; RSS, and ROO groups). On protocol days 41 and 55, mice were injected i.p. with ovalbumin complexed to Imject Alum (OVA and ROO groups) or with isotonic saline (SAL and RSS groups). These mice were then exposed to aerosolized ovalbumin or saline for 20 minutes on protocol days 65, 66, and 67. n=8/group.

Mice

BALB/c breeders were purchased from the Division of Laboratory Animal Medicine (School of Veterinary Medicine, Louisiana State University) and seven day old pups were obtained by time-mating. All mice were maintained in ventilated micro-isolator cages housed in a specific pathogen-free animal facility. Sentinel mice within this animal colony were negative for antibodies to viral and other known mouse pathogens. All animal protocols were prepared in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and approved by the Institutional Animal Care and Use Committee at Louisiana State University.

Infection of Mice with RSV and Assessment of Viral Titers

Seven day old mice (protocol day 0; Figure 2.1) were infected intranasally (i.n.) with 2×10^5 TCID₅₀ RSV / g body weight (RSV A-2, Advanced Biotechnologies Incorporated, Columbia, MD) or culture media alone (OVA group). Four days post-infection, viral titer of whole lung homogenates was determined using the TCID₅₀ method of Spearman-Kärber (Karber, 1931; Spearman, 1908).

Ovalbumin Sensitization and Challenge

Mice were sensitized and challenged with chicken ovalbumin (grade V; Sigma, St. Louis, MO) as previously described (Becnel et al., 2005). Mice (OVA and ROO groups) were intraperitoneally (i.p.) injected with 20 µg Ova complexed with 2 mg Imject Alum (Al [OH]₃/Mg [OH]₂; Pierce, Rockford, IL) or Sal (RSS and SAL groups) on protocol days 41 and 55 (Figure 2.1) and subsequently challenged with aerosolized 1% Ova (in isotonic Sal; OVA and ROO groups) or Sal (RSS and SAL groups) using an ultrasonic nebulizer (PariNeb Pro Nebulizer) on protocol days 65 to 67 (Figure 2.1).

Assessment of Pulmonary Function

A) Lung resistance in response to methacholine

On protocol day 69, lung resistance to increasing doses of methacholine (MeCh, Sigma; 0, 6.25, 12.5, and 25 mg/ml in isotonic saline) was assessed using the forced oscillation technique as previously described (Becnel et al., 2005). Anesthetized animals were mechanically ventilated with a tidal volume of 10 ml / kg and a frequency of 2.5 Hz using a computer-controlled piston ventilator (FlexiVent, SCIREQ; Montreal, Canada). Resistance data were collected using single compartment model and plotted as the percent change from the 0 mg/ml MeCh dose.

B) Airway hyperreactivity in response to methacholine

Airway hyperresponsiveness to MeCh (Sigma; 0, 6.25, 12.5, 25, and 50 mg/ml in isotonic saline) was assessed by whole body plethysmography (Buxco Electronics, Troy, NY and EMKA Technologies, Falls Church, VA) as described previously (Becnel et al., 2005). Mice were exposed to aerosolized MeCh for 3 minutes at each dose and enhanced pause (Penh) was recorded for 3 minutes and averaged for each dose. Penh data were plotted as percent change from saline per dose.

Determination of Bronchoalveolar Lavage Fluid Cellularity

On protocol day 69, BALF was isolated in 1 ml of PBS containing 2% heat-inactivated FBS. Total BAL cellularity was determined with the use of a hemocytometer. Cytospin slides were fixed and stained using the Diff-Quick kit (IMEB, Chicago, IL), and differential cell counts by two unbiased observers were obtained using standard morphological criteria to classify individual leukocyte populations. Three mice from each group were used for these analyses, and 200-300 cells were counted per cytopsin preparation.

Pulmonary Histopathology

On protocol day 69, lungs were inflated with 1 ml of 4% paraformaldehyde. The lungs were then excised and fixed in 4% paraformaldehyde for 24 hours at 4°C. These tissues were then embedded in paraffin, cut in 4 µm frontal sections and stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS) to show mucus production in airway goblet cells, or Masson's trichrome (MT) to indicate airway collagen deposition. Scores were assigned to these histological endpoints by two independent observers and were recorded on a scale of 0-3.

Cytokine Level in BALF

IL-2, IL-4, IL-5, IFN- γ , and TNF- α levels in the BALF were examined using the Mouse Th1 / Th2 Cytokine Cytometric Bead Array Kit (BD Biosciences, San Diego, CA) as per the manufacturer's instructions. Data were acquired with a BD FACScan™ flow cytometer. Data analyses were performed using the BD Cytometric Bead Array Software to generate standard curves for each cytokine and to determine sample cytokine levels. IL-13 levels in BALF were determined using the Mouse IL-13 ELISA Ready-SET-Go kit (eBioscience, San Diego, CA). The sensitivity for each cytokine is as follows: 5.0 pg/ml for IL-2, IL-4, and IL-5; 2.5 pg/ml for IFN- γ , 6.3 pg/ml for TNF- α , and 30 pg/ml for IL-13.

Statistical Analysis

Data are presented as mean \pm SEM and were obtained from experiments with n=8 for whole body plethysmography analysis of AHR, n=4-5 for invasive measurements of pulmonary mechanics, and n=3 for BALF cellularity, cytokine assays, pulmonary viral titers and histology. For AHR, lung resistance and BALF cellularity, differences between groups were evaluated by means of two-way ANOVA using GraphPad Prism (Graphpad Software Inc, San Diego, CA). Bonferroni post-tests were performed to compare between pairs of groups. A one-way ANOVA

was used to compare the mean cytokine levels among the various groups followed by the Tukey-Kramer multiple comparison tests for significance between the groups. This was repeated for each individual cytokine. Differences between means were considered significant when $p < 0.05$.

Results

Reduced Pulmonary Function Was Observed in Mice Infected with RSV as Neonates

To determine if neonatal exposure to RSV is sufficient to induce long-term pulmonary dysfunction, mice were infected with RSV (2×10^5 TCID₅₀ / g body weight) at seven days of age and then allowed to mature to adults (Figure 2.1). Four days post-infection, we assessed pulmonary viral titers for Sham and RSV infected mice. As expected, mice from the Sham infected groups displayed no evidence of viral replication. The mean viral titer in the lungs of neonatal mice exposed to RSV alone (RSS and ROO) was $2.67 \pm 1.29 \times 10^6$ TCID₅₀/g lung tissue.

After maturation, subsets of mice were then sensitized (protocol days 41 and 55) and challenged (protocol days 65, 66, and 67) with ovalbumin - OVA and ROO groups. All other mice received saline - RSS and SAL groups. AHR was assessed on protocol days 69 and 96 (2.5 and 3.5 months after infection). We observed no significant difference in baseline airway resistance between the RSV infected (0.52 ± 0.01 cm H₂O·s/ml) or sham infected mice (0.64 ± 0.06 cm H₂O·s/ml). For ease of comparison among all groups, lung resistance data was normalized by plotting the percentage difference from baseline (percent difference = $100 \times ((\text{value}-\text{baseline})/\text{baseline})$). Lung resistance in response to increasing concentrations of inhaled MeCh was significantly greater in RSV infected neonates (RSS; 121.79 ± 10.20 cm H₂O·s/ml) than control mice (SAL; 89.05 ± 6.51 cm H₂O·s/ml) at 25 mg/ml MeCh (1.4 times, $p < 0.01$)

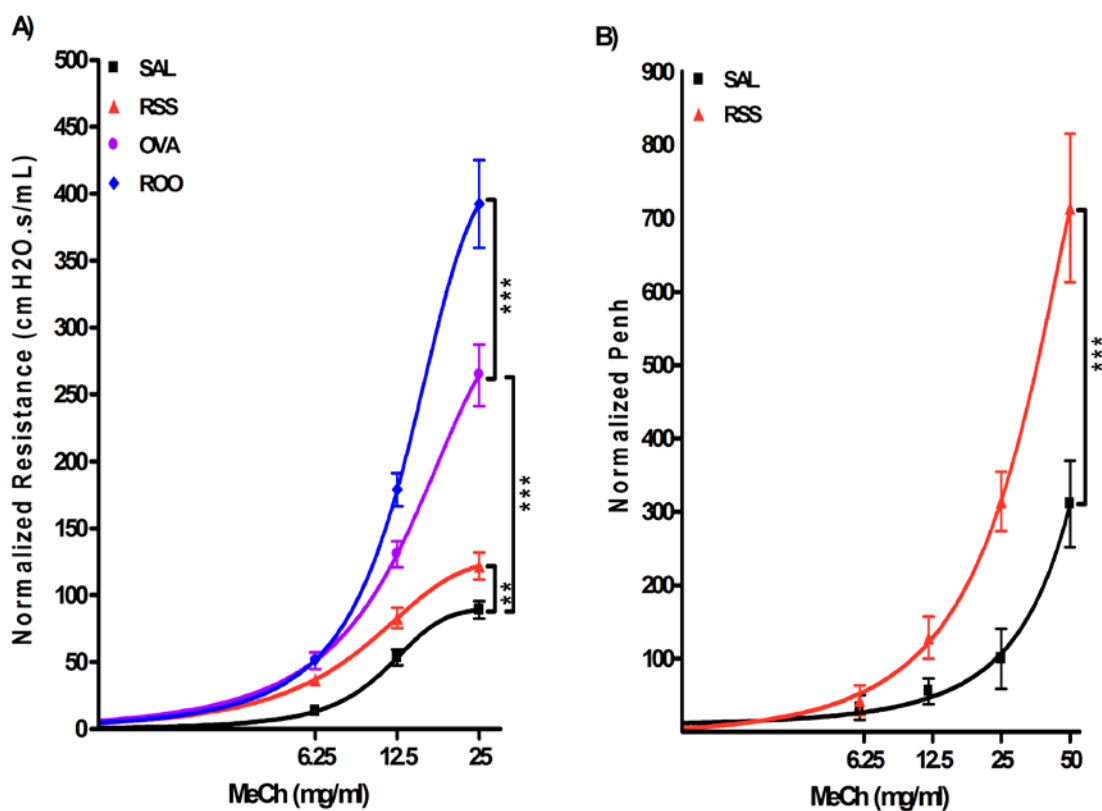


Figure 2.2 Airway hyperreactivity in RSV and/or Ova exposed mice. Mice were infected with RSV as neonates (RSS) and exposed to Ova 5 wks later (ROO). Controls were sham infected and exposed to Saline (SAL) or Ova (OVA). **A.** Lung resistance of each group is plotted as a function of increasing doses of inhaled MeCh, using single compartment model. **B.** Penh is plotted as a function of increasing dose of inhaled MeCh (0 to 50 mg / ml). Data were collected on protocol days 69 (A) or 96 (B), normalized to saline airway responses as described in methods, and expressed as mean \pm SEM. n=4-5/group. ***p< 0.001; **p<0.01.

(Figure 2.2.A). Neonatal RSV infection followed by exposure to Ova (ROO) resulted in the highest lung resistance among the four groups (392.50 ± 32.84 cm H₂O·s/ml). This increased airway resistance was greater than could be accounted for by Ova exposure alone (OVA; 264.63 ± 23.01 cm H₂O·s/ml). Thus, neonatal exposure to RSV infection appears to predispose adults to the development of airway hyperresponsiveness to subsequent allergen exposure.

To investigate the long-term influence of neonatal exposure to RSV, AHR was assessed by whole body plethysmography on protocol day 96 (103 days of age). Penh data was normalized and the percentage difference from baseline was plotted. Mice in the RSS group continued to demonstrate reduced airway function 3 months post-infection as evidenced by a significantly increased Penh compared to SAL mice at 25 and 50 mg/ml MeCh (314.30 ± 40.40 vs 100.00 ± 40.80 at 25 mg/ml; 714.30 ± 101.00 vs 311.10 ± 58.80 at 50 mg/ml; $p < 0.001$ and $p < 0.001$, respectively) (Figure 2.2.B). These data demonstrated that neonatal infection with RSV resulted in the development of chronic AHR, and furthermore, that airway function was further diminished due to an allergic phenotype.

Neonatal RSV Infection Predisposes Mice to Chronic Pulmonary Inflammation Indicated in BALF Cellularity

To evaluate the pulmonary immune responses to RSV, BALF was isolated, total cells recovered were counted, and the cellular composition of BALF was determined using morphological criteria. RSV and/or Ova exposed mice recruited significantly more leukocytes than control mice (SAL group, $p < 0.001$) (Figure 2.3). However, there was no significant difference in the total number of recovered cells among the RSS, OVA, and ROO groups. Interestingly, BALF cellularity was elevated in mice exposed to RSV as neonates (RSS) on protocol day 69. Upon allergen sensitization and challenge, total BALF cellularity was not

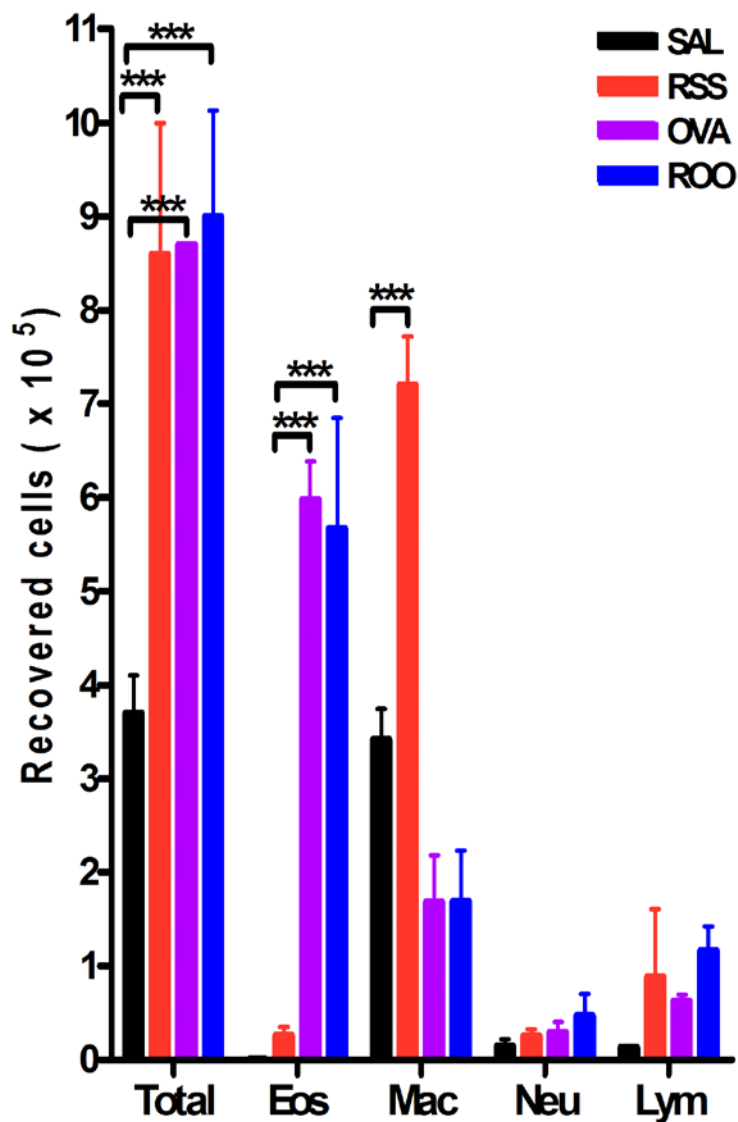


Figure 2.3 BALF cellularity in RSV and/or Ova exposed mice. Bronchoalveolar lavage fluid was isolated on protocol day 69. RSV and/or Ova induced significant increase in total BAL cellularity. In mice exposed to RSV as neonates (RSS), this increase correlated with elevated levels of macrophages; whereas, in mice exposed to Ova (ROO and Ova), this increase correlated to elevation in eosinophil numbers. Data are expressed as means \pm SEM, n=3/group. ***p < 0.001

significantly altered compared to RSS although the cell populations were changed. Neonatal RSV infection resulted in a predominantly monocyte/macrophage pulmonary infiltrate and these cells seemed to account for the elevation in the total number of recovered cells. In contrast, Ova exposure, in the absence (OVA) or presence of neonatal RSV (ROO) infection, resulted in a significant BALF eosinophilia (compared to control SAL group, $p < 0.001$). Concomitant with the observed increase in BALF eosinophil number in the OVA and ROO groups was a decrease in BALF macrophage number.

Cytokine Levels in BALF Is Altered in Mice Exposed to RSV and/or Ova

Cytokine levels in BALF were examined to study the Th1/Th2 polarization in RSV and/or Ova exposed mice. As shown in Figure 2.4.A, we assayed cytokine levels in BALF five hours post-infection, and observed greatly elevated TNF- α (21.4 times, $p < 0.001$) in RSV infected neonates. In addition, Th2 cytokines (IL-4 and IL-5) along with IL-2 were detected in BALF of neonatally infected mice whereas no IL-4, IL-5, or IL-2 was detected in control mice (SAL group). TNF- α levels declined by protocol day 69 in mice infected with RSV as neonates, but was elevated in RSV infected and Ova exposed mice (ROO group, Figure 2.4.B). Intriguingly, IL-13 levels were elevated in RSS mice at protocol day 69 compared to SAL mice ($p < 0.001$). Mice exposed to RSV and Ova (ROO group) showed increased Th2 cytokines (IL-5 and IL-13) compared to SAL group. In addition, IL-13 levels in ROO mice were significantly elevated compared to the animals exposed to Ova alone (OVA group; $p < 0.01$). Neonatal infection with RSV also resulted in increased levels of TGF- β_1 protein in the lungs 4d post-infection (RSS: 29.8 ± 0.45 ng/g lung tissue) compared to mice exposed to Sal alone (22.1 ± 0.39 ng/g lung tissue).

Enhanced Pulmonary Histopathology in Mice Exposed to RSV and/or Ova

Airway inflammation, mucus hyperproduction, and collagen deposition in the subepithelial reticular layer of the airway were observed in mice exposed to RSV and/or Ova (Figure 2.5.A: inflammation; Figure 2.5.B: mucus production; Figure 2.5.C: collagen deposition). Mice exposed to both RSV and Ova demonstrated the largest amount of inflammation, mucus production, and collagen deposition (Figure 2.5.D), and this was consistent

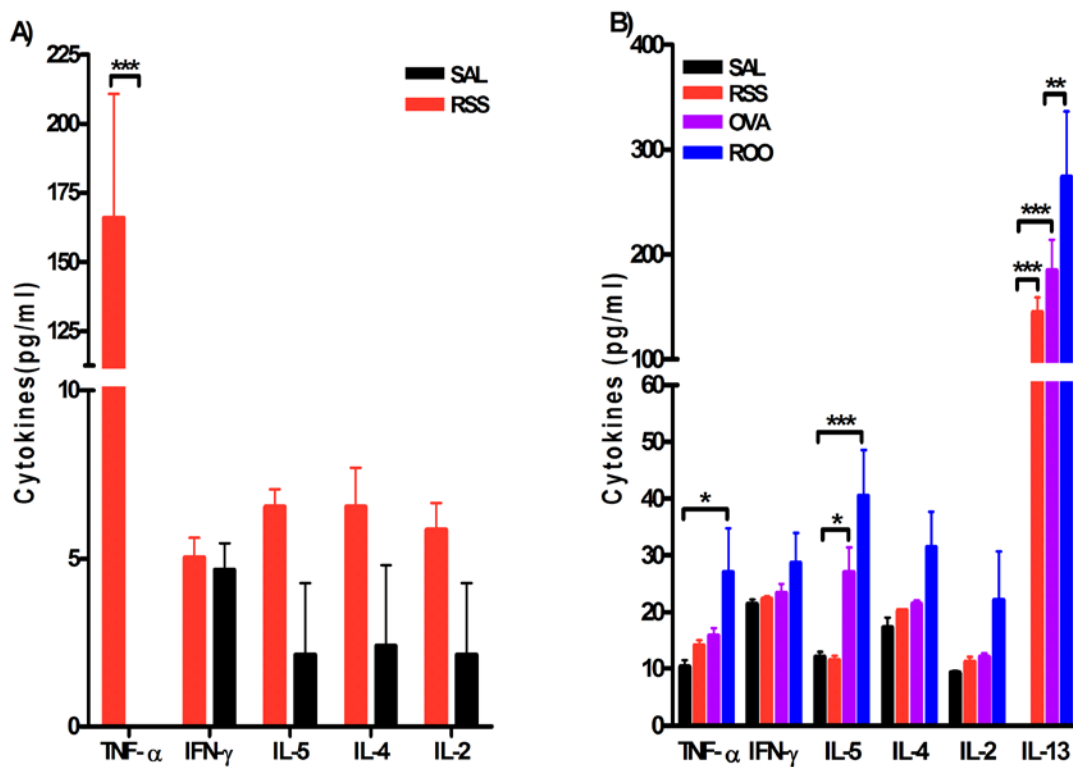


Figure 2.4 Cytokine levels in the BALF of mice exposed to RSV and/or Ova.

Bronchoalveolar lavage fluid was isolated 5 hr post-infection with RSV (A) or on protocol day 69 (B). A. Elevated levels of TNF- α , IL-5, IL-4, and IL-2 were observed as early as 5 hrs post-infection, although significance over SAL controls was observed only for TNF- α . IL-2, IL-4, and IL-5 were below the limit of detection in the SAL mice. B. IL-13 was significantly elevated in RSS mice; while TNF- α , IL-5, and IL-13 were significantly enhanced in mice exposed to RSV and Ova (ROO). IL-13 was below the limit of detection in control animals (SAL). Data are expressed as means \pm SEM, n=3/group. ***p < 0.001, **p<0.01, and *p<0.05.

with the greatest lung resistance and airway hypersensitivity (Figure 2.2). In addition, tremendous perivascular inflammation was observed in both groups of mice exposed to Ova (i.e., OVA and ROO mice; Figure 5A). Mice infected with RSV as neonates displayed chronic (69d post infection) inflammation, mucus production and collagen deposition compared to control mice (SAL group), which also correlated to enhanced lung resistance and airway hypersensitivity in the RSS group (Figure 2.2). Increased peribronchial and perivascular collagen deposition and

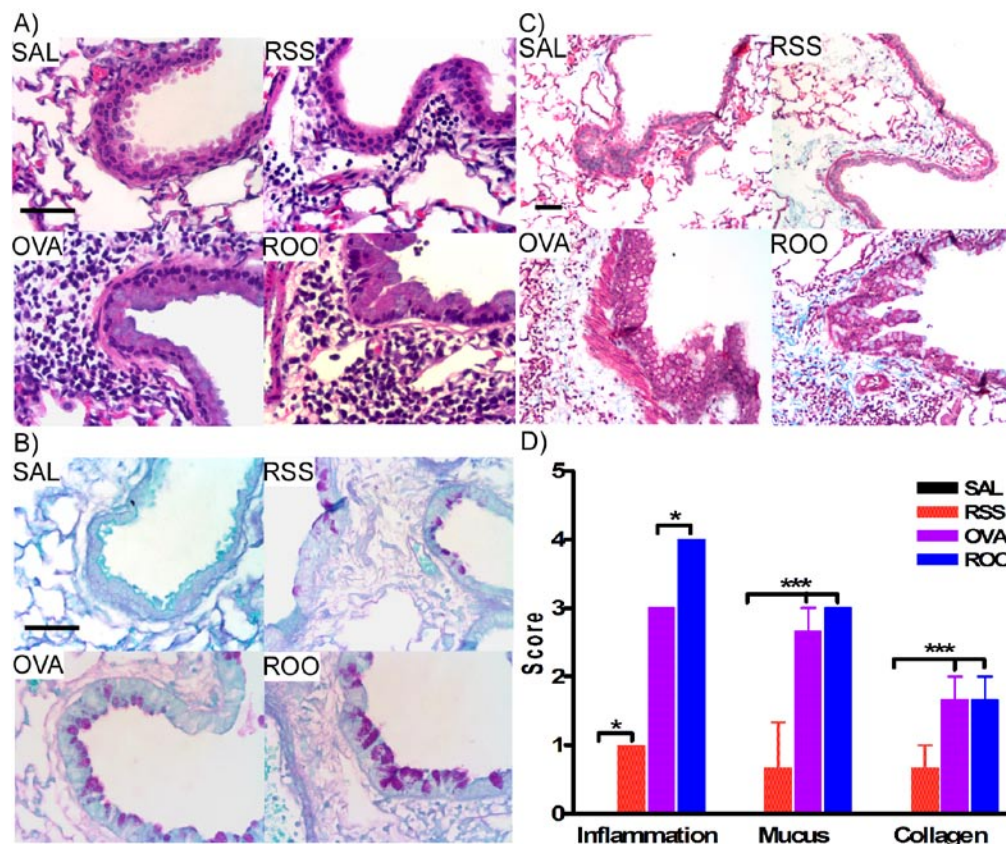


Figure 2.5 Lung histopathology of mice exposed to RSV and/or Ova. Lung tissue sections were obtained from mice on protocol day 69. Tissue sections were stained with H&E (A), PAS (B), and MT (C). **A.** Lung inflammation, **B.** mucus (purple) hyperproduction, and **C.** collagen (blue) deposition were observed in mice exposed to RSV and/or Ova. **D.** Scores were assigned to these histological endpoints by two independent observers and were recorded on a scale of 0-4. Increased deposition of peribronchial and perivascular collagen was observed in the subepithelial reticular layer of the airways in RSS, OVA and ROO mice. In all cases, neonatal RSV infection (RSS) induced persistent lung pathologies including increased peribronchial inflammation, mucus production, and subepithelial fibrosis were exacerbated by subsequent exposure to allergen (ROO). n=3/group. ***p < 0.001, *p<0.05.

peribronchial smooth muscle deposition indicated that airway remodeling occurred in mice exposed to RSV and/or Ova (Figure 2.5.B), and that neonatal RSV infection alone was sufficient to induce airway remodeling.

Discussion

In the present study, we have shown that neonatal exposure to RSV alone induced long-term airway hyperresponsiveness and pulmonary resistance in mice. This result was correlated with peribronchial inflammation, increased BALF cellularity, increased mucus production, and airway remodeling. Elevations in TNF- α and Th2 cytokines (IL-4 and IL-5) were observed in BALF immediately following neonatal RSV infection. Once these mice had matured (protocol day 69), only IL-13 remained elevated in the BALF. Neonatal RSV infection followed by adult exposure to allergen resulted in significantly higher lung resistance, along with increased total cellularity, eosinophilia, and increased TNF- α and Th2 cytokines (IL-5 and IL-13) in BALF. In addition, TNF- α and IL-13 were significantly higher compared to mice exposed to Ova alone. The most severe lung histopathology was observed in mice exposed to both RSV and Ova, as indicated by severe peribronchial and perivascular inflammation, mucus production, and collagen deposition. Collectively, these data suggest that neonatal RSV infection influences adult immune response to allergen (Ova) and exacerbates allergic pathophysiology in mice long after viral titers are no longer detectable.

In contrast to previously published studies analyzing the effect of RSV infection on allergen exposure in adult mice (Peebles et al., 2001), the present study investigated the influence of neonatal RSV infection on adult allergen sensitization. Neonatal infection with RSV alone was sufficient to induce long-term pulmonary dysfunction and inflammation. Similarly, neonatal infection of brown Norway rats with parainfluenza type 1 (Sendai) virus also

led to increases in pulmonary resistance and hyperresponsiveness to methacholine up to 65d after infection (Sorkness et al., 1991). As in our model, persistent airway dysfunction following neonatal infection with Sendai virus correlated with increased peribronchial fibrosis and pulmonary inflammation (Uhl et al., 1996). Furthermore, both models (i.e., neonatal RSV infection and neonatal Sendai virus infection) resulted in significantly increased mRNA (Uhl et al., 1996) and protein levels for TGF- β_1 . Although numerous cytokines may contribute to airway remodeling, the elevation of TGF- β_1 , a fibrogenic cytokine, in both neonatal viral infection models prior to the development of fibrosis suggests a role this cytokine in the regulation of viral-induced airway remodeling observed in neonates.

More importantly, neonatal infection with RSV predisposed mice to the development of enhanced AHR and inflammation after allergen exposure. In contrast, adult RSV infection prior to allergen exposure seemed to assert a “protective” response as evidenced by significantly decreased allergen induced pulmonary resistance, tissue eosinophilia, and IL-13 levels (Peebles et al., 2000). Our data presented here and elsewhere (Becnel et al., 2005) extend these findings and, more importantly, demonstrate that the age at initial RSV infection also determines whether RSV infection will exacerbate or prevent subsequent allergic inflammation.

A more recent study using neonatal RSV infection followed by subsequent reinfection of adults with RSV demonstrated that early RSV infection also exacerbates RSV induced diseases in the adult (Dakhama et al., 2005). Interestingly, if the primary RSV infection occurred at 3 wks of age, a protective effect upon secondary infection was observed similar to that reported by Peebles and colleagues (Peebles et al., 2001). Dakhama and colleagues further established that enhancement of AHR, pulmonary eosinophilia, and mucus hyperproduction during reinfection were dependent on IL-13 (Dakhama et al., 2005). We demonstrated that neonatal RSV infection

alone leads to elevated levels of IL-13 in the lung and that exposure to allergen significantly increases IL-13 levels over RSV exposure alone. IL-13 has emerged as a major regulatory molecule involved in mucus hyperproduction and allergen induced AHR (Kuperman et al., 2002; Taube et al., 2002; Wills-Karp and Chiaramonte, 2003). It is entirely possible that the long-term AHR, pulmonary inflammation, and mucus production observed in our neonatal RSV model is due to high levels of IL-13. In fact, elevated levels of IL-13 were observed in whole lung homogenates as early as 5 hours post-infection (data not shown) and were again observed in adult lungs on protocol day 69 suggesting that IL-13 is being chronically produced. Although the exact cellular source of IL-13 in this neonatal model of RSV infection is currently unknown, previous studies have demonstrated that epithelial cells and/or macrophages infected by RSV are a significant source of IL-13 and are capable of producing this cytokine for months after the initial infection (Schwarze et al., 2004). We are currently investigating this possibility.

In our study, TNF- α was significantly elevated in the BALF shortly after infection with RSV. TNF- α is an important cytokine for innate immune responses and a central regulator of inflammatory processes, through binding to distinct membrane receptors, referred to as p55 or TNFR1 and p75 or TNFR2 (Peschon et al., 1998). TNF- α is likely a central mediator of airway inflammation and AHR in asthma, regulating inflammatory cell infiltration, locally enhancing vascular permeability, and inducing the release of the chemokines. Ultimately, this will lead to chronic inflammation and irreversible airway remodeling. Recently, depletion studies using monoclonal antibody therapy for TNF- α have shown promising effects in viral-specific lung immunopathology (Hussell et al., 2001), rheumatoid arthritis (Lovell et al., 2000) and inflammatory bowel disease (Bell and Kamm, 2000). Moreover, a soluble TNF receptor fusion protein, etanercept, has proven efficacious in treating juvenile rheumatoid arthritis in patients as

young as 4 (Lovell et al., 2000). In viral models, TNF- α depletion reduced recruitment of inflammatory cells, reduced type 1 and type 2 cytokines in BALF, and decreased pulmonary pathology without inhibiting viral clearance (Hussell et al., 2001; Rutigliano and Graham, 2004). Although the precise mechanism by which TNF- α leads to the pathology in lungs after RSV infection remains unknown, our data and these previous studies suggest a key role for TNF- α in chronic inflammation in the lung and subsequent airway remodeling associated with asthma. Several studies along with our present data have established the correlation of severe RSV infection followed by allergen exposure and the development of allergic inflammatory disease (i.e., asthma) in mice. Although the mechanism by which the exposure causes asthma and the importance of such exposures in humans need to be further elucidated, our current and previously published data (Becnel et al., 2005) demonstrate that the initial age of the RSV infection is capable of altering adult pulmonary function and exacerbating pulmonary immunopathology when followed by subsequent allergen exposure. Furthermore, enhanced AHR correlated with chronic pulmonary inflammation, upregulation of the Th2 cytokine, IL-13, and subepithelial fibrosis of the bronchial airways. Increases in TNF- α within 5 hours of RSV infection in our mouse model also suggest a role for this cytokine in the immunopathology of RSV-induced wheeze and asthma development in humans.

Conclusion

We have demonstrated that neonatal infection with RSV in mice leads to reduced lung function, which is associated with chronic inflammation, increased mucus production, and airway remodeling in the lung. In addition, RSV infection in neonates predisposes the adult to develop enhanced airway responses upon allergen exposure. The upregulation of IL-13 and

TNF- α suggests that these cytokines may play a key role in mediating this process and highlights the importance of these cytokines as therapeutic targets for RSV induced asthma.

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CHAPTER THREE:

PREVENTION OF PULMONARY DYSFUNCTION DURING REINFECTION WITH RESPIRATORY SYNCYTIAL VIRUS BY ADMINISTRATION OF ANTISENSE OLIGONUCLEOTIDES TO IL-4RALPHA DURING PRIMARY INFECTION

Introduction

Respiratory syncytial virus (RSV) is a common cause of upper and lower respiratory infection in humans. Each year in the United States, RSV causes about 91 thousand hospitalizations (Anderson et al., 1990) and has an economic impact of about 300 million dollars (Openshaw, 2002). Although the majority of RSV infections result in only mild pulmonary diseases, about 25 to 40% of infants develop severe bronchiolitis and/or pneumonia with 0.5 to 4.4% of these requiring hospitalization (Collins and Graham, 2008; Welliver, 2003). Severe RSV bronchiolitis in infancy adversely affects lung function and respiratory health into adulthood (Korppi et al., 2004; Sigurs et al., 2000; Sigurs et al., 2005).

The pathogenesis of RSV infection is not well understood. However, data in humans and mice support the notion that a Th2-biased immune response develops which is at least partly responsible for RSV-induced pulmonary dysfunction. Th2 and Th1 cells are two subsets of T helper cells (CD4⁺ T cells). Primary RSV infection in neonatal mice (seven days of age) predisposes the response of T helper cells to a Th2-dominant response upon secondary infection. This response is characterized by pulmonary eosinophilia and goblet cell hyperplasia (Culley et al., 2002; Dakhama et al., 2005). The mechanisms by which neonatal exposure favors a Th2 response upon priming and rechallenge with RSV are unclear and are a major obstacle for the development of effective pediatric vaccines.

The first hint to why neonatal exposure usually gives rise to a Th2-biased secondary response was presented by Zaghoulani's group (Li et al., 2004). They showed that although both Th1 and Th2 responses are elicited in neonatal mice during primary challenges with allergens, secondary responses are dominated by Th2 cells. Specifically, Th1 cells are selected to undergo apoptosis in the secondary response, leaving the Th2 cells and thus a Th2-dominated secondary

response. Apoptosis correlates with increased expression of the IL-13 receptor α 1 (IL-13R α 1) subunit. Furthermore, blockade of either the IL-13R α 1 or IL-4 receptor α (IL-4R α) subunit during priming with antigen blocks apoptosis and restores Th1 recall responses. This finding provides a possible mechanism for Th2-biased secondary responses and emphasizes the importance of IL-4 and IL-13 signaling in the development of Th2 recall responses.

IL-4 is the major cytokine inducing Th2 cell differentiation and, therefore, an important regulator in Th2-biased immunopathogenesis of neonatal RSV infection. IL-13, an important Th2 cytokine, stimulates the differentiation of goblet cells (Tyner et al., 2006) and has been shown to mediate the airway hyperresponsiveness caused by both primary (Tekkanat et al., 2001; You et al., 2006) and secondary RSV infections in mice (Dakhama et al., 2005). Both IL-4 and IL-13 signal through receptors which share a common subunit, IL-4R α . The type I IL-4 receptor is composed of the IL-4R α and common γ c chains and binds only to IL-4. The type II IL-4 receptor is a heterodimer consisting of the IL-4R α and IL-13R α 1 chains and binds to both IL-4 and IL-13. Besides the type II receptor, another IL-13 receptor exists, which is thought to be a decoy receptor for negative regulation of IL-13 (Chatila, 2004).

We hypothesized that local inhibition of IL-4R α expression would prevent the Th2 dominated secondary responses to RSV infection and improve pulmonary function in adult mice originally infected with RSV as neonates. To reduce IL-4R α expression in the lung, antisense oligonucleotides (ASO) against IL-4R α were administered intranasally.

Methods

Mice

BALB/c mice were purchased from Harlan Sprague Dawley, Inc and housed in a specific-pathogen-free facility located in the Animal Care Facility at the Louisiana State

University Health Sciences Center. Breeders were time-mated, and pups born on the same date were randomized and grouped according to the study design. All animal protocols used were in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and were approved by the Institutional Animal Care and Use Committee.

Administration of Oligonucleotides

The antisense oligonucleotides against IL-4R α and the non-specific seven-base mismatch oligonucleotides (MM) were provided by Isis Pharmaceuticals, Inc. Both oligonucleotides were 20 bases in length with 2'-O-methoxyethylribose modification on bases 1 to 5 and 16 to 20. The sequences of the ASO and MM are shown below; the mismatched bases are presented in lower case (Karras et al., 2007).

ASO: 5'-CCGCTGTTCTCAGGTGACAT-3'

MM: 5'-CCaCTcaTCaCtGcTGACtT-3'

The ASO and MM were suspended in sterile saline and administered intranasally (i.n.) to mouse pups at a dose of 500 μ g/kg body weight. Control pups (i.e., SHAM, SAL, RSV, and RR) received sterile saline.

Inoculation and Titer Determination of RSV

The original RSV stock was purchased from Advanced Biotechnologies, Inc, propagated in our laboratory, and stored in aliquots at -80 °C. Seven-day-old pups were infected intranasally with RSV (2×10^5 TCID₅₀/gram body weight) in 10 μ l of Dulbecco's modified Eagle's medium (DMEM) containing 2% heat-inactivated fetal bovine serum (FBS). Similarly, adult mice were infected with RSV (2×10^5 TCID₅₀/gram body weight) in 50 μ l of the same media. SHAM mice received media alone.

To determine lung viral load, lungs were isolated, placed in media, and stored at -80 °C until processing. To process, lungs were homogenized, cellular debris was pelleted by centrifugation, and clarified supernatants were used to infect 96-well plates of Vero cells (ATCC # CCL-81). TCID₅₀ were calculated using the method of Spearman-Kärber (Karber, 1931; Spearman, 1908).

Experimental Design

The experimental design is outlined in Figure 3.1. Mice were treated with IL-4R α ASO i.n. on protocol days -5, -3, -1, and 1 and infected with RSV on protocol day 0 (seven days of age). Expression of IL-4R α on various lung cells was determined on protocol day 0 prior to RSV infection. Three groups were included in the expression study (not shown in Figure 3.1): SAL (treated with saline), ASO (treated with IL-4R α ASO), and MM (treated with MM). Viral titers and T cell populations during primary infection were determined on protocol days 4 and 6, respectively. After the treated mice matured (six weeks of age), all groups except SHAM were

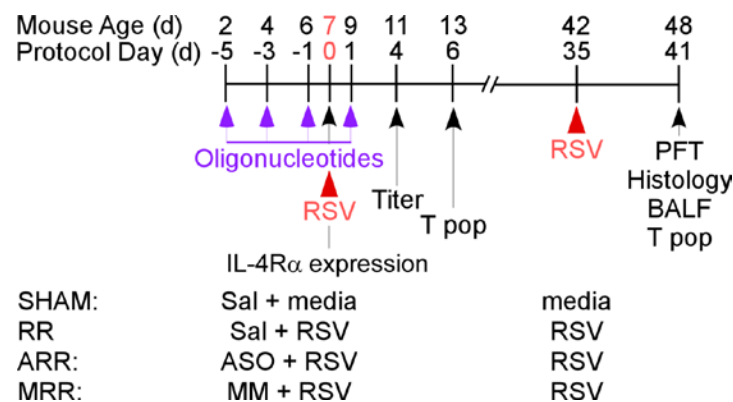


Figure 3.1 Experimental design. Four groups were included in the study: one experimental group (ARR) and three control groups (SHAM, RR, and MRR). For the ARR group, pups received IL-4R α ASO i.n. on protocol days -5, -3, -1, and 1 and were infected with RSV on protocol day 0. Mice were allowed to mature to six weeks of age and were reinfected with RSV. SHAM mice received saline and media instead of oligonucleotides and RSV. RR mice were treated with saline and infected with RSV. MRR mice received MM and RSV. Various analyses were performed at different time points, as indicated, including expression of IL-4R α , T cell populations (T pop), lung viral titers and histology, pulmonary function (PFT), and bronchoalveolar lavage fluid (BALF) cellularity and cytokine levels.

reinfected with RSV. Six days post-infection (dpi), various endpoints — including pulmonary function, bronchoalveolar lavage fluid (BALF) cellularity and cytokine levels, and T cell populations — were determined. Four groups were included in these studies (Figure 3.1): SHAM (treated with vehicles only, saline and media as appropriate); RR (treated with saline and infected with RSV); MRR (treated with MM and infected with RSV); and ARR (treated with IL-4R α ASO and infected with RSV).

Cell Surface Antigen and Intracellular Cytokine Staining

To determine the expression level of IL-4R α on various lung cells, single lung cell suspensions were prepared in RPMI-1640 supplemented with 2% heat-inactivated FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (HyClone), and then split into two subsets. One subset was stained with antibodies to IL-4R α and to CD4 and CD8 to identify T cell subsets. The other was stained with antibodies to IL-4R α and to E-cadherin, CD45, CD11c, and CD11b to identify airway epithelial cells (E-cadherin⁺ CD11b⁻ CD45⁻) and myeloid dendritic cells (mDCs; CD11c⁺ CD11b⁺).

To determine the T cell subpopulations, single lung cell suspensions were isolated in RPMI-1640 media supplemented with 2% heat-inactivated FBS, 100 U/ml penicillin and 100 mg/ml streptomycin (HyClone), and stimulated at 37 °C in the same media that was additionally fortified with 5 ng/ml phorbol-12-myristate-13-acetate (PMA; Sigma), 500 ng/ml ionomycin (Sigma), and a protein transport inhibitor (1 μ l/10⁶ cells; GolgiPlug, BD Biosciences). After stimulation for five hours, cells were washed and stained with antibodies to surface antigens (CD4 and CD8); and then fixed, permeabilized, and stained with antibodies to intracellular cytokines (IFN- γ and IL-4) to identify T cell subsets.

After staining, cells were washed and fixed in a paraformaldehyde-based fixative (BD Stabilizing Fixative; BD Biosciences). Staining profiles of these cells were acquired on an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Version 7.2.2 for Windows, Tree Star, Inc). Antibodies, including PerCP-CD4 (RM4-5), Alexa Fluor 488-CD8a (53-6.7), FITC-E-cadherin (36/E-cadherin) and PE-IL-4R α (mIL4R-M1) were purchased from BD Biosciences. Others, including PE-Cy7-CD11c (N418), APC-CD11b (M1/70), Alexa Fluor 700-CD45 (30-F11), PE-IFN- γ (XMG1.2), and PE-Cy7-IL-4 (BVD4-24G2) were purchased from eBiosciences.

Determination of BALF Cellularity and Cytokine Production

BALF was isolated in 1 ml of PBS containing 2% heat-inactivated FBS as a protein carrier. The total cell number was determined using a hemocytometer. Cells (20,000) were cytopun onto slides, stained using a HEMA-3 staining kit (Fisher Scientific), and were counted by two independent observers. Differential cell counts were based on cell morphology and staining.

Cytokine concentrations in the BALF were measured using a multiplex suspension array system (X-Plex Mouse Assay and Bio-Plex 200 platform; BioRad Laboratories). A total of seven cytokines were measured simultaneously in each sample, including IL-4, -5, -6, -10, -13, -17, and IFN- γ , as per the manufacturer's instructions. The sensitivity varied for each cytokine (0.19 to 1.66 pg/ml).

Lung Histology

Lungs were perfused with PBS containing 20 U/ml heparin, inflated to total lung capacity, and fixed in HistoChoice Tissue Fixative (Amresco, Inc). After serial dehydration, lungs were embedded in paraffin and sectioned at 4 μ m. Lung sections were then stained with

either hematoxylin and eosin (H&E) to characterize the cellular infiltrates or periodic acid-Schiff (PAS) to identify mucus-productive goblet cells in airways. Micrographs of representative areas of lungs were taken under bright field with 100 x magnification (H&E) or 400 x magnification (PAS).

Measurement of Pulmonary Function

Respiratory mechanics were measured using an invasive method on a FlexiVent system (SCIREQ Scientific Respiratory Equipment Inc). Mice were anesthetized, intubated, and ventilated using a computer controlled-ventilator. Mice were then challenged with increasing doses (0, 12.5, and 25 mg/ml) of nebulized methacholine (MeCh; Sigma). Lung pressure and volume changes after each challenge with MeCh were recorded and converted into digital signals, which the FlexiVent software fit into a single-compartment model to calculate lung resistance and compliance data (FlexiVent, version 5.2)

Statistics

Statistical calculations were performed using GraphPad Prism software (GraphPad Software, Inc.). Student's t-test was used to compare means between different groups with the exception of pulmonary function data. Two-way ANOVA was used to analyze pulmonary function data, with a Bonferroni adjustment for pair-wise comparisons. Data are expressed as means \pm SEM, and $p < 0.05$ was chosen as the significance level.

Results

Administration of IL-4R α ASO Downregulated the Expression of IL-4R α on Lung Cells

After ASO treatment, flow cytometry was performed to examine the expression levels of IL-4R α on lung cells including epithelial cells, T cells, and mDCs. The IL-4R α expression levels were quantified as the mean fluorescence intensity (MFI) of the antibody to IL-4R α . As

shown in Figure 3.2, surface IL-4R α was significantly down regulated on both CD4⁺ T cells and mDCs in mice treated with IL-4R α ASO, compared to control mice receiving saline (SAL group) or MM (MM group). In fact, IL-4R α expression in the ASO group was reduced by 15% on CD4⁺ T cells and 40% on mDCs. Expression of IL-4R α on CD8⁺ T cells and lung epithelial cells was not changed compared to the SAL group. Down regulation of IL-4R α was specific to the ASO group, since IL-4R α expression on any cell type examined did not differ significantly between the MM and SAL groups.

Administration of IL-4R α ASO Altered T Lymphocyte Responses to Primary RSV Infection

IL-4 is a Th2 cytokine important in Th2 cell maturation and effector functions. Therefore, instillation of IL-4R α ASO was expected to influence the development of Th2 cells in RSV-infected animals. To study this, mice were treated with ASO and infected with RSV (AR; Figure 3.1). Three control groups were included: SHAM (treated with saline and media, as appropriate), RSV (treated with saline and infected with RSV), and MR (treated with MM and infected with RSV). Flow cytometry was then performed to identify T helper cell populations

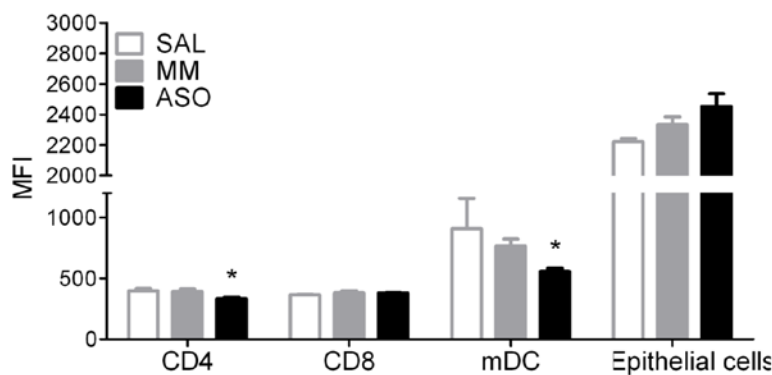


Figure 3.2 Expression of IL-4R α on lung cells. Mouse pups were treated with ASO, MM, or saline (SAL) on protocol days -5, -3, and -1. Twenty-four hours later, single lung cells were isolated and stained with antibodies to IL-4R α and specific differentiating surface markers. The levels of IL-R α expression were determined by flow cytometry and plotted as the mean fluorescence intensity (MFI) of gated cells. n=3. *p<0.05.

and cytotoxic T cell populations (CTLs; CD8⁺ T cells). Like T helper cells, CTLs are also categorized into two subsets, based on the cytokines that they produce: Tc1 (IFN- γ producing) and Tc2 (IL-4 producing). As shown in Figure 3.3, RSV-infected pups induced a mixture of Th1 (IFN- γ ⁺ CD4⁺), Th2 (IL-4⁺ CD4⁺), Tc1 (IFN- γ ⁺ CD8⁺), and Tc2 (IL-4⁺ CD8⁺) cells in the infected lungs. Instillation of IL-4R α ASO altered the primary T cell responses to RSV infection, as evidenced by the significantly increased percentage of pulmonary Th1 cells and the decreased percentage of Th2 cells. The percentage of Th1 cells in the AR mice was three fold higher than in the RSV mice, while the percentage of Th2 cells was reduced by half compared to the RSV mice. No differences were observed in either type of CD8⁺ T cells between the AR and RSV groups. Furthermore, no differences were observed in the MR and RSV groups.

Administration of IL-4R α ASO Increased Viral Load during Primary RSV Infection

Pulmonary viral load may be associated with increased severity induced by RSV infection in some human cases (Fodha et al., 2007). To address the possible effects of ASO treatment on RSV replication in the lung, pulmonary viral loads were measured at 4 dpi. Interestingly, viral loads in both the MM and ASO treated mice (MR and AR, respectively) were elevated compared to mice not receiving ASO/MM (RSV; Figure 3.4). While RSV-infected lungs had mean viral loads of 10^4 TCID₅₀/g lung tissue, both AR and MR mice exhibited significantly higher viral loads ($10^{4.8}$ and $10^{4.6}$ TCID₅₀/g lung tissue, respectively).

Treatment with IL-4R α ASO during Primary RSV Infection Prevented Airway Hyperreactivity upon Rechallenge

Neonatally infected mice and infants develop severe respiratory distress, characterized by increased airway hyperresponsiveness, upon reinfection (Dakhama et al., 2005; Kim et al., 1969). To address the effects of IL-4R α ASO treatment on pulmonary function following

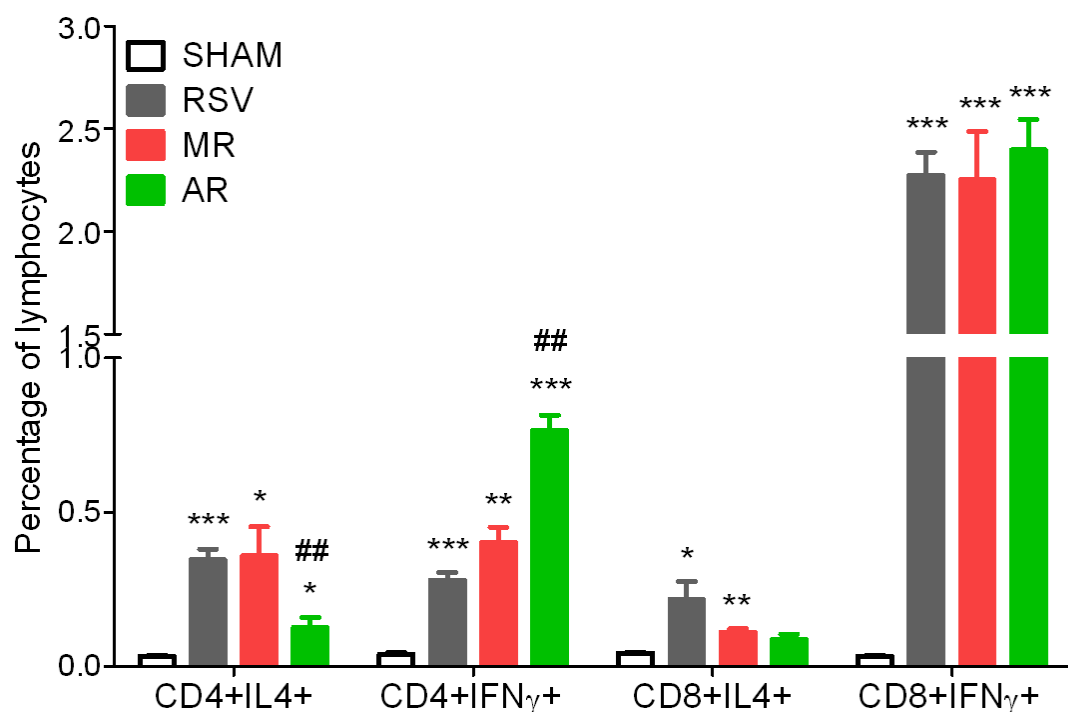


Figure 3.3 T cell responses to neonatal RSV infection in the presence or absence of ASO treatment. Four groups were incorporated in the study: one experimental group (AR) and three control groups (SHAM, RSV, and MR). In the AR group, mouse pups were treated with IL-4R α ASO on protocol days -5, -3, -1, and 1 and were then infected with RSV on protocol 0. On protocol day 6, lung cells were isolated, stimulated *in vitro*, stained with differentiating markers, and analyzed by flow cytometry. SHAM mice were treated with saline and media as appropriate; RSV mice received saline and RSV; and MR mice received MM and RSV. n=3. ***: p<0.001, **: p<0.01, *: p<0.05, compared to SHAM mice; ##: p<0.01 compared to RSV group.

secondary RSV infection, neonatally infected mice were reinfected with RSV at six weeks of age. Six days later, pulmonary function was tested. The results are shown in Figure 3.5.A. Airway resistance to MeCh was similar between ASO-treated mice (ARR) and SHAM mice (Figure 3.5.A). In contrast, non-treated (RR) and MM-treated mice (MRR) showed significant airway hyperreactivity in response to MeCh. In fact, lung resistance at 50 mg/ml of MeCh in the RR and MRR mice was about 2.5-fold higher than in the SHAM or ARR mice. Lung compliance followed the same trend (Figure 3.5.B). Compliance in the ARR mice was similar to that in the SHAM mice, while both RR and MRR mice showed substantially lower compliance at 50 mg/ml of MeCh (1.5-and 1.8-fold, respectively) compared to the SHAM or ARR group.

IL-4R α ASO Treatment Altered BALF Cellularity and Cytokine Levels upon RSV Reinfection

BALF cellularity is an indication of inflammation occurring in airways and alveolar spaces, and therefore was determined at 6 dpi. As shown in Figure 3.6.A, secondary infection

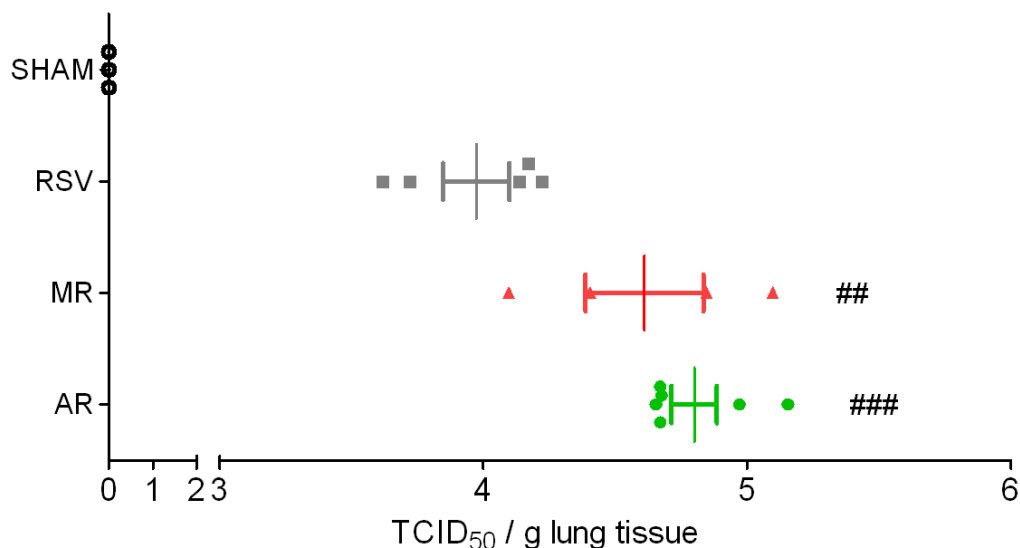


Figure 3.4 Pulmonary viral load following primary RSV infection in neonatal mice treated with IL-4R α ASO. Viral titers were measured in lung homogenates at four days post-infection (dpi), using the TCID₅₀ method. n=4-6. ##: p<0.01, ###: p<0.001, compared to RSV group.

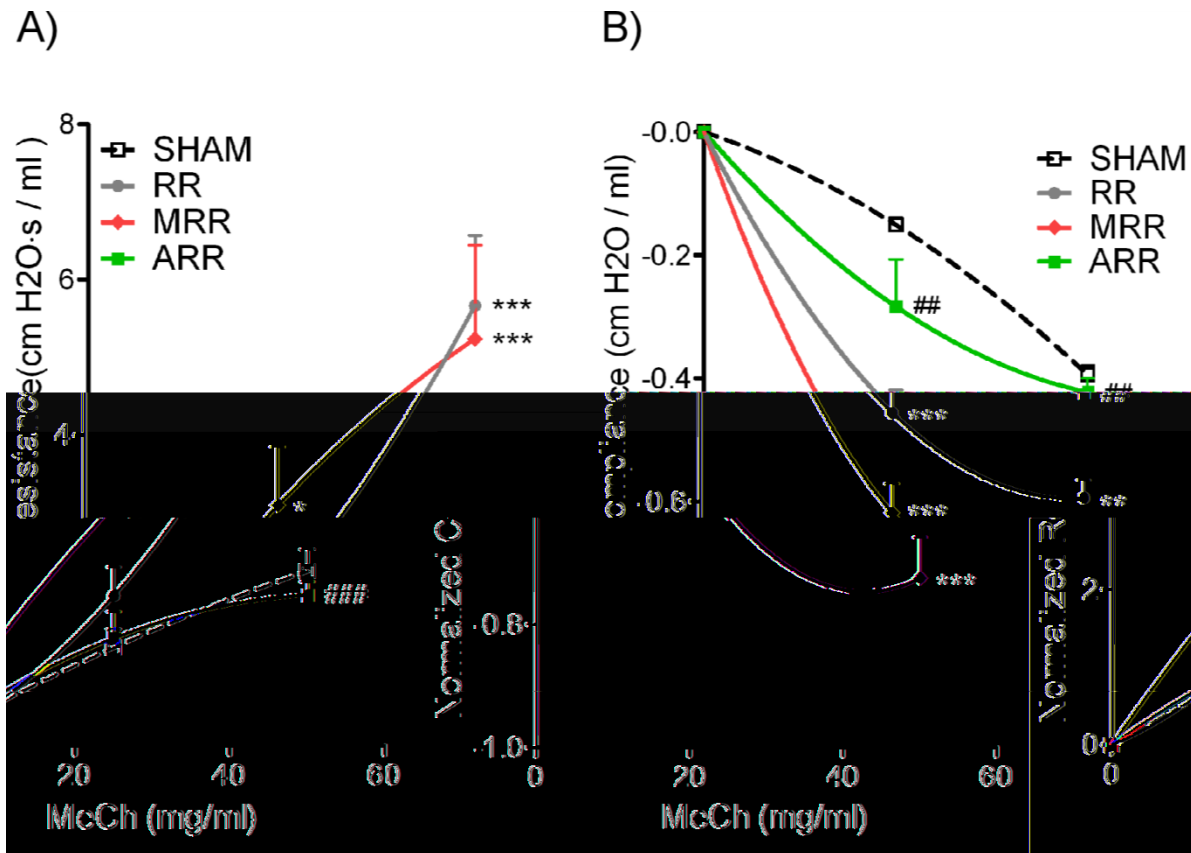


Figure 3.5 Pulmonary function after secondary RSV infection in mice receiving IL4R α ASO during primary infection as neonates. Mice infected with RSV as neonates were reinfected at six weeks of age. Six days later, pulmonary function was tested in four groups. ARR mice received ASO treatment during primary infection and were reinfected with RSV as adults. Control groups were MRR (treated with MM and RSV), RR (treated with saline and RSV), and SHAM (treated with saline and media as appropriate). **A)** Lung resistance was determined and normalized to baseline resistance at 0 mg/ml of MeCh and was plotted as normalized resistance in response to increasing doses of MeCh. **B)** Lung compliance was normalized to baseline compliance at 0 mg/ml of MeCh and was plotted as normalized compliance in response to increasing doses of MeCh. n=5-6. *: p<0.05, **: p<0.01, ***: p<0.001, compared to SHAM mice; ##: p<0.01, ###: p<0.001, compared to RR group.

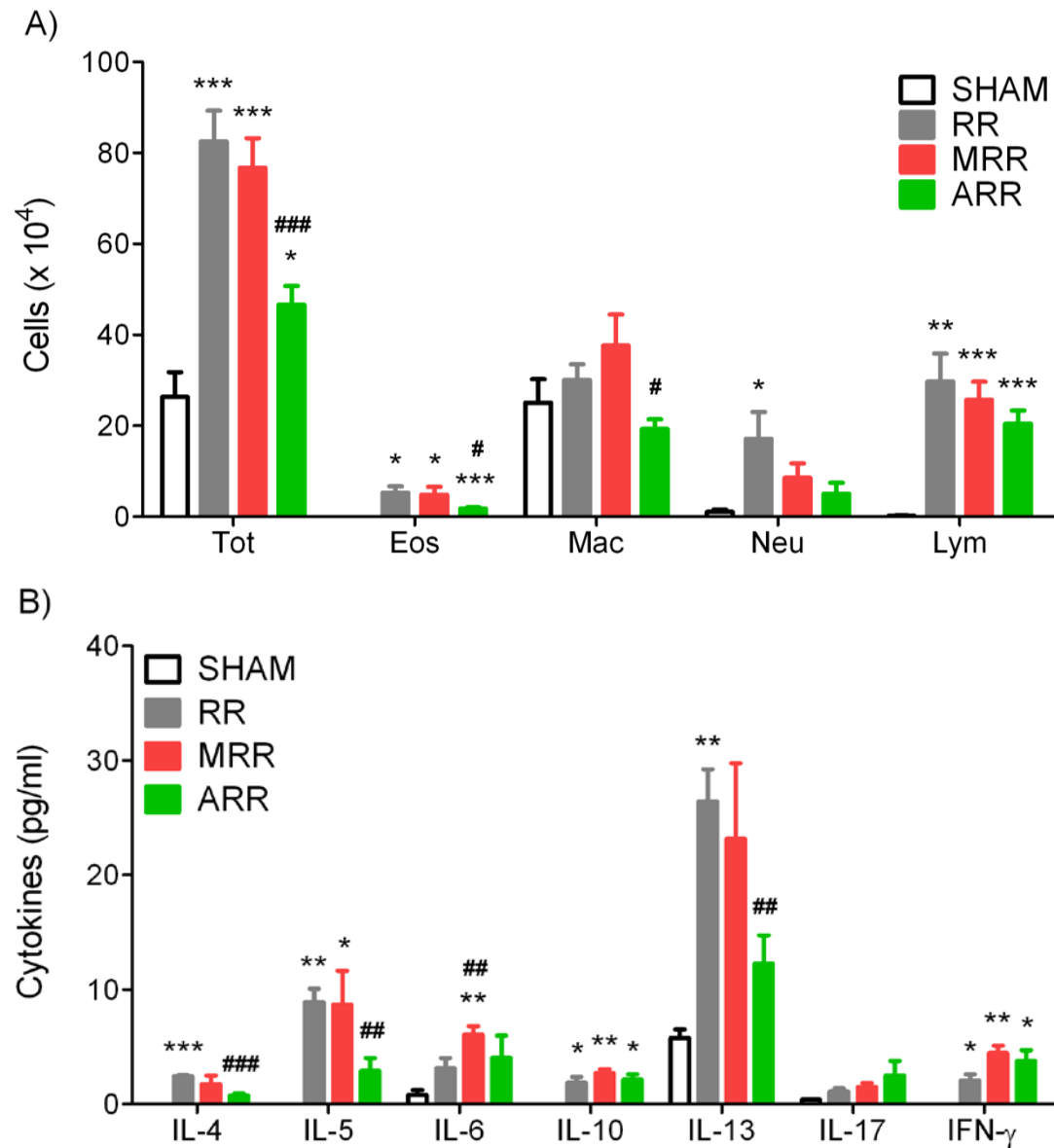


Figure 3.6 BALF cellularity and cytokine levels after secondary RSV infection.

Neonatally infected mice were reinfected with RSV at six weeks of age. BALF cellularity and cytokine production were determined at 6 dpi. **A)** BALF cellularity. Tot: total; Eos: eosinophils; Mac: monocytes/AMs; Neu: neutrophils; Lym: lymphocytes. **B)** Cytokine levels in the BALF. n=4-8; *: p<0.05, **: p<0.01, ***: p<0.001, compared to SHAM mice; #: p<0.05, ##: p<0.01, ###: p<0.001, compared to RR mice.

with RSV elicited a significant increase of BALF cellularity in all three infected groups of mice (RR, MRR, and ARR); however, IL-4R α ASO-treated mice (ARR) had significantly lower total BALF cellularity compared to the RR group. Furthermore, the composition of the BALF differed among these groups. All three infected groups of mice recruited neutrophils, lymphocytes, and eosinophils into the airway. ARR mice recruited significantly fewer eosinophils and monocytes/AMs compared to the RR mice. No significant differences were observed between MRR and RR mice in either cell types or total cellularity.

BALF cytokine levels were also measured at 6 dpi (Figure 3.6.B). RSV reinfection resulted in secretion of a variety of cytokines, including IL-4, -5, -6, -10, -13, -17, and IFN- γ . IL-4R α ASO treatment (ARR) during primary infection in neonatal mice led to a reduction of Th2 cytokines including IL-4, IL-5, and IL-13. The decreased Th2 cytokine production in the BALF was consistent with the reduced eosinophilia in the BALF of ARR mice. No differences were observed between ARR mice and non-treated mice (RR) in other cytokines, including the prominent Th1 cytokine IFN- γ . The BALF cytokine profile observed in mice receiving MM (MRR) was similar to that of the RR mice, with the exception of elevated IL-6 secretion.

Administration of IL-4R α ASO Reduced Lung Histopathology Induced by Secondary RSV Infection

Secondary RSV infection in mice infected originally as neonates typically induces severe lung pathology, characterized by substantial inflammation and goblet cell hyperplasia. This pulmonary pathology is regulated in part by Th2 cells and Th2 cytokines — in particular IL-13 (Dakhama et al., 2005). To determine if the improved pulmonary function in ASO-treated mice was associated with reduced pathology, lung histopathology was performed at 6 dpi. Interestingly, no observable differences were found in the magnitude of inflammation among the

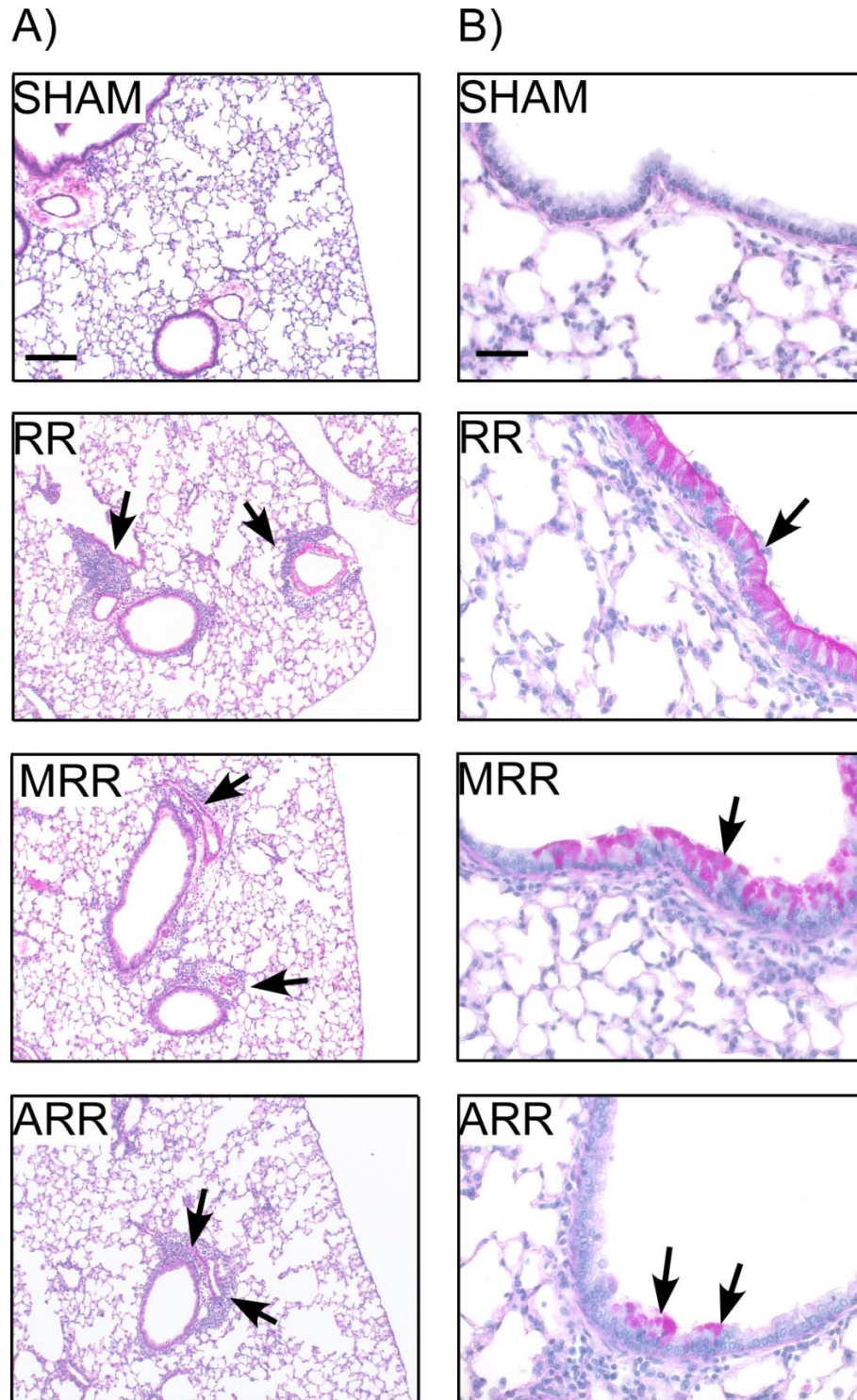


Figure 3.7 Lung histology after secondary RSV infection. Neonatally infected mice were reinfected with RSV at six weeks of age. Lung sections were prepared and stained with H&E or PAS at 6 dpi. The micrographs shown are representative of data from three mice. **A)** H&E staining to identify inflammatory cells. Bar=200 μ m. **B)** PAS staining (purple) to identify mucus in goblet cells. Bar=50 μ m.

three RSV-infected groups (RR, MRR, and ARR; Figure 3.7.A). All three groups showed perivascular and peribronchiolar foci of inflammation with occasional diffuse alveolitis. Consistent with BALF cellularity, the inflammatory cells in the lungs also included monocytes, neutrophils, eosinophils, and lymphocytes. No inflammation was observed in the lungs of SHAM mice. In addition, secondary RSV infection also led to goblet cell hyperplasia and mucus hyperproduction in the bronchioles of RR and MRR mice (Figure 3.7.B). Intriguingly, treatment with IL4R α ASO significantly reduced goblet cell hyperplasia and mucus hyperproduction, as evidenced by the fact that only one or two foci of goblet cells were observed per lung section in the ARR group. No differences in mucus staining were observed between the RR and MRR groups.

Administration of IL-4R α ASO Altered T Lymphocyte Responses to Secondary RSV Infection

To address the possible mechanisms by which IL-4R α ASO treatment alleviated pulmonary distress upon RSV reinfection, T cell responses (i.e., Th1/Th2 and Tc1/Tc2 responses) were investigated using flow cytometry. Th1, Th2, Tc1, and Tc2 cells migrated to lungs, as evidenced by significantly higher numbers of all four cell types in the lungs of RSV-infected mice (RR, MRR, and ARR) than in the SHAM mice. Treatment with IL-4R α ASO led to a significant reduction in Th2 cell recruitment (by about 50% in the RR mice) upon secondary RSV infection (Figure 3.8.A). Although not significant, ARR mice showed an increased Tc1 response compared to the RR mice. Treatment with MM (MRR) failed to show any effects on secondary T cell responses (compare MRR to RR).

Discussion

The present study demonstrated that seven day old mice infected with RSV developed severe pulmonary diseases when reinfected with RSV at six weeks of age. Pulmonary disease

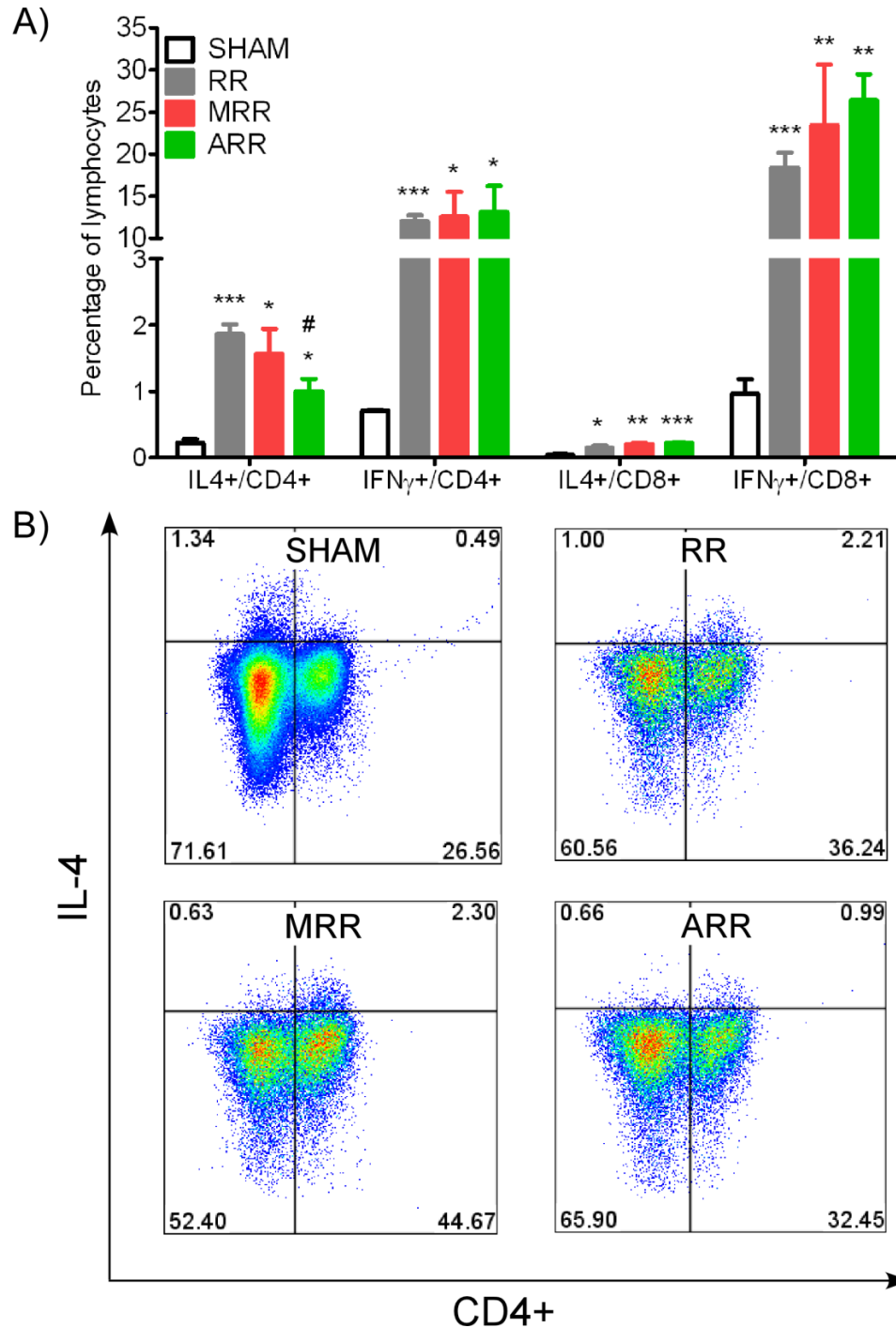


Figure 3.8 Pulmonary T cell populations following secondary infection with RSV.

Neonatally infected mice were reinfected with RSV at six weeks of age. Single lung suspensions were prepared, stimulated, stained with various antibodies, and analyzed by flow cytometry to examine T cell subsets recruited to the lung at 6 dpi. **A)** T cell subpopulations in the lungs after secondary infection. **B)** Dot plots representing IL4⁺ CD4⁺ T cell population (upper right quadrant) following secondary infection. n=3; *: p<0.05, **: p<0.01; ***: p<0.001, compared to the SHAM mice; #: p<0.05, compared to the RR group.

was characterized by airway hyperreactivity, inflammation, goblet cell hyperplasia, and mucus hyperproduction. Immunologically, the illness correlated with Th2 cell infiltration and effector functions including eosinophilia and the release of Th2 cytokines (IL-4, IL-5, and IL-13). When ASO to IL-4R α were administered during primary RSV infection, the majority of the RSV-mediated immune-physiological responses were suppressed upon reinfection. These included diminished Th2 cellular infiltration, Th2 cytokine secretion, eosinophilia, goblet cell hyperplasia, and abolished airway hyperreactivity in response to MeCh.

The most intriguing result was not that treatment with IL-4R α ASO altered the Th1/Th2 balance during primary infection in the neonates, but that the treatment completely inhibited pulmonary dysfunction upon reinfection. This ablation of pulmonary dysfunction may be attributed to the reduced Th2 primary responses, including a reduction of IL-13 in the BALF of ASO-treated mice, since depletion of IL-13 during RSV reinfection has been shown to abolish mucus hyperproduction and airway hyperreactivity (Dakhama et al., 2005). Although not tested in the present study, the reduction of primary Th2 responses most likely led to a decrease in memory Th2 responses to reinfection and may be explained as follows: ASO treatment down-regulated IL-4R α expression on mDCs, the professional antigen presenting cells in the lung. After taking up the virus, mDCs migrated to draining lymph nodes and presented the virus to, activated, and provided differentiating signals to naïve T cells (de Jong et al., 2005; Kalinski et al., 1999; Kapsenberg, 2003). The major differentiating signal for Th2 cell commitment is IL-4, and IL-4 stimulates mDCs via IL4R α to produce more IL-4 (Maroof et al., 2006). Therefore, the down regulation of IL-4R α expression on mDCs may have inhibited primary Th2 cell differentiation, which could have led to the decreased number of Th2 cells observed in the present study (Figure 3.3). After activation and differentiation, a subset of Th2 cells develops

into memory cells, which circulate in the body to respond rapidly if a secondary infection occurs. Upon reinfection, fewer Th2 recall responses were observed in ASO-treated mice (Figure 3.8), most likely due to the reduced Th2 cellular memory response upon primary infection.

Despite the moderate increase in Th1 responses in ASO-treated mice during the primary infection, we found no difference in Th1 cells upon reinfection (compare ARR and RR groups). This may be due to the recruitment of nonspecific Th1 cells to the infected lungs upon primary infection or to the failure to create memory Th1 cells; laboratory studies are currently being performed to test this hypothesis.

Notably, the present study provides a possible vaccine strategy for RSV via immunomodulation at the time of vaccination. Developing vaccines for infants is difficult, due to their relatively immature immune systems, which favor a Th2 response instead of a protective Th1 response. In the infamous RSV vaccine trial of the late 1960s, 80% of the children vaccinated with formalin-inactivated RSV developed severe bronchiolitis and/or pneumonia upon naturally acquired reinfection. Two of the thirty-one vaccinated infants died. Autopsies on the lungs from these infants showed tremendous pulmonary inflammation and eosinophilia, indicating an exacerbated Th2 response. Our data demonstrate that treatment with IL-4R α ASO increases the ratio of Th1 to Th2 cells during primary infection and, more importantly, that this mild push away from a Th2 response and toward a Th1 response during primary RSV infection leads to a balanced secondary Th1/Th2 response and ablation of pulmonary dysfunction upon reinfection. The data indicate that the rebalancing of Th1 and Th2 responses appears to have been achieved by a decrease in the number of Th2 cells in ASO-treated mice rather than an increase in the number of Th1 cells.

Excluding the possible recruitment of non-specific Th1 cells, this phenomenon actually has its advantages. Too many Th1 cells and an exaggerated Th1 response could lead to

bystander tissue damage and enhanced disease severity, as eloquently demonstrated by Openshaw and colleagues with a recombinant RSV expressing IFN- γ (rRSV/IFN- γ) (Harker et al., 2007). In these studies, overexpression of the Th1 cytokine IFN- γ led to detrimental effects on the disease severity induced by RSV reinfection, as evidenced by enhanced weight loss in mice originally infected with rRSV/IFN- γ . In fact, mice infected with rRSV/IFN- γ developed more severe pulmonary inflammation upon rechallenge, characterized by massive pulmonary inflammation of Th1-related Tc1 cells, compared to mice infected with wild-type RSV.

In summary, using antisense oligonucleotides against IL-4R α during primary RSV infection in neonatal mice abolished the pulmonary dysfunction normally observed following rechallenge in adults. This ablation of pulmonary dysfunction correlated with decreased Th2 responses including reduced eosinophilia, Th2 cytokine secretion, and goblet cell hyperplasia. In short, treatment with the IL-4R α ASO during primary RSV infection in neonatal mice succeeded where the 1960s vaccine trial failed. In combination, our data suggest that interventional therapy, during the initial RSV infection, with IL-4R α ASO may be of significant benefit.

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CHAPTER FOUR:

**INCHOATE CD8⁺ T CELL RESPONSES IN NEONATAL MICE
PERMIT INFLUENZA INDUCED PERSISTENT PULMONARY
DYSFUNCTION**

Introduction

Each year influenza viruses cause significant morbidity and mortality (2000). Influenza virus A, in particular, has been shown to elicit respiratory illnesses such as pneumonia and bronchiolitis and to exacerbate underlying respiratory diseases such as chronic obstructive pulmonary disease (COPD) and asthma (Upshur et al., 2006). In fact, last winter (Oct 2006 to May 2007), 23,753 specimens tested positive for influenza viruses in collaborating laboratories of the World Health Organization and the National Respiratory and Enteric Virus Surveillance System in the United States (<http://www.cdc.gov/flu/weekly/weeklyarchives2006-2007/06-07summary.htm>). Of the influenza positive specimens, 79% were influenza A viruses and 62% of these were of the H1 subtype (<http://www.cdc.gov/flu/weekly/weeklyarchives2006-2007/06-07summary.htm>). Interestingly, the highest incidence of illness occurs in pre-school and school age children with 20 to 30% of children in this age group being diagnosed with influenza infections each year (Chin et al., 1960; Collins and Lehmann, 1951; Glezen, 1996; Glezen et al., 1997). Infected infants younger than six months of age have higher hospitalization and mortality rates than do older children (Ajayi-Obe et al., 2007; Bhat et al., 2005). Complications of influenza infections in infants typically include lower respiratory tract involvement and although these infections occur less frequently than respiratory syncytial virus (RSV) infections in infants, they are also a significant cause of wheeze (Neuzil et al., 2002). Epidemiological data demonstrate that infection of infants with influenza can lead to chronic pulmonary distress and furthermore that the effects of influenza in this population are underestimated and may be responsible for previously unexplained chronic pulmonary dysfunction (Laraya-Cuasay et al., 1977).

To understand how influenza induces pulmonary illness, various animal models have been used, including ferrets, rats, chickens, and mice (Eichelberger, 2007). While neutralizing

antibodies to influenza are important for protection from viral infection, CD8⁺ T cells have been shown to play a pivotal role in viral clearance and recovery from the illness in adult mice. CD8⁺ T cell deficient mice exhibit delayed viral clearance and significantly higher mortality when challenged with a sub-lethal dose of influenza (Bender et al., 1992). In addition, in B cell deficient mice, adoptive transfer of CD8⁺ T cells promotes more rapid clearance of the virus and recovery from illness (Epstein et al., 1998; Graham and Braciale, 1997).

Despite the global burden of influenza infection in infants and children, very few studies have examined the pathogenesis of infection in an infant model. The infant and neonatal immune systems are immature and this immaturity contributes to the pathogenesis of various lower respiratory tract infections including influenza, RSV, and others. Lower respiratory tract viral infections in infants are often associated with acute and persistent pulmonary dysfunction, which is characterized by increased airway resistance and hyperresponsiveness (Pullan and Hey, 1982; Sigurs et al., 2000; Sigurs et al., 2005). Previously, we and other groups have described a neonatal mouse model (i.e., 7d of age at initial infection) of RSV infection (Culley et al., 2002; Dakhama et al., 2005; You et al., 2006). In this model, the age of primary infection determined the immunological nature (Th1 or Th2) of the secondary infection. Th2 immune responses dominated upon secondary infection, if the primary infection occurred at 1 week or less of age; while Th1 responses dominated if the mice were primarily infected as adults (8 wk old) (Culley et al., 2002). Interestingly, a single infection with RSV in neonatal mice induced long-term pulmonary dysfunction (You et al., 2006); which was exacerbated upon secondary infection with RSV (Dakhama et al., 2005). The RSV neonatal model demonstrated that the age at which the initial infection occurred is critical in determining the subsequent host immune response to the pathogen; and furthermore, it suggested that if the infection occurs too early, the immature immune response contributes to pathogenesis instead of protection. .

A recent study in human infants suggests that a failure to develop a cytotoxic T lymphocyte response is responsible for the high rate of infant morbidity and mortality caused by respiratory viruses (Welliver et al., 2007). The article demonstrated that in both fatal infant influenza and RSV infection, CD4⁺ and CD8⁺ T cells were present in the lung at very low frequency. In addition, granzyme-producing cells (either cytotoxic CD8⁺ T cells or NK cells) were not observed in the lungs of infants with fatal influenza virus infection and correlated with massive viral replication and apoptosis of inflammatory cells.

To understand the relationships among the type of respiratory virus, the pulmonary immune response and the long-term pulmonary pathophysiology after infection in infancy, we established a neonatal (seven day of age) mouse model of influenza A virus infection. In this model, neonatal infection induced long-term pulmonary inflammation and airway injury and dysfunction accompanied with a weak CD8⁺ T cell response (lower number and lower IFN- γ production in CD8⁺ T cells). To examine if the weak CD8⁺ T cell response played a role in the pathogenesis of influenza infection in neonates, we adoptively transferred adult CD8⁺ T cells into neonatal mice prior to infection with influenza. Our data demonstrate that the insufficient production of IFN- γ by neonatal CD8⁺ T cell may be responsible for the long-term pulmonary inflammation and lung injury in influenza infected neonatal mice.

Materials and Methods

Mice

BALB/c mice were purchased as breeders from Harlan Sprague Dawley, Inc. IFN- γ knockout mice (C.129S7 (B6)-Ifng^{tm1Ts}/J) were purchased from the Jackson Laboratory. Mice were maintained under specific pathogen-free conditions within the vivarium at Louisiana State University Health Sciences Center (New Orleans, LA). Sentinel mice within each colony were

monitored and were negative for specific known mouse pathogens. Breeders were time-mated and seven-day-old pups (neonates) were used for experiments. All animal protocols were prepared in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and approved by the Institutional Animal Care and Use Committee at Louisiana State University Health Sciences Center.

Viral Infection and Viral Titer Determination

Human Influenza A/PR/8/34 (H1N1) was purchased as a sucrose-gradient purified virus from Advanced Biotechnologies, Inc. The virus preparation was determined to be free of bacteria, yeast, and fungi. Viral titer was measured in whole lung homogenates at different time points (Figure 4.1) using the TCID₅₀ method of Spearman-Kärber (Karber, 1931; Spearman, 1908). Mardin-Darby canine kidney cells were seeded on a 96-well plate and then inoculated with a series of 10 fold dilutions of lung homogenates. Cells were then incubated at 37 °C and 5% CO₂ for four days; wells showing cytopathic effects were counted and TCID₅₀ were calculated.

Experimental Design

Seven day old pups were infected intranasally with 10 TCID₅₀/g body weight of influenza (FLU) in 10 µl of DPBS or sham infected with 10 µl DPBS (SHAM). Mouse pups were then allowed to mature and various assays performed at the indicated time points as outlined in Figure 4.1.A. For adoptive transfer studies, CD8⁺ T cells from naïve wild-type adults (FLU/CD8A), wild-type neonates (FLU/CD8N), or IFN-γ knockout mice (FLU/CD8AKO) were administered intraperitoneally to six-day-old pups; and at seven day of age, these pups were then infected with influenza (Figure 4.1.B). Four groups of mice were included as controls: vehicle treated pups (SHAM), influenza infected pups (FLU), vehicle treated adult mice (ASHAM), and influenza

infected adult mice (AFLU). A range of assays were performed at indicated time points as shown in Figure 4.1.B.

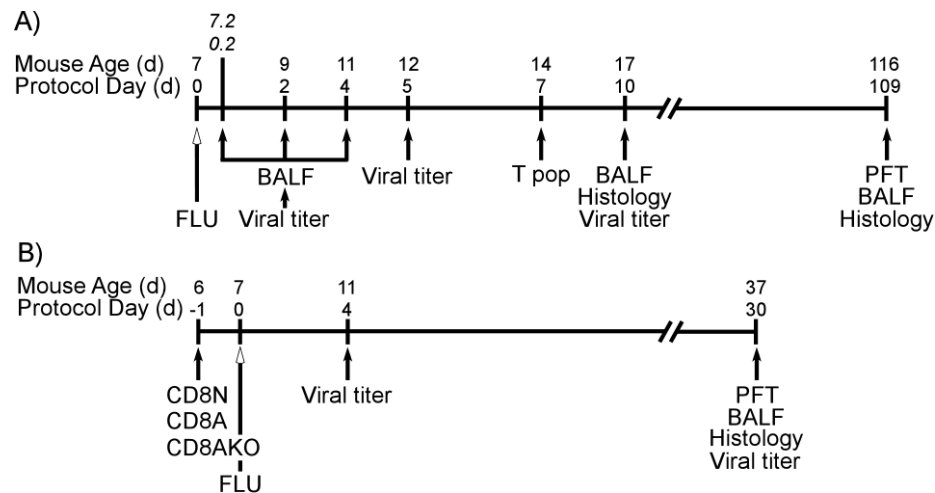


Figure 4.1 The study protocol for the experiments. A) seven day old mouse pups were infected intranasally with 10 TCID₅₀ H1N1/g whole body weight (FLU); while a control group of mice were mock-infected with vehicle (SHAM). BALF was isolated at 0.2, 2, 4, 10, and 109 dpi. Lungs were isolated for viral titer assays at 2, 5, and 10 dpi; for histopathology at 10 and 109 dpi; and for evaluation of T cell subpopulations (T pop) at 7 dpi. Pulmonary function (PFT) was tested at 109 dpi. **B)** For the adoptive transfer study, 6 d old pups were administered CD8 T cells purified from the spleens of naïve neonatal (7 d old, CD8N), adult (4 wk old, CD8A), or adult IFN- γ deficient (4 wk old, CD8AKO) mice. Pups were infected with influenza (FLU) at 7 d of age and allowed to mature until 37 d of age (30 dpi). Four control groups were included: neonatal or adult non-infected mice (SHAM and ASHAM, respectively) and neonatal or adult influenza infected (FLU and AFLU, respectively). Viral titers were measured at 4 and 30 dpi. Pulmonary function test, BALF cellularity, and lung histopathology were measured at 30 dpi.

Pulmonary Function Test

Pulmonary function, specifically the respiratory mechanics, were measured using an invasive method as previously described (You et al., 2006). Briefly, anesthetized mice were intubated and mechanically ventilated by a computer controlled piston ventilator (FlexiVent, SCIREQ). Mice were then challenged by an aerosolized bronchoconstrictor methacholine (Sigma) at increasing doses (MeCh: 0, 25, and 50 mg/ml). At each dose, lung resistance and compliance were calculated using the single compartment model.

Lung Histopathology

At different time points, lungs were perfused, inflated by gentle infusion of HistoChoice Tissue Fixative (Amresco, Inc) to tidal volume (6 ml/kg), and isolated (Figure 1). The fixed lungs were then dehydrated, embedded in paraffin, and sectioned at 4 μm . Each lung section was stained with either hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). Specific histopathological diagnoses were performed by unbiased pathologists (D. T. and D. S.).

Initial observations of the influenza infected lungs suggested emphysematous-type lesions. Morphometric analyses of the lung sections were used to quantify these changes in lung architecture including airspace enlargement (i.e., mean linear intercept; L_m) and destruction of the alveolar walls (i.e., destructive index; DI). L_m was quantified using NIH-Image (<http://rsb.info.nih.gov/nih-image>). The number of alveolar wall intersections was counted on 12 non-overlapping lung fields and was expressed as μm using a protocol adapted from Thurlbeck (Thurlbeck, 1967). To measure DI, a grid with 42 equidistant points (100 μm between each point) was placed at the center of and superimposed on the lung field. Structures lying under these points were classified as normal (N) or destroyed (D) alveolar and/or duct spaces. Points falling over other structures, such as duct walls, alveolar walls, etc. were excluded from the calculations. The DI was calculated from the formula: $DI = D / (D + N) \times 100$ (Saetta et al., 1985).

Bronchoalveolar Lavage Fluid Cellularity and Cytokine Measurement

Bronchoalveolar lavage fluid (BALF) was isolated in 1 ml of PBS containing 2% heat inactivated FBS at the indicated time points (Figure 1). The cells were then centrifuged onto slides and stained using a Hema-3 staining kit (Fisher). Two unbiased readers counted a total of 300 cells per slide and recorded the differential cell counts based on the morphology and staining

of the cells. Cytokine levels were measured from 50 μ l of cell-free BALF using a high-throughput multiplex cytokine assay system (X-Plex Mouse Assay; BioRad) according to the manufacturer's instructions. Each sample was analyzed in triplicate on the Bio-Plex 200 system (BioRad). A broad sensitivity range of standards ranging from 1.21 to 37,312 pg/ml (depending on the analyte) was used to quantitate a dynamic range of cytokine concentrations. The concentrations of analytes in these assays were quantified using a standard curve and a 5-parameter logistic regression was performed to derive an equation that was then used to predict the concentration of the unknown samples. The following cytokines were assayed: IL-2, IL-4, IL-5, IL-6, IL-12(p40), IL-13, IL-17, IFN- γ , and TNF- α . The data presented here excluded any number below the range of sensitivity for the particular analyte.

Assessment of Pulmonary T Cell Populations

A single cell suspension of lung cells was prepared using a standardized protocol (Ormerod, 2000). Briefly, lungs were perfused, excised, cut into small pieces and incubated at 37°C for 1 hour in RPMI-1640 media supplemented by 2% heat inactivated FBS, 1 mg/ml Collagenase I (Invitrogen), and 150 μ g/ml DNase I (Sigma). After incubation, single cells were obtained by mashing the lung pieces through a 40 μ m cell strainer (BD Biosciences). Red blood cells were lysed using 1X RBC lysis buffer (eBioscience) and the remaining cells were stimulated for 5 hours with 5 ng/ml phorbol-12-myristate-13-acetate (PMA; Sigma) and 500 ng/ml ionomycin (Sigma) in the presence of a protein transport inhibitor (1 μ l/1 x 10⁶ cells; GolgiPlug, BD Biosciences). After stimulation, cells were harvested, fixed, and permeabilized (fixation and permeabilization buffer; eBioscience), and stained with the following antibodies purchased from eBioscience: Pacific Blue-CD3 (17A2), PerCP-CD4 (RM4-5), Alexa Fluor 488-CD8a (53-6.7), PE-IFN- γ (XMG1.2), and PE-Cy7-IL-4 (11B11). In order to evaluate influenza

specific CD8⁺ T cell response, lung cells were stained with APC-labeled H-2K^d tetramer complexed with the immunodominant epitope TYQQRTRALV (H-2K^d/TYQQRTRALV, Immunomics) from influenza A nucleoprotein (NP) without PMA/ionomycin stimulation. After staining, cells were fixed in a paraformaldehyde-based fixative (BD Stabilizing Fixative) and assessed with an LSRII (BD Biosciences) flow cytometer. The staining profiles of different cells were analyzed and plotted using FlowJo software (Version 7.2.2 for Windows, Tree Star, Inc) after gating on the lymphocyte population as determined by forward and side scatter properties.

Adoptive Transfer of CD8⁺ T Cells

The protocol is modified from the method developed by Wells et al (Wells et al., 1981). In brief, single cell suspensions were prepared from the spleens of adult (4 wks of age), adult IFN- γ knockout mice (4 wks of age) or neonatal (7 day of age) mice using a standardized protocol as described in the method section — “assessment of pulmonary T cell populations”. CD8⁺T cells were isolated using a negative selection strategy according to the manufacturer’s instructions (Mouse CD8⁺ T cell enrichment kit; Stem Cell). Neonatal, adult, or IFN- γ knockout adult CD8⁺ T cells (4×10^6) were then resuspended in 25 μ l of sterile PBS, and injected intraperitoneally into six-day-old pups (CD8N, CD8A, CD8AKO, respectively).

Statistical Analysis

All data were plotted as mean \pm SEM and analyzed using GraphPad Prism (GraphPad Software Inc., Version 5.0.0). Two-way ANOVA and Bonferroni post-tests were used to test for differences between the groups for the pulmonary function, BALF cellularity, cytokine assays, and T cell populations. Student’s T test was used to analyze the differences in lung histopathology parameters (L_m and DI). Differences between groups were considered statistically significant if $p < 0.05$.

Results

Neonatal Influenza Infection Resulted in Acute Pulmonary Inflammation

BALB/c pups were infected at 7 d of age with 10 TCID₅₀ of influenza A virus/gram body weight (FLU); controls received sterile DPBS (SHAM). Mice infected with this sublethal dose (>90% survival) of influenza developed mild-to-moderate illness, which was characterized by ruffled fur and significant reductions in weight gain compared to SHAM. By 18 dpi, mice infected with influenza weighed 12% less than SHAM animals ($p < 0.05$). Infectious influenza virus was detected in whole lung homogenates in neonatal mice as early as 2 dpi ($10^{4.49 \pm 0.03}$ TCID₅₀/g lung tissue) and peaked at 5 dpi ($10^{6.93 \pm 0.25}$ TCID₅₀/g lung tissue). No infectious viruses were detected in neonatal or adult mice after 7 dpi.

To measure pulmonary inflammation, bronchoalveolar lavage fluid (BALF) cellularity, BALF cytokine levels, and lung histopathology were monitored throughout the course of the infection (Figures 4.2 & 4.3). All results from SHAM mice at different time points (5 hours, 1, 2, 4, and 10 days post-infection) were similar and showed no difference (data not shown), therefore only data from 10dpi were presented as a representative. Mice infected with influenza recruited significantly more inflammatory cells to the lung as observed in both the lung and the BALF. Monocytes/AMs were shown to be recruited to the bronchoalveolar space as early as 5 hours post-infection (hpi, Figure 4.2.A), and significantly more cells compared to SHAM were observed at 4 and 10 dpi with the peak at 10 dpi (44.09 ± 6.83 vs. $13.19 \pm 1.92 \times 10^4$). Neutrophils in the BALF showed the same trend. They peaked at 2 dpi (3.20 ± 1.11 vs. $0.12 \pm 0.02 \times 10^4$); and remained elevated at 10 dpi compared to SHAM (2.58 ± 0.75 vs. $0.08 \pm 0.03 \times 10^4$). A significant increase in lymphocytes was observed at 10 dpi (8.47 ± 3.79 vs. $0.01 \pm 0.01 \times 10^4$). Total BALF cell numbers were five-fold higher in the FLU group compared to the SHAM group at 10 dpi (61.30 ± 10.70 vs. $13.30 \pm 1.92 \times 10^4$, $p < 0.05$).

Cytokines in the BALF peaked at various times during the infection (Figure 4.2.B). Although a total of ten cytokines (IL-2, -4, -5, -6, -12(p40), -12(p70), -13, -17, IFN- γ , and TNF- α) were measured, only six (IL-5, -6, -12(p40), -13, IFN- γ , and TNF- α) were detectable at the time points tested. Of those cytokines detected (with the exception of IFN- γ), all were elevated at 5 hpi. TNF- α was one of the earliest cytokines detected and was present in the highest concentration at 5 hpi (72.0 ± 2.55 pg/ml). It remained significantly elevated at 2 dpi (65.0 ± 28.3 pg/ml) and was undetectable by 10 dpi. IL-12(p40) gradually increased from 5 hpi to 10 dpi; where it became significantly elevated over SHAM (61.4 ± 7.51 pg/ml). IFN- γ was only detectable at 2 (20.1 ± 10.9 pg/ml) and 4 dpi (21.5 ± 0.00 pg/ml). The other three cytokines (IL-5, -6, and -13) were detected at all time points but not significantly different than in SHAM treated mice.

Histopathologic examination of lungs from neonatally infected mice revealed marked pulmonary inflammation that was observed in the perivascular, peribronchial, and alveolar spaces of the lung at 10 dpi (Figure 4.3.A, right panel). The inflammatory infiltrates consisted of lymphocytes and occasional plasma cells. In addition, extensive mucus production was observed in the goblet cells of the infected lungs, along with proliferation of peribronchial glands within the hilum (Figure 4.3.B, right panel). Diffuse emphysematous changes with a slight thickening of the alveolar walls and vascular congestion were also observed. In contrast, SHAM lungs showed normal thickening of the alveolar wall with no inflammation (Figure 4.3.A, left panel) and no mucus staining (Figure 4.3.B, left panel).

Neonatal Influenza Infection Led to Long-term Pulmonary Dysfunction and Injury

In order to investigate the long-term effects of neonatal influenza infection, a group of 7 d old pups were infected with the virus and allowed to mature to 116 d of age (109 dpi, Figure

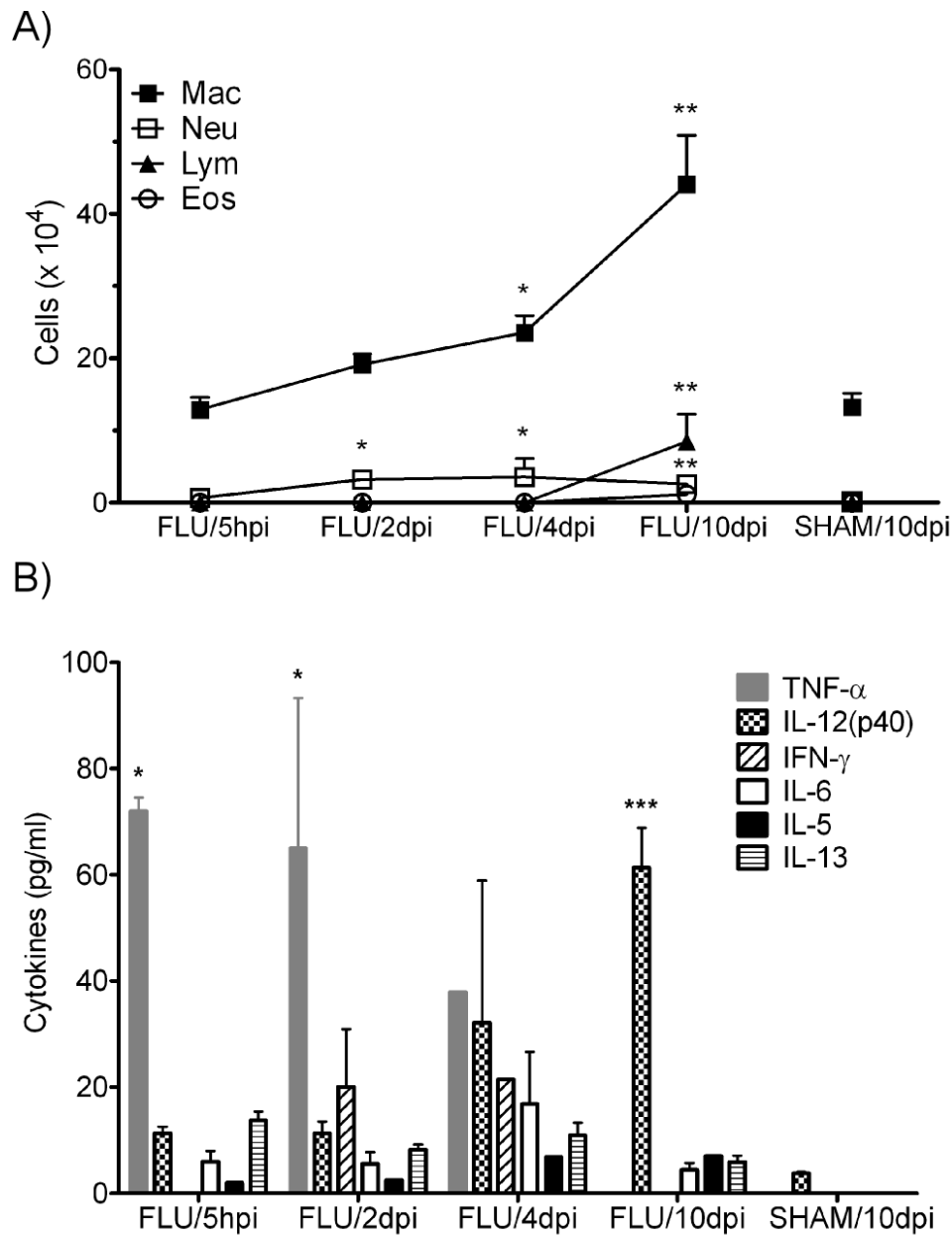


Figure 4.2 BALF cellularity and cytokine levels in neonatal mice after infection. **A)** BALF was isolated and the cellularity determined at 5 hpi and at 2, 4, and 10 dpi. Absolute cell numbers were plotted versus time post-infection. n=4-5/group. **B)** Cytokine levels in the BALF supernatant was determined for the following cytokines: IL-2, -4, -5, -6, -12(p40) - 12(p70), -13, -17, IFN- γ , and TNF- α . Only detected cytokines are displayed on the graph. n=3-5/group. Data are expressed as mean \pm SEM, *: p<0.05, **: p<0.01, ***: p<0.001, compared to SHAM.

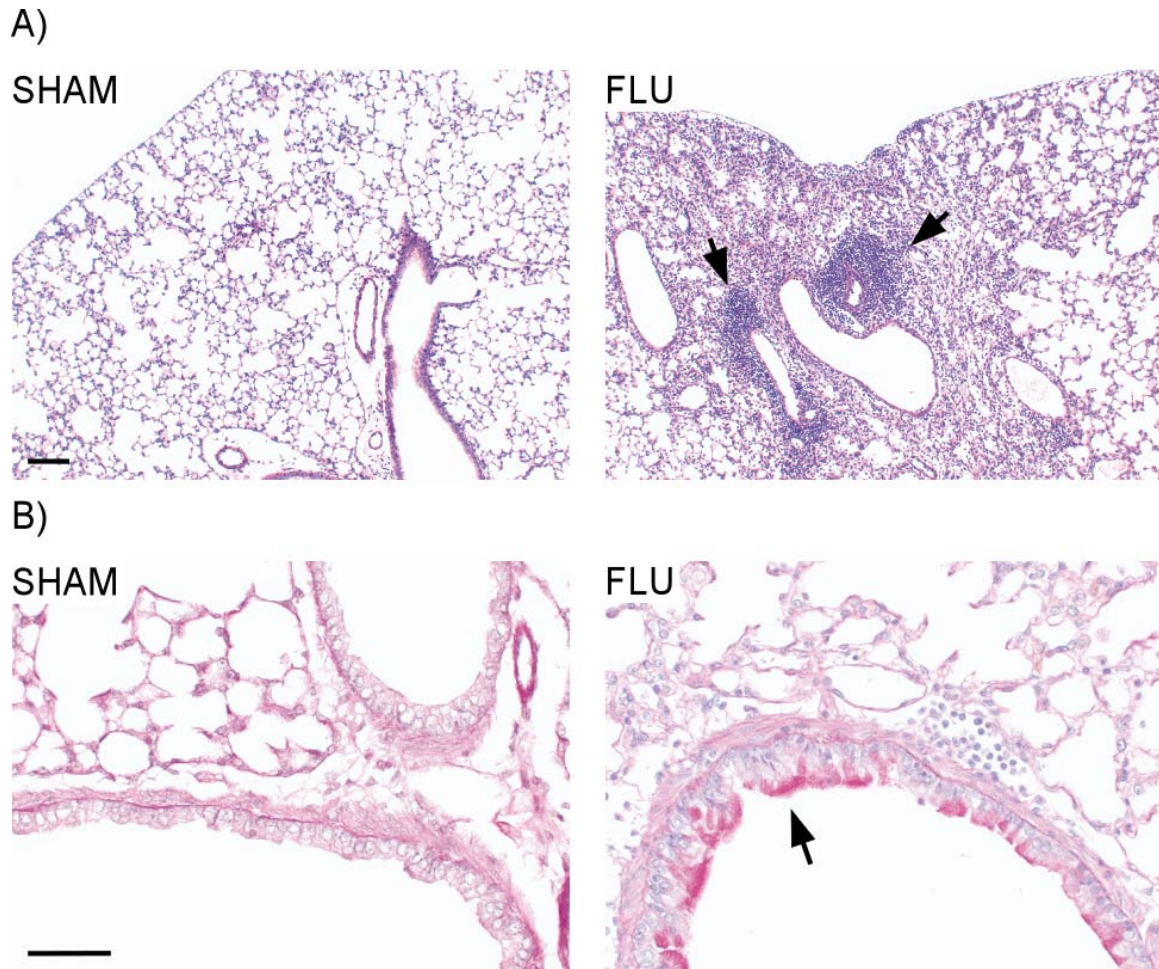


Figure 4.3 Lung histopathology following neonatal influenza infection. Lungs were isolated, fixed and stained with H&E and PAS at 10 dpi. **A)** H&E staining showed marked inflammation in the perivascular, peribronchial, and alveolar spaces of the lung (right panel). Diffuse emphysematous changes were also observed in the infected lungs along with slight thickening of the alveolar walls. SHAM mice showed no inflammation or emphysematous changes (left panel). Scale bar=100 μ m. **B)** PAS staining demonstrated widespread mucus production and proliferation of peribronchial glands within the hilum in the influenza infected mice (right panel). SHAM mice showed no mucus staining. Scale bar=50 μ m. Each picture is a representative of three different animals.

4.1.A). As with the acute studies, BALF cellularity, BALF cytokine levels, and lung histopathology were recorded at 109 dpi.

Significantly more total cells were present in the BALF of previously infected mice compared to SHAM animals at 109 dpi (6.05 ± 1.89 vs. $2.59 \pm 0.70 \times 10^5$, $p < 0.05$). As shown in Figure 4.4, there were significantly more monocytes/AMs ($51.02 \pm 8.79 \times 10^4$) present in the BALF of infected animals compared to SHAM mice ($25.08 \pm 5.17 \times 10^4$). Neutrophils ($14.42 \pm 6.47 \times 10^4$) and lymphocytes ($6.20 \pm 3.24 \times 10^4$) were higher than SHAM (1.08 ± 0.53 and $0.25 \pm 0.07 \times 10^4$, respectively), although not significantly different ($p = 0.074$). Eosinophils were not observed in the BALF of either FLU or SHAM mice. None of the cytokines assayed in the BALF at 109 dpi were significantly different than that of controls (data not shown).

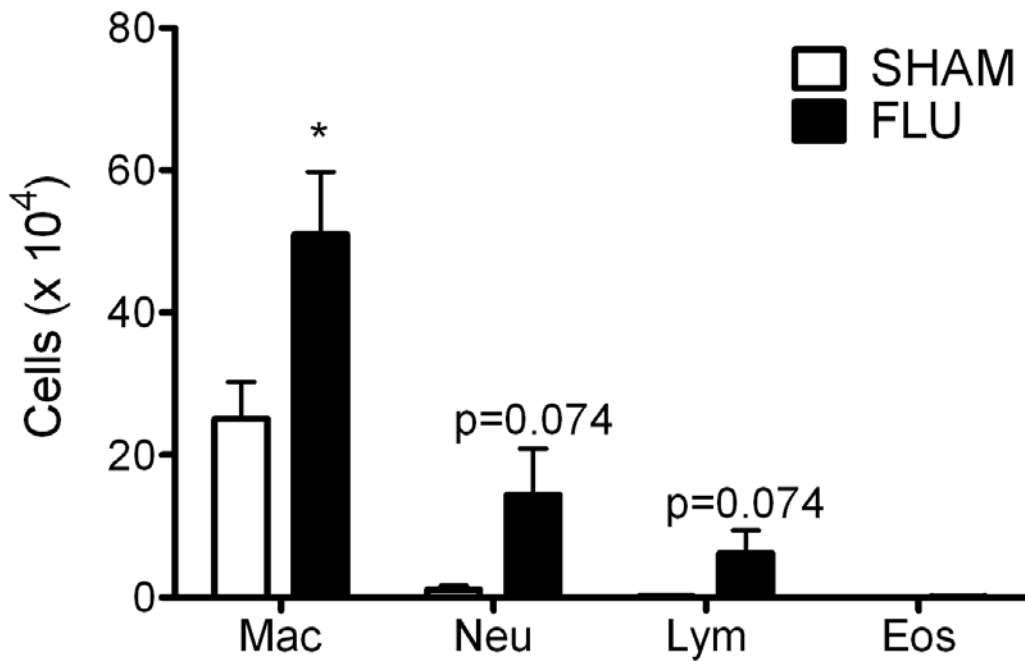


Figure 4.4 BALF cellularity in adult mice infected with influenza as neonates. BALF was isolated at 109 dpi and leukocyte populations determined. Data were expressed as mean \pm SEM, $n = 4-5$ /group. *: $p < 0.05$.

The persistent change in BALF cellularity at 109 dpi suggested that there might also be unresolved pulmonary inflammation in the lungs of the adult mice initially infected with influenza as neonates. Indeed, lung histopathologic examination (Figure 4.5.A, right panel) confirmed mild-to-moderate peribronchial chronic inflammation consisting mostly of lymphocytes. Furthermore, moderate mucus production was still present in the goblet cells of infected lungs (Figure 4.5.B, right panel). Diffuse emphysematous changes with focal areas of minimal thickening within the alveolar walls were also observed in the infected lungs.

Emphysematous lesions were quantified by evaluating both the airspace enlargement in terms of L_m and the destruction of the alveolar walls by measurement of DI. Influenza infected lungs exhibited a marked increase of alveolar enlargement (L_m $79.0 \pm 2.89 \mu m$) compared to SHAM ($60.0 \pm 5.15 \mu m$) and a significant increase destroyed alveolar walls (DI: $57.1 \pm 2.91 \mu m$) versus SHAM ($14.5 \pm 0.58 \mu m$).

The change in the lung structure associated with the persistence of inflammatory cells in the lung and the BALF suggested alterations in pulmonary function. To determine if neonatal infection with influenza produced long-term effects on lung function, lung mechanics were measured in mechanically ventilated animals at 109 dpi. Mice infected with influenza as neonates showed significantly impaired lung function compared to the SHAM mice as evidenced by increased airway hyperreactivity with decreased dynamic compliance in response to MeCh (Figure 4.6.A & B). Baseline resistance was similar in these two groups at this time point (0.49 ± 0.03 vs. 0.47 ± 0.08 cm H₂O·s/ml). Although there was no significant shift to the left in the MeCh dose response curve; influenza infection increased pulmonary resistance in response to 50 mg/ml of MeCh (4.12 ± 0.93 vs. 1.25 ± 0.21 cm H₂O·s/ml, Figure 4.6.A) and therefore, airway hyperreactivity.

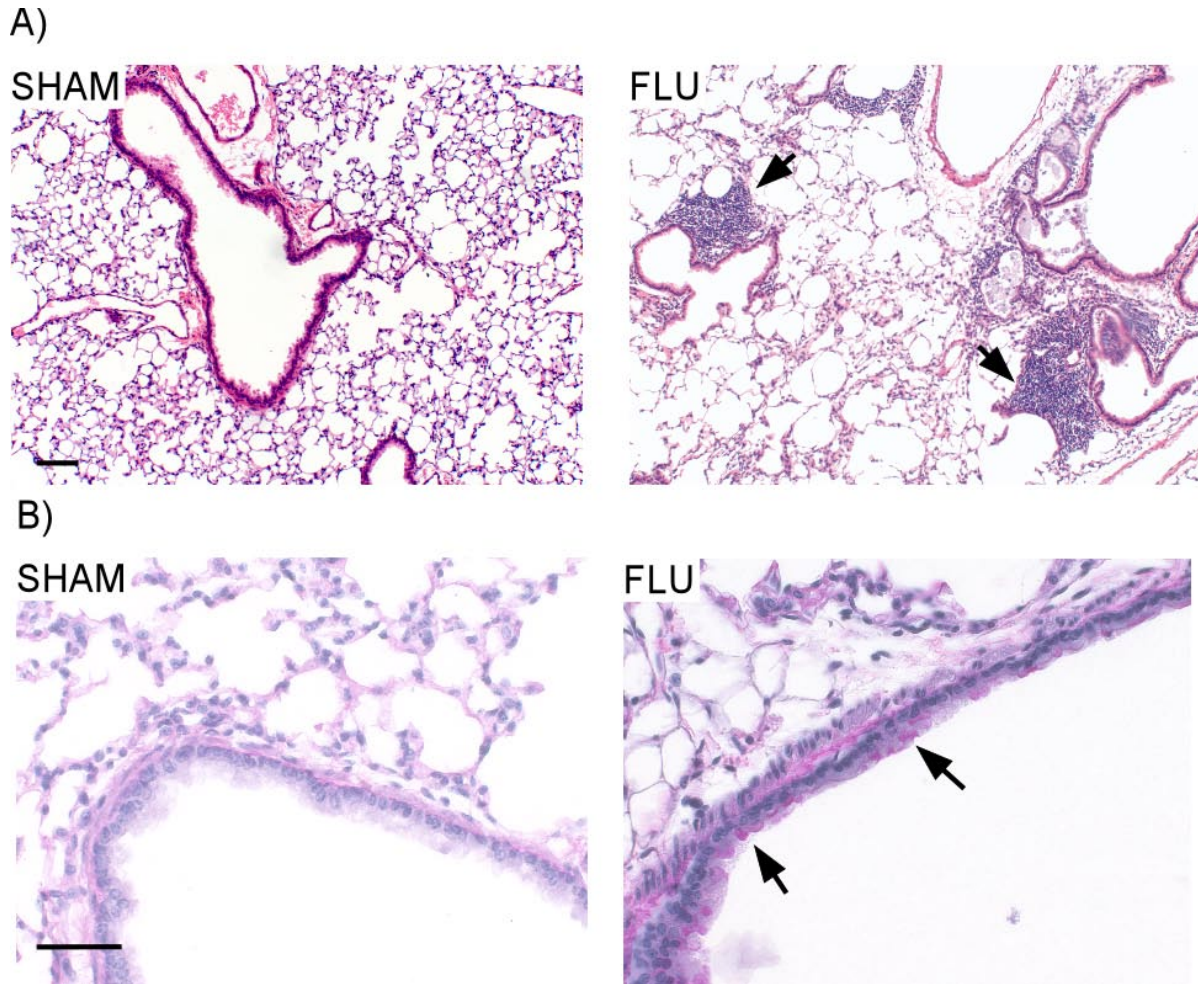


Figure 4.5 Adult lung histopathology of mice infected with influenza as neonates. Lungs were isolated, fixed and stained by H&E and PAS at 109 dpi. **A)** Mild-to-moderate peribronchial chronic inflammation consisting mostly of lymphocytes and diffuse emphysematous changes were observed in the lungs of mice infected with influenza as neonates (right panel). No inflammation or emphysematous changes was observed in SHAM mice (left panel). Scale bar=100 μ m. **B)** Moderate mucus production persisted in airway epithelial cells of influenza infected mice (right panel); while no mucus staining was observed in the lungs of SHAM infected mice. Scale bar=50 μ m. Each picture is a representative of three animals.

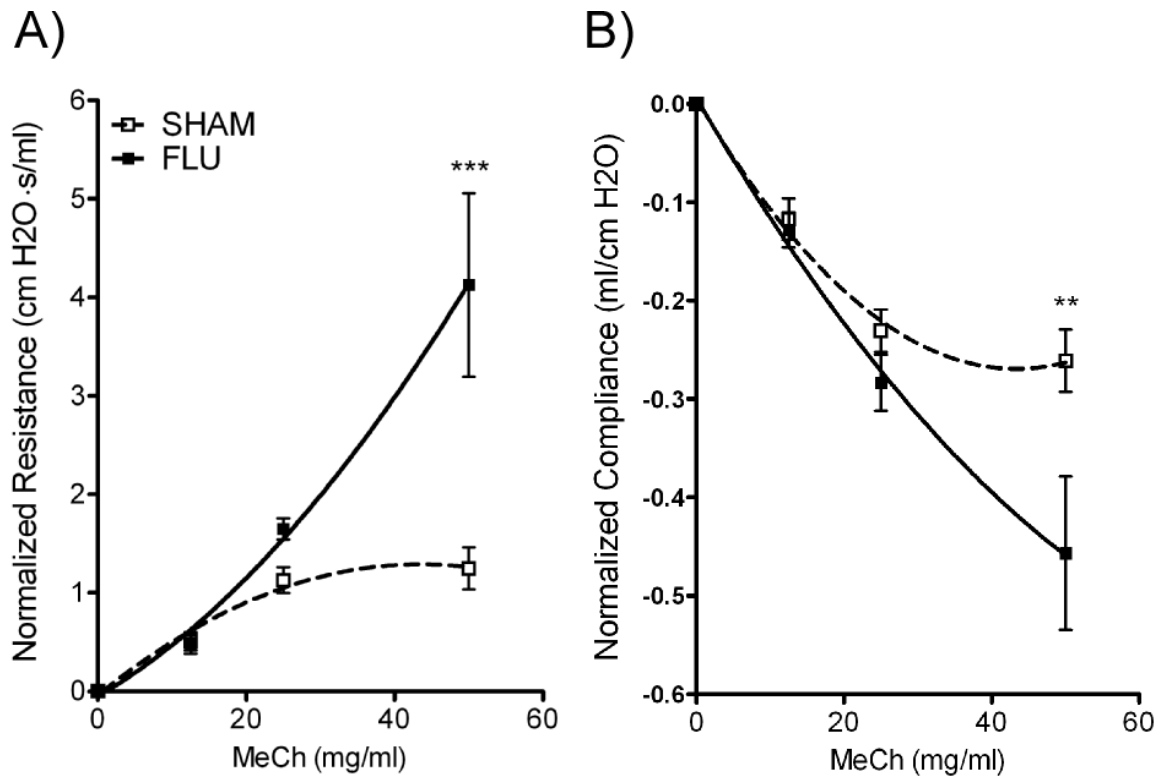


Figure 4.6 Persistent airway dysfunction in adult mice infected with influenza as neonates. Pulmonary function was assayed at 109 dpi. **A)** Influenza infected mice showed a significant increase in lung resistance compared to sham-inoculated mice when challenged by 50 mg/ml methacholine (MeCh). There was no difference in baseline resistance between the FLU and SHAM groups. Data were normalized to baseline (0 mg/ml MeCh) and are expressed as mean \pm SEM, n=5/group. ***: p<0.001. **B)** These same mice exhibited a marked decrease in lung compliance if they were infected. For simplicity in interpretation, data are presented as normalized to baseline (0 mg/ml MeCh) and expressed as mean \pm SEM, n=5/group. **: p<0.01.

Reduced IFN- γ + T Cells Are Observed in the Neonates in Response to Influenza Infection

To dissect the possible mechanism(s) responsible for the observed long-term pulmonary dysfunction, pulmonary T cell responses were measured using intracellular cytokine staining in influenza infected neonates (FLU) and adults (AFLU; 4wk old) at 7 dpi. Approximately, two-fold more CD8+ T cells (9.7 ± 1.1 vs. $5.2 \pm 1.3 \times 10^5$) and slightly more CD4+ T cells (1.67 ± 0.18 vs. $1.41 \pm 0.48 \times 10^6$) were present in the adult lungs compared to the neonatal lungs (Figure 4.7.B, left panel). In addition, neonates infected with influenza appeared to mount a weak IFN- response to the virus, compared to that of adults (Figure 4.7.B, middle panel). Adult mice infected with influenza (AFLU) were able to recruit almost six-fold more CD8+IFN- + T cells (12.3 ± 1.92 vs. 2.76 ± 1.92 % of CD8+ T cells) and two-fold more CD4+IFN- + T cells (4.04 ± 0.67 vs. 1.92 ± 0.34 % of CD4+ T cells) to the lung as compared to the neonates. CD4+ IFN- γ + T cells in neonates were statistically different from SHAM levels (0.61 ± 0.07 % of CD4+ T cells). No difference was observed in the number of CD4+IL-4+ T cells recruited to the lungs of mice infected with influenza as neonates or adults (data not shown). To examine the specificity of the CD8+ T cells recruited to the lung, we stained lung lymphocytes with H-2K^d tetramers containing the immunodominant epitope from the influenza nucleoprotein (Figure 4.7.B right panel). Influenza infection of adult mice resulted in the recruitment of about two-fold more CD8+ T cells that bound the influenza tetramer compared to infection of neonatal mice ($2.29 \pm 0.16\%$ vs $1.24 \pm 0.04\%$).

Reversal of Long-term Pulmonary Dysfunction after Adoptive Transfer of Adult CD8+T Cells but not Neonatal CD8+ T Cells

To determine whether the persistent pulmonary inflammation, altered lung structure, and long-term lung dysfunction were due to the functional immaturity of host lymphocytes or other factors (e.g., IFN- γ), CD8+ T cells were purified from the spleen of naïve neonatal, adult, or

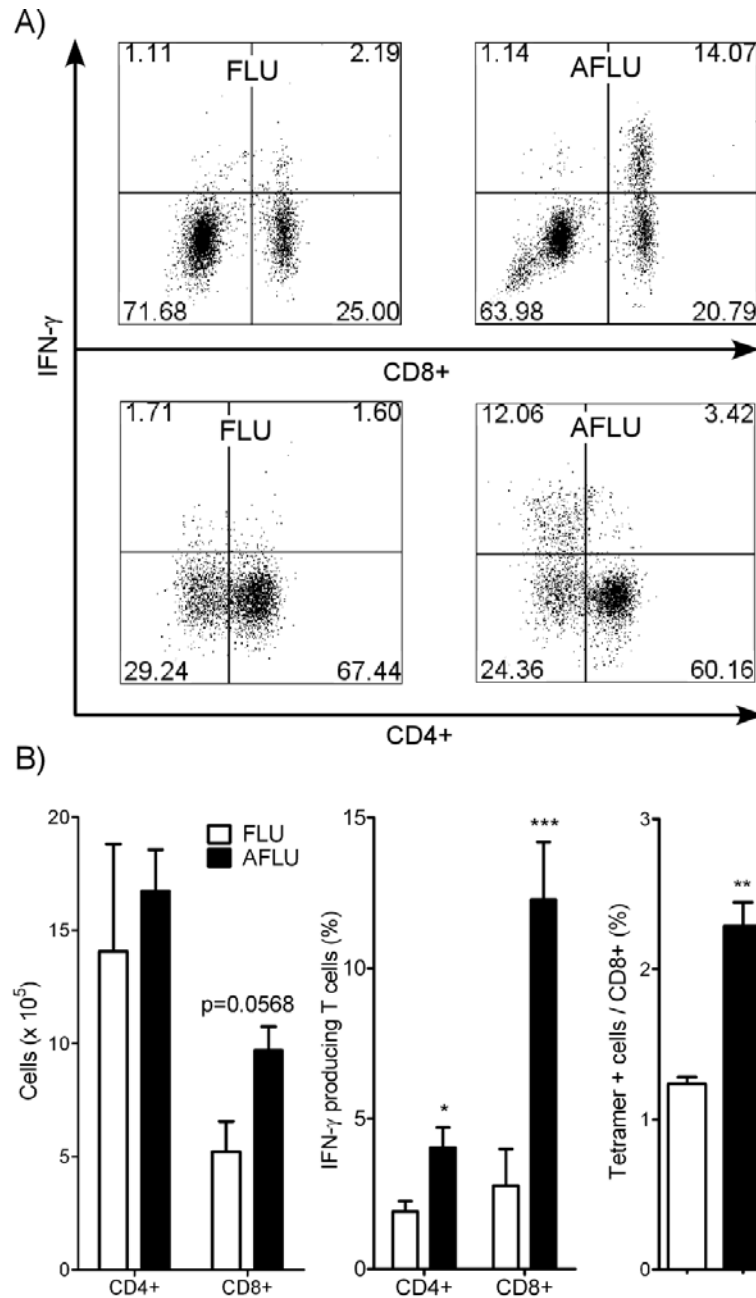


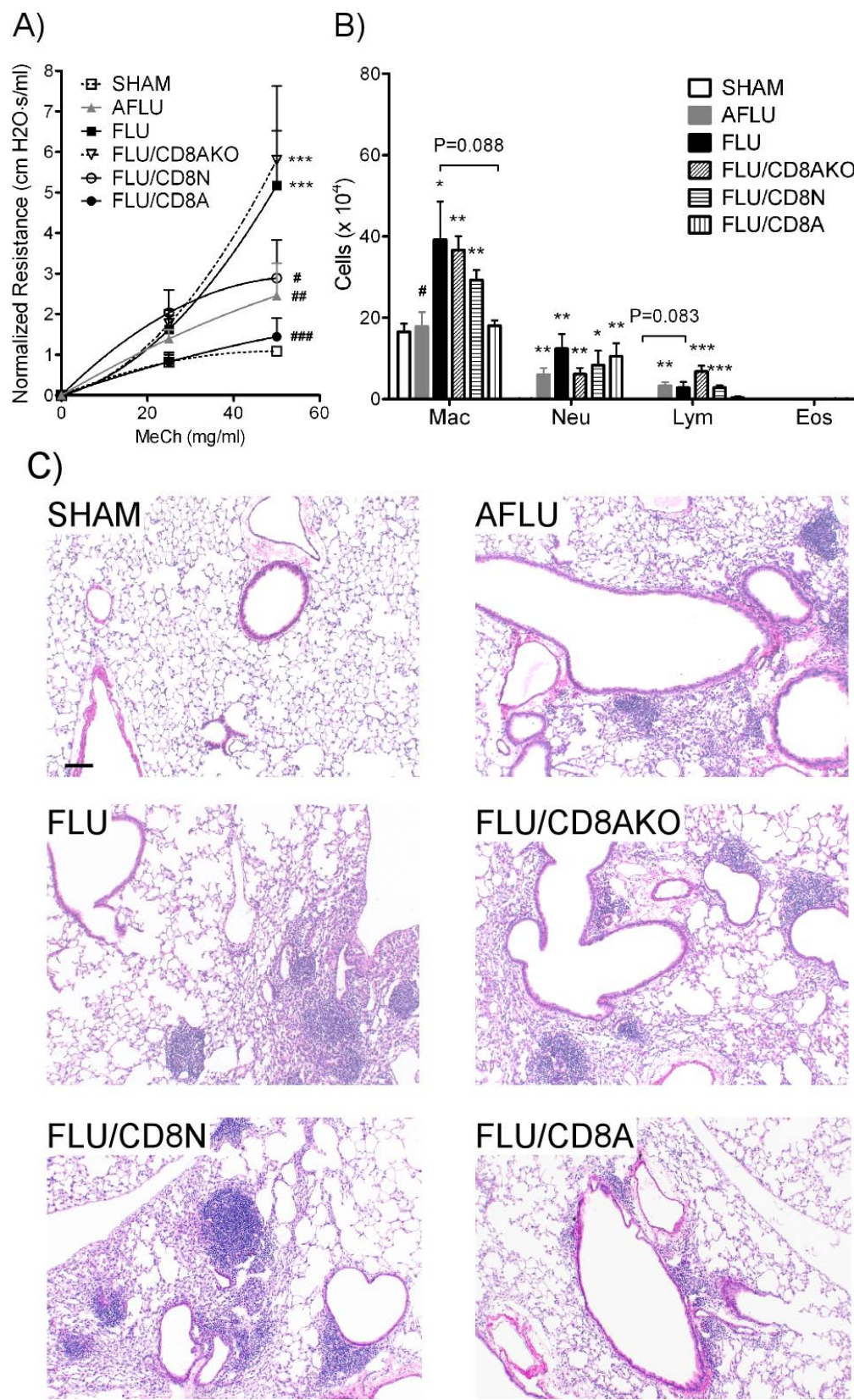
Figure 4.7 Lymphocyte populations in the lungs of mice infected with influenza as neonates and adults. T cell subpopulations were analyzed in lung homogenates at 7 dpi in mice infected at either 7 d (FLU) or 4 w (AFLU) of age using intracellular cytokine staining and flow cytometry. **A)** A representative flow cytometry dot plot of IFN- γ + CD4+ and CD8+ T cells in infected adult or neonatal mice. **B)** Both CD4+ and CD8+ T cells appeared to be elevated in influenza infected adults (AFLU) compared to mice infected as neonates (FLU, left panel). IFN- γ + CD4+ and CD8+ T cells were significantly greater in influenza infected adults as compared to infected neonates (middle panel). Finally, CD8+ T cells specific for an immunodominant epitope of influenza in AFLU mice were also significantly greater than in FLU mice (right panel).

adult IFN- γ deficient mice and adoptively transferred to 6 d old pups (Figure 4.1.B). One day later, these mice were then infected with 10 TCID₅₀/g body weight of influenza (FLU/CD8N, FLU/CD8A, or FLU/CD8AKO, respectively). In addition, neonatal and adult mice were simply infected with influenza (FLU and AFLU, respectively). Pulmonary function testing, BALF cellularity and lung histopathology were performed at 30 dpi.

Both FLU/CD8N and FLU/CD8A groups showed an improvement in pulmonary function, as evidenced by lower airway hyperreactivity compared to control FLU mice (2.89 ± 0.94 and 1.45 ± 0.46 vs. 5.17 ± 1.35 H₂O·s/ml at 50 mg/ml MeCh, Figure 4.8.A). Since non-infected control mice, either adult or neonate at time of sham infection, exhibited almost identical responses (data not shown), therefore only the non-infected control mice that were sham infected as neonates (SHAM) are presented. Compared to the FLU/CD8N group, the FLU/CD8A group showed significantly lower pulmonary resistance at 50 mg/ml MeCh (1.00 ± 0.17 cm H₂O·s/ml). Moreover, the resistance of FLU/CD8A mice was comparable to that of SHAM mice (1.25 ± 0.21 cm H₂O·s/ml). In contrast, FLU/CD8N mice had higher resistance compared to SHAM mice, although pulmonary resistance was lower than that of the FLU mice (2.05 ± 0.39 vs. 4.12 ± 0.93 cm H₂O·s/ml). Interestingly, AFLU mice showed medium airway hyperreactivity - lower than mice infected as neonates (FLU) and higher than FLU/CD8A mice. Finally, adoptive transfer of adult CD8⁺ T cells deficient in IFN- γ prior infection (FLU/CD8AKO) was unable to reverse the effects on pulmonary dysfunction observed in mice infected with influenza as neonates (resistance at 50 mg/ml MeCh of 5.80 ± 1.83 vs. 5.17 ± 1.35 H₂O·s/ml).

Lung histopathology was also greatly improved in the FLU/CD8A mice as compared to FLU, FLU/CD8N, and FLU/CD8AKO mice. These improvements included reduced pulmonary

Figure 4.8 Pulmonary function, BALF cellularity and lung histopathology following adoptive transfer of CD8+ T cells. Neonatal (FLU/CD8N), adult (FLU/CD8A), or adult IFN- γ deficient CD8+ T cells (FLU/CD8AKO) were administered one day prior to influenza infection of neonatal mice. **A)** Pulmonary function was assayed at 30 dpi. Mice receiving either adult or neonatal CD8+ T cells exhibited improved pulmonary function as demonstrated by a reduction in airway hyperreactivity compared to control mice infected with influenza (FLU), while mice receiving adult IFN- γ deficient CD8+ T cells showed no improvement in lung function. Adoptive transfer of adult CD8+ T cells completely reversed airway hyperreactivity to SHAM levels, while FLU/CD8N mice still showed increased airway hyperreactivity compared to SHAM. Data were normalized to baseline (0 mg/ml MeCh) and are expressed as mean \pm SEM, n=4-7/group. ***: p<0.001, compared to SHAM; #: p<0.05, ##: p<0.01, ###: p<0.001, compared to FLU. **B)** BALF cellularity. There were less monocytes / AMs and lymphocytes in FLU/CD8A mice compared to FLU mice. BALF cellularity for FLU/CD8N and FLU/CD8AKO mice was similar to FLU mice. Data were expressed as mean \pm SEM, n=4-7/group. *: p<0.05, **:p<0.01, ***:p<0.001, compared to SHAM; #: p<0.05, compared to FLU mice. **C)** Lung pathology was assessed at 30 dpi. Substantial reductions in pulmonary infiltrates are observable in the lungs of mice receiving adult CD8+ T cells (FLU/CD8A) prior to infection as compared to all other groups. Other groups included: mice infected as neonates (FLU); mice receiving CD8+ T cells from naïve wild-type adults (FLU/CD8A), wild-type neonates (FLU/CD8N), or IFN- γ knockout mice (FLU/CD8AKO) prior to infection; and adult infected mice (AFLU). Scale bar=100 μ m.



inflammation (Figure 4.8.C) and mucus production (data not shown) in the infected lungs at 30 dpi. Adoptive transfer of adult CD8⁺ T cells was not able to completely reverse pulmonary inflammation at 30 dpi, since small foci of inflammatory cells were still observed in the peribronchial and perivascular areas of the lung (Figure 4.8.C). Although the lungs from AFLU mice exhibited similar levels of inflammation as observed in the FLU, FLU/CD8N, and FLU/CD8AKO mice (Figure 4.8.C), there were no emphysematous-type lesions and little-to-no mucus (data not shown).

BALF cellularity showed the same trend (Figure 4.8.B). FLU/CD8A had less monocytes/AMs (18.07 ± 1.32 vs. $39.24 \pm 9.35 \times 10^4$) and lymphocytes (0.37 ± 0.24 vs. 2.82 ± 1.41) in BALF compared to FLU controls, although a significant amount of neutrophils were still present (10.54 ± 3.23 vs. $12.40 \pm 3.61 \times 10^4$). Conversely, transfer of neonatal CD8⁺ T cells (FLU/CD8N) or adult IFN- γ deficient CD8⁺ T cells (FLU/CD8AKO) prior infection did not reduce cellular infiltrates in BALF, as there were no differences observed between these two groups and FLU controls. Interestingly, adult mice infected with influenza (AFLU) had significant elevated numbers of neutrophils and lymphocytes, but not monocytes/AMs, in their BALF compared to SHAM mice.

The ability of neonatal mice to resolve infection following adoptive transfer of the adult or neonatal CD8⁺ T cells was then analyzed. Viral titers were determined at 4 and 30 dpi from lung homogenates. The FLU/CD8A group showed a significantly lower viral load compared to FLU mice ($10^{2.80 \pm 0.040}$ vs. $10^{6.42 \pm 0.53}$ TCID₅₀/g lung tissue). AFLU mice also had lower viral titer than FLU mice ($10^{3.57 \pm 0.18}$ vs. $10^{6.42 \pm 0.53}$ TCID₅₀/g lung tissue). In addition, FLU/CD8A mice had a significantly lower viral load than AFLU mice at 4dpi ($10^{2.80 \pm 0.040}$ vs. $10^{3.57 \pm 0.18}$ TCID₅₀/g lung tissue). No significant differences were observed between FLU/CD8N and FLU

or FLU/CD8AKO and FLU mice, although titers in both groups were slightly lower than FLU group (data not shown). At 30 dpi, virus was no longer detectable in any group.

Discussion

In the present study, we described a neonatal mouse model of influenza A infection. In this model, BALB/c mice were infected with influenza virus at 7 d of age and allowed to mature. The mice developed acute, severe pulmonary inflammation, which remained unresolved four months later and long after influenza virus was no longer detectable. This correlated with significant increases in pulmonary resistance in response to increasing doses of methacholine (i.e., airway hyperreactivity) and decreases in compliance at four months. Histopathologic analysis of the lungs from adult mice infected as neonates revealed emphysematous-type lesions characterized by airspace enlargement and destruction of alveolar walls. Adoptive transfer of adult CD8⁺ T cell into the pups before infection with influenza reversed the effects of neonatal influenza infection as evidenced by enhanced pulmonary function (returning airway resistance to SHAM levels) and reduced pulmonary inflammation and reduced viral load compared to pups receiving neonatal CD8⁺ T cells. Adoptive transfer of adult CD8⁺ T cells deficient in IFN- γ indicated that IFN- γ was critical in determining disease outcome.

The inflammatory cells in the BALF during the acute phase (5 hpi to 10 dpi) and chronic phase (109 dpi) included monocytes/AMs, lymphocytes, and neutrophils. Interestingly, although there were more cells in the BALF at 10 dpi than at 109 dpi (~50% more), the composition of the BALF was not significantly altered (i.e., both were comprised of ~80% of monocytes/AMs and 20% of lymphocytes and neutrophils) suggesting the continued presence or secretion of chemokines responsible for neutrophil and lymphocyte recruitment even in the absence of detectable infectious virus or viral antigen. At 109 dpi, we were unable to detect differences in

cytokines between mice infected as neonates and SHAM controls; however, the role of other cytokines or inflammatory mediators (e.g., leukotrienes, etc.) cannot be excluded.

In addition to the chronic inflammation and airway hyperreactivity to MeCh, histopathologic analysis of the lungs from adult mice originally infected as neonates revealed emphysematous-type lesions within the lung architecture. Intriguingly, there were no differences in baseline resistance between this group and SHAM controls at 109 dpi despite significant differences in lung architecture and inflammatory state. The reason(s) for this are unclear, but may represent masking due to the persistent inflammation or a limitation of the model chosen (i.e., single-compartment) to measure respiratory mechanics.

In general, the immune system of a neonate is quite different from that of an adult, in that the innate and adaptive immune responses are immature (Billingham et al., 1953; Bona, 2005). Naïve, neonatal T cells are also functionally distinct from adult T cells (Adkins, 1999); and effector, neonatal T cells are less able to lyse antigen bearing cells or produce cytokines (Granberg et al., 1979). Although neonatal T cells are able to mount comparable proliferative responses to mitogens (Yarchoan and Nelson, 1983), they have intrinsically lower levels of CD3/TCR, adhesion molecules, and costimulatory molecules (Velilla et al., 2006). Data from our studies indicate that recruitment of T cells is different in the infected neonates versus the adult and although both T cell populations are induced in the neonate, CD8⁺ T cell numbers are doubled in the adults. This suggested that cell number alone may have been responsible for the pathophysiological impact of neonatal influenza infection. Although adoptive transfer of neonatal CD8⁺ T cells prior to infection did help to control the infection (i.e., reduced viral load and improved pulmonary function compared to neonates infected with influenza); it was not as effective as adoptive transfer of adult CD8⁺ T cells (i.e., further reduction in viral load and

pulmonary function equivalent to that of SHAM). These data suggest that neonatal CD8⁺ T cells are functionally impaired compared to adult CD8⁺ T cells.

In addition to the lower magnitude of the T cell response in the neonates, the number of IFN- γ ⁺ CD8⁺ T cells was significantly lower than that of their adult counterparts after influenza infection. Previous studies have showed that IFN- γ plays an important role in recovery from influenza infection by helping to clear the virus (Bruder et al., 2006; Moskophidis and Kioussis, 1998) and that adoptive transfer of Tc1 cells (IFN- γ ^{hi} cytotoxic T cells) promotes clearance of the influenza virus, while transfer of Tc2 cells (IFN- γ ^{lo} cytotoxic T cells) does not affect viral clearance (Wiley et al., 2001). Our data confirmed that IFN- γ produced by CD8⁺ T cells was important to effectively clear influenza from the neonatal lung, since mice receiving IFN- γ deficient adult CD8⁺ T cells showed higher viral loads than mice receiving wild-type adult CD8⁺ T cells. Moreover, adoptive transfer of IFN- γ deficient CD8⁺ T cells totally abolished the benefits observed upon administration of adult CD8⁺ T cells, as demonstrated by increased airway hyperreactivity, BALF cellularity, and lung histopathology (similar to neonatal infection controls). In total, our data further demonstrate the importance of IFN- γ in the resolution of infection and inflammation initiated upon infection of neonatal mice.

Viral load and immune function are inescapably linked. Also, it is readily apparent that CD8⁺ T cells do not directly affect disease outcome and that they alter the course of pathogenesis by acting against virus-infected cells (i.e., decreasing viral load) through production of IFN- γ . This contention is strengthened by our observation that introduction of poorly functional neonatal CD8 T-cells does not ameliorate disease, or act against virus-infected cells, thereby permitting a higher viral load in the host. Mice receiving wild-type adult CD8⁺ T cells

exhibited lower viral loads, improved pulmonary function, a reduction in total BALF cellularity, and a reduction in pulmonary inflammation compared to mice infected as adults.

Influenza and another common respiratory virus, RSV, infect the same human population (infants) but elicit different pulmonary diseases. It has been reported that Th2 (IL-4+ T helper cells) responses dominate in neonatal immunity; while Th1 (IFN- γ + T helper cells) responses dominate in adults (Adkins et al., 2000; Adkins et al., 1996; Adkins et al., 1993; Min et al., 1998; Pack et al., 2001). However, studies from our lab and other groups clearly demonstrate that the immune response initiated by neonates is more complex (You et al., 2006). A previous study showed that RSV infected neonatal mice mount a Th2 biased response when rechallenged as adults with RSV (Culley et al., 2002). Although a mixture of Th1 and Th2 cells is elicited in lungs during reinfection, there were significantly more (4 fold) Th2 cells in lungs compared to mice primarily infected as adults (Culley et al., 2002). Data from our lab showed that even at primary infection, neonatal RSV infection mounts a Th2-skewed response (You et al., 2006) compared to influenza infection (data presented here). In fact, both infections mount a mixed Th1/Th2 response. Following influenza infection about 5 fold more Th1 cells than Th2 cells were recruited to the lungs, while RSV infection recruited similar numbers of Th1 and Th2 cells. These data suggest that the immune response initiated in neonates is not predestined toward a Th2 response, as previously implied, and appears to depend on the antigen encountered. Besides the differences in responses of helper T cells to RSV and influenza, both viruses induce a weak CD8+ T cell response similar in magnitude and function. Finally during neonatal RSV infections, although airway remodeling is present (i.e., increased basement membrane thickness, smooth muscle hypertrophy, subepithelial fibrosis), there is relatively no tissue destruction (You et al., 2006). In contrast, neonates infected with influenza exhibited a tremendous amount of

tissue destruction, which may be the principal determinant of the severity of airway symptoms. Taken together, there is similarity and disparity in the immune responses induced by RSV and influenza, and the immune and cytopathic differences may explain the specific pulmonary diseases elicited by these two viruses.

In summary, our data demonstrate that infection of newborn mice with influenza has long-term consequences for the host inducing diffuse emphysematous changes in the lung and marked pulmonary inflammation. These alterations were persistent and associated with increased airway resistance and reduced compliance. The adaptive T cell response was markedly reduced in the neonates, with the most striking difference being observed among the CD8⁺ T cell population. Our adoptive transfer data suggest that the immaturity of this cell population is an important factor in determining disease outcome in the context of the pulmonary microenvironment. These data, along with recent data suggesting that one lung infection has the potential to modify immunity for extended periods of time (Didierlaurent et al., 2008), emphasize the importance of delaying the time of initial influenza infection, and therefore the importance of vaccination in infants and young children. Future studies to elucidate the molecular mechanisms responsible for the persistent inflammation and structural alterations observed with neonatal influenza infection should identify important therapeutic targets capable of controlling long-term complications due to viral bronchiolitis in infancy. Our observations (i.e., that CD8⁺ T cell responses in neonate are functionally different than that of adults) have significant implications for human infants beyond just influenza infection including infant responses to nosocomial infections and even responses to vaccination.

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CHAPTER FIVE:
GENERAL DISCUSSION

Respiratory viral infections continue to be a significant cause of morbidity and mortality worldwide. Respiratory syncytial virus and influenza virus are the two major causes of lower respiratory tract infections (LRTI) in infants and children (Denny, 1995), and disease severity is inversely related to age at infection (Welliver, 2003). In particular, infants under two years of age, especially those younger than six months of age, develop severe LRTI involving bronchi, bronchioles, and alveoli (Welliver, 2003).

Despite the intense research on RSV and influenza, the pathogenesis of these two viruses is unclear, partly due to the usage of age-unmatched animal models. The majority of the studies on the pathogenesis of RSV and influenza are carried out in adult mouse models. However, discrepancies between pathogenesis in adult mouse models and in human infants have been observed. RSV infections in infants are usually Th2 biased (Roman et al., 1997), while RSV infections in adult mice are Th1 biased (Peebles et al., 2001). Influenza infections in infants exhibit a lack of adaptive immune responses (Welliver et al., 2007), while influenza infections in adult mice induce a substantial number of adaptive immune cells (Allan et al., 1990). The inconsistency of immune responses between human and mouse infections indicate that an age-matched mouse model must be used to match the maturity of immune systems in both organisms.

Therefore, we established two neonatal mouse models of RSV and influenza infection in the research for the present dissertation. In our models, both viruses led to long-term pulmonary dysfunction; however, the mechanisms behind this phenomenon were quite different. The following discussion will detail the relevance of these two models to infections in human infants and the similarities and dissimilarities between the pathogenesis of these two viruses in neonatal mice. We will also propose a possible model for the pathogenesis of both viruses in neonatal mice and discuss the implications of our study of antisense oligonucleotides (ASO) to IL-4R α (Chapter 3) on the development of RSV vaccines and therapies for infants.

Neonatal Mouse Models and Infant Infections

Respiratory Syncytial Virus

In our neonatal mouse model, infection with RSV at seven day of age elicited a long-term airway hyperreactivity associated with unresolved pulmonary inflammation, goblet cell hyperplasia, and airway remodeling. In combination with an allergic model (ovalbumin), we demonstrated that neonatal RSV infection exacerbated many symptoms observed in allergic asthma including airway hyperreactivity and eosinophilic pulmonary inflammation. These data mimic epidemiologic data demonstrating that infantile RSV infections correlate with long-term pulmonary function abnormalities and increased risk of developing recurrent wheezing and asthma in later life (Castro-Rodriguez et al., 1999; Hall et al., 1984; Korppi et al., 2004; Sigurs et al., 2000; Sigurs et al., 2005; Stein et al., 1999). Although the duration of pulmonary dysfunction (i.e., 13 to 20 years) due to RSV infections during infancy is controversial, it is generally well accepted that severe RSV infection does induce long-term pulmonary dysfunction and increased sensitivity to aeroallergens.

In humans, the question remains: does severe RSV infection during infancy predispose to long-term pulmonary function abnormalities or does it unmask some genetic or pulmonary abnormalities that may already exist? In our neonatal mouse model, RSV infection appears to be an independent cause inducing persistent pulmonary dysfunction and exacerbating allergic pulmonary diseases well into adulthood.

To better address this issue in humans, clinical studies using interventions such as anti-viral drugs (i.e., ribavirin) and RSV antibodies (i.e., RSV-IGIV and palivizumab) have been carried out. Current data suggest that ribavirin therapy does not protect against long-term pulmonary dysfunction in infants following RSV LRTI (Krillov et al., 1997; Long et al., 1997;

Lugo and Nahata, 1993; Rodriguez et al., 1999), which may be explained by the controversial effects of ribavirin on treating RSV (Ventre and Randolph, 2007), whereas clinical studies with RSV-IGIV suggest that a cause-effect relationship exists between RSV LRTI and the subsequent development of asthma. In these studies, using RSV-IGIV in infants to prevent LRTI caused by RSV decreases the incidence of asthma five to nine years after treatment (Simoes, 2001).

Interestingly, mice originally infected with RSV as neonates and then reinfected as adults developed Th2-biased immune responses, including pulmonary eosinophilia and IL-13-mediated mucus hyperproduction. It is difficult to know what responses occur in humans upon reinfection because: 1) only 25 to 40% of infected infants develop severe LRTI (Collins and Graham, 2008); 2) of those, only 40% develop long-term wheeze or asthma (Sigurs et al., 2005); and 3) recurrent infections can occur throughout life (Hall et al., 1991; Scott et al., 2006). However, the tragic formalin-inactivated-RSV vaccine trial of the late 1960s suggests that a similar Th2-biased response occurred when the vaccinated infants later became naturally reinfected. In fact, 80% of those vaccinated infants developed severe bronchiolitis and/or pneumonia. Two of the vaccinated infants died, and the postmortem examinations revealed significant pulmonary eosinophilia in their lungs (Kim et al., 1969).

Influenza

In our neonatal mouse model of influenza, infection of seven-day-old mice induced persistent pulmonary pathophysiology, including airway hyperreactivity, emphysematous-type lesions, and severe pulmonary inflammation with alveolitis and consolidation. Few studies have investigated long-term effects of influenza infection in infants, probably because it occurs less frequently. However, an in-depth literature search revealed that these studies, although old, exist. The data suggest that influenza infection in infants causes chronic (2.5 to 5 years post-

infection) pulmonary dysfunction, and therefore the long-term effects of infantile influenza infection may be underestimated (Laraya-Cuasay et al., 1977).

Many components of our model mimic the responses in influenza-induced LRTI in human infants, including influx of neutrophils and mononuclear cells into the airways, necrosis of epithelial cells, and alveolar edema. More importantly, deficient adaptive immunity to influenza infection in neonatal mice is also consistent with that in human infants. Limited CD4+ and CD8+ T cell responses are observed in fatal infantile infections of influenza (Welliver et al., 2007). Repeated infection in humans with the same strain is usually asymptomatic; antigenically similar but different strains are a frequent cause of reinfection and manifest similar symptoms as primary infection (Sonoguchi et al., 1986). It would be interesting to assess reinfection with influenza in our neonatal mouse model to investigate whether reinfection with the same strain is similar to that observed in humans. We expect prior neonatal infection would be protective against subsequent infection but, as with RSV memory, that protection may be incomplete due to early exposure to the virus (Lawrence and Vorderstrasse, 2004). For all these reasons, neonatal mice provide a good model to study the immunological aspects of influenza infection in infants.

RSV versus Influenza in Neonatal Mice

Similarities between RSV and influenza infections in neonatal mice are obvious. Both neonatal infections led to long-term airway hyperreactivity, along with persistent pulmonary inflammation and goblet cell hyperplasia in matured adults. However, the magnitude of these responses was quite different, with influenza inducing more severe pulmonary dysfunction and inflammation. Furthermore, lung injury following neonatal influenza infection was more prominent. Emphysematous-type lesions were readily apparent in influenza-infected lungs, while RSV infection in neonates led to extensive subepithelial fibrosis (i.e., airway remodeling).

Cellular inflammation in the BALF during the acute phase of the infection (during active infection) was also more prominent in influenza infection in terms of scale. Both viruses led to the recruitment of neutrophils, monocytes/alveolar macrophages (AMs), and lymphocytes; however, two-fold more cells were present in the BALFs of influenza-infected mice. In addition, the composition of the inflammatory cells in the convalescent phase (months after virus was no longer detectable) was different between RSV and influenza infections: monocytes or AMs were the major cells observed in RSV-infected lungs, whereas neutrophils and lymphocytes comprised a significant portion of the inflammatory cells observed in influenza-infected lungs. The difference between neonatal RSV and influenza infections, in this respect, may be attributed to the fact that RSV is less virulent than influenza in mice. RSV infects mice less efficiently than influenza and requires 20,000-fold more infectious virions than influenza to elicit a productive infection compared to influenza.

Furthermore, the time course and the amount of cytokine production in the BALF were quite different for RSV and influenza infections. Influenza infection induced more robust cytokine responses than RSV, with the exception of higher levels of TNF- α , IL-13, and IL-17 secreted in RSV-infected lungs. The proinflammatory cytokine TNF- α was secreted and peaked as early as five hours post-infection in both RSV and influenza and was undetectable ten days post-infection (dpi). Another proinflammatory cytokine, IL-6, was a bit different. It peaked within five hours of RSV infection and was no longer detectable 24 hours after infection. With influenza, IL-6 peaked four dpi and was undetectable six days later. The Th2 cytokine IL-13 peaked within 24 hours of both infections, with RSV inducing two-fold IL-13 secretion over influenza. As expected, the Th1 cytokines IFN- γ and IL-12 were much higher in influenza infection and were barely detected in the BALF of neonates infected with RSV.

T cell responses in neonatal RSV and influenza infections also showed similarities and dissimilarities. RSV infection in neonatal mice elicited a Th2-biased response (1:1 ratio of Th1/Th2 cells in the lungs) compared to influenza infection (5:1 ratio of Th1/Th2 cells). Responses of cytotoxic T cells (CTLs) were similar between RSV and influenza with both infections eliciting prominent Tc1 (IFN- γ -producing CTL) responses. Interestingly, the magnitude of T cell responses in both infections was weaker than the magnitude of T cell responses observed in adult infections.

Current Model for the Pathogenesis of RSV and Influenza

Combining data from our neonatal infection studies, we propose a model for the pathogenesis of RSV and influenza infections (Figure 5.1). For ease of discussion, unless otherwise stated, “primary infection” here means infection in neonates, and “reinfection” means infection in adults who had originally been infected as neonates.

The outcome of primary infection is determined by the virulence of the invading viruses, the infectious doses, and the state of the host’s immunity. Epithelial cells of the airways are the major targets for both viruses. The replication of viruses in epithelial cells leads to two consequences: 1) the necrosis of epithelial cells and 2) the release of a series of inflammatory signals. Destruction of the epithelial layer is more prominent in influenza infection, probably due to the fact that influenza is more virulent than RSV and replicates more efficiently in mice. Combined with the weak adaptive immune responses in neonates, the control of influenza infection is poor. The cytopathic effects of the virus, therefore, play major roles in the pathogenesis of influenza infection. In the case of RSV primary infection, both the cytopathic effects of the virus and the Th2-biased immune responses are responsible for the pathogenesis. However, since RSV is less virulent, the pulmonary disease is less severe.

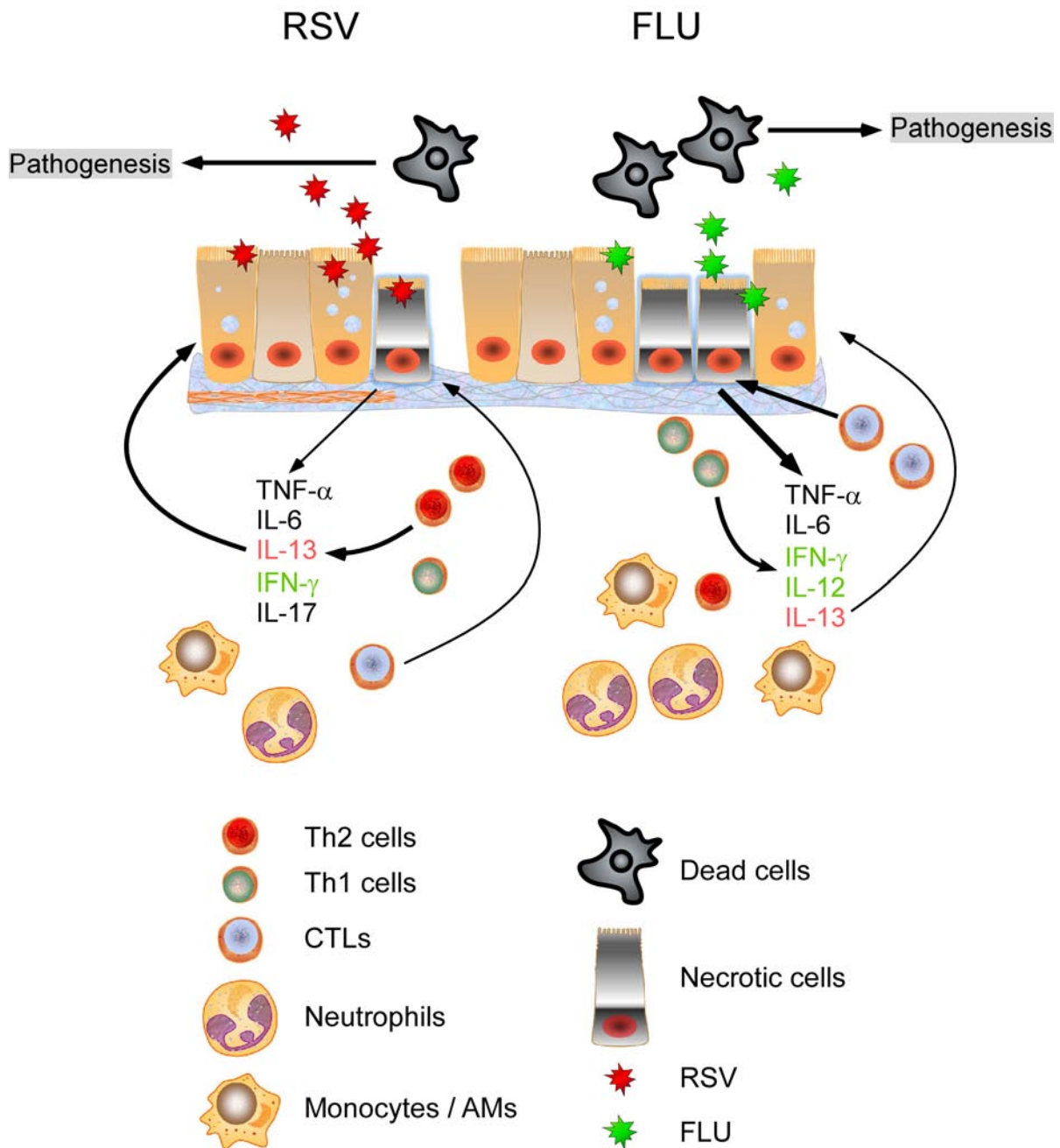


Figure 5.1 A proposed model for the pathogenesis of primary infections of RSV and influenza in neonatal mice. Both RSV and influenza infections in neonatal mice result in the necrosis of epithelial cells and the recruitment of inflammatory cells into the lung. Inflammation and destruction of the epithelial layer is more prominent in influenza infection, while RSV induces more Th2 cells to the lung.

The second consequence of viral replication in epithelial cells (i.e., the release of inflammatory signals) comes to the forefront when adult mice are infected. In general, pulmonary disease induced by these two viruses is milder in adults than in neonates, most likely due to a more mature and efficient adaptive immune system and response. However, it is possible that the benefit of the adult immune response could be overcome with a higher infectious dose. A higher infectious dose would result in more replication of the virus and, subsequently, greater destruction of epithelial cells, with considerable release of inflammatory signals. Large numbers of inflammatory cells, including cytotoxic T cells, are recruited to the infected area and exert their killing function on infected epithelial cells. However, the bystander cytotoxicity of immune cells adds more destructive burden to the infected animals and is therefore more pathogenic than protective.

In the case of reinfection, the pathogenesis of RSV and influenza infections differs drastically. Although not tested in this dissertation research, reinfection with influenza is thought to be consistent with the canonical model of reinfection, based on the adult infection data from other groups (Lawrence and Vorderstrasse, 2004): memory of adaptive immunity is established during primary infection and protects the host during secondary infection. In RSV reinfection, Th2-biased immune responses play major roles in the pathogenesis of secondary infection. Abundant inflammatory cells, including CTLs, are recruited to the infected area to clear the virus; however, other recruited cells, e.g., eosinophils and Th2 cytokines, such as IL-5 and IL-13, erroneously add inflammatory burden to the lungs and airways, and induce mucus hyperproduction and airway hyperresponsiveness, which are hall marks of RSV-induced illness. This discrepancy between RSV and influenza reinfections is possibly due to undefined molecular mechanisms by which RSV induces a Th2-biased memory response in neonates. IL-4R α is a

candidate for study, since this molecule has been shown to regulate selective apoptosis of Th1 cells, leaving Th2-biased responses upon rechallenge with the same antigen (Li et al., 2004). Toll-like receptors (TLRs) are other good candidates, since they are at the first line of defense against viruses, and the interplay between these receptors and the invading virus in innate cells signals the down-stream adaptive immune responses, including T cell responses.

In summary, both the cytopathic effects of the virus and immunopathology play roles in the pathogenesis of primary infections with RSV and influenza in mice; Th2-dominated memory responses contribute to the exacerbated respiratory distress upon RSV reinfection.

There Is Hope

The fact that no vaccines are available for RSV infection is disappointing given the burden of this virus in infants and the long-term effects of severe infantile infections on pulmonary function in children and adolescents. The tragic vaccine trial of the late 1960s has intimidated vaccinologists, and the difficulty of inducing protective immune responses in infants with live attenuated vaccines has hampered the progress of vaccine development.

The usual vaccination strategy uses an attenuated virus to inoculate neonates in the hope of eliciting protective memory responses in a rather immature or weakened immune system. In contrast, we used a wild-type virus to infect neonatal mice, along with immunomodulation at the time of infection. The results, so far, are promising. We demonstrated that treating neonates with ASO against IL-4R α during primary RSV infection completely protects against the pulmonary dysfunction usually seen in RSV reinfection. This protection was achieved by inducing a strong-enough immune response with a wild-type virus instead of an attenuated one and by delicately balancing between Th1 and Th2 responses with IL-4R α ASO treatment during

infection. We believe that our IL-4R α ASO treatment offers a good opportunity for future therapies and vaccine development strategies.

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APPENDIX:
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VITA

Dahui You is the daughter of Mr. Jiang You and Ms. Yu Chen. She was born in a small village in China in 1979, where she lived until she went to Sichuan University in 1997. Dahui You graduated with a Bachelor of Science degree in biology from Sichuan University, Sichuan in 2001 and a Master of Science degree in genetics from Sichuan University in 2004. After her graduation, she began her doctoral research under the direction of Dr. Stephania Cormier in the Department of Biological Sciences at Louisiana State University in Baton Rouge, Louisiana. Dahui You will graduate with the degree of Doctor of Philosophy in August, 2008.