Respiratory Syncytial Virus: Rodent Models and Vaccine Development

DISSERTATION

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By

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ABSTRACT

Respiratory syncytial virus (RSV) is the leading cause of lower airway disease in infants worldwide. Progress toward an effective RSV vaccine has been hampered by lack of rodent models which accurately recapitulate human disease. We developed and throughout characterized two rodent models of RSV infection, the chinchilla and the cotton rat. We found the chinchilla to be a useful model of upper airway RSV infection and the cotton rat to be a useful model of both upper and lower airway RSV infection. Additionally, we found that RSV infection of the cotton rat was associated with allergic-like lung disease. This is of particular interest given the association between RSV infection during infancy and development of asthma or recurrent wheezing in later childhood. We utilized these newly characterized rodent models to evaluate an RSV vaccine that was developed in our lab based on the hypothesis that the poor IFN response to RSV infection observed in mice and humans contributed to the relatively ineffective adaptive immune response to viral infection. To test this idea, we inserted the RSV F protein gene into the genome of Newcastle disease virus (NDV), an avian paramyxovirus that is nonpathogenic in mammals but is known to induce very high IFN levels in mice. We found that our vaccine construct, NDV-F, induced robust, long-lasting adaptive immune responses and protected chinchillas and cotton rats against RSV infection. These studies significantly contribute to the field of RSV research by characterizing rodent
models that can be utilized to study mechanisms of RSV pathogenesis and by advancing a promising RSV vaccine candidate toward clinical trials.
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# Table of Contents

Abstract ........................................................................................................................................... ii
Acknowledgements ......................................................................................................................... iv
Vita ..................................................................................................................................................... v
List of Tables ..................................................................................................................................... viii
List of Figures ................................................................................................................................... ix

**Chapter 1: Introduction** ................................................................................................................. 1
Respiratory Syncytial Virus ............................................................................................................... 2
The Clinical Problem ......................................................................................................................... 3
The Immune Response to RSV Infection ......................................................................................... 5
Animal Models of RSV Disease ....................................................................................................... 7
RSV Vaccination ............................................................................................................................... 10
Summary ........................................................................................................................................... 17

**Chapter 2: The Chinchilla Model of RSV Infection** ..................................................................... 18
Abstract ........................................................................................................................................... 19
Introduction ....................................................................................................................................... 20
Materials and Methods ..................................................................................................................... 23
Results ............................................................................................................................................... 26
Discussion .......................................................................................................................................... 31

**Chapter 3: The Cotton Rat Model of RSV Infection** ................................................................. 42
Abstract ........................................................................................................................................... 43
Introduction ....................................................................................................................................... 44
Materials and Methods ..................................................................................................................... 48
Results ............................................................................................................................................... 51
Discussion .......................................................................................................................................... 59
<table>
<thead>
<tr>
<th>Chapter 4: Pre-clinical Development of an RSV Vaccine Candidate</th>
<th>82</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>83</td>
</tr>
<tr>
<td>Introduction</td>
<td>85</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>89</td>
</tr>
<tr>
<td>Results</td>
<td>97</td>
</tr>
<tr>
<td>Discussion</td>
<td>106</td>
</tr>
</tbody>
</table>

| Chapter 5: Conclusions and Future Directions                   | 127|

| References                                                   | 132|
List of Tables

Chapter 2

Table 2.1. Infectious rrHRSV in nasopharyngeal lavage fluids ........................................... 35
List of Figures

Chapter 2
Figure 2.1 Gross anatomy of the chinchilla nasal cavity.................................36
Figure 2.2 Microscopic anatomy of the chinchilla nasal cavity.............................37
Figure 2.3 Eustachian tube and nasopharynx of rrHRSV-infected animals, confocal
microscopy and immunohistochemistry.....................................................38
Figure 2.4 Naso- and ethmoid turbinates of rrHRSV-infected animals, confocal
microscopy and immunohistochemistry.....................................................40
Figure 2.5 Ethmoid turbinate 14 d after rrHRSV infection, immunohistochemistry…..41

Chapter 3
Figure 3.1 Upper and lower airway viral load after acute RSV infection..................68
Figure 3.2 Distribution and extent of acute RSV infection of the cotton rat upper
airway............................................................................................................69
Figure 3.3 Distribution and extent of acute RSV infection of the cotton rat lower
airway............................................................................................................71
Figure 3.4 RSV infection persists in the cotton rat upper airway............................73
Figure 3.5 Histopathology of acute and chronic RSV infection of the cotton rat upper
airway............................................................................................................74
Figure 3.6 Histopathology of acute RSV infection of the cotton rat lower airway……..76
Figure 3.7 Cytology of RSV infection of the cotton rat lower airway……………………..78
Figure 3.8 Chronic lung histopathology after RSV infection…………………………….80

Chapter 4

Figure 4.1 Histology of the nasal cavity and lung following NDV-F vaccination of cotton rats………………………………………………………………………………..111
Figure 4.2 Histology of the nasal cavity and lung following primary RSV challenge of NDV-F vaccinated cotton rats……………………………………………………113
Figure 4.3 Histology of the nasal cavity following primary RSV challenge of NDV-F vaccinated chinchillas……………………………………………………115
Figure 4.4 Nasal cavity and lung immunohistochemistry and plaque assay following primary RSV challenge of NDV-F vaccinated cotton rats…………………..117
Figure 4.5 Nasal cavity immunohistochemistry and plaque assay following primary RSV challenge of NDV-F vaccinated chinchillas……………………………..119
Figure 4.6 Cotton rat humoral immune response to NDV-F vaccination and RSV challenge………………………………………………………………………………120
Figure 4.7 Chinchilla serum antibody response to NDV-F vaccination and RSV challenge………………………………………………………………………………123
Figure 4.8 Chinchilla humoral immune response to NDV-F vaccination and RSV challenge………………………………………………………………………………124
Figure 4.9 Histology of the lung following secondary RSV challenge of NDV-F vaccinated cotton rats………………………………………………………125

Figure 4.10 BAL fluid cellularity and cellular differential following vaccination and RSV challenge of cotton rats…………………………………………………126
Chapter 1:

Introduction
Respiratory Syncytial Virus

RSV is an enveloped, negative sense, non-segmented, single strand RNA virus of the family Paramyxoviridae and the genus Pneumovirus. The genus Pneumovirus also includes bovine RSV, ovine RSV and pneumonia virus of mice. There are two RSV subtypes, A and B, and 10 viral genes which encode 11 proteins including three surface proteins (F, G and SH), a matrix protein (M1), two polymerase cofactors (M2-1 and M2-2), three nucleocapsid proteins (N, P, and L) and two nonstructural proteins (NS1 and NS2). Genes are linearly ordered from the 3` to the 5` end of the genome with the NS1 and NS2 genes positioned at the 3` end and the L gene positioned at the 5` end. Positioning within the genome is of significance because, as with other non-segmented RNA genomes, a transcriptional gradient dictates that genes positioned closest to the 3` end are the most abundantly expressed while genes positioned closest to the 5` end are the least abundantly expressed. The G and F genes, which encode for proteins that are essential for viral attachment (G and F) and fusion (F) are situated close to the 5` end of the genome and are, thus, not amongst the most highly transcribed genes.

The surface-exposed G and F glycoproteins are the only RSV antigens to which neutralizing antibodies are produced. The G protein is much more heavily glycosylated than the F protein and is produced both as a transmembrane protein and as a secreted protein. The G protein is highly variable within and amongst RSV subtypes. The F protein is synthesized as an inactive precursor which is cleaved by a furin-like protease in
the trans-Golgi of an infected cell. The F protein can mediate cell fusion to form syncytia. In contrast to the G protein, the F protein is highly conserved and is thus widely considered to be an ideal vaccine antigen. The receptors for the G and F proteins are not fully defined.

**The clinical problem**

Respiratory infections are responsible for the largest burden of disease worldwide ([1] and WHO). This is particularly true amongst the pediatric population. The 2002 WHO Burden of Disease Project determined that 18% of mortality for children less than 5 years of age was due to respiratory infections. Diarrhea and malaria were the second and third most common causes of death in this age group, respectively. The economic impact of respiratory infection is significant. In the developed world, the direct and indirect costs associated with each episode of acute respiratory infection ranges from $140 - $240 USD, depending several factors including the pathogen [2,3]. Cases which require hospitalization are considerably more costly. For example, the average cost to treat one case of RSV bronchiolitis was estimated at $5,250 USD [4].

Major viral pathogens of the human respiratory tract include influenza virus, rhinovirus, metapneumovirus, parainfluenza viruses and RSV. In 1956, RSV was isolated from a chimpanzee with clinical signs of upper respiratory tract disease and was soon after
recognized as a major cause of respiratory illness in humans of all age groups [5]. It is now firmly established that RSV is the most common cause of lower respiratory tract infection in children and that RSV is second only to influenza virus as a cause of pneumonia in the elderly [6-9]. It was recently estimated that 34 million cases of RSV-associated lower respiratory tract infection occur annually in children under 5 years of age and that up to 10% of those cases require hospitalization [10]. RSV outbreaks occur on an annual basis and, given its ubiquity and highly-infectious nature, RSV infects essentially all persons within the first two years of life. Newborns are partially protected by RSV-specific maternal antibodies, but passive protection is short-lived [11]. Decline of RSV-specific maternal antibodies occurs prior to maturation of the immune system, putting infants 2-6 months of age at particularly high risk for severe RSV-associated disease [8,12]. Although premature infants and infants with congenital cardiopulmonary disorders are predisposed to severe RSV infection, the vast majority of severe RSV bronchiolitis cases occur in previously healthy infants with no known predisposing factors [13].

Although the first RSV infection is the most severe, reinfections occur commonly. Reinfections, which are generally limited to the upper respiratory tract, are usually associated with mild cold-like symptoms in healthy adults, but are commonly associated with otitis media in young children [14,15]. In addition, severe lower airway RSV infection during infancy has been associated with the development of asthma and wheezing in later childhood and with exacerbation of wheezing in asthmatic patients.
It remains unclear, however, whether RSV is a cause or an early marker of infants that are predisposed to allergic lung disease [18,19].

While supportive care remains the basis for treatment of severe lower airway RSV infection, monthly intravenous administration of the RSV-neutralizing monoclonal antibody palivizumab (Synagis®, MedImmune, Gaithersburg, MD) during the RSV season is highly effective at preventing severe disease. However, palivizumab is cost-effective and indicated for use only in at-risk infants [20-22]. Additionally, palivizumab is only useful as a prophylactic agent; administration after the onset of disease is not efficacious [23]. The only specific treatment approved for clinically ill patients is ribavirin, an antiviral compound. Ribavirin is only marginally effective, however, and routine use is neither employed nor recommended [24]. Clearly, a more widely applicable prophylactic such as a vaccine and a more effective therapeutic are needed.

**The immune response to RSV infection**

Immunity to RSV is remarkably ineffective. This is illustrated by the fact that immunocompetent, seropositive children and adults can be repeatedly infected [25,26]. Unlike other viral pathogens, serum antibody levels are very slow to rise following RSV infection, with a gradual accumulation of protective antibodies only after multiple re-infections [27,28]. Thus, it appears that it is the weakness of the B cell response to RSV
that allows for continual reinfection. The importance of neutralizing antibodies is demonstrated by substantial protection of infants that receive palivizumab, the monoclonal antibody to the RSV F protein prior to virus exposure. The protective nature of passive immunity has been reported in multiple animal models [29-32]. Furthermore, susceptibility of healthy adults, children and the elderly to reinfection of the lower airway correlates with serum neutralizing antibody titers [14,33,34].

While high serum neutralizing antibody titers protect the lower airway against reinfection, it is the local secretory antibody response that protects the upper airway against reinfection. Mucosal antibody responses to RSV are less durable and shorter lived than serum antibody responses, leaving the upper airway vulnerable to reinfection. The importance of mucosal antibodies in preventing reinfection was demonstrated by the observation that nasal secretions collected from adult volunteers that did not develop clinical signs or symptoms after upper airway RSV challenge contained significantly more RSV-specific antibody compared to nasal secretions collected from volunteers that did develop clinical disease following RSV challenge [14]. The lower airway is generally considered resistant to reinfection by RSV, but when lower airway resistance of adults experiencing natural reinfection of the upper airway was monitored, increased resistance for up to 8 weeks was observed [35]. The lack of robust, long-lived immunity following natural RSV infection likely underlies the difficulties that have been encountered in attempts to design effective, attenuated vaccine strains [36-40].
Animal models of RSV disease

Humans are the natural hosts for RSV, but chimpanzees in contact with humans are readily infected and do display clinical signs similar to that observed in humans. The level of attenuation of live-attenuated RSV vaccine candidates has been evaluated in chimpanzees, but the results have not always accurately predicted the level of attenuation in infants [41]. A cold-passaged, temperature-sensitive live-attenuated RSV vaccine candidate developed at the National Institute of Allergy and Infectious Diseases, for example, was highly attenuated in chimpanzees, but caused significant nasal congestion in infants that interfered with eating and sleeping [40]. Inconsistent prediction of attenuation and efficacy of vaccine candidates and ethical considerations make chimpanzees a seldom used and inappropriate model for RSV infection and for pre-clinical RSV vaccine development.

In addition to chimpanzees, several other nonhuman primate species have been used to study RSV infection and to evaluate vaccine candidates. African green monkeys, the most heavily utilized nonhuman primate species, are semi-permissive to RSV infection. This species has been used to model the enhanced disease observed following vaccination of infants with a formalin-inactivated RSV vaccine (detailed below) [42]. Rhesus macaques have also been used to study RSV disease, but the virus replicates poorly in this species, so PCR to detect viral replication products is typically required [43].
Considering the ethical and economic cost, nonhuman primates are not sufficiently susceptible to RSV infection to warrant study of this disease in these species.

A major road block in elucidating the mechanism of RSV pathogenesis has been lack of a small animal model in which accurately recapitulates human disease. RSV disease has been extensively studied in small animals, each of which has benefits and limitations [44]. Mice have been utilized most heavily, but other small animal species used to study RSV infection include the cotton rat and, rarely, the guinea pig, the hamster and the ferret. When interpreting data from rodent models, one should keep in mind that using highly inbred, or even outbred, rodents will not capture all of the potential responses of a genetically heterogeneous human population.

The strengths of the mouse model stem from the low cost to acquire and house this species, the availability of immunological reagents and the ease with which this species is genetically modified. The mouse model of RSV infection, however, has major limitations. First, mice are poorly susceptible to human RSV infection and, in contrast to human patients, primarily the lower, not the upper airway is infected. Second, virus replication takes place in mouse alveolar lining cells and only rarely bronchial lining cells, the cell type targeted by RSV in human infection [45]. Finally, the mouse upper airway is resistant to RSV infection and a large volume, high virus titer inoculum is required to induce even minimal lower airway virus replication. Thus, the natural progression of infection seen in humans from the upper to the lower airway is bypassed.
in the mouse. This likely has significant consequences regarding the pulmonary response to RSV infection, as the early window for innate and adaptive immune responses that occur during the upper airway phase of the disease are not allowed to develop. Additionally, RSV tends to produce a large number of replication deficient antigen particles that would be confined to the upper airway in natural disease, but contribute to the immense antigen load encountered by the pulmonary parenchyma when viral inoculums are delivered directly to the lung. Overall, the mouse model of RSV disease has significant limitations and does not recapitulate the human disease, but has been useful to elucidate certain mechanisms of RSV pathogenesis, including viral antagonism of the interferon pathway, and to evaluate the contribution of certain cellular subsets and cytokines to the disease process.

A more robust rodent model of RSV infection than the mouse is the cotton rat (*Sigmodon hispidus*). Cotton rats are relatively permissive for RSV infection and, as is seen in human infection, it is the cells lining the nose and bronchial tree which are infected in this species [46]. Unlike mice, cotton rats are susceptible to secondary RSV infection. Permissiveness of the cotton rat upper airway to secondary RSV infection increased after 8 months and appeared to correlate with a decline in neutralizing antibody titers [47]. These results are similar to those found in adult human reinfection trials, where the interval required before reinfection with the same RSV strain varied between 2 and 18 months [14]. The cotton rat has proven useful for evaluation of potential RSV therapeutics and vaccine candidates. The current dosing regime for palivizumab, a
monoclonal antibody licensed for prevention of severe RSV disease in at-risk infants, was accurately predicted by studies first performed in cotton rats. While the cotton rat has been, and will continue to be, useful to study many aspects of RSV infection, including pre-clinical evaluation of RSV vaccine candidates, the pathology and immune response to RSV infection in this species has not been characterized. A better understanding of disease progression following RSV challenge of the cotton rat could increase the utility of this species for study of RSV disease.

**RSV vaccination**

The impact of vaccination on human health cannot be overstated. Vaccination trumps even antibiotics as the single most important medical intervention in human history and only access to clean drinking water has more positively impacted mortality rates. Classical vaccine approaches include injection with a heterologous organism from another species (the Jenner approach), killing of the organism (the Salk approach), attenuation of the organism, and injection of subunits or purified protein antigens from the organism. These approaches have been highly effective against a wide array of diverse pathogens and have led to the global eradication of one human (smallpox) and one veterinary (rinderpest) disease as well as the significant control of many others including measles and polio. Classical approaches have not been successful for all pathogens, however, and in some cases have been associated with significant pathology.
It has become clear that innovative strategies using sophisticated molecular techniques will be required to combat infectious diseases caused by particular pathogens such as HIV, hepatitis C virus and RSV and non-infectious diseases such as cancer and autoimmune disease.

The advantage of live-attenuated vaccines is that all antigenic determinants, in their native conformations, are present. For alternative RSV vaccine approaches, discussed below, antigenic selection is a major consideration. Given that antibodies to the F and G glycoproteins are neutralizing and that the monoclonal antibody against the F protein is efficacious, the F and G proteins are widely regarded as ideal vaccine antigens. Because the F, but not the G, protein is highly conserved and because soluble G protein may act as a decoy receptor, thereby reducing the effectiveness of neutralizing antibodies, the F protein is likely the more desirable antigen for inclusion in an RSV vaccine. Additionally, an effective RSV vaccine will likely depend on robust antigen-specific humoral as well as cellular immunity, so inclusion of T cell epitopes is desirable. In addition to the glycoproteins, the highly conserved internal viral proteins such as M2-2 are sources of many T cell epitopes in humans and mice. Characterization of more human B and T cell epitopes will aid future rational RSV vaccine design.

The epidemiology of RSV infection is ideal for eradication through vaccination. Spread of the virus is dependent on close contact with infected individuals and there is no known animal reservoir. Epidemiology, however, may be the only major advantage for RSV
vaccinologists. RSV vaccine development faces many challenges including the need to vaccinate at a time when the immune system is immature and immunosuppressant maternal antibodies are present, the weak and short-lived immune response to natural infection, lack of rodent models which recapitulate human infection, and the legacy of vaccine-enhanced disease in an early clinical trial.

RSV vaccination efforts began almost immediately after the virus was discovered. Based on the recent success of the killed polio vaccine, the initial approach to RSV vaccination was formalin-inactivation of live virus followed by precipitation with alum (FI-RSV). Unexpectedly, intramuscular FI-RSV vaccination of RSV naïve infants resulted in enhanced disease upon subsequent natural RSV infection [48]. The hospitalization rate was markedly increased in infants that had been FI-RSV vaccinated prior to nature RSV infection and two FI-RSV vaccinated infants died. The basis of enhanced disease observed in FI-RSV-vaccinated children has been attributed to a number of factors, but is not yet completely understood. It has been suggested that formalin inactivation altered key antigenic epitopes, which led to production of non-neutralizing antibodies, and that cellular immune responses were minimal due to the inert nature of the vaccine [49]. Taken together, poor cellular and humoral immune responses may have led to an exceptionally high viral burden in the lung and consequent recruitment of inflammatory cells. This hypothesis is supported by autopsy findings from the two fatal cases in the FI-RSV vaccine trial which revealed pulmonary infiltration by eosinophils and neutrophils and small airway obstruction [48]. The peripheral eosinophilia observed in hospitalized
FI-RSV vaccinated infants and the eosinophilic pulmonary inflammation observed in the two fatal cases has led to the widely accepted hypothesis that FI-RSV induced a skewed T\textsubscript{H}2 response. This idea has been supported in multiple animal studies in which increased T\textsubscript{H}2 (e.g. IL-4, IL-5, IL-13) and decreased T\textsubscript{H}1 (e.g. IFN-\textgamma) cytokine production following RSV infection of FI-RSV vaccinated animals has been demonstrated [50-54]. Other animal studies, however, have found a balanced, yet increased, cytokine response to RSV following FI-RSV vaccination [55]. Additionally, it is not universally accepted that eosinophils contributed to vaccine-enhanced disease. One study demonstrated a protective role of eosinophils during RSV infection [56]. Another study found no decrease in pulmonary disease when eosinophil-deficient mice were FI-RSV vaccinated prior to RSV challenge [57]. Furthermore, many investigators have stressed that T\textsubscript{H}2 responses have numerous positive effects including class-switching to IgA, an essential event for effective mucosal immunity. Given the conflicting evidence, decisions regarding advancement of RSV vaccine candidates toward clinical trials should be based on many factors and not just the presence or absence of T\textsubscript{H}2 cytokines and eosinophils in the lungs of vaccinated animals.

Cautious as a result of the disastrous FI-RSV trial, investigators have since focused on development of live-attenuated RSV vaccines, which is considered to be the safest approach. Thus, only live-attenuated RSV vaccine candidates have been evaluated in seronegative infants. In the two clinical trials in which seronegative infants received a live-attenuated RSV vaccine, enhanced disease was not observed, but vaccination was
minimal immunogenic [58, 59]. This is not surprising given that natural RSV infection does not induce long-term adaptive immunity. Additionally, there are significant practical considerations for production, storage and distribution of a live-attenuated vaccine. First, RSV is technically challenging to culture to high titer and produces a high proportion of defective, non-replicating particles. Second, RSV is an inherently unstable virus and even when stored at subfreezing temperatures, replicating virus titers decrease over time. The World Health Organization recommends that all vaccines, except the oral polio vaccine, be stored and transported at 2-8º C, a temperature range at which RSV is unstable [60]. An appropriate cold-chain may be achievable in the developed world, but proper storage and transport of a live-attenuated RSV vaccine may not be possible in the regions of the world where RSV vaccination is most needed.

The disadvantages of a live-attenuated approach to RSV vaccination have led investigators to develop several alternative approaches. Alternatives to live-attenuation to reach human clinical trials include viral-vectored vaccines and protein subunit vaccines. These strategies are discussed in more detail below. Alternatives to live-attenuation that are still in the early stages of development include nanoparticle vaccines, virus-like particle vaccines and DNA vaccines.

Viral-vectored (chimeric) vaccines are advantageous because, as they are live, replication competent viruses capable of stimulating multiple pattern recognition receptors and because they induce robust innate and adaptive (cellular and humoral) immune responses.
Several viral-vectored vaccines have been licensed for use in veterinary medicine including the highly-efficacious vaccinia-vectored oral rabies virus vaccine (Raboral V-RG®, Merial, Duluth, GA) [61]. To date, Imojev® (Sanofi-Pasteur, Bridgewater, NJ), a yellow fever virus-vectored Japanese encephalitis virus vaccine, is the only viral-vectored vaccine licensed for human use. However, the value of this approach has been increasingly appreciated and it is likely that more viral-vectored vaccines will be licensed for human use in the coming years [62]. Several RSV vaccine candidates, in which selected RSV genes are expressed by a different virus, have been developed. Viruses utilized for this approach include bovine parainfluenza virus (PIV3), Sendai virus and alphaviruses, which are host-restricted viruses that replicate minimally in humans, and human adenovirus, a virus to which the majority of the population has been exposed and thus possesses neutralizing antibodies. The only chimeric RSV vaccine to advance to clinical trials is MEDI-534 (MedImmune, Gaithersburg, MD), a bovine PIV3 that expresses the human RSV F and human PIV3 F and HN genes. This construct protected African green monkeys from RSV challenge, but was minimally immunogenic in seropositive children [63,64]. Safety and immunogenicity is MEDI-534 is currently being evaluated in healthy adults, children and seronegative infants (clinicaltrials.gov, accessed 9/25/12).

Purified protein, or subunit, vaccines are a simple approach, but have thus far been licensed only for hepatitis B and human papillomavirus [65]. Given that subunit protein vaccines induce primarily CD4+ T cell and neutralizing antibody responses, with little to
no induction of CD8+ T cell responses, concern that skewed T_{H2} responses would lead to enhanced disease would make licensure of a purified protein RSV vaccine for use in seronegative infants difficult. However, purified protein RSV vaccines have been developed and evaluated in older children and adults, in which they have been generally safe and modestly immunogenic ([66-68]. Despite induction of neutralizing antibody responses, purified F protein vaccination did not significantly impact the incidence of lower airway RSV infection and, when administered to expectant mothers, did not reduce the incidence of RSV infection in infants [69]. Thus, development of an effective purified F protein vaccine will require further investigation, with recent interest focused on the more stable postfusion form of the F protein [70].

Given the wide age range of susceptibility and the individual challenges associated with vaccination at the extremes of age, it seems likely that a combination of approaches, each tailored to a different age group, will be required to eliminate RSV as a major human pathogen. Although RSV vaccine development faces many challenges, optimism that an RSV vaccine is in sight stems from the high priority that governments and pharmaceutical companies place on licensure of an effective RSV vaccine.
Summary

RSV is certainly one of the most important human pathogens for which a vaccine does not yet exist. Rational RSV vaccine design is dependent on a more thorough understanding of RSV pathogenesis, which is dependent on development of small animal models which more accurately recapitulate human disease. Therefore, the goals of the studies outlined in chapters 2-4 are to develop better rodent models of RSV disease and to pre-clinically evaluate an RSV vaccine candidate developed based on our understanding of RSV pathogenesis.
Chapter 2:
The Chinchilla Model of RSV Infection
ABSTRACT

Although most upper respiratory tract viruses can predispose to bacterial otitis media, human respiratory syncytial virus (HRSV) is the predominant viral co-pathogen of this highly prevalent pediatric polymicrobial disease. Rigorous study of the specific mechanisms by which HRSV predisposes to otitis media is hindered by lack of a relevant animal model. We recently reported that the chinchilla, the preferred rodent host for studying otitis media, is semi-permissive for upper-airway HRSV infection. In the current study, we defined the anatomy and kinetics of HRSV infection and spread in the upper airway of chinchilla hosts. Chinchillas were challenged intranasally with a fluorescent protein-expressing HRSV. Upper-airway tissues were recovered at multiple time points after viral challenge and examined by confocal microscopy and immunohistochemistry. HRSV replication was observed from the rostral- to caudalmost regions of the nasal cavity as well as throughout the Eustachian tube in a time-dependent manner. Although fluorescence was not observed and virus was not detected in nasopharyngeal lavage fluids 14 d after infection, the latest time point examined in this study, occasional clusters of immunopositive cells were present, suggesting that the nasal cavity may serve as a reservoir for HRSV. These data provide important new information concerning the time course of HRSV infection of the uppermost airway and suggest that chinchillas may be useful for modeling the HRSV-induced changes that predispose to secondary bacterial infection.
INTRODUCTION

Human respiratory syncytial virus (HRSV), an enveloped, negative-strand, nonsegmented RNA virus of the family *Paramyxoviridae*, is the single greatest causative agent of acute respiratory tract infections in infants and children worldwide [71]. Although HRSV infection is generally limited to the upper respiratory tract (URT), in the United States, primary HRSV infection has a 0.5 % hospitalization rate for those children who develop severe bronchiolitis or pneumonia [72]. One of the most interesting aspects of HRSV is its ubiquity: there are annual winter/spring outbreaks in temperate climates [73], and approximately 90% of all children have experienced infection by their second birthday [72]. Although immunity to HRSV infection is sufficient to prevent reinfection of the lower airway in most human patients, this response is incomplete, resulting in reinfection of the upper airway throughout life [72]. Although URT infection by HRSV alone does not constitute a major problem for healthy adults, its association with the development of bacterial otitis media in children [74-84], and exacerbation of asthma in all age groups [85], make it an important health concern.

Despite ubiquity of the virus, the epidemiology of HRSV is not well understood. There is no known animal reservoir, and although new strains emerge over time, many remain in circulation over several seasons or reappear many years after they were first detected [86,87]. Therefore, although antigenic variation driven by development of HRSV immunity in a given population is possible, this hypothesis has not yet been proven. In
fact, in one study, [14] it was demonstrated that human subjects could be repeatedly infected with the same HRSV strain and that the presence of virus-specific antibody provided only short-lived and incomplete protection. Therefore, HRSV may circulate among seropositive individuals, and it has been suggested that persistently infected individuals may harbor the virus between seasonal outbreaks [88,89]. Therefore, in addition to the important clinical issues surrounding the prevention of HRSV disease, basic scientific questions regarding HRSV circulation and mechanisms of viral immunoevasion remain unanswered.

A key hurdle in the study of HRSV pathogenesis has been the lack of a suitable animal model. Most published studies have utilized BALB/c mice, which have the advantage of many reagents available for the study of immune responses but the disadvantage of relative resistance to human HRSV infection [90]. Although pulmonary infection is easily detected in HRSV-infected mice, primary infection of the upper airway in this species is minimal [91,92] and secondary infection of the URT does not occur. More susceptible rodent species include the cotton rat (Sigmodon hispidus) [46] and the chinchilla (Chinchilla lanigera) [91], which are both relatively permissive for HRSV infection of the upper airway. Given the paucity of URT specimens encountered in general pathology practice, the development of a robust small animal model for the study of HRSV infection and spread in the uppermost airway is particularly important. Moreover, effective vaccine development depends upon a better understanding of why this compartment remains susceptible to reinfection in immune hosts.
Here we describe the anatomy of HRSV infection in the chinchilla URT over a 2-wk period, using confocal microscopy to monitor the retrograde spread of a recombinant red fluorescent protein-expressing RSV construct (rrHRSV) [93] from the site of inoculation. Although rrHRSV has previously been used to study the susceptibility of various cell types to virus infection in vitro [93,94], our current report is the first wherein this biological agent has been used to trace the route and extent of infection after intranasal instillation of virus in vivo. To establish the usefulness of this approach, immunohistochemistry and plaque assay were used to verify the sensitivity and specificity of fluorescence detected at 2, 3, 5, and 14 days after infection. By these combined methods, we were able to follow the retrograde spread of virus infection from the respiratory epithelium of the nasoturbinates and nasopharynx (at the earliest time point) to the Eustachian tubes and ethmoid turbinates at later time points. The ability to visualize the anatomy and kinetics of HRSV replication in the uppermost airway can now form the basis for future studies of upper-airway susceptibility to virus reinfection and bacterial coinfection.
MATERIALS AND METHODS

Chinchillas. Juvenile male and female chinchillas (*Chinchilla lanigera*, n=12, weight, 347 +/- 37g, Rauscher Chinchilla Ranch, La Rue, OH) were included in this study. On the basis of physical examination by a licensed veterinarian, all animals were considered healthy upon arrival at our facility. Testing for specific pathogens was not performed. Animals were housed individually in a BSL2 facility and were offered a commercial pelleted chow and water ad libitum. Animals were acclimated for 7 to 10 d prior to challenge with rrHRSV. Video otoscopy and tympanometry revealed that all animals were free of middle ear disease at the time of rrHRSV challenge. All procedures used here were conducted humanely and have been described in detail elsewhere [91]. Animal housing and experimental procedures were approved by the Institutional Animal Care and Use Committee at the Research Institute at Nationwide Children’s Hospital.

Infection. The rrHRSV was constructed and rescued as described previously [93]. Anesthetized chinchillas were challenged intranasally with $1 \times 10^7$ pfu rrHRSV delivered in 80 µl sterile PBS, with the total dose divided equally between nares. Three animals were euthanized on each of the following days after rrHRSV challenge: 2, 3, 5, and 14. Chinchillas were monitored by a licensed veterinarian daily after rrHRSV challenge for signs of clinical disease as we as by video otoscopy and tympanometry for signs of middle ear inflammation (otitis media) [91].

23
Nasopharyngeal Lavage and Plaque Assay. Prior to euthanasia, chinchillas were anesthetized, and nasopharyngeal lavage fluids were collected as previously described [91]. Briefly, anesthetized prone chinchillas were allowed to passively inhale 500 µl of sterile PBS in droplets delivered by sterile pipet tip. Nasopharyngeal lavage fluids were collected from the contralateral naris as they were exhaled and stored at –80ºC. Viral titers in nasopharyngeal lavage fluids were determined by plaque assay performed as previously described [91].

Confocal Microscopy. At euthanasia, the skull was sectioned in a sagittal plane after removal of extraneous tissues. Whole sagittal sections of the chinchilla head included the nasopharynx, naso- and ethmoid turbinates, and olfactory bulb. The Eustachian tube was carefully dissected away from the inferior bulla then sectioned longitudinally. The middle ear was not examined in this study. The sagittal head and longitudinal Eustachian tube sections underwent confocal microscopy (LSM510 Meta laser module attached to an Axiovert 200M inverted microscope, Carl Zeiss, Thornasal washood, NJ). Thresholds were set using uninfected chinchilla tissue and then were maintained for evaluation of rrHRSV-infected tissues to significantly reduce or eliminate the contribution of background fluorescence.

Immunohistochemistry. After confocal microscopic analysis, tissues were fixed in neutral buffered formalin and decalcified with 0.35M EDTA in 0.1M Tris (pH 6.95) until no calcium could be detected via chemical endpoint assay [95]. Decalcified tissues were
paraffin-embedded, sectioned at 5 µm and stained with Hematoxylin and Eosin or, for α-RSV immunohistochemistry, were incubated with goat polyclonal RSV antiserum (Biodesign, Saco, ME) diluted 1:500 in buffer followed by incubation with biotinylated rabbit antigoat antibody (ScyTek, Logan, UT). Virus detection was accomplished by means of a streptavidin-horse radish peroxidase complex and aminoethyl carbazole as a chromagen (Scytek).
RESULTS

Nasopharyngeal lavage fluids contain infectious virus.

Unlike the mouse model, wherein instillation of HRSV is generally intratracheal following intranasal delivery of 50 to 100 µl virus suspension, a similar volume containing $10^7$ pfu virus remains in the nasopharynx of chinchillas. We did not detect lower respiratory tract infection in the chinchilla by plaque assay of lung homogenates following intranasal virus inoculation (data not shown).

We sought to assess both the path of virus infection in the chinchilla URT and the utility of the reagents available for these studies. To ensure that infection with rrHRSV was comparable to that observed with previously characterized RSV A2 strain [91], viral load in NP lavage fluids collected on days 2, 3, 5 and 14 after virus instillation was determined by plaque assay (Table 2.1). In all but one animal, virus was detected in nasopharyngeal lavage fluids by this method at one or more time points after HRSV challenge. Virus was found in nasopharyngeal lavage fluids on days 2, 3 and 5 after rrHRSV challenge but had cleared by day 14. Viral titers were not measured in all animals at all time points to avoid repeated exposure to anesthesia.

Gross and microscopic anatomy of the chinchilla nasal cavity.

The nasal cavity of the chinchilla is composed of the naso- and ethmoid turbinates rostrally and caudally, respectively, and the nasopharynx ventrally (Figures 2.1 and
2.2A). The nasoturbinates form the rostralmost portion of the nasal cavity and are composed of thin scroll-like bones lined by ciliated columnar respiratory epithelium interspersed with goblet cells. The ethmoid turbinates form the caudalmost portion of the nasal cavity and, like the nasoturbinates, are composed of thin scroll-like bones. The ethmoid turbinates, however, are lined predominantly by olfactory epithelium, but regions with respiratory and transitional epithelium are also present. The olfactory epithelium is composed of neuronal cell bodies and processes, superficial non-ciliated sustentacular cells, which cover the neuronal cell bodies and surround their processes, as well as the ducts from the submucosal Bowman glands. Figure 2.2B is centered on the ethmoid turbinate mucosa and demonstrates the transition from respiratory to olfactory epithelium. The respiratory epithelium just before the transition consists of tall columnar ciliated cells as well as basal cells. The relatively sharp transition between these epithelial types is marked in Figure 2.2B but can be easily appreciated by noting the abrupt absence of cilia. The nasopharynx connects the nasal cavity to the middle and lower respiratory tracts and is lined by ciliated columnar respiratory epithelium with interspersed goblet cells. The Eustachian tube, a tubal organ, connects the nasopharynx and the middle ear space. The mucosal surface of the Eustachian tube is lined by ciliated columnar epithelial cells interspersed with goblet cells proximally and transitions to low columnar to squamoid nonglandular nonciliated epithelium distally (Figure 2.2C) [46].
**Expression of red fluorescent protein marks the sites and progression of RSV infection.**

On day 2 after challenge with rrHRSV, the time point in which fluorescence was most intense at this site, red fluorescence was detected along the entire length of the nasopharynx (Figure 2.3). Fluorescence was observed as far retrograde as the nasoturbinates at this time but had not yet reached the ethmoid turbinates (Figure 2.4). By 3 d after challenge, the distribution of red fluorescence had expanded to involve all areas of the nasal cavity (Figures 2.3C and 2.4C, D), except for the nasopharynx, which was positive on days 2 and 5 (Figures 2.3B, D, and F). Red fluorescence on day 5 was similar in intensity and distribution to that observed on day 3 but was more intense in the ethmoid turbinates as the infection progressed (Figure 2.4).

To determine the kinetics of ascension of rrHRSV from the nares to the proximal (nasopharyngeal) and distal (bullar) Eustachian tube openings, we analyzed these structures. Red fluorescence was observed at the proximal orifice to the midpoint of the Eustachian tube by day 2 (Figure 2.3A) and had progressed to the distal portion of the Eustachian tube by day 5 (Figure 2.3E). Fluorescence was not detected in any portion of the nasal cavity or Eustachian tube on day 14 after HRSV challenge, the latest time point examined in this study.

There was remarkable consistency of virus infection kinetics between animals in this study, with rapid spread of infection from the nasopharynx to the nasoturbinates,
proximal Eustachian tube, and then to the distal Eustachian tube and ethmoid turbinates. In addition rrHRSV continues to replicate in the naso- and ethmoid turbinates after it is largely cleared from the nasopharynx. Although the human nasal cavity is much less complex than that of the chinchilla, HRV may persist in permissive locations in the human as well.

**Confirmation of RSV infection by immunohistochemistry.**

Unlike fluorescent imaging, immunohistochemistry offers an opportunity to determine the specific cell types infected within the upper airway and is thus an essential adjunct to confocal microscopy. Immunohistochemical labeling and confocal microscopic analysis of the Eustachian tube and the decalcified nasal cavity of rrHRSV-infected chinchillas produced similar results, with multiple small foci of virus infection detected with both techniques. Compared with confocal imaging, immunohistochemistry was slightly more sensitive, in that small clusters of antigen positive cells were found in the nasopharynx and nasoturbinate 3d (Figure 2.3) and in the ethmoid turbinate 14 d (Figure 2.5) after infection, in the absence of a fluorescent signal. These data indicate that use of red fluorescent protein-expressing HRV is a valid method for tracing the spread of infection but also demonstrate the importance of the complementary histologic information provided by immunohistochemistry.

Taken together, our data show that the red fluorescence observed by confocal microscopy was in fact due to the presence of intracellular rrHRSV protein expression in all types of
mucosal epithelium present in the nasal cavity including tall, ciliated, pseudostratified respiratory epithelium, transitional epithelium and olfactory epithelium (Figure 2.4F and 5). The ciliated respiratory epithelium is thought to be the primary target for RSV in the human host [45,94], but because nasal epithelium from infected patients is not examined in the course of routine medical practice, whether human olfactory epithelium is susceptible to RSV infection is unknown. We have previously described RSV-infected olfactory neurons in BALB/c mice, and we confirm here that olfactory epithelium is also a target for RSV infection in the chinchilla. Although specific markers for each cell type are not available for chinchillas, the RSV-infected cells within the olfactory mucosa are morphologically most consistent with neuronal cell bodies and processes.
DISCUSSION

Our interest in modeling upper airway infection with RSV arises from our interest in the pathogenesis of otitis media. Chinchillas, rats, gerbils and hamsters have been used to experimentally model human otitis media; however, to date only in chinchillas and ferrets [96] has it been possible to demonstrate the polymicrobial nature of otitis media, which always involves bacterial superinfection of a virus-compromised upper airway. Development of the first viral-bacterial superinfection model of otitis media, contributed to our understanding of the mechanisms by which influenza A virus predisposes to secondary invasion of the chinchilla middle ear by Streptococcus pneumoniae, [97-99] and our laboratory has used the chinchilla model similarly to study adenovirus-mediated predisposition to nontypeable Haemophilus influenzae of the Eustachian tube [100]. However, despite its predominance as a copathogen of this highly prevalent pediatric disease, HRSV infection of the URT and its association with bacterial otitis media has not been well studied.

Our limited understanding of URT infection by RSV is largely due to lack of a relevant animal model in which to explore questions regarding RSV pathogenesis in the Eustachian tube and middle ear (tubotympanum). In an attempt to develop such a model, we have characterized HRSV infection of the uppermost airway in both murine and chinchilla hosts [91] challenged in a manner designed to restrict viral delivery to the nasal cavity without dose-loss to either the GI tract or lungs [101]. In the cited study,
chinchillas proved to be more permissive to upper airway infection than were BALB/c mice, in which infection is largely restricted to the lungs [91]. In the chinchilla, as in human hosts, HRSV is primarily an upper airway infection, and in the current study, we have used a variety of tools to map the anatomy of URT infection by this virus. Although chinchillas did not develop signs of clinical disease at any time during the study period, microscopic examination of nasal mucosa revealed mild multifocal neutrophilic and lymphocytic infiltrates (data not shown).

HRSV spread from the site of inoculation to the pharyngeal orifice of the Eustachian tube by 48 h after challenge in chinchillas, but 5 d were required before virus could be detected in the distal-most aspect of the Eustachian tube. Virus was present in the nasoturbinates (equivalent to the human concha) 2 d after challenge and as far retrograde as the ethmoid turbinates by day 3. Immunohistochemistry allowed us to determine the microanatomy of infection, revealing not just the location of infection but also the cell types infected. In general, as has been reported for well-differentiated, polarized, pseudostratified human airway epithelial cells grown at the air-liquid interface [94], infection was limited to ciliated respiratory epithelial cells in chinchillas. However, in the ethmoid turbinates, immunopositive cells were identified within olfactory as well as respiratory epithelial mucosa. Although there are no reports of olfactory epithelial infection by RSV in human subjects, we previously demonstrated this localization in BALB/c mice, wherein we demonstrated foci of RSV antigen-positive cells in ductal epithelium as well as in bipolar neurons of the olfactory epithelium [91]. In the current
study, we also observed infection of bipolar neurons within the olfactory epithelium, demonstrating susceptibility of that cell type to infection in a second rodent species.

Viral clearance of rrHRSV, as measured by plaque assay and confocal microscopy, was complete by 5 days after infection, although clusters of immunopositive epithelial cells were still present in sections taken from the nasoturbinates and ethmoid turbinates on day 14. This result suggests that low-level upper airway infection continues beyond the observation period of the present study, and we are currently exploring the possibility of long term persistence at this site. Given the annual appearance of RSV in the absence of a known animal reservoir, it is certainly possible that some individuals may serve as persistently infected carriers. Although the site(s) of persistent infection in the mouse are unknown, viral genomic and mRNA were detected in lung homogenates of BALB/c mice that had been challenged intranasally with HRSV more than 100 days earlier [88]. The ease with which persistently infected primary human airway epithelial [94] and dendritic cell [102] cultures have been established also supports the idea that persistent HRSV infection in the absence of cytopathology may be possible, but an in vivo source of persistently infected cells has not yet been identified. Our previous demonstration of infected olfactory neurons and the identification of antigen-positive neurons in infected mouse lungs [103] suggests another possible virus reservoir.

In conclusion, unlike the relatively resistant BALB/c mouse, the more susceptible chinchilla likely will prove to be a useful model for studies of RSV infection of the upper
airway. Now that the kinetics of Eustachian tube compromise by the virus have been established, we can explore both bacterial superinfection in that compartment as well as the mechanisms by which HRSV infection allows middle ear invasion by bacterial organisms that are normally nonpathogenic including nontypeable *Haemophilus influenzae* and *Moraxella catarrhalis*. In addition, the chinchilla model of URT infection provides a new platform to address host defense mechanisms against reinfection, including why these defenses are inadequate in the case of HRSV.
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**Table 2.1. Infectious rrHRSV in nasopharyngeal lavage fluids.** Nasopharyngeal lavage fluids were recovered as indicated after intranasal infection with $1 \times 10^7$ pfu rrHRSV. Viral load ($x 10^3$ pfu rrHRSV/ mL) was determined by plaque assay. Virus load in the current study correlated with that found in nasopharyngeal lavage fluids from chinchillas challenged intranasally with the rrHRSV parent A2 strain (data not shown).
Figure 2.1. **Gross anatomy of the chinchilla nasal cavity.** Parasagittal section of an unfixed chinchilla head. The nasal cavity is composed of nasoturbinates rostrally, ethmoid turbinates caudally and the nasopharynx ventrally. Bar, 5 mm.
Figure 2.2. Microscopic anatomy of the chinchilla nasal cavity. (A) Chinchilla head, parasagittal section. The naso- and ethmoid turbinates are separated by the sub-ethmoid shelf. (B) Ethmoid turbinate. The relatively sharp transition to olfactory epithelium is marked by the abrupt absence of cilia. (C) Eustachian tube, longitudinal section. RSV infection of the Eustachian tube begins at the nasopharyngeal opening and spreads retrograde toward the middle ear space as infection progresses. Hematoxylin and eosin stain; bar: 5 mm (A), 100 µm (B), 500 µm (C).
Figure 2.3. Eustachian tube and nasopharynx of rrHRV-infected animals, confocal microscopy and immunohistochemistry. Multifocal punctate red fluorescence was detected in the proximal portion of the Eustachian tube on days 2 and 3 (A, C) and in the distal portion of the Eustachian tube on day 5 (E). Multifocal to widespread punctate red fluorescence was detected in the nasopharynx on days 2 (B) and 5 (F) and was most intense on day 2. HRSV-infected cells were not detected at these sites by confocal
microscopy or immunohistochemistry on day 14 (not shown). The degree and location of RSV infection determined using these two detection methods were highly correlative. The single exception was the nasopharynx on day 3 (D), in which red fluorescence was absent whereas clusters of RSV infected cells were detected by immunohistochemistry. Insets depict increased magnification of the same field. Immunohistochemistry and confocal microscopy of sham-infected (saline only) chinchillas was negative in all sections examined (data not shown). Bar, 100 µm.
Figure 2.4. Naso- and ethmoid turbinates of rrHRSV-infected animals, confocal microscopy and immunohistochemistry. Widespread punctuate red fluorescence was detected throughout the nasoturbinates at similar intensity on days 2 (A), 3 (C), and 5 (E). Neither red fluorescence (B) nor immunopositive cells (D) were detected in the ethmoid turbinates on day 2. However, by day 3 (D) and increasing in intensity by day 5 (F), multifocal punctate red fluorescence as well as immunopositive cells were detected at this anatomic site. HRSV-infected cells were not seen by confocal microscopy on day 14 (not shown). Insets depict increased magnification of the same field. Bar, 100 µm.
Figure 2.5. Ethmoid turbinate 14 d after rrHRSV infection, immunohistochemistry.

Although red fluorescence was not detected at any site on day 14, occasional immunopositive cells were observed in the ethmoid (as shown) or nasoturbinates (not shown) of 2/3 of animals at this time point, suggesting that the nasal cavity may serve as a site for prolonged HRSV replication in the chinchilla. Bar, 100 μm.
Chapter 3:
The Cotton Rat Model of RSV Infection
Human Respiratory Syncytial Virus (RSV) is the cause of a mild upper airway disease characterized by symptoms similar to the “common cold”. However, in neonates and the elderly, a large proportion of RSV infections spread to the lower airway, which can result in severe, even fatal, pneumonia. Mounting evidence suggests that infants who recover from severe RSV pneumonia are highly predisposed to asthma and recurrent wheezing later in childhood. Progress toward understanding the mechanisms of RSV pathogenesis has been hampered by lack of relevant rodent models. The mouse, the species most utilized to study RSV infection, is minimally susceptible to infection of the upper airway and infection of the mouse lower airway is only accomplished by administration of viral loads many orders of magnitude greater than required for human infection. These large viral loads must be delivered in large volume inoculums that circumvent natural spread of infection from the upper to the lower airway. To address the need for better rodent models of RSV infection, we characterized the acute and chronic pathology of RSV infection of the cotton rat. We demonstrate that virus delivered to the upper airway results in robust RSV replication in ciliated respiratory epithelial cells of the nasal cavity and, to a lesser extent, of the lung. Unexpectedly, we observed chronic lung pathology reminiscent of human asthma. These data support use of the cotton rat as a robust rodent model to examine the pathogenesis of RSV infection including the association between RSV pneumonia and subsequent development of asthmatic lung disease.
Human Respiratory Syncytial Virus (RSV) is the most important cause of lower airway disease in children worldwide and is second only to influenza virus as a cause of pneumonia in the elderly [104]. While RSV infection is generally mild and limited to the upper airway, significant sequelae can occur including severe pneumonia and middle ear infections (otitis media) [105,106]. More recently, it has been demonstrated that infants suffering from severe RSV pneumonia are predisposed to development of asthma and recurrent wheezing later in childhood [107-109].

RSV is ubiquitous worldwide, but the incidence of upper airway infection is difficult to quantify, as treatment for such infections is generally not sought. Given that lung involvement occurs in only a minority of cases, however, it is clear that upper airway infections greatly outnumber lower airway infections. The incidence of lower airway infection, on the other hand, can be estimated with reasonable accuracy, as medical care is much more commonly required compared to upper airway infection. The global significance of lower airway RSV infection in children was highlighted by a recent estimate that annually there at least 33.8 million episodes of RSV-induced acute pulmonary infection in children less than 5 years of age, and that as many as 200,000 of those episodes are fatal [10]. While supportive treatment for severe RSV pneumonia is highly effective in infants, access to such treatment is generally only available in
industrialized countries. Thus, more than 90% of fatal RSV pneumonia cases occur in the developing world [10].

Mice have been used extensively to study RSV infection, yet there are major limitations to using the mouse to study human RSV disease and immunity. The most important limitation is the poor permissivity of the mouse; while RSV will replicate to a limited extent in the lung of this host, large viral loads are required and virus must be delivered by “drowning” the lung with large volume inoculums, a method which circumvents spread of infection from the upper to the lower airway. Additionally, the pattern of lung infection in the human and mouse are markedly divergent. In the human, RSV primarily infects ciliated bronchiolar epithelial cells and, to a much less extent, alveolar lining cells [45]. In contrast, bronchiolar epithelium is only rarely infected in the mouse lung; RSV instead targets flattened alveolar lining cells [110]. Finally, unlike the human, in which the upper airway is the primary target of RSV replication, the mouse is poorly susceptible to infection of this compartment, with no virus detected in nasal washes by plaque assay and only rare RSV-antigen positive cells detected by immunohistochemistry [91,92,111]. Conversely, the cotton rat airway is quite susceptible to RSV infection of the upper and lower airway and the pattern of infection mirrors that seen in humans. The cotton rat was established as a model of RSV infection over three decades ago and has since emerged as the preferred rodent model in which to evaluate RSV therapeutics and vaccine candidates [46]. The relevance of the cotton rat as a model of RSV infection is highlighted by the FDA-approved advancement of RSV-specific antibodies, currently the only prophylaxis
for severe RSV pneumonia, to clinical trials based on safety and efficacy studies performed in the cotton rat [112-114]. Additionally, as availability of cotton rat specific reagents has improved, the cotton rat has become an increasingly useful model in which to study RSV pathogenesis [115]. While the susceptibility of the cotton rat to RSV infection has been firmly established, the acute and chronic pathology of RSV infection in this species have not been adequately characterized.

While it is clear that asthma is a multifactorial disease, dependent on both genetic and environmental factors (see [18,116] for recent reviews), many studies have pointed to a role for respiratory virus infection in the induction of asthma and exacerbation of wheezing. Gern et al. [117] have calculated that the combination of atopy and the presence of virus in nasal secretions synergistically increased the odds ratio for wheezing in children 25-fold and Jackson et al. [118] found that repeated rhinovirus infections in the first 3 years of life increased the risk of developing asthma by age 6 years 26-fold. This relationship is obviously a complex one, influenced both by the nature and the timing of the virus infection. Nonetheless, up to 80% of acute asthma exacerbations in children and approximately 50% in adults, are related to virus infection [113,119]. The majority of these infections are attributed to rhinoviruses, a large family of related viruses which generally result in a relatively mild upper airway infection in normal subjects – also known as the “common cold”. Other respiratory virus infections can also provoke these attacks including influenza virus, RSV and coronavirus. Beyond exacerbation of established asthma, there is mounting evidence that severe RSV pneumonia as an infant
is associated with development of asthma and recurrent wheezing in later childhood. An association between lower airway RSV infection and subsequent development of recurrent wheezing and asthma was demonstrated over 30 years ago [120] and several recent prospective studies have firmly established this link [108,121].

Here we expand upon existing knowledge of the cotton rat model of RSV infection by fully characterizing the early (acute) and late (chronic) pathology of the upper and lower airway following viral challenge. We confirm that RSV robustly replicates in the cotton rat respiratory tract and demonstrate that infection of the species is primarily an upper airway phenomenon. We also demonstrate that primary RSV infection of the cotton rat lung, even at the low levels observed in this study, induces pathology that resembles that observed in cases of human allergic asthma. Thus, our expanded characterization of the cotton rat model of RSV infection provides important information that will guide future use of this model for evaluation of RSV vaccine candidates and therapeutics, for exploring mechanisms of RSV pathogenesis and for novel purposes such as study of the association between RSV pneumonia and subsequent development of asthma and recurrent wheezing.
MATERIALS AND METHODS

Cotton rats. 6-10 week old cotton rats (*Sigmodon hispidus*) were used in this study (Sigmovir Biosystems, Inc., Bethesda, MD, USA). Animals were housed in groups of 3-4 in a BSL 2 facility and were offered a commercial pelleted rat chow and water ad libitum. On the basis of physical examination by a licensed veterinarian, all animals were considered healthy upon arrival at our facility. Testing for specific pathogens was not performed. Animals were acclimated for 7 to 10 days prior to viral challenge. All procedures used here were conducted humanely. Animal housing and experimental procedures were approved by the Institutional Animal Care and Use Committee at the New York University School of Medicine.

Viral Infection. Human RSV strain A2, originally obtained from ATCC (VR-1540), was passaged on murine STAT1−/− fibroblast monolayers as previously described [122]. While under isofluorane anesthesia, cotton rats were intranasally (i.n.) challenged with 1 x 10^6 PFU RSV, or were mock challenged with sterile saline, in a 50 µl total volume divided equally between nares. The viral inoculum was delivered slowly, over approximately 15 seconds, in a small volume in order to restrict initial virus infection to the upper airway. For each time point, 8 cotton rats were challenged. For saline controls only one time point, 1 day after saline challenge, was performed. Nasal wash and bronchoalveolar lavage (BAL) fluids were collected from 4 animals while lung (right lung for plaque assay and left lung for histology and immunohistochemistry) and nasal
cavities (for histology and immunohistochemistry) were collected from the other 4 animals. Thus, each sample/tissue was used for only one purpose (e.g. lung histology was not performed on lungs that had been lavaged with saline).

**Viral Plaque Assay.** Cotton rats were euthanized by CO\textsubscript{2} asphyxiation at multiple time points, up to 28 days, after viral challenge. Nasal wash fluids and lungs were collected immediately after euthanasia and stored at \(-80^\circ\text{C}\) until use in viral plaque assay. Viral plaque assay was performed on murine STAT1 \(-/-\) fibroblast monolayers as previously described [91].

**BAL cell analysis.** BAL fluids were collected from cotton rats immediately after CO\textsubscript{2} asphyxiation by washing the lung with 2 ml of sterile saline. BAL fluid cell counts were determined by hemocytometer and BAL differentials were determined on Wright-Geimsa stained CytoSpin preparations (Thermo Scientific, Waltham, MA, USA).

**Histology & Immunohistochemistry.** Nasal cavities and lung were collected immediately following CO\textsubscript{2} asphyxiation. Nasal cavities were fixed in neutral buffered formalin then decalcified with 0.35M EDTA in 0.1M Tris (pH 6.95). The right lung and decalcified nasal cavities were processed routinely, paraffin-embedded and sectioned at 5 \(\mu\text{m}\). Tissues sections were stained with hematoxylin and eosin (H&E) or were left unstained for immunohistochemistry. For IHC, tissue sections were incubated with goat polyclonal RSV antiserum (Biodesign, Saco, Maine, USA) diluted 1:500 followed by.
incubation with biotinylated α-goat (rabbit) antibody then HRP (ScyTek, Logan, UT, USA). Virus detection was accomplished with the streptavidin link and AEC chromagen (Scytek, Logan, UT, USA). IHC-labeled tissue sections were counterstained with hematoxylin. Slides were scanned to 400x magnification with a Leica SCN400 digital slide scanner and images were captured with Leica image viewer software (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

**Statistical Analysis.** SigmaPlot 12.0 (Systat Software, Inc., San Jose, CA, USA) was used to perform student t-tests where appropriate. A p-value < 0.05 was considered statistically significant.
### RESULTS

**RSV robustly infects the cotton rat upper airway and only sporadically infects the cotton rat lung.**

While the cotton rat has been used extensively to pre-clinical evaluate RSV therapeutics and vaccine candidates, RSV infection of this species has not been extensively characterized. To address this gap, we performed intranasal RSV infections of the cotton rat using small volume viral inoculums, delivered slowly, to restrict initial infection to the upper airway. We then tracked viral replication and associated pathology during the acute infection and at a late time point, 28 days after infection, when active virus infection was expected to be resolved.

Virus replication was detected in nasal cavity and lung tissues via plaque assay and immunohistochemistry. Quantitative plaque assay revealed that viral load in both the upper and lower airway was greatest on day 5 after viral challenge (Fig. 3.1). Maximum viral load was equivalent in nasal cavity and lung tissues.

Immunohistochemistry for RSV antigens revealed robust, diffuse virus replication in the nasal cavity, which peaked on day 5 (Fig. 3.2) and only scattered virus replication in the lung, which peaked on day 4 (Fig. 3.3). While 100% of cotton rats in this study had robust and widespread RSV infections of the upper airway, lung infection was always sporadic, with only a few clusters of immunopositive cells detected, even at the peak of
infection. Although RSV antigen was detected in 100% of cotton rat lungs 2 days after viral challenge, the number of infected cells was very low. By 4 days after RSV infection, the peak of viral replication in the lung, RSV antigen was detected in the lung of only 75% of animals. RSV antigen was detected in the lung of only 25% of animals by day 6, again at very low levels, a time point at which infection of the upper airway was still robust in 100% of animals. RSV antigen was not detected in the lung beyond the 6 day time point (data not shown).

Importantly, just as is seen in the human, but in contrast to the mouse, ciliated respiratory epithelial cells which line the nasal cavity and conducting airways of the lung were the primary target for RSV infection in the cotton rat [45,123]. In addition to infection of ciliated respiratory epithelial cells, we also observed RSV infection of olfactory epithelial cells of the caudal upper respiratory tract, which was often associated with syncytia formation and collapse of the mucosa (Fig. 3.2 B). As acute infection progressed through day 6, the upper airway contained abundant mucus with sloughed epithelial cells, many of which were positive for RSV antigens (Fig. 3.2 D and F). Abundant mucus production is observed clinically in cases of upper airway RSV infection [25], thus the excessive mucus production observed in the cotton rat upper airway provides further validation that RSV infection of the cotton rat recapitulates human infection.

The result of the plaque assay and the immunohistochemistry findings on these cotton rat tissues are seemingly contradictory, with robust lung infection suggested by plaque assay
data and limited lung infection demonstrated by immunohistochemistry. Differences in tissue density and collection methods between the upper and lower airway may affect plaque assay results by artificially decreasing viral load in the upper airway and artificially increasing viral load in the lower airway. Thus, we feel that immunohistochemistry is a more accurate reflection of the intensity of viral infection in each compartment.

**RSV infection persists in the upper airway.**

One of the last remaining uncertainties of RSV epidemiology is the basis for seasonal outbreaks. RSV outbreaks occur only in the winter months, yet no animal reservoir, which could sustain the virus between human outbreaks, has been identified. While not convincingly demonstrated, it is suspected that the upper respiratory tract of some individuals harbors low-levels of virus throughout the year and that during seasonal outbreaks, for reasons unknown, virus replication resumes, virus particles are shed and infection of the human population is maintained [88,89]. In support of this hypothesis, we previously demonstrated that virus is detected in the chinchilla upper respiratory tract as late as 14 days after RSV infection, a time point at which productive infection is not be detected by plaque assay [124]. We wished to determine if the cotton rat upper airway is similarly permissive to persistent RSV infection. To accomplish this, we collected nasal cavities 28 days after RSV infection and examined mucosal tissue RSV antigen by immunohistochemistry (Fig. 3.4). Surprisingly, 100% of animals displayed low-level viral antigen expression, suggesting low-level RSV replication, in the upper airway at this
time point. In contrast, RSV antigens were not detected in the lung beyond the 6 day time point (data not shown).

**RSV infection of the cotton rat upper airway incites intense acute inflammation with rapid resolution.**

It has been long recognized that RSV replicates robustly in the cotton rat upper airway, but the inflammatory response to RSV infection in the cotton rat upper airway is generally not evaluated in studies involving RSV infection of the cotton rat [125-128]. One early report briefly described upper airway inflammation in the RSV-challenged cotton rat, but in this, as in many other studies in which RSV infections are performed, a large viral inoculum designed for direct infection of the lung was used, making interpretation of these findings difficult [46]. Thus, here we thoroughly characterize the pathology in the cotton rat upper airway following intranasal delivery of a small volume RSV inoculum.

We found that RSV infection of the cotton rat airway incited marked inflammation and mucosal pathology that peaked 5 days after infection and was essentially resolved by the 28 day time point. At the peak of the inflammatory response (Fig. 3.5 C), to a lesser extent one day prior to the peak (Fig. 3.4B) and for up to one week (Fig. 3.5 D and E) after RSV infection, the nasal cavity mucosa, including the epithelium and the underlying lamina propria, was infiltrated by large numbers of lymphocytes with fewer histiocytes, eosinophils and neutrophils. Additionally, mucosal epithelial changes including
disorganization, atrophy and loss of cilia were pronounced (Fig. 3.5 C). Evidence of mucosal epithelial regeneration was observed as early as 6 days after RSV infection (Fig. 3.5 D) and was well underway by the 7 day time point (Fig. 3.5 E). Epithelial regeneration was characterized by increased mitoses and hypertrophy of basal cells. Resolution of nasal mucosal inflammation was essentially complete by 28 days after RSV infection, with moderate widespread hyperplasia of mucus-producing goblet cells (Fig. 3.5 F, top) and minor scattered foci of mucosal inflammation (Fig. 3.5 F, bottom) serving as the only evidence of prior injury.

**Acute RSV infection of the cotton rat lung is characterized by bronchiolitis with eosinophilic infiltrates, regenerative bronchiolar epithelial changes and rapid resolution.**

Because of the clinical significance of lower airway RSV infection resulting in bronchiolitis and pneumonia, RSV vaccination strategies are primarily targeted at prevention of spread of RSV infection from the upper to the lower airway. Consequently, RSV infection of the cotton rat lung has been much more extensively documented than RSV infection of the cotton rat upper airway. Despite this, RSV infection of the cotton rat lower airway remains poorly described. Thus, we sought to thoroughly characterize acute RSV infection in the cotton rat lung.

We observed moderate multifocal peribronchiolar inflammation with occasional inflammation of the alveolar septae that was maximal 5 days after RSV infection (Fig.
Inflammation was characterized by influx of lymphocytes with fewer histiocytes and eosinophils that surrounded bronchioles and migrated through the mucosal epithelium or, in the case of alveolar infiltrates, expanded alveolar septae and filled alveolar spaces. Coincident with inflammatory influx was the appearance of bronchiolar epithelial changes including disorganization, loss of cilia, occasional necrosis characterized by shrunken hypereosinophilic cytoplasm and pyknotic nuclei and hypertrophy of basal cells. The acute inflammatory response was rapidly resolved, with few lymphocytes and only rare eosinophils present in the epithelial mucosa 10 days after RSV infection (Fig. 3.6 G-I). While inflammation was largely resolved, evidence of ongoing epithelial repair, characterized by flattened, squamoid bronchiolar epithelium, loss of cilia and frequent hypertrophy of epithelial cells, was observed at the 10 day time point.

**BAL Cytology reveals long-lasting pulmonary eosinophilia in the cotton rat following RSV infection.**

Histopathology is an invaluable tool when evaluating any animal model of disease and BAL fluid analysis is an important complementary method to assess the lung inflammatory process. The BAL procedure, which can be performed on live animals and humans, samples cells free in the airways, cells which can then be identified and quantified. To provide quantitative information regarding the cellularity and cellular composition of the lung airways in response to RSV infection, we collected BAL fluid up to 28 days after viral challenge. We observed a mild increase in BAL fluid cellularity
(Fig. 3.6A) that peaked on day 6 and essentially returned to baseline by the 28 day time point. Despite only mild changes in overall cellularity, BAL fluid cytology revealed marked shifts in cellular composition in response to RSV infection (Fig. 3.7 B). At baseline, after saline challenge, BAL fluid was composed predominately of monocytes. This was not an unexpected finding, as BAL fluid from healthy animals of many other species is composed predominately of this cell type. What was unexpected, and is unreported, was that the percentage of eosinophils in the lung of unchallenged cotton rats hovered around 30%, a much higher percentage than is observed in other species. Interestingly, in response to RSV challenge, there was a complete reversal of the predominant BAL fluid cell type from monocytes to eosinophils. The predominance of eosinophils in the BAL fluid was highly significant and persisted to the 28 day time point.

**RSV infection of the cotton rat is characterized by chronic asthma-like lung pathology.**

It has been increasingly recognized that a major predisposing factor for development of asthma and recurrent wheezing in childhood is severe RSV pneumonia as an infant [108,109]. To determine if primary RSV infection was associated with chronic allergic-type lung disease in cotton rats, we collected lungs 28 days after RSV infection and examined them by histology. Chronic changes in the cotton rat lung following RSV infection were characterized by mucosal and goblet cell hyperplasia (Fig. 3.8, A-F), eosinophilic peribronchiolar inflammation (Fig. 3.8, B and C) with increased
peribronchiolar mast cells (Fig. 3.8 E). Additionally smooth muscle surrounding bronchioles was often hypertrophied and hyperplastic (Fig. 3.8, A, D and G). Finally, the lumen of terminal airways was sometimes plugged by accumulations of eosinophils, sloughed epithelial cells and mucus (Fig. 3.8, H and I).
DISCUSSION

Here, we describe the acute and chronic lung pathology following RSV infection of cotton rats. We found that, after slow delivery of small volume viral inoculums, RSV replication occurs primarily in the cotton rat upper airway. RSV infection incited intense acute upper airway inflammation accompanied by degenerative epithelial changes. Acute inflammatory lesions in the lung, limited primarily to the bronchioles and adjacent surrounding parenchyma, were much less severe but were also accompanied by degenerative bronchiolar epithelial changes. In both compartments, acute inflammatory responses were rapidly resolved. Unexpectedly, we found that BAL fluid collected from naïve cotton rats contained a high baseline level of eosinophils and that the percentage of eosinophils increased dramatically in response to acute RSV infection. While goblet cell hyperplasia was the only evidence of prior injury of the upper airway a month after RSV infection, low numbers of cells containing RSV antigen were observed at this late time point. In contrast, chronic lung pathology was more severe than that seen acutely, despite only minimal RSV replication which was undetectable beyond the 6 day time point. Chronic lung changes were characterized by bronchiolitis with infiltration of eosinophils and mast cells as well as airway remodeling as evidenced by bronchiolar smooth muscle hyperplasia. Overall, these findings expand the current description of RSV infection of the cotton rat and demonstrate that primary infection is associated with an acute eosinophilic inflammatory response in the lung with chronic pathology similar to what has been described in human asthma patients.
Safety issues limit study of RSV infection in humans, so animal models have been developed in which to study the mechanisms by which RSV causes disease. The most common animal models utilized to study RSV disease include rodents, primarily the mouse, and, much less frequently, non-human primates [44]. Heterologous models, in which a related virus infection in its natural host is studied with the goal of making correlates to human RSV infection, including bovine RSV (bRSV) infection of calves and pneumonia virus of mice infection of mice, have also been developed [129-131]. While none of these models reproduces all aspects of human disease, each has its strengths. An understanding of the advantages and disadvantages of each model will guide animal model choice for a particular study (for a recent review of animal models of RSV disease see [132]). For example, studies focused on immune mechanisms of disease are generally performed in the mouse because of the plethora of reagents and genetically modified strains available, as well as the relatively low cost to purchase and house this species. The bovine, whose genome has been sequenced and reagent availability continues to increase, has been similarly used to study immune mechanisms of bRSV infection. Evaluation of RSV vaccine candidates and therapeutics has been commonly performed in cotton rats because of the permissivity of the upper and lower airway of this species to RSV replication.

The cotton rat is susceptible to a wide range of human pathogens. While there are no genetically modified strains, inbred and microbiologically defined cotton rats are commercially available. The list of immunologic reagents available for the cotton rat is
much shorter than for the mouse, but as demand increases, the availability of cotton rat- 
specific reagents continues to expand (see [55,133,134] for reviews of the cotton rat in 
biomedical research and as a model of human RSV infection).

Experimental RSV infection of the cotton rat was first reported in 1971 and the 
inflammatory response to RSV infection of the cotton rat was first described in 1978 
[46,135]. Prince and colleagues described mild epithelial changes in the nasal cavity 
mucosa and bronchioles with mild neutrophilic intraepithelial infiltrates that were most 
severe in the cotton rat upper and lower airway 4-6 days after challenge with the Long 
strain of RSV. Prince and colleagues then went on to describe the pulmonary lesions of 
RSV infection, re-infection and vaccine-enhanced disease in the cotton rat as mild 
lymphocytic peribronchitis and perivasculitis, neutrophilic bronchitis which was more 
severe after secondary virus challenge and, in those animals that received an inactivated 
RSV vaccine prior to viral challenge, low numbers of neutrophils in the lung interstitium 
and alveoli [136]. Most subsequent studies which utilize the cotton rat to study aspects of 
RSV infection are focused on the effect of a specified preventative or treatment on virus 
replication and clearance and focus little or no attention on the pathologic process 
[113,125-127,137,138]. Thus, the two early reports of RSV infection in the cotton rat are 
the most extensive descriptions of the pathologic lesions associated with infection of this 
species.
Of note, in these initial descriptions of cotton rat RSV infection, and in the majority of subsequent studies in which RSV infection of cotton rats and mice was performed, large volume viral inoculums were delivered in order to deposit virus directly to the lung. Direct infection of the lung in this manner bypasses the natural kinetics of infection from the upper to the lower airway. While others have reported RSV replication in the lung that is equivalent to that seen in the upper airway, we observed much lower levels of lung infection compared to nasal cavity infection. This was likely due to our method of virus delivery, which was designed to anatomically restrict initial virus replication and to allow for physiologic viral spread from the upper to lower airway.

Limited access to clinical specimens has prevented study of the histologic changes related to RSV infection of the human upper airway. Thus, it is not possible to compare the pathologic changes in response to RSV infection of the human and cotton rat upper airway. It is reasonable to assume, however, that RSV infection of its natural human host incites an inflammatory response in the upper airway that is equal to or greater than that observed in the cotton rat. The character of the inflammatory response following RSV infection of the human upper airway could be approximated by evaluation of nasal washes collected during acute upper airway infection, but such evaluations have not been performed in humans. However, the goblet cell hyperplasia observed in the cotton rat upper airway during the chronic response to RSV infection is in accordance with the intense mucus production observed clinically following RSV infection of humans [25].
It has been suggested that persistent human infections may be the basis for the seasonality of RSV outbreaks [88,89]. Studies in animal models suggest that RSV may remain “latent” or replicate at very low levels for prolonged periods in the lung [88,139-141]. In these animal studies, sensitive techniques (RT-PCR) were able to detect viral genomic RNA in the lung as long as 100 days after RSV infection. We have previously demonstrated by immunohistochemistry that RSV antigens can be found in the chinchilla upper airway for at least two weeks after RSV challenge [124] and in this study we used the same method to demonstrate RSV antigens in the cotton rat upper airway one month after challenge. RSV antigens were detected for a maximum of only 6 days in the lung following viral challenge of cotton rats in this study. It is possible, however, that a more RT-PCR would have detected viral transcripts in the lung at later time points. Overall, detection of RSV antigens in the upper airway at late time points, both in the chinchilla and cotton rat, support the hypothesis that RSV circulates at low levels between outbreaks and/or remains “latent” in persistently infected individuals.

RSV replication in the lung was restricted to only a few scattered clusters of bronchiolar epithelial cells and only at early time points after challenge. This finding is of interest because, in both children and adults, RSV infection is most often limited to the upper airway, with only 40%-50% of primary infections in infants resulting in clinically-significant lower airway infection [106]. Therefore, it appears that RSV infection follows the same course in the cotton rat as it does in the human, making the cotton rat a very attractive model for studying RSV pathogenesis.
It is interesting that significant chronic pulmonary pathology resembling human allergic asthma was observed in all animals with similar severity even though virus replication in the lung was minimal. This suggests that either minimal levels of RSV replication for only a few days is sufficient to induce significant asthma-like lung pathology or that the severity of lung pathology is due to a combination of limited lower airway infection and cytokine effects of marked upper airway infection.

The predominance of eosinophils in BAL fluid and the appearance of eosinophils surrounding bronchioles and migrating through bronchiolar epithelium in response to primary RSV challenge of the cotton rat was an unexpected finding and is contrary to what has been reported in the human, in which primary RSV infection of the lung is mediated predominately by lymphocytes and neutrophils [45]. Notably, in this study, eosinophils comprised approximately 30% of the BAL fluid cells of cotton rats challenged only with saline. We observed a similar eosinophil percentage in BAL fluid collected from naïve, unchallenged cotton rats (data not shown), which leads us to conclude that the high baseline level of eosinophils in BAL fluids is a real phenomenon and not due to the challenge procedure itself. The baseline numbers of circulating eosinophils, however, is not elevated above what has been reported in other laboratory species [142,143] or humans [144] nor is it increased in response to RSV infection (our unpublished observation). The eosinophilic response we observed in the cotton rat lung may be a feature of paramyxovirus infection in this species, as high levels of eosinophils in BAL fluid has been demonstrated in cotton rats infected with measles virus [145].
Pulmonary eosinophilia, however, doesn’t appear to be a general reaction to virus infection in the cotton rat, as the pulmonary inflammatory response to the Hantavirus Black Creek Canal Virus, a natural cotton rat pathogen, is mediated by mononuclear cells [146].

Human asthma is a complex disease with an incompletely understood pathogenesis. While there are many potential inciting causes, including infection with a respiratory tract virus, the associated lung pathology in chronic cases is essentially identical, regardless of inciting cause. Pathologic hallmarks of chronic human asthma include goblet cell hyperplasia of the bronchiole and bronchiolar epithelium, airway infiltration by eosinophils and mast cells and hypertrophy and hyperplasia of bronchiolar smooth muscle [147,148]. In a long-term study in which children with severe RSV bronchiolitis as infants were followed for 20 years, more than 30% developed persistent wheezing up to 13 years of age which may extent into early adulthood [107].

The sole existing model for studying the association between pulmonary virus infection and allergic asthma is the mouse. The mouse has been utilized primarily to study the acute effects of virus infection following allergen-sensitization, but the long-term effects have also been studied. For example, long-term pulmonary hyperresponsiveness, as indicated by exaggerated response to the cholinergic agonist methacholine, and pulmonary inflammation for up to 154 days after RSV infection has been demonstrated [139,149]. The chronic inflammatory response observed in this long-term study was
characterized by accumulations of lymphocytes, histiocytes and plasma cells around large vessels and airways. While these findings are intriguing, the chronic changes reported in the mouse model do not correlate with the histologic picture of chronic asthma in humans. On the other hand, the chronic lung lesions following RSV infection in the cotton rat reported here much more closely mirror those seen in the human.

While the mechanisms by which RSV predisposed to allergic lung disease are unclear, our data indicate that this virus is a poor inducer of Th1 responses [150]. We propose that this may be due, at least in part, to the very limited type I and type III IFN response to this pathogen in mouse and man [150-153]. In support of this hypothesis, very recent work by a number of laboratories demonstrating a deficient type I (IFN-α/β) and type III (IFN-λ) response to virus infection in asthmatic and atopic patients [154-157]. These reports, along with the findings reported here, have encouraged us to begin studies of the relationship between, RSV infection, IFN induction, allergic sensitization, and the development of asthma in the cotton rat.

In summary, our expanded description of the cotton rat model of RSV infection confirms that RSV infection of this species is similar to that observed in humans. Our findings will aid in interpretation of future studies that use of this species to evaluate potential RSV therapeutics and vaccine candidates as well as to determine mechanisms of RSV pathogenesis. Additionally, our findings support future studies which utilize the cotton rat
to examine the relationship between RSV infection and development of asthma and recurrent wheezing.
Figure 3.1. Upper and lower airway viral load after acute RSV infection. To quantitatively evaluate viral load after RSV infection, nasal cavity and lung tissues were collected at multiple early time points after viral challenge. Viral load was determined by plaque assay. For both nasal cavity and lung tissues, viral load peaked 5 days after challenge. Peak viral titers were equivalent in the upper and lower airway. Data points represent means and error bars represent standard error of the mean.
Figure 3.2. Distribution and extent of acute RSV infection of the cotton rat upper airway. To determine the distribution and extent of upper airway RSV infection, nasal cavity tissues were collected from cotton rats at multiple early time points after RSV infection and examined for RSV antigens by immunohistochemistry. Widespread infection of ciliated respiratory epithelial cells was detected in 100% of animals 4-6 days after infection.
(A, C, E) after RSV infection with peak infection observed at the 5 day time point. Additionally, RSV infection was not limited to ciliated respiratory epithelial cells, but was also detected in olfactory epithelial cells of the caudal upper airway, where it was often associated with syncytia formation and collapse of mucosa (B, 4 days after RSV infection). Finally, as RSV infection progressed through days 5 and 6, mucus with embedded RSV antigen-positive sloughed epithelial cells was frequently present in upper airway lumens (D and F). Images were captured at 100x (A, C, E), 200x (B, D, F) or 400x (insets) magnification.
Figure 3.3. Distribution and extent of acute RSV infection of the cotton rat lower airway. To determine the distribution and extent of lower airway RSV infection, lung was collected from cotton rats at multiple early time points after RSV infection and examined for RSV antigens by immunohistochemistry. Sporadic infection of ciliated
respiratory epithelial cells lining bronchioles was detected in the lungs of 100%, 75% and 25% of animals 2 (not shown), 4 (A) and 6 (B) days after RSV infection, respectively. Lung infection, which was always low-level, decreased markedly by 6 after RSV infection as demonstrated by detection of only few weakly antigen-positive cells, with antigen restricted to the cilia. RSV antigen was not detected in lung collected after the 6 day time point (not shown). Images were captured at 200x or 400x (insets) magnification.
Figure 3.4. **RSV infection persists in the cotton rat upper airway.** To determine the duration of upper airway RSV infection, nasal cavity tissues were collected from cotton rats 28 days after RSV infection and examined for RSV antigens by immunohistochemistry. Sporadic antigen-positive ciliated respiratory epithelial cells (shown here) as well as sporadic antigen-positive olfactory epithelial cells in the caudal upper airway (not shown) were detected. This finding demonstrates RSV can be harbored in the upper airway for extended periods of time and supports the hypothesis that RSV remains dormant or latent in the human airway between seasonal outbreaks. Images were captured at 200x or 400x (inset) magnification.
Figure 3.5. Histopathology of acute and chronic RSV infection of the cotton rat upper airway. To determine the extent of mucosal pathology induced by RSV infection, nasal cavity tissues were collected from cotton rats up to 28 days after viral challenge and examined by histopathology. Naïve upper respiratory mucosa (A) is characterized by an orderly arrangement of ciliated respiratory epithelial cells, low numbers of basophilic, granular mucus-producing goblet cells infrequently scattered between ciliated cells and a poorly-cellular underlying lamina propria containing few stromal cells and mononuclear cells including lymphocytes and histiocytes. RSV infection induced marked epithelial and mucosal changes were most severe at the 5 day time point and were resolving by the
6 day time point. The nasal mucosa collected 4 days after infection (B) was disorganized, mildly atrophic with intermittent loss of cilia and infiltrated by mild to moderate numbers of lymphocytes, eosinophils and neutrophils. Similar, but more severe, histopathologic changes were observed in nasal mucosa collected 5 days after RSV infection (C). At the 5 day time point, epithelial disorganization was more pronounced and large numbers of inflammatory cells infiltrated the lamina propria as well as the epithelium. By 6 days after RSV infection (D), pathologic changes as described for days 4 and 5 persisted, but early evidence of epithelial regeneration was also observed characterized by hypertrophy of basal cells which were sometime undergoing mitosis (asterisk). Epithelial regeneration, characterized by cellular hypertrophy, was more pronounced by 7 days after RSV infection (E). Lingering histopathologic evidence of inflammation including intraepithelial abscesses (asterisk) as well as cilia loss were also observed at the 7 day time point. Finally, regeneration of nasal cavity mucosa and resolution of inflammation was essentially complete by 28 days after RSV infection, with goblet cell hyperplasia (F, top) and occasional intraepithelial inflammation (F, bottom) being the only histopathologic changes observed. Images were captured at 200x or 400x (insets) magnification.
Figure 3.6. Histopathology of acute RSV infection of the cotton rat lower airway. To determine the extent of lung pathology induced by acute RSV infection, lung was collected up to 10 days after viral challenge and examined by histopathology. Naïve lung (A-C) is characterized by thin alveolar septate and bronchioles which are lined by an orderly arrangement of ciliated respiratory epithelial cells and surrounded by a thin layer of connective tissue.
of smooth muscle as well as scattered stromal cells and mononuclear inflammatory cells including lymphocytes and histiocytes. By 5 days after RSV infection (D-F), there were many foci of moderate hypercellularity, particularly surrounding bronchioles, but also sometimes involving alveolar septae. Inflammatory cells, which migrated through the bronchiolar epithelium, consisted mainly of mononuclear cells including lymphocytes and histiocytes with fewer eosinophils (asterisk). In addition to inflammation, mucosal hyperplasia, characterized by disorganization, loss of cilia and hypertrophy of bronchiolar epithelial cells, was also observed. By 10 days after RSV infection (G-I), inflammation had largely resolved with only rare eosinophils present within the bronchiolar epithelium (asterisk). Mucosal repair was ongoing at the 10 day time point characterized by a flattened, squamoid appearance of the bronchiolar epithelium with cilia loss and frequent hypertrophy of epithelial cells. Images were captured at 40x (top panels), 100x (middle panels) or 400x (bottom panels) magnification. Boxes in middle panel images indicate the area of higher magnification in lower panel images.
Figure 3.7. Cytology of RSV infection of the cotton rat lower airway. To examine cellular responses to RSV infection, BAL fluid was collected from cotton rats up to 28 days after RSV infection and examined by cytology. RSV infection induced a mild, not statistically significant, increase in total number of BAL cells (A) that was most pronounced at the 6 day time point. BAL cell differentials to determine the cell types present (B) revealed a marked increase in eosinophil % as early as two days after RSV infection. The increase in eosinophil %, which was accompanied by a corresponding decrease in monocyte %, was highly statistically significant and persisted to the 28 day
time point. The quantitative increase in eosinophil % shown graphically in B is supported by BAL cytology (C) where eosinophils (E) were differentiated from neutrophils (N) by the presence of abundant brightly eosinophilic cytoplasmic granules. Monocytes (M), the second most predominant BAL cell type observed, are large cells characterized by abundant basophilic granular to vacuolated cytoplasm and frequently ruffled cell borders.

*p<0.05; **p<0.001; Data points represent the mean and error bars represent SEM; Image in C was captured at 400x magnification; abbreviations in C: E= eosinophil; N= neutrophil; M= monocyte.
Figure 3.8. Chronic lung histopathology after RSV infection. To determine if RSV infection induces chronic lung pathology, lungs were collected 28 days after viral challenge and examined by histopathology. In marked contrast to the upper airway, which was much more extensively infected by RSV compared to the lower airway yet was essentially normal by the 28 day time point, marked changes were observed in the
lung 28 days after RSV infection. Chronic lung changes included mucosal and goblet cell hyperplasia (A-F), peribronchiolar inflammation which was mediated by eosinophils (B and C) as well as mast cells (asterisk) (E). Additionally smooth muscle surrounding bronchioles was often hyperplastic (A, D, G) and mucus containing eosinophils and sloughed epithelial cells was frequently filling the lumen of terminal airways (H and I). Taken together, these changes are reminiscent of those observed in humans with chronic asthma and support the broadly recognized association between RSV lower airway disease and later onset of asthma and recurrent wheezing. Images were taken at 100x (left column) or 400x (middle and right columns). Boxes in A are areas of magnification in B and C; Boxes in D are areas of magnification in E-G.
Chapter 4:

Pre-clinical Evaluation of an RSV Vaccine Candidate
ABSTRACT

Human Respiratory Syncytial Virus (RSV) is the leading cause of lower airway disease in infants worldwide. Although RSV infection during infancy may lead to severe, even life-threatening, lower airway disease, the majority of these primary RSV infections are mild and limited to the upper airway or induce only mild pulmonary disease. Essentially all subsequent RSV infections are limited to the upper airway and such infections of healthy, immunocompetent individuals are repeated throughout life. The basis for the inadequate, short-lived adaptive immune response to RSV infection is poorly understood. It is likely that the ability of RSV to antagonize type I interferon (IFN) production leads to a weak immune response. IFN production during viral infection induces the anti-viral state and is critical for downstream development of robust, long-lived immunity. Based on the hypothesis that an RSV vaccine that induces robust IFN production would be protective, we previously constructed a Newcastle disease virus-vectored vaccine that expresses the F glycoprotein of RSV (NDV-F) and demonstrated that vaccinated mice that were challenged with RSV had reduced viral load in their lungs. Here we show that vaccination with NDV-F significantly reduced viral load in the respiratory tract of both chinchillas and cotton rats that were challenged with RSV. Additionally, vaccination induced robust, long-lived antigen-specific antibody production and was not associated with enhanced pathology. Finally, pulmonary eosinophilia and allergic-type airway disease induced by RSV infection of unvaccinated cotton rats were prevented by vaccination with NDV-F. This finding is of particular interest given the mounting
evidence that severe RSV pneumonia during infancy predisposes to asthma and recurrent wheezing in later childhood. Overall, these data demonstrate the feasibility of our approach and support progression of this RSV vaccine toward clinical trials.
INTRODUCTION

Human Respiratory Syncytial Virus (RSV), a negative sense RNA virus in Paramyxoviridae family, is the most important cause of lower airway disease in infants worldwide. Despite intense effort, an RSV vaccine has yet to be licensed. While RSV infection is limited to the upper respiratory tract in most healthy adults and children, it is the major cause of bronchiolitis and pneumonia in infants [8,9]. RSV outbreaks occur on an annual basis and essentially all persons are infected within the first two years of life. The most severely affected are infants under 6 months of age, but severe, even fatal, RSV pneumonia occurs in transplant recipients and the elderly [8]. Subsequent RSV infections, which are generally limited to the upper respiratory tract, are usually associated with mild cold-like symptoms in healthy adults, but are commonly associated with otitis media in young children [14,105,106]. In addition, severe RSV pneumonia as an infant has been associated with the development of asthma and exacerbation of wheezing in asthmatic patients [16,17,107,108].

Otitis media is the most common pediatric disease. There are multiple risk factors for development of otitis media such as attendance at day care and antimicrobial peptide gene polymorphisms, but recent or concurrent infection with an upper respiratory tract virus is overwhelmingly the most important predictor of otitis media development [158-160]. As sample collection and viral identification techniques have improved, RSV has
emerged as the most commonly isolated upper respiratory tract virus in cases of acute otitis media [75,81,161].

Because of the severity of RSV pneumonia, vaccination and research efforts have focused on prevention and understanding of lower airway infection. Given the viruses’ potential to cause severe pneumonia in infants, the strong association between severe RSV pneumonia in infancy and development of asthma and recurrent wheezing in later childhood, and the role of RSV in upper airway infection and in the development of otitis media, a vaccine that effectively prevents RSV infection of the upper airway would be predicted to have a significant impact on pediatric health.

Immunity to RSV is remarkably ineffective, even in immunocompetent children and adults, allowing for repeated infection throughout life [25,26]. Unlike other viral pathogens, serum antibody levels are very slow to rise following RSV infection, with a gradual accumulation of protective antibodies only after multiple re-infections [27,28]. Serum neutralizing antibodies develop following primary infection and tend to protect the lower airway against re-infection [14,162-164]. Serum antibodies are ineffective in the upper airway, however, and consequently the upper airway of healthy children and adults is infected repeatedly throughout life [14,25,72]. The inability of RSV to induce robust immunity following repeated natural infections very likely underlies the difficulties encountered in attempts to design effective, attenuated vaccine strains [36].
Our group hypothesized that the relative inability of RSV to generate a potent IFN response in mouse [153] or man [151,152,165] contributes to the relatively ineffective T and B cell responses to this virus. We tested this idea by constructing a vaccine vector based on the Hitchner B1 vaccine strain of Newcastle disease virus (NDV), an avian paramyxovirus that is nonpathogenic to humans or mice but is known to induce very high IFN levels in mice and mammalian cells [166,167]. IFNs, produced early in viral infection, induce the anti-viral state and are potent immunomodulators. IFNs are known to activate natural killer (NK) cells [168,169], upregulate DC costimulatory molecule expression [167,170,171] and to act directly on CD8+ T cells to stimulate clonal expansion and memory formation [172,173]. There is recent evidence to suggest that IFNs also have a role in antibody production by, and IgA class-switching of, B cells [174,175]. In addition to potent IFN production, NDV is advantageous as a vaccine vector because the vast majority of the human population is immunologically naïve to this virus and because clinical trials evaluating NDV as an oncolytic agent have demonstrated that this virus is safe for use in humans [176].

Previously, we demonstrated reduced viral load in the lungs of mice that had been vaccinated with NDV expressing the F protein of RSV (NDV-F) prior to RSV challenge and demonstrated that protection of mice correlated with a robust F-specific CD8+ T cell response [110]. In the present study, we evaluated NDV-F as an RSV vaccine candidate in the chinchilla and cotton rat, species which are more susceptible to RSV infection than the mouse ([91,124] see [177] for a recent review of the chinchilla as a rodent model of
human otitis media). We found that intranasal delivery of NDV-F induced robust long-lived antigen-specific serum and mucosal humoral immunity, largely eliminated RSV replication in the upper airway and completely prevented RSV replication in the lower airway. Additionally, we found no evidence of pathology associated with NDV-F vaccination or of enhanced pathology following RSV infection of NDV-F vaccinated animals. Finally, the eosinophilic, allergic-type, inflammatory response observed in the lung after RSV infection of the cotton rat was abrogated by NDV-F vaccination.
MATERIALS AND METHODS

Animals. Cotton rats. 6-10 week old female cotton rats (Sigmodon hispidus) were used in this study (Sigmovir Biosystems, Inc., Bethesda, MD, USA). Animals were housed in groups of 3 in a BSL 2 facility and were offered a commercial pelleted rat chow and water ad libitum. Animals were acclimated for 7 to 10 days prior to vaccination or viral challenge. Animal housing and experimental procedures were approved by the Institutional Animal Care and Use Committee at the New York University School of Medicine. All procedures used here were conducted humanely. Data were collected from 3-4 cotton rats per cohort per time point, unless otherwise specified.

Chinchillas. Sixteen Juvenile chinchillas (Chinchilla lanigera) (441 +/- 45 g) obtained from Rauscher Chinchilla Ranch, Llc (La Rue, OH, USA) were included in this study. Animals were acclimated for 7 - 10 days prior to use. Animal housing and experimental procedures were approved by the Institutional Animal Care and Use Committee at the Research Institute at Nationwide Children’s Hospital. All procedures were conducted humanely and have been described in detail elsewhere [91].

Virus stocks. NDV-F was constructed as previously described [110] then amplified in 10-day-old embryonated chicken eggs. Human RSV, strain A2, originally obtained from ATCC (VR-1540), was passaged on murine STAT1 +/- fibroblast monolayers as previously described [122].
**Vaccination.** *Cotton Rats.* While under isofluorane anesthesia, cotton rats were intranasally (i.n.) vaccinated with $1 \times 10^6$ PFU NDV-F, $1 \times 10^6$ PFU NDV vector alone, or allantoic fluid as a mock control in a 100 µl total volume divided equally between nares. Twenty-eight days after the priming vaccination, cotton rats were boosted in the same manner as for priming.

*Chinchillas.* Lightly anesthetized chinchillas were immunized i.n. by passive inhalation of $5 \times 10^5$ PFU NDV vector alone, $5 \times 10^5$ NDV-F or allantoic fluid (mock) in 80 µl total volume, divided equally between nares.

**Challenge Infection.** *Cotton rats.* Four (the 4 days after RSV infection time point only) or 10 weeks (all other time points) after the boosting vaccination, cotton rats in all vaccination cohorts (allantoic fluid, NDV vector only, NDV-F) were i.n. RSV challenged. The nasal cavities of cotton rats under isofluorane anesthesia were instilled with $1 \times 10^6$ PFU RSV in a 50 µl total volume, divided equally between nares. The viral inoculums were delivered slowly, over approximately 15 seconds, in a small volume in order to restrict initial virus infection to the upper airway. Seven months after the primary RSV challenge, all cotton rats received a secondary RSV challenge. Secondary RSV challenge was performed as for the primary RSV challenge.
*Chinchillas.* Twenty-eight days after immunization, chinchillas were i.n. challenged with $1 \times 10^7$ PFU RSV in 80 µl total volume by passive inhalation as described for immunization.

**Viral plaque assay.** Nasal lavage fluid was collected from anesthetized chinchillas as previously described [91] prior to and after immunization and after RSV challenge. Briefly, anesthetized prone chinchillas were allowed to passively inhale 500 µl of sterile pyrogen-free phosphate buffered saline in droplets delivered by sterile pipet tip. Nasopharyngeal lavage fluids were collected from the contralateral naris as they were exhaled. Nasal wash fluid and lungs were collected immediately after euthanasia of cotton rats. Samples were stored at $-80^\circ$C until use in viral plaque assay. Viral plaque assay was performed on murine STAT1 -/- fibroblast monolayers as previously described [91].

**BAL fluid analysis.** BAL fluids were collected from cotton rats immediately after CO$_2$ asphyxiation by washing the lung with 2 ml of sterile saline. BAL fluid cell counts were determined by hemocytometer and BAL differentials were determined on Wright-Geimsa stained CytoSpin preparations (ThermoScientific, Waltham, MA, USA). For the 1 month time point, BAL fluid data was available for only 2 NDV vaccinated animals because BAL fluid collected from one of the NDV vaccinated animals was of poor quality and thus excluded. For the 2 month time point, BAL fluid data was available for only 2 mock vaccinated animals because one of the mock vaccinated animals died prior to sampling.

91
For the 4 days after secondary RSV infection time point, BAL fluid data was available for only 2 mock vaccinated animals because necropsy of one mock vaccinated animal revealed pyometra, an abdominal mass and fibrinous pericarditis. BAL fluid analysis was not performed on that animal.

**Serum neutralization assay.** Blood was collected via cardiac puncture from anesthetized chinchillas or from euthanized cotton rats prior to and after immunization and after RSV challenge. Serum was stored at -80°C until use in neutralization assay. For serum neutralization assay, serum dilutions pre-incubated with ~200 PFU RSV were used as the inoculum for plaque assay on murine STAT1-/- fibroblast monolayers as previously described [91].

**RSV- and NDV-specific antibody isotype ELISA.** Nasal wash fluid, serum and BAL fluid (cotton rat only) were collected as described above and stored at -80°C until use in ELISA assay. For chinchillas studies, RSV glycoproteins purified from whole cell lysates of RSV A2-infected murine STAT1-/- fibroblasts via ion-exchange chromatography were used to coat wells of Nunc maxisorb immunoplates (ThermoScientific, Waltham, MA, USA) overnight at 4°C (serum, 0.25 µg/well; NP lavage fluid, 0.30 µg/well diluted in 0.1M sodium carbonate buffer). For cotton rat studies, the RSV F glycoprotein purified from RSV-infected whole cell lysates via nickel affinity chromatography was used to coat wells overnight at 4°C (0.3 µg purified F protein per well diluted in 0.1M sodium carbonate buffer). Coated wells were blocked overnight with 10% fetal bovine serum in
PBS (PBS-F). Blocked wells were incubated with serial sample dilutions for 1 hour at 25°C. Secondary antibody diluted in PBS-F was added to wells (goat α-guinea pig IgG conjugated to HRP, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; sheep α-guinea pig IgA conjugated to HRP, Immunology Consultants Laboratory, Inc., Newberg, OR; chicken anti-cotton rat IgG, Immunology Consultants Laboratory, Inc., Portland, OR, USA; goat anti-mouse IgA conjugated to HRP, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Plates were incubated at 25°C for 60 minutes. For cotton rat IgG isotype ELISA only, after incubation with secondary antibody, tertiary antibody diluted in PBS-F was added to wells (goat anti-chicken IgG conjugated to HRP, Southern Biotech, Birmingham, AL, USA). After incubation with secondary or tertiary antibody, tetramethylbenzidine peroxidase substrate was added to wells (ScyTek, Logan, UT, USA) and plates were incubated at 25°C for 30 minutes. The reaction was stopped by addition of 2N H₂SO₄. Absorbance at 450 nm was determined using an Epoch Microplate Spectrophotomer (BioTek, Winooki, VT, USA).

For NDV-specific ELISA, plates were coated overnight with whole cell lysate of NDV-infected STAT 1 -/- mouse fibroblasts. Samples were added to coated plates and were then treated as for F-specific ELISA.

**T cell depletion and F-specific ELISpot.** For T cell depletion, mouse anti-cotton rat CD3 and mouse anti-cotton rat CD4 antibodies were labeled with DSB-X according to the manufacturer’s protocol (Dynabeads FlowComp Flexi, Invitrogen Life Technologies,
Grand Island, NY, USA). Single cell suspensions of cervical lymph node, pooled by cohort, were incubated with 0.5 µg each of anti-cotton rat CD3 and anti-cotton rat CD4 DSB-X labeled antibody per sample, then T cells were depleted according to the manufacturer’s protocol (Dynabeads FlowComp Flexi, Invitrogen Life Technologies, Grand Island, NY, USA). Cervical lymph node single cell suspensions, depleted of T cells and pooled by cohort, were used in ELISpot assays.

For F-specific ELISpot, multiscreen HTS HA plates (Millipore, Jaffrey, NH, USA) were coated overnight at 4°C with 0.3 µg purified F protein diluted in 0.1M sodium carbonate buffer. Plates were washed with RPMI supplemented with 10% fetal calf serum (cRPMI) and then were blocked with cRPMI for 1 hr at 37°C in a 5% CO₂ incubator. Plates were washed with RPMI then cervical lymph node single cell suspensions were added in duplicate. Plates were incubated 4 – 6 hrs at 37°C in a 5% CO₂ incubator. Plates were washed with PBS then PBS containing 0.05% Tween-20 (PBS-T). Secondary antibody diluted in PBS-T (chicken anti-cotton rat IgG, Immunology Consultants Laboratory, Inc., Portland, OR, USA: or goat anti-mouse IgA, Southern Biotech, Birmingham, AL, USA) was added and plates were incubated overnight at 4°C. Plates were washed with PBS-T. Tertiary antibody diluted in PBS-T was added (goat anti-chicken AP, Southern Biotech, Birmingham, AL, USA; or donkey anti-goat AP, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and plates were incubated at 25°C for 60 minutes. Plates were washed with PBS-T. NBT/BCIP substrate (Millipore, Jaffrey, NH, USA) was added and plates
were incubated in the dark for 30 minutes at 25°C. Plates were washed with tap water. Spots were counted visually.

**Histology and immunohistochemistry.** Nasal cavities and lung (cotton rat only) collected immediately following euthanasia were fixed in neutral buffered formalin. Nasal cavities were decalcified with 0.35M EDTA in 0.1M Tris (pH 6.95). After decalcification, the nasal cavity was sectioned in a coronal plane at four standard sites used for rodent toxicology studies [178]. Paraffin-embedded tissues were sectioned at 5 µm then stained with hematoxylin and eosin or Alcian blue PAS or were left unstained for immunohistochemistry. For immunohistochemistry, tissue sections were incubated with goat polyclonal RSV antiserum (Biodesign, Saco, Maine, USA) diluted 1:500 followed by incubation with biotinylated α-goat (rabbit) antibody (ScyTek, Logan, UT, USA). Virus detection was accomplished with the streptavidin link and AEC chromagen (Scytek, Logan, UT, USA). Tissue sections were counterstained with hematoxylin. For chinchilla studies, images were captured with an AxioCam HRc using Axio Vision software (Zeiss, Oberkochen, Germany). For cotton rat studies, slides were scanned to 400x magnification with a SCN400 digital slide scanner and images were captured with Leica image viewer software (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

**Statistics.** One-way ANOVA, rank-based one-way ANOVA (for non-normal distributions), or Dunn’s test of rank-based one-way ANOVA (for non-normal distributions with treatment groups of unequal size) was applied to data when appropriate.
using SigmaPlot 12 software (Systat Software, Inc., San Jose, CA, USA). A p value < 0.05 was considered statistically significant. In all graphs data points represent means and error bars represent standard error of the mean.
RESULTS

NDV-F vaccination does not induce adverse histopathology in the cotton rat respiratory tract.

Cotton rat nasal cavity and lung tissues were examined histologically following each of the two small volume intranasal vaccine doses. In the nasal cavity, no histologic changes were observed following delivery of allantoic fluid as a mock control or NDV vector alone (Fig. 4.1 A, B and E, F). In nasal cavity tissues collected from NDV-F vaccinated animals, however, lymphoplasmacytic mucosal infiltrates, indicative of an acquired immune response, accompanied by epithelial disorganization and hypertrophy, indicative of epithelial regeneration, were observed following priming and were of increased intensity following boosting (Fig. 4.1 C, D and G, H).

In the lung, no histologic changes were observed following delivery of allantoic fluid or following a priming dose of NDV vector alone (Fig. 4.1 I, L, and J). In response to NDV-F priming, lymphoplasmacytic infiltrates, similar to those observed in the nasal cavity mucosa, were observed surrounding bronchioles (Fig. 4.1 K). The number of lymphocytes and plasma cells surrounding bronchioles was increased following NDV-F boosting (Fig. 4.1 M). Similar peribronchiolar infiltrates were observed in response to a boosting dose of NDV vector alone (Fig. 4.1 M), but the number of cells was fewer when compared to the first NDV-F vaccination. Additionally, following a second dose with NDV vector alone or either dose of NDV-F, eosinophils were occasionally observed
intermixed with lymphocytes and plasma cells surrounding bronchioles (Fig. 4.1 K, M, and N).

**NDV-F vaccination prevents upper and lower airway histopathology associated with RSV infection.**

Access to clinical specimens has limited investigation of the inflammatory response to RSV in the human upper airway. However, intense rhinorrhea observed clinically likely corresponds to increased mucus production and goblet cell hyperplasia [25]. In our experience, while goblet cell hyperplasia in response to RSV infection of the mouse upper airway does occur to a limited extent, essentially no inflammatory response is observed. In contrast, as described below, we observed inflammation of the cotton rat and chinchilla upper airway and of the cotton rat lower airway in response to RSV infection.

Nasal cavity and lung tissues were examined histologically 4 days after RSV challenge of cotton rats that had received two intranasal doses of allantoic fluid, NDV vector alone or NDV-F. In nasal cavity tissues collected from cotton rats that had received allantoic fluid or NDV vector alone prior to RSV challenge, marked mucosal pathology was observed (Fig. 4.2 A and B). Mucosal epithelium was highly disorganized with loss of apical cilia, attenuated (flattened) and markedly infiltrated by lymphocytes. Additionally, the submucosa underlying the epithelium was infiltrated by lymphocytes and plasma cells with fewer histiocytes and neutrophils. In contrast, nasal cavity tissues collected from cotton rats that were NDV-F vaccinated prior to RSV challenge exhibited mild histologic
changes similar to those observed in response to vaccination alone including submucosal lymphoplasmacytic infiltrates and mild epithelial disorganization, (Fig. 4.2 C).

In lung tissues collected from cotton rats that had received two doses of allantoic fluid or NDV vector alone prior to RSV challenge, small airways were sometimes plugged with mucus containing sloughed epithelial cells as well as inflammatory cells and mild peribronchiolar infiltrates of lymphocytes, plasma cells and fewer eosinophils were observed (Fig. 4.2 D, E). In contrast, excess mucus production with small airway plugging was not observed in lungs collected from cotton rats that were NDV-F vaccinated prior to RSV challenge. Histologic changes in response to RSV infection of NDV-F vaccinated animals, which included mild peribronchiolar and occasionally alveolar infiltrates of lymphocytes and plasma cells, were similar to changes observed in response to vaccination alone (Fig. 4.2 F).

A similar reduction in nasal cavity inflammation was observed in upper airway tissues collected from NDV-F vaccinated chinchillas. Upper airway tissues were examined 4 days after RSV challenge of chinchillas that had received a single intranasal dose of allantoic fluid, NDV or NDV-F 28 days prior to viral challenge. We found that the semi-permissive chinchilla upper airway responds to RSV infection both by marked goblet cell hyperplasia and by mucosal infiltration of inflammatory cells (Fig. 4.3). The upper airway inflammatory response to RSV infection in control chinchillas was mediated predominately by lymphocytes and occasional neutrophils. The inflammatory response to
RSV infection of NDV-F vaccinated chinchillas was milder and mediated primarily by lymphocytes and plasma cells. Additionally, mucosal changes including epithelial disorganization, loss of cilia and squamous metaplasia were observed following RSV infection of control but not NDV-F vaccinated chinchillas. Finally, Alcian-blue PAS staining revealed marked goblet cell hyperplasia in response to RSV infection of control animals, while goblet cell hyperplasia was absent to mild after RSV infection of NDV-F vaccinated animals (Fig 4.3).

**NDV-F vaccination protects against RSV infection.**

Nasal cavity and lung tissues were collected 4 days after RSV challenge of cotton rats that had received two intranasal doses of allantoic fluid, NDV vector alone or NDV-F. RSV antigen was detected by immunohistochemistry and viral load was determined by plaque assay. Immunohistochemistry revealed many RSV antigen positive cells in the upper and lower airway of mock and NDV vaccinated cotton rats while only rare RSV antigen positive cells were detected in the nasal cavity and no RSV antigen positive cells were detected in the lung of NDV-F vaccinated cotton rats (Fig. 4.4 A-F). RSV antigen was restricted to the ciliated respiratory epithelium (Fig. 4.4 D-F) and to olfactory epithelium (not shown) of the nasal mucosa and to ciliated respiratory epithelium of the bronchioles (Fig. 4.4 A, B). Rarely RSV antigen was detected in alveolar lining cells (not shown). In support of immunohistochemistry findings, viable, replicating virions were not detected by plaque assay of nasal mucosa and lung collected from cotton rats that had been NDV-F vaccinated prior to RSV challenge (Fig. 4.4 G).
Similar reduction in viral load was observed in the upper airway of chinchillas that had been NDV-F vaccinated 28 days prior to RSV challenge. Plaque assay (Fig. 4.5 A) of nasal wash fluids collected after RSV challenge revealed a marked reduction in viral load in samples collected from NDV-F vaccinated chinchillas compared to controls. Reduction in viral load was statistically significant on day 4 and nearly so (p=0.055) on day 7 after viral challenge. Additionally, plaque assay revealed more rapid viral clearance of RSV in NDV-F vaccinated chinchillas. Plaque assay results were supported by immunohistochemical detection of RSV antigen in nasal mucosa specimens (Fig. 4.5 B). While many clusters of strongly immunopositive ciliated respiratory and olfactory epithelial cells were observed in nasal mucosa collected from control chinchillas 4 days after RSV infection, only occasional immunopositive cells were observed in nasal mucosa collected from NDV-F vaccinated chinchillas at the same time point.

**NDV-F vaccination induces a robust, long-lived mucosal and systemic antibody response.**

Serum, BAL fluid and nasal wash fluid were collected from cotton rats at multiple time points after vaccination and RSV challenge for determination of F-specific IgG and IgA levels by ELISA (Fig. 4.6 A-F). NDV-F vaccination induced robust F-specific IgG and IgA responses that were boosted following RSV challenge and then maintained at high levels. While serum antibody levels were similar amongst cohorts 1-2 months after RSV infection, mucosal F-specific antibody responses remained significantly greater in BAL and nasal wash fluid samples collected from NDV-F vaccinated cotton rats compared to
controls. Increased mucosal F-specific antibody responses in samples collected from NDV-F vaccinated animals were maintained for at least 6 months after RSV infection, the latest time point examined. Serum neutralization assay revealed that antibodies produced in response to NDV-F vaccination were neutralizing (Fig. 4.6 H).

Cervical lymph nodes were collected 4 days and 5 months after RSV challenge of primed and boosted cotton rats. F-specific IgG and IgA antibody producing cells (ASCs) were enumerated by ELISpot (Fig. 4.6 I, J). While a response was not detected or was minimal in cervical lymph nodes collected from mock and NDV vaccinated cotton rats, robust F-specific ASC responses were observed in cervical lymph nodes collected from NDV-F vaccinated cotton rats at both the 4 day and 5 month time points.

NDV-specific IgG antibody levels in cotton rat serum were determined by ELISA after each vaccination and 5 months after RSV challenge (Fig. 4.6 K). After priming and boosting, NDV-specific antibody levels were significantly greater in serum collected from NDV-F vaccinated cotton rats compared to mock and NDV vaccinated cotton rats. By 5 months after RSV challenge, however, NDV-specific IgG antibody levels in serum collected from NDV and NDV-F vaccinated cotton rats were similar and were significantly greater than NDV-specific IgG antibody levels in serum collected from mock vaccinated cotton rats.
Similarly, we found that RSV-specific serum antibody production (Fig. 4.7 B) was more robust following RSV infection of NDV-F vaccinated chinchillas compared to controls. However, we did not detect differences in neutralizing antibody levels (Fig. 4.7 A) between chinchilla cohorts. Additionally, RSV-specific IgA levels (Fig. 4.8 A) were significantly increased 10 days after RSV infection in nasal wash fluids collected from NDV-F vaccinated chinchillas compared to controls. While not statistically significant, RSV-specific IgG levels (Fig. 4.8 B) were increased in nasal wash fluids collected from NDV-F vaccinated chinchillas compared to controls at the time of RSV challenge and throughout the remainder of the study.

**Secondary RSV infection of NDV and NDV-F vaccinated cotton rats induces lymphoplasmacytic peribronchiolar infiltrates.**

Lung was collected from cotton rats following secondary RSV challenge of animals that had been RSV challenged 7 months earlier and had received 2 intranasal doses of allantoic fluid, NDV vector only or NDV-F prior to primary RSV challenge. Histology revealed minimal peribronchiolar inflammatory infiltrates in response to secondary RSV infection of cotton rats that had received allantoic fluid only, while moderate to marked lymphoplasmacytic peribronchiolar infiltrates were observed in lungs collected from both NDV and NDV-F vaccinated cotton rats (Fig. 4.9 A-C). Immunohistochemistry of nasal cavity and lung collected 4 days after secondary RSV challenge revealed few scattered antigen-positive cells in the nasal mucosa of all cohorts and in the lung of allantoic fluid and NDV vector only, but not NDV-F, vaccinated cotton rats (not shown). Plaque assay
of nasal cavity mucosa and lung collected after secondary RSV challenge was negative in all animals (not shown).

**NDV-F vaccination prevents RSV-induced eosinophilic inflammation in the cotton rat lung.**

Cellularity and percentage of eosinophils in BAL fluid collected from cotton rats at multiple time points after vaccination and RSV challenges were determined by hemocytometer counts and evaluation of Wright-Giemsa stained cytospin preparations, respectively (Fig. 4.10 A, B). While vaccination alone did not increase total BAL fluid cellularity, a small, but not statistically significant, increase in total BAL fluid cellularity was detected in all cohorts after primary RSV challenge. After secondary RSV infection, a much more striking, and statistically significant, increase in cellularity was detected in BAL fluid collected from cotton rats that had been vaccinated with allantoic fluid or NDV vector alone compared to BAL fluid collected from NDV-F vaccinated cotton rats.

BAL fluid eosinophil percentage was not altered by vaccination alone, but was significantly increased one month after primary RSV challenge of cotton rats that had received allantoic fluid or NDV vector alone compared to cotton rats that had been NDV-F vaccinated. BAL fluid eosinophil percentage decreased nearly to baseline by 2 months after primary RSV challenge, but was again increased in response to secondary RSV challenge of cotton rats that had received allantoic fluid or NDV vector alone compared
to NDV-F vaccinated animals. This increase in BAL fluid eosinophil percentage following secondary RSV infection, while striking, did not reach statistical significance.
DISCUSSION

RSV is a major cause of lower respiratory tract disease in infants and the elderly worldwide. While an effective RSV vaccine has been eagerly sought, no licensed vaccine yet exists. RSV vaccine development has focused primarily on live-attenuation, a strategy with demonstrated safety in infants, the target population for most RSV vaccines. Unfortunately, the live-attenuated RSV vaccines evaluated clinically to date have been poorly immunogenic, an outcome that could be predicted from the weak immune response to natural RSV infection [40,59,63]. Disappointing immunogenicity of live-attenuated RSV vaccines has led investigators to diversify RSV vaccine strategies (recently reviewed in [179]). Here, we demonstrate that an NDV vectored RSV vaccine, designed to stimulate robust IFN production at the time of RSV antigen encounter, protects against RSV infection and induces a protective, long-lived humoral immune response.

Although prevention of lung infection is a major goal of all RSV vaccine candidates, the importance of eliminating upper airway infection cannot be understated. First, lung infection occurs solely as a consequence of upper airway infection. Second, virus shedding during upper airway infection of otherwise healthy children and adults is the major source of infection for those most at risk for severe lower airway disease. Thus, it is essential that any RSV vaccine candidate prevent infection of both the upper and lower airway. In this study, NDV-F vaccination completely protected the cotton rat lower
airway against RSV infection, but rare antigen-positive cells were detected at the peak of RSV infection in nasal mucosa collected from NDV-F vaccinated cotton rats and chinchillas. However, no replicating virus was detected in these animals and the clinical significance of rare antigen-positive cells without productive viral shedding is uncertain. It may be possible to completely abrogate viral infection of the upper airway by adjusting the dosing regime or by combining vaccine approaches, i.e. NDV-F prime following by DNA vaccine boost.

A major goal of pre-clinical vaccine evaluation is to establish the safety profile of a vaccine candidate. Given the history of the 1960’s vaccine trial in which enhanced pathology characterized by pulmonary eosinophilia was observed following natural RSV infection of infants that had previously received a formalin-inactivated RSV vaccine [48], this is particular true for all subsequent RSV vaccine candidates. Importantly, delivery of NDV-F was not associated with adverse histopathology. The lymphoplasmacytic nasal and pulmonary infiltrates observed in response to vaccination likely indicate induction of an appropriate adaptive immune response. Similar, but much milder, inflammatory infiltrates were observed in response to vaccination with NDV vector alone, suggesting that NDV is more immunostimulatory when engineered to express the F protein of RSV. Additionally, the eosinophilic pulmonary infiltrates detected in cotton rats that had been mock vaccinated with allantoic fluid or vaccinated with NDV vector alone prior to primary and secondary RSV challenge were not detected in the lung of NDV-F vaccinated cotton rats. This finding is of particular interest when considered in light of
mounting evidence that severe RSV lung disease during infancy is associated with asthma and recurrent wheezing later in childhood [16,17,107,108]. While our preliminary safety evaluation is promising, a more thorough safety assessment, including dose escalation and repeated dosing in animals of various ages, will be necessary to firmly establish the safety profile of NDV-F.

RSV-specific secretory and serum antibodies have been correlated with protection of the upper and lower airway, respectively, against re-infection with RSV [180,181]. Thus, it is essential that any RSV vaccine candidate induce both systemic and mucosal RSV-specific antibodies. In this study, NDV-F vaccination induced serum and mucosal F-specific antibodies that were maintained for at least 6 months. Additionally, the mucosal F-specific antibody response induced by NDV-F vaccination was more robust and longer lived than that induced by RSV infection alone. We previously demonstrated that NDV-F vaccination protected mice against lower airway RSV infection and that protection was mediated by CD8+ T cells [110]. It is likely that T cells play an important role in protection in cotton rats and chinchillas as well. Studies to assess the contribution of T cells to protection against RSV infection of NDV-F vaccinated cotton rats are ongoing.

To assess the duration of protection afforded by NDV-F vaccination, cotton rats were challenged with RSV a second time, seven months after the primary viral challenge. Immunohistochemistry revealed only rare antigen-positive cells after secondary RSV challenge. Despite this, secondary RSV infection induced pulmonary eosinophilia in
mock and NDV vaccinated cotton rats to the same extent as was observed following primary RSV infection of these cohorts. In one recent study of RSV reinfection of cotton rats, replicating virus was not detected following secondary RSV infection, but abortive viral replication was detected by PCR [182]. Interestingly, Boukhvalova et al found that abortive replication was sufficient to induce pulmonary inflammation in the cotton rat. It is possible that abortive replication was responsible for the pulmonary eosinophilia observed in response to secondary RSV infection of mock and NDV vaccinated animals in this study. While pulmonary eosinophilia was not observed following primary or secondary RSV infection of NDV-F vaccinated cotton rats, primary and secondary RSV infection incited lymphoplasmacytic peribronchial infiltration that resembled that observed in response to NDV-F vaccination alone. Peribronchial infiltrates in response to RSV infection of NDV-F vaccinated animals could be explained by boosting of the adaptive immune response to the RSV F protein. Lymphoplasmacytic peribronchial infiltrates observed in response to secondary RSV infection of NDV vaccination animals, however, can not be explained by an antigen recall response. Given that peribronchial infiltrates were not observed in the lung in response to secondary RSV infection of mock vaccinated animals, a general response to RSV infection is unlikely. It is possible that lymphoplasmacytic peribronchial infiltrates observed in the lung of NDV vaccinated animals following secondary RSV infection was due to non-specific polyclonal stimulation [183,184]. The short and long-term responses to NDV will be more thoroughly evaluated in future pre-clinical studies.
In conclusion, these data support the hypothesis that the ability of RSV to antagonize IFN production is key to the ability of this virus to circumvent host immunity. By providing a strong stimulus for IFN production, NDV-F vaccination conferred protection against RSV infection and induced long-lived humoral immunity. These data support our approach to RSV vaccination and provide a sound foundation upon which to more thoroughly evaluate NDV-F as an RSV vaccine candidate in the pre-clinical and then the clinical setting.
Figure 4.1. Histology of the nasal cavity and lung following NDV-F vaccination of cotton rats. Nasal cavity (A-H) and lung (I-N) samples were collected 28 days after priming and 28 days after boosting. The nasal cavity submucosa of animals that had been NDV-F primed and boosted was multifocally mildly to markedly infiltrated by lymphocytes and plasma cells, with migration of fewer cells through the epithelium (C and higher magnification in D; G and higher magnification in H). Additionally, following NDV-F priming and boosting, the nasal epithelium was mildly disorganized and occasionally hypertrophic, indicating an active reparative process. These changes were not observed the nasal mucosa collected from control animals (A-F). Following priming
and boosting with NDV-F (K and N) and, to a much milder extent, boosting with NDV vector only (M), small bronchioles were multifocally surrounded by mild to moderate infiltrates similar to those observed in the nasal mucosa. In addition to lymphocytes and plasma cells, occasional eosinophils were also observed in peribronchiolar infiltrates (* in insets of K and M). Images were taken at 100x or 400x (insets) magnification.
Figure 4.2. Histology of the nasal cavity and lung following primary RSV challenge of NDV-F vaccinated cotton rats. Nasal cavity (A-C) and lung (D-F) were collected 4 days after primary RSV challenge from animals that had been previously vaccinated. Virus infection induced widespread marked nasal mucosal changes including attenuation, disorganization, loss of cilia and lymphoplasmacytic infiltrates (A and B). NDV-F vaccination protected against these nasal mucosal changes (C); rather, nasal mucosa changes following RSV challenge of NDV-F vaccinated animals were similar to those observed following vaccination alone (Fig. 4.1). RSV infection of the lung induced mild peribronchiolar infiltrates of lymphocytes, plasma cells and fewer eosinophils and increased mucus production, resulting in plugging of small airways with mucus,
inflammatory cells and sloughed epithelial cells (D and E). RSV challenge of NDV-F vaccinated animals resulted in lymphoplasmacytic peribronchiolar, and occasionally alveolar, pulmonary infiltrates (F). Increased mucus production and plugging of small airways was not observed in lungs collected from animals that had been NDV-F vaccinated prior to RSV challenge. Images were taken at 100x (D-E), 200x (A-C) or 400x (insets) magnification.
Figure 4.3. Histology of the nasal cavity following primary RSV challenge of NDV-F vaccinated chinchillas. Nasal cavity was collected 4 days after RSV challenge from chinchillas that had been mock, NDV or NDV-F vaccinated twenty-eight days prior to viral challenge. H&E staining revealed that, in control groups, mucosal epithelium was disorganized with frequent loss of cilia and squamous metaplasia and that moderate numbers of mononuclear cells, predominately lymphocytes, infiltrated the lamina propria and migrated through the epithelium. Histologic changes observed in nasal mucosa
collected from NDV-F vaccinated animals, however, was limited to mild influx of lymphocytes and plasma cells. Alcian Blue PAS staining, which stains goblet cells intensely blue, revealed marked goblet cell hyperplasia in nasal mucosa collected from control animals, but not from NDV-F vaccinated animals. Bar = 50 µm (H&E) or 200 µm (AB-PAS)
**Figure 4.4. Nasal cavity and lung immunohistochemistry and plaque assay following primary RSV challenge of NDV-F vaccinated cotton rats.** Nasal cavity and lung were collected 4 days after primary RSV challenge from animals that had been previously vaccinated. RSV immunohistochemistry revealed marked to complete reduction in antigen-positive cells in nasal cavity (A-C) and lung (D-F) collected from NDV-F vaccinated animals compared to controls. Virus was not detected by plaque assay (G) in samples collected from animals that had been NDV-F vaccinated prior to RSV challenge. Images were taken at 200x (A-C), 100x (D-F) or 400x (insets) magnification.
Figure 4.4
Figure 4.5. Nasal cavity immunohistochemistry and plaque assay following primary RSV challenge of NDV-F vaccinated chinchillas. Nasal wash fluid and nasal mucosa were collected from chinchillas that had been mock, NDV or NDV-F vaccinated twenty-eight days prior to RSV challenge. Plaque assay (A) revealed that NDV-F vaccination resulted in reduced nasal cavity viral load and earlier viral clearance compared to controls. In support of plaque assay results, immunohistochemistry to detect RSV antigen (B) revealed many clusters of antigen positive cells in mucosa collected from controls 4 days after viral challenge while only rare antigen positive cells were detected in mucosa collected from NDV-F vaccinated animals at the same time point. Bars = 100 µm
**Figure 4.6. Cotton rat humoral immune response to NDV-F vaccination and RSV challenge.** Serum, BAL fluid and nasal wash fluid were collected at multiple time points after vaccination and RSV challenge and F-specific IgG and IgA levels were determined by ELISA (A-F). NDV-F vaccination induced long-lived F-specific antibody production that was more robust than RSV infection alone. Virus neutralization assay (H) revealed that F-specific antibodies induced by NDV-F vaccination were neutralizing. NDV-specific serum IgG antibody levels were determined by ELISA (I). NDV-F priming induced NDV-specific antibody production that was slightly increased after boosting. Cervical lymph nodes (CLNs) were collected 4 days and 5 months after RSV challenge of vaccinated animals and F-specific IgG and IgA antibody secreting cells (ASCs) were enumerated by ELISpot (J and K). F-specific ASC numbers were greater in CLNs collected from NDV-F vaccinated animals compared to controls.
Figure 4.6
Figure 4.6 cont.
Figure 4.7. Chinchilla serum antibody response to NDV-F vaccination and RSV challenge. Serum was collected from chinchillas that had been mock, NDV or NDV-F vaccinated twenty-eight days prior to viral challenge. While neutralization assay (A) did not reveal differences between cohorts, RSV-specific IgG ELISA (B) revealed a significant increase in RSV-specific IgG in serum collected from NDV-F vaccinated animals compared to controls on days 7 and 28 after RSV challenge. Data points represent means from 2-6 animals and error bars represent standard error. *p<0.05, **p<0.005
Figure 4.8. Chinchilla humoral immune response to NDV-F vaccination and RSV challenge. Nasal wash fluid was collected from chinchillas that had been mock, NDV or NDV-F vaccinated twenty-eight days prior to viral challenge. RSV-specific isotype ELISA revealed a significant increase in IgA (A) antibody levels in samples collected 10 days after RSV challenge from NDV-F vaccinated animals compared to controls. RSV-specific IgG antibody levels (B), although not statistically significant, were increased in samples collected from NDV-F vaccinated animals at the time of RSV challenge (day 0) and throughout the remainder of the study. Data points represent means from 2-6 animals and error bars represent standard error.
Figure 4.9. **Histology of the lung following secondary RSV challenge of NDV-F vaccinated cotton rats.** Lungs were collected 4 days after secondary RSV challenge from animals that had been previously vaccinated and challenged with RSV. Secondary RSV challenge induced minimal pulmonary inflammation in lungs of animals that had been vaccinated with allantoic fluid only (A) whereas moderate peribronchial infiltrates of lymphocytes and plasma cells were observed in lungs of animals that had been vaccinated with NDV (B) or NDV-F (C). Images were taken at 100x magnification.
Figure 4.10. BAL fluid cellularity and cellular differential following vaccination and RSV challenge of cotton rats. BAL fluid was collected at multiple time points after vaccination and RSV challenge. Cellularity (A) of BAL fluid collected from animals in all cohorts increased slightly, but not significantly, in response to primary RSV challenge then decreased gradually over time. Cellularity increased in BAL fluid collected from mock and NDV, but not NDV-F, vaccinated animals in response to secondary RSV challenge. The percentage of eosinophils (B) in BAL fluid collected from mock and NDV, but not NDV-F vaccinated animals increased in response to primary and RSV challenge. BAL fluid cellularity and eosinophil percentage were not affected by vaccination alone. * p<0.05
Chapter 5:

Conclusions and Future Directions
The major barrier to successful RSV vaccine development is a limited understanding of RSV pathogenesis. The major barrier to a more complete understanding RSV pathogenesis is the lack of rodent models which accurately recapitulate human disease. In these studies we have thoroughly characterized two rodent models which more faithfully reflect human RSV disease. These models can be used to explore the mechanisms by which RSV evades the host immune response. Knowledge gained from use of these rodent models can be applied to rational RSV vaccine design. Additionally, each of these rodent models has unique strengths that provide a means to study specific aspects of RSV disease. The chinchilla, for example, is a robust model of polymicrobial otitis media and is susceptible to upper airway RSV infection. A chinchilla model of polymicrobial otitis media in which RSV serves as the inciting viral co-pathogen would provide a system in which to study the mechanisms by which RSV predisposes to otitis media. The cotton rat, for example, is prone to allergic-type airway disease in response to RSV infection and thus may serve as a useful model in which to study to the association between RSV pneumonia and later development of asthma and recurrent wheezing.

We utilized the chinchilla and cotton rat models to evaluate an RSV vaccine candidate developed in our lab. Safety and efficacy studies performed here indicate that NDV-F vaccination protects against RSV infection without inducing enhanced disease. These promising results support future pre-clinical evaluation of our RSV vaccine candidate. Future work will include more extensive evaluation of NDV-F safety and efficacy in cotton rats. Promising results will lead to initiation of human clinical trials. Additionally,
cotton rats will be utilized to examine the B and T cell responses to RSV infection and NDV-F vaccination. These studies will expand our current understanding of RSV pathogenesis and will elucidate mechanisms by which NDV-F vaccination protects against RSV infection.

Study of closely related nonhuman pneumoviruses in their natural host, including bovine RSV (bRSV) infection of calves and pneumonia virus of mice infection of mice, has contributed to our understanding of RSV disease [131,185-188]. The value of these model systems is that they utilize natural host-pathogen relationships and thus accurately reflect pathogenesis. Study of bRSV infection of calves has been particularly informative. bRSV is a major cause of respiratory disease in cattle, particularly in the young [189]. Just as with RSV infection, bRSV outbreaks occur on an annual basis with up to 70% of calves infected by 1 year of age [190,191]. Microscopic lesions also parallel one another, with bronchiolitis predominating in both RSV and bRSV infection [192-195]. In contrast to RSV, licensed bRSV vaccines have been on the market for decades. Despite widespread use, however, bRSV vaccines have had minimal impact on BRSV disease incidence and severity or have been associated with enhanced disease [196-198]. Development of effective RSV and bRSV vaccines faces similar challenges including the need to vaccinate the immunologically immature in the presence of immunosuppressing maternal antibodies and the poor adaptive immune response to natural infection [129]. While differences do exist between RSV and bRSV and the diseases they induce, our understanding of RSV pathogenesis has been significantly advanced by study of bRSV
infection of its natural host. The antagonism of IFN production by the NS1 and NS2 proteins, for example, was first demonstrated for bRSV and was then similarly demonstrated for human RSV [199,200]. Therefore, cattle may be an ideal host in which to elucidate mechanisms of RSV pathogenesis and in which to evaluate candidate vaccines and therapeutics.

As cattle are the species in which RSV disease is most similar to humans, we plan to conduct safety and efficacy studies of our vaccine candidate in calves. Like humans, cattle are not a natural host for NDV and do not have preexisting antibody against this virus. Subbiah et al. [201] have shown that NDV infection of calves induced both humoral and mucosal antibodies against NDV in the absence of disease or viral shedding, underscoring the feasibility of this approach. In addition to these experimental considerations, there are several practical benefits to using the bovine host. First, there are many techniques and reagents available for viral immunology studies in this species [202]. Second, demonstration of safety and efficacy in cattle will partially fulfill pre-clinical trial requirements for advancement to human clinical trials. Thirdly, demonstration of safety and efficacy could lead to widespread use in veterinary medicine. A single vaccine strategy that would protect both humans and cattle is an intriguing possibility.

Beyond pre-clinical evaluation of RSV vaccine candidates, the bovine host is ideal to study mechanisms of RSV pathogenesis. We are particularly interested in B cell
responses to RSV infection and plan to examine the intensity and quality of the B cell response after acute bRSV infection of cattle and also plan to evaluate the longevity and level of the bRSV-specific plasma and memory B cell response in adult seropositive cattle.

In conclusion, these studies have laid the foundation for future work to elucidate mechanisms of RSV pathogenesis and to progress our RSV vaccine candidate toward clinical trials.
REFERENCES


induced by double-stranded RNA or viral infection. Proc Natl Acad Sci U S A 100: 10872-10877.


