INTERVENTIONS FOR PREVENTION OF RESPIRATORY SYNCYTIAL VIRUS BRONCHIOLITIS AND SEQUELAE IN GUINEA PIGS

by

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
(DEPARTMENT OF EXPERIMENTAL MEDICINE)

We accept this thesis as conforming
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THE UNIVERSITY OF BRITISH COLUMBIA

June 2004

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Title of Thesis: INTERVENTIONS FOR PREVENTION OF RESPIRATORY SYNCYTIAL VIRUS BRONCHIOLITIS AND SEQUELAE IN GUINEA PIGS.

Degree: M.Sc EXPERIMENTAL MEDICINE Year: 2004

Department of EXPERIMENTAL MEDICINE
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Vancouver, BC Canada

ABSTRACT

Respiratory syncytial virus (RSV) is the leading cause of infant hospitalization and children who recover from RSV bronchiolitis often develop episodes of recurrent wheezing and "asthma"-like symptoms. Despite considerable research, the mechanisms causing both RSV bronchiolitis and its sequelae are poorly understood. Recent clinical studies suggest a T helper 2 (Th2) immune response may be involved in the pathogenesis of RSV bronchiolitis. By applying the T helper 1 (Th1)/T helper 2 (Th2) paradigm, we examined the effects of two interventions, (administration of "low" dose RSV or CpG-ODN) designed to shift the Th1/Th2 balance towards a Th1 response, on the development of RSV bronchiolitis and post-bronchiolitis sequelae in a guinea pig model of experimental RSV infection. There were four groups of animals: a Sham-Inoculated Group, a Challenge Dose RSV Group (receiving ~10^4 syncytial forming units (s.f.u.)), an Intervention Dose RSV (~10^2 s.f.u.) + Challenge Dose RSV Group, and a CpG-ODN + Challenge Dose RSV Group. We compared the four groups (n =11-12) during acute and chronic (days 7 and 60 post-challenge dose inoculation, respectively) RSV infection. Airway hyperresponsiveness (AHR) was measured using an acetylcholine challenge method in a whole body plethysmograph. Airway infiltration by T cells and eosinophils was assessed by point counting stained lung sections. Using a semi-quantitative RT-PCR method we compared lung IFN-γ/IL-5 mRNA ratios as an index of the Th1/Th2 balance and, using a RSV immunostaining technique, we assessed the proportion of animals in each group with RSV antigens in the lungs. Results on day 7 showed that the Challenge Dose RSV Group developed AHR, increased levels of airway T cells and eosinophils, a Th2 shift in lung IFN-γ/IL-5 mRNA ratio and showed 9/11 animals with RSV positive

immunostaining. The Intervention Dose Group differed from the Challenge Dose Group in that it had a significantly lower proportion of animals with RSV positive immunostaining (1/11) and had an intermediate level of eosinophils compared to the Sham-Inoculated and Challenge Dose RSV Groups. The CpG-ODN Group did not develop AHR, a Th2 shift, or increased levels of eosinophils, but did develop an intermediate level of T cells. The CpG-ODN Group, like the Intervention Dose Group, had a significantly lower proportion of animals with RSV positive immunostaining (3/12) compared to the Challenge Dose RSV (positive control) Group, suggesting that both interventions prevented RSV infection and/or enhanced viral clearance.

On day 60, the Challenge Dose RSV Group maintained AHR, elevated levels of airway T cells and eosinophils, and a Th2 shift observed on day 7, and 7/12 animals had RSV positive immunostaining. The Intervention Dose Group, on the other hand, did not maintain AHR and increased levels of T cells and eosinophils seen on day 7 and had a Th1/Th2 ratio that was not significantly different from the Sham-Inoculated or Challenge Dose RSV Group. The CpG-ODN Group was unaltered with respect to AHR, airway eosinophils, or a Th2 shift, but did have a decrease in airway T cells to a level similar to that seen in the Sham-Inoculated Group. In both the Intervention Dose Group and CpG-ODN Group no animals (0/12 and 0/11 respectively) had RSV positive immunostaining, suggesting that the interventions prevented the development of a persistent RSV infection. In conclusion, CpG-ODN immunotherapy protected against the development of RSV bronchiolitis, while the "low" dose intervention did not, and both interventions provided protection against the development of post-bronchiolitis sequelae in guinea pigs.

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LIST OF SPECIALIZED ABBREVIATIONS

ACh	Acetylcholine
AHR	Airway hyperresponsiveness
APC	Antigen presenting cell
AOI	Area of interest
BALT	Bronchus-associated lymphoid tissue
BCG vaccine	Bacillus of Calmette and Guerin vaccine
BRSV	Bovine respiratory syncytial virus
CpG-ODN	CpG-oligodeoxynucleotides
CTL	Cytotoxic T lymphocyte
DTH	Delayed-type hypersensitivity
EIA	Enzyme immunoassay
FI-RSV	Formalin-inactivated RSV vaccine
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
IFN-γ	Interferon-γ
IL	Interleukin
IP-10	Inflammatory protein-10
LT	Lymphotoxin
MAP kinase	Mitogen activated protein kinase
MCP-1	Macrophage chemoattractant protein-1
MIP-1α	Macrophage inflammatory protein-1α

MIP-2	Macrophage inflammatory protein-2
$NANC_e$	Nonadrenergic, noncholinergic excitatory neurons
NK1 receptor	Neurokinin 1 receptor
NO	Nitric Oxide
OA	Ovalbumin
P_aCO_2	Partial pressure of arterial carbon dioxide
P_{O2}	Partial pressure of oxygen
s.f.u.	Syncytial forming units
RAD	Reactive airway disease
RANTES	Regulated on Activation, Normally T cell Expressed and Secreted
R_L	Pulmonary Resistance
RSV	Respiratory syncytial virus
RSV-IGIV	RSV immune globulin intravenous
RT-PCR	Reverse transcription – polymerase chain reaction
SP-A	Surfactant protein A
STAT	Signal transducer and activator of transcription
TLR	Toll-like receptor
Th1	T helper 1
Th2	T helper 2
TNF	Tumor necrosis factor

Acknowledgments

I would like to thank my supervisor, Dr. R.G. Hegele for his continuous support, encouragement and interest in my professional development. I would like to thank my supervisory committee Drs. R. R. Schellenberg, S. Hayashi, and J.C. Hogg for their constructive comments; Dr. A. Bramley for his collaboration in measuring guinea pig lung mechanics; Dr. F. Tayyari and J. McDonough for their assistance with molecular biology techniques; Drs. M.K. Ashfaq, P.K. Kaan, T. Sedgewick, L. Ding, and Mr. M. Sekhon, S. Greene, and D. English for their helpful comments and technical advice. I would like to thank Ms D. Minshall, L. Carter, C. Collier, and K. Groden, from the Animal Care Facility; Ms. A. Samra for processing histological sections; Dr. H. E. Manson for statistical advice; and Janel, my family, and fellow graduate students for their support, encouragement, and companionship.

This project was funded by the Canadian Institutes of Health Research (CIHR) and British Columbia Lung Association.

CHAPTER 1: RESPIRATORY SYNCYTIAL VIRUS (RSV) BRONCHIOLITIS AND SEQUELAE

1.1 GENERAL INTRODUCTION

Respiratory syncytial virus (RSV) bronchiolitis is the leading cause of infant hospitalization [1]. Nearly all children are exposed to RSV by two years of age; however, for unknown reasons only a minority of children develop RSV bronchiolitis requiring hospitalization [2, 3]. Between 40 to 92% of children hospitalized for bronchiolitis later develop episodes of recurrent wheezing and symptoms considered by some to be asthma [4-11]. To date, no vaccine or pharmacological therapy is effective in preventing or effectively treating RSV bronchiolitis and post-bronchiolitis sequelae and, despite considerable research, the mechanisms of bronchiolitis and post-bronchiolitis wheezing remain poorly understood.

1.2 DEFINITION AND CLASSIFICATION

Bronchiolitis is a common, lower respiratory tract infection/disease that usually affects infants less than 24 months of age [12]. Viral infection of the small airways causes inflammation leading to airway obstruction and produces clinical manifestation of the disease [13]. RSV is the causative agent in the majority of cases of bronchiolitis [12]. However, it should also be noted that other infectious agents, including parainfluenza virus, adenovirus, *Mycoplasma*, rhinoviruses, mumps, metapneumovirus, and influenza viruses are capable of causing bronchiolitis [12, 14].

1.3 EPIDEMIOLOGY

Within the first two years of life all children are exposed to RSV, with infection rates of approximately 66% during the first year of life and 80% during the second year [2]. Many children develop bronchiolitis; however, only 1-3% of children develop disease severe enough to require hospitalization [3]. As a result, in the United States between 60,000 - 100,000 infants are hospitalized each year with RSV bronchiolitis at an estimated annual cost of $341-449 million [15]. RSV bronchiolitis has a mortality rate of 8.3 deaths per 100,000 female infants and 10.4 deaths per 100,000 male infants [1]. Bronchiolitis and RSV epidemics occur in parallel with annual peaks in midwinter to spring (December through April) in the Northern Hemisphere. During epidemics RSV is estimated to be the causative agent in 80% of cases requiring hospitalization and during nonepidemic periods RSV is estimated to account 50% of these cases [12].

Male infants are 1.3-1.4 times more likely to develop severe disease and children who are born premature, have bronchopulmonary dysplasia, congenital heart disease, chronic respiratory conditions (e.g., cystic fibrosis), and immunodeficiency (e.g., chemotherapy, HIV) are at increased risk of developing bronchiolitis requiring hospitalization [16-18]. Other risk factors for severe bronchiolitis include lack of breast feeding, crowded living conditions, indoor air pollution (i.e., cigarette smoke) and a family history of atopy/asthma [16, 19].

Children who recover from an episode of RSV bronchiolitis often develop episodes of recurrent wheezing and "asthma"- like symptoms. Depending on the population studied and study design, between 40-92% of children develop post-bronchiolitis sequelae [4-11]. Whether RSV bronchiolitis predisposes children specifically to asthma is controversial,

with some studies reporting an increased incidence of asthma in children after RSV bronchiolitis and other studies finding no such association [9, 11, 20]. Part of this controversy is that some children clearly "outgrow" their asthma, and secondly it is difficult to diagnose asthma in young children because bronchoprovocation tests cannot be performed accurately [21].

1.4 PATHOGENESIS

RSV is trophic for ciliated airway epithelium. RSV initially infects the nasopharynx and has an incubation period of 5-7 days. If the infection is contained in the nose then symptoms of upper respiratory tract infection ensue (see section 1.5). Bronchiolitis develops when RSV spreads to the lower airways. The mechanism of spread to the lower airways is unknown. However, it is speculated that RSV spreads by direct cell-to-cell contact or by aspiration of nasopharyngeal secretions into the lungs. RSV infection of lower airways results in bronchiolar epithelial necrosis [13, 22].

Necrosis of the epithelial cells lining the lower airways induces the release of pro-inflammatory substances including cytokines, chemokines and leukotrienes from macrophages and epithelial cells [13, 19]. Cytokines released include histamine, interleukin (IL)-1, IL-6, and interferon-γ (IFN-γ), which promote increased capillary permeability and production of secretions. Chemokines released include IL-8, macrophage inflammatory protein (MIP) 1α, and RANTES (Regulated on Activation, Normally T-cell Expressed and Secreted). These chemokines attract pro-inflammatory cells including macrophages, neutrophils, eosinophils, and natural killer cells to the site of infection [13, 19].

Increased capillary permeability results in leakage of plasma proteins into interstitial space, small airways, and alveoli, in turn leading to interstitial swelling. The presence of plasma proteins and exudates in the airway inhibits the function of pulmonary surfactant. Leukotrienes released include C_4 and D_4, which are potent bronchoconstrictors [13, 19]. The combination of increased secretion production, decreased secretion clearance due to destruction of the ciliated epithelium, and ineffective surfactant function leads to filling of the small airways with secretions and debris. In combination with such mediators as leukotrienes C_4 and D_4 causing further bronchoconstriction of the small airways, there is increased airway resistance, air trapping, airway obstruction, and wheezing [13, 19, 22].

1.5 CLINICAL FEATURES

The hallmark features of bronchiolitis are hyperinflation, atelectasis and wheezing [23]. These features develop as the disease progresses to involve the lower respiratory tract. Initial RSV infection is limited to the upper respiratory tract resulting in sneezing, clear rhinorrhea, fever (38.5-39°C), productive cough and diminished appetite. These symptoms may persist for several weeks and resolve without incident [19, 22]. Bronchiolitis develops when RSV spreads into the lower respiratory tract. Cough becomes more severe, secretions are thicker and more copious, and respiratory distress develops. Signs of respiratory distress include wheezing, tachypnea, increased work of breathing with nasal flaring and retractions, and a prolonged expiratory phase [12, 19, 22]. Increased respiratory effort, fever and cough often lead to poor feeding and vomiting. Lethargy and dehydration are often observed particularly in young infants [12].

1.6 DIAGNOSTIC PROCEDURES

Diagnostics include a chest radiograph and laboratory screening for RSV. Chest radiograph reveals hyperinflation with flattened diaphragm and patchy atelectasis as well as air trapping, peribronchial thickening, and consolidation [12, 19].

A specific diagnosis of RSV allows for the elimination of bacterial pneumonia, prevents administration of antibiotics and unnecessary alternative interventions, permits infection control, and allows for epidemiological monitoring [22]. Three main laboratory techniques are used for the detection of RSV. These include culture, antigen detection, and reverse transcription-polymerase chain reaction (RT-PCR). Sterile collection of a nasal washing is required for all techniques. Culture is highly sensitive and specific, and is the gold standard to which all other techniques are compared. In the clinical setting, culture is of limited diagnostic use because RSV grows slowly (requiring 5-7 days before viral diagnosis [22]), thus by the time results become available, the clinical course of the patient is far advanced. The most commonly used diagnostic tests are the immunofluorescence assay (IFA) and enzyme immunoassay (EIA) for RSV antigen detection. The sensitivity of these tests varies between 74% and 94%, however a rapid turn around time of 15-30 minutes makes them a useful diagnostic tool [13, 22]. RT-PCR detects viral RNA and a study comparing RSV RT-PCR to RSV culture positive nasopharyngeal washings found that RT-PCR had a sensitivity of 97.5% [24].

1.7 TREATMENT OF RSV BRONCHIOLITIS

Infants with bronchiolitis and signs of respiratory distress are hospitalized. Treatment for these infants is mainly supportive. Arterial blood gas measurements are made and oxygen saturation is monitored [12]. Hypoxic infants are given cool, humidified oxygen, and fluids are given intravenously to hydrate the infant. Infants with bronchiolitis related apnea, rising P_aCO_2 values, and listlessness and retractions indicating impending respiratory failure are intubated and mechanically ventilated. For infants at high risk of aspirating their food, a nasogastric tube is placed to facilitate feeding [12].

Several pharmacological therapies exist to treat RSV bronchiolitis, however many of them are of little or questionable benefit. β_2 agonists are delivered as an aerosol and are used to treat wheezing and bronchoconstriction. Black [13] reviewed the meta-analysis studies conducted since 1996 evaluating β_2 agonists and found that 2 of 4 studies showed a positive effect while the remaining studies found no effect. Thus β_2 agonists are currently recommended on a trial basis. If the patient responds to an initial dose then therapy is continued [13]. Epinephrine is used to treat interstitial edema and is delivered either as a nebulized aerosol or by injection. Black also reviewed the literature on the use of epinephrine during bronchiolitis and found that 8 of 10 studies showed improved oxygenation, transcutaneously measured P_{O2}, respiratory distress score, and pulmonary function measure [13]; however, many of theses studies enrolled small numbers of patients. In contrast, a recent multicenter randomized double-blind control trial comparing nebulized epinephrine to placebo found that epinephrine treatment had no significant effect on the primary outcome variable of length of hospital stay [25]. Additionally, on the secondary outcome measures of respiratory rate, heart rate,

respiratory effort score and time on supplemental oxygen there were no significant differences between the placebo and epinephrine treated groups indicating that epinephrine is not effective in treating bronchiolitis [25].

Inhaled and systemic corticosteroids have also been used to treat bronchiolitis. Three of 17 studies have shown positive results for corticosteroid treatment [13]. However Garrison *et al.*, [26] performed a meta-analysis that allowed for the combination of six studies, four of which showed no benefit to the use of steroid treatment. The analysis of the combined data showed that corticosteroids decreased the length of hospitalization by 0.43 days with slightly improved clinical scores [26]. Thus Garrison concluded that some positive benefit may be derived from steroid administration, however larger clinical trials are required to properly evaluate their efficacy and effectiveness.

RSV immunoglobulin has also been tested as a therapeutic agent. RSV immunoglobulin is used as a prophylactic agent (discussed below); however when administered during RSV bronchiolitis there were no significant differences between the treatment group and placebo in duration of hospitalization and intensive care stay, number of infants requiring mechanical ventilation, supplemental oxygen requirement, and the number of adverse side effects [27].

Ribavirin is the only agent specifically licensed by the US Food and Drug Administration (FDA) for treating RSV infection. Ribavirin is a synthetic guanosine nucleoside analogue with antiviral properties and is administered as an aerosol for several hours a day [13]. Early clinical trials were promising and showed positive results. However more recent trials have shown that ribavirin does not reduce mortality or duration of hospitalization in infants suffering from bronchiolitis [28] and ribavirin is now

administered only to infants with exceptionally severe bronchiolitis or with substantial comorbid conditions such as cardiopulmonary disease or immunodeficiency [29]. Thus despite many trials evaluating therapies for bronchiolitis, no therapy is capable of limiting the extent or severity of the disease.

1.8 PREVENTION OF RSV BRONCHIOLITIS

Current strategies for preventing RSV bronchiolitis are focused on vaccine development and passive immunization or prophylaxis.

1.8.1 Vaccine

In the 1960's, after success with a killed polio virus vaccine, clinical trials were conducted using a formalin-inactivated RSV vaccine. Children between 2 months and 9 years of age were vaccinated. Upon subsequent RSV infection, 80% of the vaccinees required hospitalization compared to 5% of controls, and sadly two infants died [30]. The failure of this clinical trial has greatly hindered vaccine development and has led to the development of several animal models of RSV bronchiolitis (see Chapter 3). Despite this setback, vaccine development has continued and currently live attenuated virus vaccines and protein subunit vaccines are in clinical trials [31]. Despite these advances, the major challenge facing vaccine development is effectively immunizing a population (infants) that is immunologically immature [31], thus alternative methods of immunotherapy are required.

1.8.2 Passive Immunization

There are currently two immunoglobulin preparations available for prophylactic treatment of RSV bronchiolitis: 1) RSV immune globulin intravenous (RSV-IGIV) (RespiGam) and 2) Palivizumab (Synagis). RSV-IGIV was introduced in the 1990's and is a polyclonal antibody prepared from the sera of adult humans [13]. RSV-IGIV is administered once a month for 5 months during the RSV season. RSV-IGIV clinical trials have shown that RSV-IGIV prophylaxis of high-risk children reduces RSV hospitalization rates by 41% and the total number of days spent in the hospital by 53% [32]. Palivizumab is a humanized mouse monoclonal antibody and was approved by the FDA in 1998. Palivizumab like RSV-IGIV is given monthly however it is administered by an intramuscular injection. In clinical trials Palivizumab reduced hospitalization rates in high-risk infants by 55% and like RSV-IGIV reduced the total number of hospital days [33]. Given that both immunoglobulins have similar efficacy, the American Academy of Pediatrics recommends that Palivizumab be used instead of RSV-IGIV due to the ease of intramuscular injections and decreased risk of transfer of blood-borne pathogens. The Academy further outlines that due to the high cost of prophylaxis, only high-risk infants (infants with at least two risk factors) should receive treatment and recognizes that prophylaxis of all at-risk children (infants with one risk factor) is simply not cost effective [34].

1.9 TREATMENT AND PREVENTION OF POST-BRONCHIOLITIS WHEEZING

Treatment and prevention of post-bronchiolitis wheezing has only been examined by a small number of investigators. The approaches have been to determine: 1) if treating or lessening the severity of RSV bronchiolitis decreases post-bronchiolitis wheezing, and 2) if prophylaxis for RSV bronchiolitis decreases post-bronchiolitis wheezing [35]. Two studies examined "reactive airway disease" (RAD) in children with RSV bronchiolitis treated with ribavirin compared to those that received conventional treatment. In the ribavirin treated group there was a significant decrease in the incidence and severity of RAD one year after treatment [36, 37]. Three studies have examined factors such as incidence of recurrent wheezing and reactive airway disease (asthma), and pulmonary function in children treated with ribavirin compared to children treated by conventional methods at least 6 years after recovery from bronchiolitis [38-40]. In these long-term studies there were no significant differences between the two groups with respect to any of the variables measured [38-40]. Taken together with the results from the 1 year follow-up studies, these results indicate that there is a transient improvement with ribavirin treatment. Kimpen reviewed the literature on corticosteroid use and concluded that the current studies have failed to show that treatment with corticosteroids either during or after RSV bronchiolitis reduced or prevented recurrent wheezing [35]. Wenzel *et al.* [41] examined whether prophylaxis with RSV-IGIV would decrease the post-bronchiolitis wheezing and found that children who received RSV-IGIV had a better forced expiratory volume/forced vital capacity (FEV/FVC) ratio, lower incidence of atopy and were less likely to have missed school or have had an asthma attack compared

to age-matched controls. However due to the small number of patients enrolled in the study, the authors could only conclude that RSV-IGIV may reduce the long-term complications of RSV bronchiolitis and that larger studies are needed [41]. No analogous studies have been completed with Palivizumab, although studies are currently underway [35].

1.10 NATURAL HISTORY

RSV bronchiolitis is a naturally self-limiting condition and infants that do not require hospitalization recover from the infection [28]. Children developing respiratory distress are hospitalized for 2-5 days and have a low mortality rate [1, 3]. Immunity to RSV infection is not complete and thus infants and adults develop recurrent infections throughout life, however the initial infection usually produces severe disease while subsequent infections are limited to the upper respiratory tract [23].

Of children hospitalized for RSV bronchiolitis, 40 to 92% develop episodes of post-bronchiolitis wheezing and "asthma" –like symptoms up to age 13 [4-11, 20, 42] and these complications have been shown to decrease the relative-quality of life of children at least 3 years after resolution of bronchiolitis [43].

1.11 SUMMARY

In summary, RSV bronchiolitis is the leading cause of infant hospitalization [1]. RSV bronchiolitis develops when RSV infects the lower respiratory tract [13]. The hallmark features of RSV bronchiolitis are atelectasis, wheezing, and hyperinflation [23]. Children with respiratory distress are hospitalized and treatment for these infants is mainly

supportive [19]. Most pharmacological treatments for RSV bronchiolitis are of questionable benefit and to date no cost effective preventative measure has been developed. Children who recover from RSV bronchiolitis often develop episodes of recurrent wheezing and "asthma"-like symptoms and the pathogenesis of these sequelae is poorly understood [4-11].

CHAPTER 2: RESPIRATORY SYNCYTIAL VIRUS

2.1 HISTORICAL BACKGROUND

RSV was first isolated in 1956 from a colony of chimpanzees suffering from cold symptoms and was originally designated as the "chimpanzee coryza agent" [44]. One year later, a similar virus was isolated from infants with respiratory illness [45], and on the basis of syncytia formation in tissue culture and similarities between the monkey and human strains, the term "respiratory syncytial virus" was coined [45]. Through several epidemiological studies, RSV quickly became recognized as "the most important viral agent of serious lower respiratory tract disease in the pediatric population worldwide" [46]. RSV is the leading cause of bronchiolitis and causes 40% of cases of pneumonia in young children [3]. Additionally RSV is now recognized as an important pathogen in the elderly and immunocompromised [3, 47]. Reinfection is common throughout life and RSV causes colds and ear infections in children and adults [48].

2.2 CLASSIFICATION

RSV is classified in the family *Paramyxoviridae* of the order *Mononegavirales* [46]. *Paramyxoviridae* is divided into two subfamilies: *Pneumovirinae* and *Paramyxovirinae*. The various strains of RSV (e.g., human RSV A &B, bovine RSV) comprise the *Pneumovirinae* subfamily and the *Paramyxovirinae* subfamily is comprised of Sendai virus, measles, mumps, human parainfluenza virus and others [46]. Members of the *Paramyxoviridae* family share several features. All members have a nonsegmented, single-stranded, negative-polarity RNA genome that is found exclusively in an RNase resistant helical nucleocapsid with the viral polymerase. The genome is transcribed in a

sequential stop-restart mode producing subgenomic mRNA's, and the virus replicates in and is restricted to the cytoplasm of the host cell. All progeny virions have a lipid envelope acquired by budding at the cell plasma membrane and virions enter new host cells by fusing with the plasma membrane [46].

2.3 RSV STRUCTURE

The RSV virion has an irregular spherical shape and ranges in size from 150-300 nm in diameter [46]. Each virion consists of a nucleocapsid contained within a lipid envelope and has a single functional copy of the RNA genome. The RSV genome is 15,222 nucleotides long (strain A2) and encodes 11 proteins [46]. The virion envelope is a lipid bilayer that contains three virally encoded transmembrane surface glycoproteins. These proteins include the attachment (G) protein, the fusion (F) protein, and the small hydrophobic (SH) protein. These glycoproteins form "spikes" on the virion surface 11-20 nm long and 6-10 nm apart [46]. The G protein is responsible for attachment to human airway cells [49] and the two major strains of RSV (A and B) are distinguished based on variations in this protein [23]. The F protein is responsible for viral fusion with the host cell membrane and viral penetration, and the SH protein is a short integral membrane protein with an unknown function [46].

The matrix (M) protein is also encoded by the viral genome and is thought to form a layer on the inner surface of the virion envelope. The M protein is thought to have two functions: (1) to render the nucleocapsid transcriptionally inactive before packaging and (2) to mediate the assembly of the virion [46].

The nucleocapsid is a symmetrical helix, 12-15 nm in diameter, comprised of 4 nucleocapsid/polymerase proteins. The four proteins in the nucleocapsid are the major nucleocapsid (N) protein, the phosphoprotein P, the antitermination factor M2-1, and the L protein [46]. The N protein binds to the genomic RNA to form the RNase resistant nucleocapsid and phosphoprotein P is a chaperone for protein N. The L protein is the viral RNA polymerase, and the M2-1 protein regulates transcription of the viral genome [46]. The RSV genome also encodes the M2-2 protein and the nonstructural proteins NS1 and NS2. M2-2 also regulates viral transcription [49], and although the NS1 and NS2 proteins have no known function, deletion of their sequences from the RSV genome results in a reduction in replication efficiency *in vivo* [49].

2.4 REPLICATIVE CYCLE

RSV is spread by transmission or self-inoculation of large-particle aerosols on to the nasal mucosa or conjunctivae. RSV can survive on inanimate objects such as linens, tissue, and countertops for several hours, and infection occurs when an individual contacts infected secretions by cuddling an infected child or handling their toys. RSV does not spread by aerosolization of droplet nuclei, thus sitting in the room with an infected infant does not result in transmission of RSV [50]. RSV infects ciliated epithelial cells in the respiratory tract [13]. The G protein binds to the cell surface, through an unknown receptor, and the F protein induces fusion of the virus envelope with the plasma membrane. During fusion, the nucleocapsid is inserted into the cytoplasm, and the M protein dissociates from the nucleocapsid allowing viral replication to begin [46].

Viral RNA acts as a template for mRNA and mRNA is the template for translation of viral proteins. Complementary RNA serves as the template for replication of the viral genome and the M protein coordinates the assembly of the various proteins into a virion [49]. Multiple virions are assembled within a cell and are released by budding from the plasma membrane [13]. Viral antigens can be detected in cell culture 9 hours after infection and infectious particles are present 11 to 13 hours after infection [51]. In response to RSV infection the host cell ultimately dies or fuses with adjacent cells to form syncytia [49].

2.5 HOST RESPONSE TO RSV INFECTION

The importance of the host response to RSV was demonstrated by the failed formalin-inactivated vaccine trials in the 1960's [30] and later by the experimental induction of vaccine-enhanced disease in cotton rats [52]. Despite recognition of the importance of the host immune response, it is not known why only a small percentage of children develop severe bronchiolitis requiring hospitalization. Several genetics studies have examined various chemokine, cytokine, and surfactant gene polymorphisms and found some associations. Polymorphisms in genes coding for interleukin-8 (IL-8), IL-4, and IL-10 have been reported to occur at higher frequencies in children hospitalized with RSV bronchiolitis [53-55] and an IL-4α receptor polymorphism has been reported to occur at a higher frequency in children hospitalized after 6 months of age when compared to controls and to children hospitalized at less than 6 months of age [55]. This latter finding suggests a gain-of-function in the IL-4α receptor gene that does not have pronounced effects until after 6 months of age [55]. Surfactant protein polymorphisms

and alleles have also been examined due to the role of surfactant in the innate host defense and pathogenesis of RSV bronchiolitis. A surfactant protein D polymorphism and surfactant protein A (SP-A) allele $1A^3$ have been found to occur at higher frequencies in children with RSV bronchiolitis, while the SP-A allele 1A has been found to be at a lower frequency in children with RSV bronchiolitis, suggesting that there is an association between the SP-A gene locus and RSV bronchiolitis [56, 57].

The finding that atopy is a risk factor for RSV bronchiolitis [16] and the discovery of RSV-specific IgE antibodies in children recovering from RSV bronchiolitis [58] have led to the idea that RSV bronchiolitis may occur in children who develop a polarized T helper 2 (Th2) cytokine response to RSV infection. Th2 responses are characterized by the production of IL-4, IL-5, and IL-13, and mediate allergic inflammatory responses. T helper 1 (Th1) responses are characterized by the production IFN-γ, IL-2, IL-12, and IL-18 and clear intracellular pathogens such as bacteria and viruses. The hypothesis that RSV may induce a Th2 response led to several of the genetic studies already discussed, as well as several animal experiments examining vaccine-enhanced illness, and clinical studies. Results of early studies in mice contradicted this hypothesis, since RSV induced a decrease in the IL-4/ IFN-γ ratio; however studies in guinea pigs provided evidence of a Th2 shift in the lung with evidence of a decrease in the IFN-γ/IL-5 mRNA [59, 60]. Initial clinical studies examining a Th2 shift during acute bronchiolitis yielded contrasting results with some studies showing a Th2 cytokine polarization and other studies showing the opposite effect [61]. However these studies failed to investigate cytokine response in the airway, control for age, assess viral load, or prospectively sample bronchiolitis and control subjects at the same time [61]. When Legg *et al.*[61]

controlled for these factors, they found that infants hospitalized with RSV bronchiolitis compared to those with RSV infection not requiring hospitalization, had elevated IL-4/IFN-γ and IL-10/IL-12 ratios in their nasal washings, indicative of a Th2 response. The authors further investigated these cytokine responses in stimulated peripheral blood mononuclear cells where IL-18 levels were reduced and the IL-4/IFN-γ was elevated, again, indicative of a Th2 response [61]. Additionally, there was a trend for children hospitalized with RSV bronchiolitis towards decreased viral clearance compared to RSV infected controls without bronchiolitis, despite equivalent initial viral loads [61].

2.6 INNATE IMMUNITY

Respiratory epithelial cells, while being permissive to RSV, are also the first line of defense in the innate immune response [49]. RSV infected epithelial cells produce opsonins and collectins, release nitric oxide (NO), and secrete cytokines, chemokines and leukotrienes. SP-A is a collectin and is secreted by respiratory epithelial cells. *In vitro,* SP-A has been shown to bind and neutralize RSV while mice deficient in SP-A have impaired clearance of the virus [49]. Respiratory epithelial cells release NO and NO production has been shown to inversely correlate with RSV titers suggesting anti-viral activity for NO against RSV [62]. Cytokines released in response to RSV infection include IL-1β and IL-6 and chemokines released include IL-8, RANTES, macrophage inflammatory proteins (MIP) –1α and β, MIP-2, IP-10, eotaxin, and macrophage chemoattractant protein (MCP) –1 [49, 62, 63]. These various cytokines and chemokines induce neutrophil, CD4+ helper cell and eosinophil chemotaxis to the lungs [62]. The recruitment of CD4+ T helper cells leads to the development of an adaptive immune

response (discussed in section 2.7.2) [62]. Studies in mice also show that natural killer cells are recruited to the lung during RSV infection and produce IFN-γ [63]. Macrophages also play an important role in innate immunity to RSV and, along with epithelial cells, are the first cells encountered by RSV [63]. *In vitro*, the F protein of RSV binds to Toll-like receptor 4 (TLR-4) and the CD14 receptor on purified monocytes stimulating the release of IL-1β, IL-6, IL-8 and tumor necrosis factor (TNF) -α [63]. In addition, RSV infected macrophages release IL-10 and IL-12. The release of these cytokines further up-regulates the immune response and promotes the recruitment of lymphocytes, neutrophils, and eosinophils to the site of infection [63].

2.7 ADAPTIVE IMMUNITY

Adaptive immunity can broadly be divided into cell-mediated and humoral immunity. Adaptive immunity differs from innate immunity in that an adaptive response is antigen or pathogen specific. Studies in mice show that RSV-specific CD4+ and CD8+ T cell subsets are important in clearing the primary RSV infection [64] while RSV-specific B lymphocytes are not required for initial RSV clearance, but are important in limiting viral replication during subsequent infections [65].

2.7.1 Humoral Immunity

Humans develop antibodies to most RSV proteins, with the viral F and G proteins stimulating the induction of potent neutralizing antibodies [63]. During primary infection IgM antibodies are produced within the first 1-2 days and IgG production follows beginning the second week of infection, peaking at 4 weeks and declining over 1-2

months. IgA is also secreted into respiratory secretions, but its production is more variable and may not always occur [63].

Full-term infants have maternal RSV-neutralizing antibodies in their serum. Neutralizing antibodies are transferred across the placenta during the last few weeks of gestation and also by breast feeding [63, 66]. After birth the titer of maternal antibodies decreases during the first few months of life and high concentrations of maternal antibodies are thought to result in the relative sparing from RSV infections in children less than 6 weeks of age [63, 66]. This idea is supported by a study of native Alaskan children, which found RSV bronchiolitis incidence rates in children < 6 months of age of 55% when children were bottle-fed compared to 38% when children were breast-fed [67]. High titers of anti-RSV antibody found in the serum of children who received the formalin-inactivated vaccine suggested that neutralizing antibodies to RSV may enhance disease [68]. However, the results of studies in the cotton rat contradicted this hypothesis [69-71], and the reduced likelihood of severe RSV infection associated with breast feeding and efficacy of prophylaxis with anti-RSV antibodies demonstrate that anti-RSV antibodies are protective against RSV [16, 18, 32, 33].

In contrast to these findings, RSV-specific IgE antibodies have been isolated in children recovering from RSV bronchiolitis, and higher levels of RSV-IgE have been associated with more severe disease and recurrent wheezing following hospitalization. The identification of RSV-specific IgE antibodies in children with more severe disease and the correlation with post-bronchiolitis sequelae suggests that an allergic response to RSV may result in more severe disease and sequelae [5, 58].

2.7.2 Cell-Mediated Immunity

The importance of cell-mediated immunity during RSV infection is demonstrated by children with deficient cellular immunity who shed virus and have symptoms of RSV infection for months compared to immunocompetent individuals who recover from the infection and clear RSV within weeks [72, 73]. Immunocompetent infants develop a cellular response within 10 days of RSV infection [74]. Both RSV-specific CD4+ and CD8+ T cells are thought to be important in the immune response to RSV infection. When RSV-infected mice received virus-specific CD4+ or CD8+ T cells via passive transfer, both cell types reduced pulmonary shedding of the virus but also increased pulmonary damage [75]. Relatively few studies have examined the role of CD8+ T cells (cytotoxic T lymphocytes (CTL)) in RSV immunity, although it is known that CTL's recognize the viral N, SH, F, M, M2 and NS2 proteins, but not the G protein [76]. Mouse studies suggest that failure of CTL's to recognize the RSV G protein may be a factor in disease pathogenesis [77]. In human studies, RSV-specific CTL levels correlate positively with IFN-γ concentrations and inversely with IL-4 concentrations, and infants with detectable CTL activity in the first year of life are less likely to have significant lower respiratory tract disease during subsequent RSV epidemics [78].

In contrast to CD8+ T cells, CD4+ T helper cells have been studied more extensively in relation to RSV infection. CD4+ T helper cells can be divided into subsets based on the cytokines they secrete [79]. Major subsets of CD4+ T cells include T helper 1 (Th1) and T helper 2 (Th2) cells. Th1 and Th2 cells have opposing roles and negatively regulate each other [80]. Naïve CD4+ cells can differentiate into either Th1 or Th2 cells. Differentiation into predominantly Th1 cells results in an immune response termed

delayed-type hypersensitivity (DTH) and is protective against intracellular pathogens and viruses [80]. Differentiation into Th2 cells results in a humoral immune response, allergic reactions to environmental antigens, and protection against helminthes and other extracellular pathogens [81].

Murine Th1 cells secrete IL-2, IFN-γ, lymphotoxin (LT), and TNF-β where as murine Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 [80, 82]. Human Th1 and Th2 cells have similar profiles of cytokine secretion to the mouse, however IL-2, IL-6, IL-10 and IL-13 release is not tightly restricted to a single subset of cells [80]. Th1 cells, through the release of IFN-γ, IL-2, and TNF-β, promote the development of cytotoxic T cells and macrophages that promote pathogen clearance. Th2 cells, through the release of IL-4, IL-5, and IL-13, mediate antibody production, specifically IgE, and activate mast cells and eosinophils that cause allergic-type responses and promote the removal of extracellular parasites [82].

Differentiation into Th1 or Th2 cells is dependent upon several factors including local cytokine milieu, type of antigen presenting cell (APC), dose and form of the antigen, costimulatory signals from the APC to the T cell, and affinity of the antigen-T cell receptor interaction [83]. IL-12 is the dominant cytokine in Th1 differentiation and is produced by dendritic cells and macrophages [82]. IL-12 induces Th1 differentiation by activating the signal transducer and activator of transcription (STAT)-4. Differentiated Th1 cells in turn produce IFN-γ which up-regulates expression of the IL-12 receptor and inhibits the growth of Th2 cells [82]. IL-4 is produced by mast cells, basophils, eosinophils, and NK 1.1. T cells and induces the differentiation of Th2 cells by activation of STAT-6 [80, 82]. IL-4 like IFN-γ, also has an inhibitory function on the opposing T

helper cell subset, and down regulates IL-12 receptor expression on developing T cells, thus promoting a commitment to Th2 differentiation [82]. Taken together, Th1 and Th2 responses negatively regulate each other and attain a Th1/Th2 balance in the body that can be polarized in different disease conditions. Polarization towards a Th2 cytokine profile is thought to contribute to the etiology of atopic diseases such as asthma, whereas Th1 polarization is implicated in the development of autoimmune diseases including type-1 diabetes and multiple sclerosis [84].

2.8 ANTIGEN DOSE AND Th1/Th2 DIFFERENTIATION

The role of antigen dose in Th1/Th2 differentiation has been studied in mice using *Leishmania major* and *Mycobacterium bovis*, extracellular and intracellular pathogens respectively. A study in BALB/c mice showed that initial low doses (~10^2 infectious units) of *Leishmania* induced a delayed-type hypersensitivity (Th1) response, while high pathogen doses (~10^5 infectious units) induced a mixed Th1/Th2 response [85]. Upon subsequent challenge with either a high or low dose of pathogen, the immune response elicited was the same as that induced by the initial challenge. Thus it appeared that the initial immune response was "imprinted" and determined the subsequent host response [85]. These studies were then extended and the "imprinting" hypothesis was tested using different strains of mice and *Leishmania*, and *Mycobacterium bovis* [86, 87]. These subsequent studies confirmed that pathogen dose, not type of pathogen, determined the initial immune response and that the initial response was "imprinted". To our knowledge, this hypothesis has not been examined with viruses and we propose that

initial low doses of RSV may "imprint" a Th1 response that upon subsequent challenge with a high dose of RSV may be protective.

2.9 CpG-MOTIFS – Th1 STIMULI

CpG-motifs are unmethylated cytosine-phosphate-guanine (CG) deoxynucleotide motifs [88]. They are found at a high frequency in bacterial DNA and in vertebrates they are prevalent at much lower frequencies and are almost always methylated [89]. CpG-motifs were first identified in *M.tuberculosis* DNA, when it was found that purified DNA elicited a stronger immune response than the crude Bacillus of Calmette and Guerin (BCG)-vaccine preparation [90]. CpG-oligodeoxynucleotides (CpG-ODN) are synthetic CpG-motifs and have been shown to prime Th1 immune responses in several species of experimental animals, including guinea pigs [91, 92]. The vertebrate immune system recognizes CpG-motifs by Toll-like receptor (TLR)-9 [93] and TLR-9 recognition of CpG-motifs results in the activation of MAP kinase cascades that activate transcription factors AP-1 and NF-κB [88]. In response to activation through TLR-9, monocytes and dendritic cells secrete IL-12. Natural killer cells are activated by IL-12 and produce IFN-γ [89] which promotes Th1 cell differentiation and feeds back on macrophages and dendritic cells further enhancing a Th-1 response. CpG-motifs do not directly activate resting T cells, instead CpG-motifs induce a Th1-cytokine environment that promotes Th1 differentiation and suppresses Th2 differentiation [89].

Because of their ability to induce a Th1 response, CpG-ODN have been administered to animals to test whether they would prevent the development of Th2-associated diseases. Several studies have shown that CpG administration results in the suppression of

allergen-induced asthma in mice and that this suppression is associated with a shift of Th1/Th2 balance toward Th1 responses [94-96]. With current clinical studies indicating that bronchiolitis may result from a Th2 shift [61], we propose that immunotherapy with CpG-ODN may be protective against RSV bronchiolitis and post-bronchiolitis sequelae.

2.10 MECHANISMS OF POST-BRONCHIOLITIS WHEEZING

For unknown reasons a proportion of children who develop RSV bronchiolitis develop post-bronchiolitis episodes of recurrent wheezing and "asthma"-like symptoms [4-9, 11, 72]. The pathogenesis of these sequelae is poorly understood; however several hypotheses have been proposed. These hypotheses are as follows:

1) Neuroimmune airway inflammation and remodeling. This hypothesis has originated from studies using RSV-infected weanling F344 rats and has not been examined in any other experimental models of RSV infection. In this model, rats clear RSV within 30 days of RSV inoculation at which time RSV is undetectable by RT-PCR or viral culture [97]. The lung is vastly innervated and there is a network of nonadrenergic, noncholinergic excitatory ($NANC_e$) neurons located below the respiratory epithelium [98]. These nerves induce smooth muscle contraction and have proinflammatory and immunostimulatory effects mediated by tackykinins. Substance P is a tackykinin released by these nerves in response to stimulation by airborne irritants (air pollutants and allergens) and substance P acts on the neurokinin 1 receptor (NK1) to exert its effects [98]. The NK1 receptor is located on T and B lymphocytes, on monocytes and macrophages, and on mast cells [98]. Five days after RSV-inoculation, there is an increase in airway

vascular permeability in association with an increase in NK1 mRNA and protein levels in the lung. The increase in vascular permeability is thought to promote the development of exudative edema [97]. Increased NK1 expression is thought to occur on lymphocytes in bronchus-associated lymphoid tissue, monocytes, and mast cells, thus making these cells more sensitive to substance P [99]. It is hypothesized that the increase in vascular permeability in the airways is enduring and that repeated irritation of the airway by airborne stimuli causes release of substance P, that acts on more sensitive inflammatory cells leading to recurrent cycles of inflammation [98]. The combination of recurrent cycles of inflammation with an enhanced propensity for exudative edema is thought to result in airway narrowing and the manifestation of post-bronchiolitis wheezing.

2) Enhancement of allergen sensitization by viral infection. This hypothesis proposes that viral infections in early life enhance later allergic sensitization. Evidence in support of this hypothesis comes from studies in mice where allergen sensitization following RSV infection lead to increased airway hyperresponsiveness (AHR) and lung inflammation [100]. Evidence for this mechanism also comes from a guinea pig study in which subsequent ovalbumin sensitization following RSV infection resulted in elevated titers of OA-specific IgG1 antibody (IgG1 in guinea pigs is the equivalent of IgE) [101].
3) Persistent viral infection. *In vitro* studies showing that RSV could persistently infect HEp-2 cells lead to the hypothesis that RSV may persist *in vivo* [102]. The first animal study to examine this hypothesis found that RSV RNA and protein persisted in the lung of Cam Hartley guinea pigs 60 days after experimental RSV

infection [103]. Subsequent studies showed that replicating virus could be isolated from the lungs of guinea pigs 60 days after RSV infection, and that AHR developed in association with viral protein persistence and chronic airway inflammation 100 days after RSV inoculation [104, 105]. Given these findings, the authors speculated that viral persistence leads to chronic airway inflammation that in turn promotes the development of chronic AHR [105]. The idea of viral persistence as mechanism of post-bronchiolitis sequelae has been further supported by a recent study that found RSV RNA could be detected in the lungs of mice up to 150 days after experimental RSV infection [106]. This latter study further reported that low levels of infectious virus were recoverable 150 days after infection from the lungs of mice depleted of T cells, indicating that RSV persists by means of low-grade replication. This persistence even in the presence of RSV-specific CTL's and RSV specific IgG suggests that RSV may persist in immunologically privileged sites such as neurons [106].

Based on studies that found RSV induced a Th2 response during acute infection in two strains of guinea pigs [60], taken together with the clinical finding that a Th2 shift during RSV bronchiolitis is associated with a trend towards decreased viral clearance [61], we propose that in guinea pigs this Th2 shift is maintained leading to decreased viral clearance. Further we propose that interventions to prevent this Th2 shift during bronchiolitis may prevent RSV persistence and the associated post-bronchiolitis sequelae.

2.11 SUMMARY

In summary, RSV is member of the *Paramxyoviridae* family of viruses that have a nonsegmented, negative-polarity RNA genome [46]. Early vaccination trials demonstrated the importance of the host response to RSV [30]. RSV induces both cell-mediated and humoral immune responses [63] and there is increasing evidence that children with severe RSV bronchiolitis develop a Th2 response to the virus [61]. We propose that either priming the host with a low infectious dose of RSV or administration of CpG-ODN prior to RSV infection may be protective against this Th2 cytokine polarization. Further we suggest that although there is little known about the mechanisms of post-bronchiolitis wheezing that prevention of a Th2 shift during bronchiolitis will prevent the establishment of the acute infection and/or prevent viral persistence and its associated sequelae.

CHAPTER 3: ANIMAL MODELS OF RSV BRONCHIOLITIS AND SEQUELAE

3.1 ANIMAL MODELS OF RSV BRONCHIOLITIS

Failure of the formalin-inactivated RSV vaccine trials in the 1960's stimulated the development of animal models of RSV disease [107]. Numerous animal models of experimental RSV infection have been described, including large animals such as chimpanzees [108], other primates [107], calves [109], and sheep [110] and small animals such as cotton rats [111, 112], F344 rats [97], mice [113], guinea pigs [114], ferrets [113] and Syrian hamsters [115].

RSV infection was first described in chimpanzees, in which chimpanzees developed significant rhinnorhea that also occurs in humans [44, 108]. The high degree of genetic similarity between humans and chimpanzees makes chimpanzees an appealing animal model; however, terminal experiments are not permitted and pulmonary disease has not been described in these animals [107, 116]. Further, limited availability of chimpanzees makes it difficult to acquire sufficient numbers of animals for experimentation and high purchase and maintenance costs limit the number of laboratories that can conduct research on these animals [107]. Several other primate models of RSV infection include several monkey species and the baboon [107]. Terminal experiments are permitted in these animals allowing for detailed virological and histological studies of pulmonary disease, however pulmonary infection has only been described in the owl monkey [117, 118]. These models have disadvantages similar to the chimpanzee model: limited numbers of animals, high purchase and maintenance costs, as well as lack of inbred strains and lack of species-specific reagents [107].

Experimental infection of calves with bovine RSV (BRSV) is another large animal model of RSV disease. The diseases caused by both BRSV and RSV have several similar characteristics. Both viruses in their respective host cause acute disease limited to the upper respiratory tract. RSV and BRSV both induce incomplete immunity and reinfect their hosts throughout life. Bovine RSV, like human RSV, causes severe disease primarily in neonates and high titers of maternal antibody in the serum are protective against RSV disease [109]. BRSV also parallels human RSV epidemiologically with BRSV epidemics occurring annually in winter months [109].

The calf model has the unique advantage of studying the virus in its natural host. However limited homology between RSV and BRSV proteins and greater difficulty in propagating and tittering BRSV *in vitro* illustrates that although these viruses are related, they are not identical [107]. The calf model also has drawbacks similar to other large animal models such as high purchase and maintenance cost, lack of inbred strains, and lack of species-specific reagents [107].

The mouse model of RSV disease was first described in 1962 and the cotton rat model was later described in 1971 [113, 119]. The cotton rat model was originally designed to study how the formalin-inactivated RSV vaccine (FI-RSV) enhanced disease. Cotton rats develop FI-RSV enhanced pulmonary disease similar to that found in humans, while mice do not develop enhanced disease [52, 120]. However, results of experiments in mice showed that immunization with FI-RSV primed a Th2 response (indicated by increased expression of IL-5, IL-13, and IL-10 mRNA and decreased expression of IL-12 mRNA) upon subsequent challenge with live RSV [121].

The cotton rat has two advantages over the mouse model: (1) cotton rats are 10-100 fold more permissive to RSV [111, 113] and (2) cotton rats are more immunologically responsive to the virus, producing 10-fold greater serum concentrations of anti-RSV antibodies [122, 123]. Initial studies in the cotton rat disproved the hypothesis that anti-RSV antibodies were responsible for the pathogenesis of vaccine-enhanced disease and produced the first evidence that prophylaxis with anti-RSV antibodies could protect against severe RSV disease [69-71]. Limitations of the cotton rat model, in contrast to mice, are lack of species-specific reagents and of transgenic or knockout strains [107]. A criticism of both of these models is that neither the cotton rat nor the mouse develops acute lower respiratory tract disease or impressive bronchiolar inflammation [111, 123]. Further, the cotton rat is susceptible to RSV infection throughout life whereas the mouse is only susceptible to RSV after maturity, neither of which represents the human condition in which infants and young children primarily suffer from severe clinical disease [111, 123]. In contrast, the guinea pig and ferret only develop pulmonary infection before maturity and thereby more effectively model the human condition [113, 124].

The Syrian hamster is another model of RSV infection. Hamsters have similar RSV permissiveness to mice and RSV has been shown to infect both the nose and lungs of these animals [115]. Unfortunately, the ferret and Syrian hamster models are not well characterized, limiting their use.

Experimental infection of guinea pigs with RSV results in disease characteristics that model bronchiolitis and presents another small animal model of RSV disease [114]. The guinea pig model was originally designed to study the interaction between the allergic

background of the Cam Hartley guinea pig and RSV infection. The well-characterized airway physiology in these animals and their relative ease of sensitization to ovalbumin [125] made them an attractive model to study post-bronchiolitis wheezing and virus-enhanced allergic sensitization. Guinea pigs are more permissive to RSV than cotton rats or mice requiring 10^3-10^4 s.f.u. to induce disease while the cotton rat requires 10^5-10^6 s.f.u. and mice require between 10^6-10^8 s.f.u. of RSV [111, 114, 123]. In comparison humans become infected with RSV after exposure to only 500 s.f.u. [126].

RSV replicates in the lungs of guinea pigs and guinea pigs develop acute lower respiratory tract disease with airway inflammation [114]. Both guinea pigs and mice develop airway hyperresponsiveness (AHR) during acute RSV infection, however significant airway inflammation and increased viral permissiveness suggests that guinea pigs more closely resemble the human condition [127, 128]. Additionally guinea pigs develop a Th2 response during acute RSV infection while mice develop a Th1 response [59, 60]. Given that recent clinical studies show a Th2 response in a considerable proportion of infants hospitalized with RSV bronchiolitis, the guinea pig model is particularly appealing. The limitations of the guinea pig model are similar to those of the cotton rat: there is a shortage of inbred guinea pigs strains, limited species-specific reagents, and no transgenic or knockout animals [107]. Further, relative to the mouse and rat, the guinea pig genome and protein sequences are less well characterized; however, the number of guinea pig sequences submitted to the Genbank database is increasing.

3.2 GUINEA PIGS AS A MODEL OF POST-BRONCHIOLITIS WHEEZING

The association between RSV bronchiolitis and post-bronchiolitis wheezing [4-11] led to the examination of whether chronic airway hyperresponsiveness (AHR) occurs in guinea pigs. Guinea pigs develop AHR that lasts for at least 100 days post-RSV inoculation, and this AHR can be considered to model post-bronchiolitis wheezing [105]. Further, in guinea pigs chronic AHR was found in association with persistence of viral protein in the lungs, suggesting that viral persistence may be involved in the pathogenesis of post-bronchiolitis wheezing. Additionally, replicating virus and viral RNA have also been isolated from the lungs of guinea pigs 60 days after RSV infection [104, 105]. Recently, RSV has also been shown to persist in the lungs of BALB/c mice, as replicating virus was isolated from lungs of mice 150 days post-RSV inoculation [106]. However, whether this persistent RSV infection occurs in association with chronic AHR is controversial. Jafri *et al.* showed that when BALB/c mice were inoculated with 10^7 s.f.u. of RSV they developed transient AHR up to 154 days post-RSV inoculation [128]. In contrast, when Schwarze *et al.,* inoculated mice with 10^5 s.f.u. of RSV, AHR was not present 21 days after RSV inoculation [129, 130], thus it appears that the development of chronic AHR in mice depends on the initial virus dose. Importantly, viral persistence was shown in BALB/c mice inoculated with 10^5 s.f.u. of RSV [106], thus the presence or absence of virus cannot explain the development of chronic AHR in mice. To date, viral persistence has not been examined in mice inoculated with 10^7 s.f.u. of RSV.

The guinea pig model presents another advantage over the mouse model. Similar to infants with RSV bronchiolitis, guinea pigs develop a Th2 cytokine shift during acute

RSV infection, whereas the mouse develops a Th1 response [59-61]; thus the guinea pig more closely resembles the human disease.

3.3 SUMMARY

In summary, several animal models of RSV bronchiolitis have been developed. The major limitations of large animal models are high cost and relatively few numbers of experimental animals [107]. The three most prominent small animal models of RSV bronchiolitis are the cotton rat, mouse, and guinea pig. Guinea pigs are more permissive to RSV than the cotton rat or mouse and develop airway inflammation and lower respiratory tract disease not seen in the cotton rat and mice [111, 114, 123]. Additionally guinea pigs develop a Th2 shift during acute RSV infection similar to clinical findings in infants, while mice develop a Th1 shift [59-61]. Limitations of the guinea pig model are a paucity of inbred strains, limited species-specific reagents, limited genomic and protein sequences, and lack of knockout and transgenic animals [107].

Guinea pigs also provide a model of post-bronchiolitis wheezing that has recently been supported by studies in mice [105, 106, 128]; however the Th2 response seen during acute infection in guinea pigs, that is not observed in mice, makes the guinea pig model particularly relevant to the human disease [59-61].

CHAPTER 4: WORKING HYPOTHESIS, SPECIFIC AIMS, AND STRATEGY

The working hypothesis for this thesis was based on the general hypothesis that interventions designed to shift/optimize the Th1/Th2 balance away from a Th2 response will prevent (or lessen the severity of) RSV bronchiolitis and post-bronchiolitis sequelae.

4.1 WORKING HYPOTHESIS

Interventions with "low" dose RSV inoculation or CpG-ODN administration of guinea pigs will decrease both acute and chronic airway hyperresponsiveness, airway infiltration by T cells and eosinophils, a Th2 shift, and the proportion of animals with RSV antigens in the lung.

4.2 GOALS

In this thesis we sought to examine two interventions for the prevention of RSV bronchiolitis and post-bronchiolitis sequelae in guinea pigs. The first proposed intervention was inoculation with a relatively "low" dose of RSV. Based on studies in mice with *Leishmania* and *Mycobacterium* [85-87], we proposed that a "low" dose of RSV would "imprint" a Th1 response that would be protective against a Th2 response induced by challenge with a "high" dose of RSV. The second intervention was the administration of CpG-ODN. CpG-ODN induce a Th1 response in guinea pigs and several experimental animals [91, 92]. Based on the knowledge that Th1 and Th2 responses are mutually inhibitory [80], we proposed that CpG-ODN administration would induce a Th1 response that would inhibit the development of a Th2 response induced by RSV.

The first goal was to determine whether "low" dose RSV inoculation (a virus-specific intervention) or CpG-ODN administration (a general intervention) could prevent the development of RSV-associated acute (Day 7) and chronic (Day 60) airway hyperresponsiveness (AHR), airway inflammation, and Th2 shift.

Given that Th1 responses are considered relevant to effective anti-viral immunity, the second goal was to determine whether "low" dose RSV inoculation or CpG-ODN administration would decrease the proportion of animals with RSV antigen in the lung, during either acute (Day 7) or chronic (Day 60) RSV infection.

4.3 SPECIFIC AIMS

1. To determine the effects of "low" (intervention) dose RSV inoculation or CpG-ODN administration on RSV-associated acute (Day 7) and chronic (Day 60) airway hyperresponsiveness;
2. To determine the effects of "low" (intervention) dose RSV inoculation or CpG-ODN administration on RSV-associated acute (Day 7) and chronic (Day 60) airway inflammation;
3. To determine the effects of "low" (intervention) dose RSV inoculation or CpG-ODN administration on lung IFN-γ/IL-5 ratios during acute (Day 7) and chronic (Day 60) RSV infection;
4. To determine the effect of "low" (intervention) dose RSV inoculation or CpG-ODN administration on the proportion of animals with RSV antigen in the lungs during acute (Day 7) and chronic (Day 60) RSV infection.

4.4 STRATEGY

4.4.1 Experimental Time Points

Animals were killed on days 7 and 60 post-inoculation with a "high" (challenge) dose of RSV. These time points were chosen because they represent respectively, maximal increases in AHR and airway inflammation ("bronchiolitis") and a time point in which there is persistent RSV infection and chronic airway inflammation.

4.4.2 Airway Hyperresponsiveness (AHR)

Acute (Day 7) and chronic (Day 60) AHR was measured by using acetylcholine (ACh) challenge in a whole-body plethysmograph as described by Ishida *et al.* [125].

4.4.3 Airway Inflammation

The small airways of asthmatics are rich in eosinophils and T cells [131, 132], as are the airways of guinea pigs during acute RSV infection [60]. Thus we assessed airway inflammation in small airways during acute (Day 7) and chronic (Day 60) RSV infection by measuring the percentage of the airway wall occupied by T cells and eosinophils (see methods section 5.10). Paraffin-embedded lung sections were stained appropriately, and digital images of small airways were analyzed using the Image Pro PLUS analysis system (Media Cybernetic Inc; Silver Spring, MD, USA).

4.4.4 Lung IFN-γ/IL-5 mRNA Ratios

RNA was extracted from homogenized lung sections of animals during acute (Day 7) and chronic (Day 60) RSV infection, and the ratio of IFN-γ to IL-5 messenger RNA was measured using semi-quantitative RT-PCR to assess Th1/Th2 balance [60].

4.4.5 RSV Immunostaining

To determine the effects of the two interventions on viral protein within the lung, paraffin-embedded lung sections underwent immunostaining for RSV antigens and the proportion of RSV-positive animals in each group during acute (Day 7) and chronic (Day 60) RSV infection were compared.

4.5 SUMMARY

A Cam Hartley guinea pig model of RSV bronchiolitis and post-bronchiolitis AHR was used to test the working hypothesis that interventions designed to prevent a shift in the lung Th1/Th2 balance towards a Th2 response would prevent (or lessen the severity of) RSV bronchiolitis and post-bronchiolitis sequelae. "Low" (intervention) dose RSV inoculation and CpG-ODN administration were interventions used to prevent a Th2 shift in the lung Th1/Th2 balance. The time points of day 7 and 60 post-"high" (challenge) dose RSV inoculation were chosen to represent the bronchiolitis and post-bronchiolitis wheezing/persistent infection. AHR was measured by using ACh challenge and airway inflammation was assessed by a quantitative histology method. Lung Th1/Th2 balance was measured using a semi-quantitative RT-PCR for IFN-γ and IL-5 mRNA expression,

and the proportion of animals with RSV antigens in the lung was assessed by immunostaining of lung tissue sections.

CHAPTER 5: MATERIALS AND METHODS

In this chapter, the experimental design and the methods used to test our hypothesis will be described.

5.1 EXPERIMENTAL DESIGN

Ninety-six Cam Hartley guinea pigs were divided into four groups (n=24 for each group). Group 1 received a sham-inoculum consisting of lysed uninfected HEp-2 cells in cell culture medium. Group 2 received a "high" infectious (challenge) dose of RSV consisting of $5.3 \pm 2.4 \times 10^4$ syncytial forming units (s.f.u.) (Mean $\pm$ Standard Error (SE)). Group 3 received a "low" (intervention) dose of RSV consisting of 54 ± 16 s.f.u. (Mean $\pm$ SE) and then 28 days later received the challenge dose of RSV. An interval of 28 days between inoculation with the intervention and challenge dose of RSV was chosen to allow effects of the intervention dose to subside [114, 127]. Group 4 received 100 μg of CpG-Oligodeoxynucleotides (CpG-ODN), biweekly for 4 weeks (3 doses). One week following the last CpG-ODN dose the challenge dose of RSV was given. A one week interval between the last CpG-ODN administration and inoculation with a challenge dose of RSV was chosen to avoid potentially confounding effects of CpG-ODN interacting with RSV [133].

Twelve guinea pigs from each group were subject to acetylcholine challenge at 7 days (representative of acute infection) and at 60 days (representative of chronic infection) post-"high" dose RSV inoculation or sham-inoculation and then sacrificed.

5.2 ANIMALS

Ninety-six juvenile female Cam Hartley guinea pigs (1-month old, body weight 250-300 g) were purchased from Charles River Laboratories (Montreal, QC, Canada). RSV infected animals were housed in a different room from uninfected animals but were otherwise kept under the same conditions of alternating 12 hour light-dark cycles and free access to guinea-pig chow (Ralston Purina Corp., St. Louis, MO, USA) and water. Animals receiving either a "high" or "low" dose of RSV were housed in filter-isolated large polycarbonate cages and all animals were allowed to acclimatize for 5 days prior to use. Two weeks after inoculation with either a "high" or "low" dose of RSV, animals were moved into the same room as uninfected animals and housed in large stainless steel cages. Animals that did not receive virus were also housed in stainless steel cages.

5.3 VIRUS PREPARATION

Human Long strain, type A RSV (American Type Culture Collection (ATCC)), Rockville, MD, USA) was propagated on HEp-2 (ATCC) cell monolayers in a humidified incubator (Fisher) at 37 °C containing 5% CO_2. Cell culture medium consisted of 1640 RPMI (Gibco, Buffalo, NY, USA) supplemented with 2% fetal bovine serum (FBS) (Gibco), 0.292 $mg \cdot mL^{-1}$ penicillin G (Gibco), 100 $\mu g \cdot mL^{-1}$ streptomycin (Gibco), and 0.25 $\mu g \cdot mL^{-1}$ amphotericin B (Gibco).

RSV stocks for inoculation of guinea pigs were prepared by addition of autoclaved 3 mm diameter glass beads to infected HEp-2 cell monolayers and placing the flask on a vortex. The resulting suspension underwent centrifugation at 1000 x *g* for 5 minutes at 22°C. The supernatant was then transferred into a 15 mL conical tube (Becton Dickinson,

Franklin Lakes, NJ, USA), placed on a vortex, and dispensed into aliquots in 2 mL cryotubes (Nalgene, Nalge Nunc International, Milwaukee, WI, USA). The sham-inoculum consisted of uninfected HEp-2 cells subject to the same conditions as infected cells. Stocks of RSV aliquots were then frozen and stored at -70 °C. One week prior to RSV inoculation of animals one stock was thawed and subjected to RSV plaque assay (described below) to determine the viral titer.

5.4 VIRAL PLAQUE ASSAY

Prior to inoculation of guinea pigs, the viral titer of RSV stocks was determined using a plaque assay. Twenty-four well plates (Corning, Corning NY, USA) were seeded with 2.5×10^5 HEp-2 cells/well overnight at 37 °C in 5% CO_2 in cell culture medium that was supplemented with 10% FBS. One aliquot of RSV stock was thawed and serially diluted (10^{-1}–10^{-8}) in 2% FBS cell culture media. The 24 well plates were rinsed once with sterile phosphate-buffered saline (PBS) (Sigma, St. Louis, MO, USA), and then exposed to 0.4 mL of serially diluted RSV. The plates that were exposed to RSV were agitated every 15 minutes to ensure even distribution of virus over the cells. After 90 minutes the plates were rinsed with sterile PBS, and overlaid with 1 mL of pre-warmed 1:1 solution of 1% sterile methylcellulose to 2 x Minimal Essential Media (MEM) (Gibco) supplemented with 2 x vitamins, L-glutamine (Gibco) and 0.05 $mg \cdot mL^{-1}$ gentamicin (Gibco). The plates were examined daily under an inverted microscope. Five to seven days post-RSV exposure (once syncytia had developed), the plates were fixed with 10% formalin for 30 minutes, rinsed with distilled water, and then fixed again in methanol (Fisher Scientific, Pittsburgh, PA, USA) for 1 minute. The plates were allowed to air dry

and were stained with 0.1% neutral red (Gibco) for 1 minute. After staining the plates were rinsed with tap water and the syncytia were counted to determine the virus titer. Viral titer was expressed in syncytial forming units per milliliter (s.f.u. /mL) of the RSV stock.

5.5 "LOW" (INTERVENTION) DOSE RSV

A "low" (intervention) dose of ~10^2 s.f.u. of RSV was chosen as an appropriate "low" dose based on a study that showed inoculation of guinea pigs with this dose of RSV did not induce a Th2 response or the production of RSV-specific antibodies [134].

5.6 CpG-OLIGODEOXYNUCLEOTIDES (CpG-ODN)

Ten mg of CpG-oligodeoxynucleotides (CpG-ODN) type B [135] single-stranded, code 2007 of the sequence 5'-TCGTCGTTGTCGTTTTGTCGTT-3' was purchased from Coley Pharmaceutical Inc. (Wellesley, MA, USA). Guinea pigs were inoculated intranasally with 100 μg of CpG-ODN biweekly for 4 weeks (3 doses). The dosing regimen of CpG-ODN was determined based on the results of a previous study [92] that showed this dose and route of administration induced the largest increase in the IFN-γ/IL-5 mRNA ratio. On the day of CpG-ODN administration, a sample of the stock solution was diluted in sterile saline to give a concentration of 0.333 $\mu g \cdot \mu L^{-1}$ or 100 μg in 300 μL.

5.7 INOCULATION PROTOCOL

On the designated days for inoculation, guinea pigs were anaesthetized *via* inhalation of 3-5% halothane. Each guinea pig received 300 μL of the appropriate inoculum by

intranasal instillation. The CpG-ODN inoculum consisted of CpG-ODN in sterile saline. The RSV inoculums consisted of RSV in cell culture media, and the sham- inoculum consisted of uninfected HEp-2 cells in cell culture media subject to the same conditions as RSV inoculum.

5.8 ACETYLCHOLINE (ACh) CHALLENGE

The protocol for measurement of lung mechanics involved inhaled acetylcholine (ACh) challenge and was based on previous studies in ovalbumin-sensitized and RSV-infected guinea pigs [60, 125, 127, 136]. On day 7 and day 60 post-"high" dose RSV inoculation, guinea pigs were weighed and anesthetized with an intraperitoneal injection of 100 $mg \cdot kg^{-1}$ of ketamine (Biomeda-MTC, Cambridge, ON) and 10 $mg \cdot kg^{-1}$ of xylazine (Biomeda-MTC). The neck region of the guinea pigs was shaved and the guinea pigs were then exposed to 2-5% inhalational halothane through a nasal mask. The neck was further anesthetized with 4 $mg \cdot kg^{-1}$ of lidocaine (Abbott Laboratories, Saint-Laurent, QC) and a tracheotomy was performed. Following exposure of the trachea, a water filled catheter was inserted through the mouth into the distal esophagus and the guinea pig was then placed inside a whole-body plethysmograph. The trachea was cannulated, electrocardiograph leads were placed, and the animal was paralyzed with an intramuscular injection of 4 $mg \cdot kg^{-1}$ of succinylcholine (Bioniche Pharma, London ON). The guinea pig was ventilated at a rate of 60 $breaths \cdot min^{-1}$ with a small animal ventilator (Harvard Instruments Corporation, South Natick, MA, USA). At day 7, animals were ventilated with a 3 mL tidal volume; and at day 60, animals were ventilated with a 4.5 mL tidal volume [60, 105].

Pulmonary Resistance (R_L) was measured at baseline, following delivery of nebulized saline, and after each dose of ACh (Sigma-Alderich, St. Louis, MO, USA) (0.5, 1.5, 5, 15, 50, and 150 $mg \cdot mL^{-1}$). ACh was nebulized in a jet nebulizer with an input oxygen flow rate of 8 $L \cdot min^{-1}$ and administered as six tidal breaths [105]. Immediately following each dose of ACh, volume changes in the box and pressure differences between the trachea and esophageal catheter were recorded for 20 seconds. Prior to subsequent challenge with the next higher dose of ACh, it was ensured that transpulmonary pressure either returned to baseline or, at higher ACh doses, stabilized. The flow and transpulmonary pressure signals were measured by a DIREC physiological recording system incorporating ANADAT software (Raytech Instruments, Vancouver, BC, Canada). Flow was calculated from the electronically differentiated volume signal with respect to time and transpulmonary pressure was calculated as the difference between the tracheal pressure and the pressure in the distal esophagus. R_L was calculated by the ANADAT software according to the method of Neergaard and Wirz [137] as follows: R_L = Transpulmonary Pressure / Airflow. For each animal, results were expressed as the mean of the three highest clustered values of R_L measured at a given challenge concentration of ACh over the 20 second interval. R_L was expressed as the absolute change in pulmonary resistance ($cmH_2O \cdot mL^{-1} \cdot s$). After completing the ACh challenges, guinea pigs were killed with an overdose of sodium pentobarbital (150 $mg \cdot kg^{-1}$) (MTC Pharmaceuticals Inc. Cambridge, ON).

5.9 LUNG TISSUE PROCESSING

Lungs were removed under aseptic conditions. Two lung lobes were cut into small pieces less than 0.5 cm in thickness and 600-700 mg of the tissue was placed into 7 mL of RNAlater (Qiagen GmbH, Hilden, Germany) in a 15 mL cryotube (Nalgene). Tissue in RNAlater was refrigerated at 4°C overnight and then stored at -20 °C for archival storage. The remaining small pieces of the lung tissue were wrapped in sterile aluminum foil and flash frozen in liquid nitrogen. Another lobe was inflated with a 1:1 solution of optimal cutting temperature compound (OCT) (Sakura Finetek, Torrance, CA) in sterile saline [138]. The inflated lung was then sectioned along the sagittal plane into 2-4 mm thick sections using a sterile scalpel. Two alternate sections were placed cut-side down in a Tissue-Tek® (Sakura, Tokyo, Japan) cassette and fixed in 10% formalin (Fisher).

5.10 LUNG HISTOLOGY

Airway inflammation was assessed in membranous bronchioles (non-cartilaginous, muscular airways) by comparing the area of the airway occupied by eosinophils and CD3+ cells to total airway area to determine a percentage of airway area occupied by these cells. These values were then compared between groups [132].

5.10.1 Eosinophil Staining

Following formalin fixation, lung specimens were embedded in paraffin and 4 μm sections were mounted on glass Histobond (Marienfeld, Germany) slides. One lung section was stained per animal. Eosinophils were stained by Hansel's method that stains basic arginine residues of eosinophil granules [139]. Slides were stained using an

Eosinophil Stain Kit (ENG Scientific Inc., Clifton, NJ). Slides were flooded with eosin solution for 60 seconds and phosphate buffer (pH 6.8) was added to the slides for 5 minutes. After washing with distilled water, the slides were counterstained with diluted methylene blue and then quickly washed in distilled water, dehydrated, cleared and mounted.

5.10.2 T cell (CD3+) Staining

Lung specimens were embedded and sectioned as above. Slides were baked at 60°C for 30-40 minutes then pretreated with Dako Target Retrieval Buffer for 22 minutes in an autoclave at 122 °C. The slide was then rinsed with distilled water and normal rabbit serum (Dako Cytomation, Carpintera, CA) was added for 20 minutes. T cell specific CD3+ polyclonal antibody (Cell Marque, Hot Springs, AK) (1/250 dilution in TBS-BSA buffer) was then added to slide for 60 minutes and the slide was washed with Tris-buffered saline (TBS) plus 0.1% Tween (TBS/Tween) (Sigma). Biotinylated goat anti-rabbit secondary antibody was then added for 30 minutes and the slide was again rinsed with TBS/Tween. Anti-alkaline phosphatase (APAAP) (Dako) was then added for 30 minutes and the slide was rinsed with TBS/Tween. Subsequently, new fuchsin was added for 20 minutes, and the slide was given a final rinse with distilled water and counterstained with Gill's Hematoxylin for 2 minutes.

5.10.3 Image Capture and Cell Counting

Slides were coded such that the microscopist was blinded with respect to the study group from which the slide originated. Digital photographs of five to ten membranous

bronchioles per animal were captured using a digital camera mounted on a light-microscope (Nikon, Japan) [105]. Images were saved as uncompressed tiff files. Image Pro PLUS image analysis software (Media Cybernetic, Inc.; Silver Spring MD, USA) was used to place a blue grid (60x60 pixels) over each image. The airway was then traced to designate an area of interest (AOI) and a computer macro was designed to calculate the number of grid points within the AOI. The grid points that were on positively stained cells and on luminal space were then counted and the percentage of airway occupied by CD3+ cells or eosinophils was calculated as follows:

(Positive Points/ (Total Number of Grid Points in AOI – Luminal Points)) * 100

Values per airway were then expressed as a percentage.

5.11 CYTOKINE MESSENGER RNA EXPRESSION

5.11.1 RNA Extraction

RNA was extracted from 140-160 mg of lung tissue preserved in RNAlater (Qiagen) using an RNeasy Midi Kit (Qiagen) according to the manufacturer's protocol. Lung tissue was placed in 4 mL of Buffer RLT in a 50 mL conical tube (Becton Dickenson) and homogenized at 30,000 rpm with a Tissue-Tearor® (BIOSPEC Products Inc, Racine, WI, USA) for 2 minutes. The homogenate then underwent centrifugation at 4, 000 x *g*, for 10 minutes, at 22°C. The supernatant was then transferred to a 15 mL conical tube (Becton Dickenson) and 4 mL of 70% ethanol in 0.1% diethylpyrocarbonate (DEPC) treated distilled deionized water was added to the sample. The sample was shaken vigorously and added to an RNeasy Midi column. The column was then centrifuged for 5 minutes as above. The flow through liquid was discarded and then 2 mL of Buffer RW1

were added to the column. The column was spun again for 5 minutes and the flow through liquid was discarded. At this point, RNase Free DNase (Qiagen) was applied to column and allowed to incubate at room temperature for 15 minutes. Following incubation the column was again rinsed with RW1 and then rinsed twice with 5 mL of RPE. After the last RPE rinse, 250 µL of RNase Free water (Qiagen) was added to the column and allowed to incubate at room temperature for 1 minute. The column was then spun at 4,000 x *g* for 1 minute at 22°C to elute the RNA. This step was repeated to yield ~ 500 µL of RNA and 100 µL aliquots were stored at -70 °C.

5.11.2 Reverse Transcription (RT)

The RNA concentration was determined at 1:50 dilution in 0.1% DEPC (Sigma) treated water using a spectrophotometer (Pharmacia Biotech, Cambridge, England). For each animal 0.5 µg of RNA underwent reverse transcription using random hexamers and Superscript II RNase H^- Reverse Transcriptase (Invitrogen Life Technology, Carlsbad, CA, USA). Reverse transcription was performed according to the manufacturer's instructions as follows: 0.2 µg of random hexamers (Invitrogen) and 10 mM of deoxynucleotide triphosphate (dNTP) (Invitrogen) mix were added to 0.5 µg of RNA and RNase Free water (Qiagen) was added to a total volume of 12 µL. The mixture was then incubated for 5 minutes at 65°C in a Robocycler 40 (Stratagene; La Jolla, CA), quickly chilled on ice and underwent brief centrifugation. Following centrifugation, 4 µL of First Strand Buffer, 2 µL of 0.1 M DTT (both included with Superscript II), and 1 µL of RNaseOUT Recombinant Ribonuclease Inhibitor ((40 units/µL) Invitrogen) were added to the mixture and mixed by pipetting. The mixture was then incubated for 2 minutes at

42°C and 1 μL of Superscript II ((200 units/μL) Invitrogen) was added. The mixture was then incubated for 10 minutes at 25°C and then 42°C for 50 minutes. Finally the mixture was heated at 70°C for 15 minutes to inactivate the Superscript enzyme. The obtained complementary DNA (cDNA) was then stored at –20°C for later amplification by PCR. Reverse transcription was performed in duplicate for each animal. For every 12 samples, a control consisting of 0.5 μg of RNA from one of the samples and all of the necessary reagents except the reverse transcription enzyme was run in parallel. This reaction controlled for DNA contamination of the RNA. Additionally, for every 24 samples a control containing RNase-free H_2O instead of RNA was run to control for contamination of reagents. Thus RT was run in batches of 27 reactions consisting of 24 samples and 3 controls.

5.11.3 Polymerase Chain Reaction (PCR)

As target messenger RNA (mRNA) was expressed at low levels, we used a semi-quantitative PCR method to amplify and quantify nucleic acid. The two genes of interest were IFN-γ and IL-5. β-actin was used as a housekeeping gene. Primers were designed by Tayyari *et al.* [140] so that the target sequence spanned an intron to distinguish the gene from cDNA and a Genbank (National Institutes of Health, Bethesda, MD, USA) search did not show homology between the primer sequences and any other guinea pig or mammalian sequences. PCR reactions were carried out in 50 μL reaction volumes. Each reaction contained 2 μL of cDNA (undiluted for IFN-γ and IL-5 and 1/100 serially diluted in Buffer EB (Qiagen) for β-actin), 5 μL of 10 X PCR Buffer (Qiagen), 2 μL of 25 mM $MgCl_2$ (Qiagen), 1 μL of 10 mM dNTP mix (Invitrogen), 2.5 μL of each 10 mM

primer (Sigma Genosis, St. Louis, MO, USA), 34.875 μL of distilled deionized water, and 0.125 μL of HotStar *Taq* DNA Polymerase ((5 units/μL) Qiagen). Reaction mixtures were incubated in a Robocycler 96 (Stratagene) according to conditions outlined in Table 5.2 and primer sequences are listed in Table 5.3.

For each cDNA sample or RT control, one PCR reaction was performed for each gene and, for every batch of RT reactions (27 reactions) subject to PCR, two PCR controls were run in parallel with the cDNA samples. One control was a negative control containing distilled deionized water, and the second control was a positive control containing a known number of PCR amplicons to control for variation in batch efficiencies. The IFN-γ and β-actin controls contained 10^6 PCR amplicons and the IL-5 internal control contained 10^5 amplicons. The number of amplicons was based upon preliminary studies that compared the intensity of serially diluted amplicons to cDNA from three untreated guinea pigs. The number of amplicons used in the positive control was chosen based on similar optical density (OD) readings of PCR products on ethidium bromide stained 2% agarose gels visualized under ultraviolet light (see section 5.11.4 for details on measuring OD).

5.11.4 Gel Electrophoresis, Digital Image Capture and Densitometry

For a given replicate per animal, 20 μL of PCR product for each gene was mixed together and 12 μL of 6X Xylene Cyanol loading dye was added and mixed by pipetting. Thirty μL of this mixture then underwent electrophoresis on ethidium bromide-stained 2% agarose gels in 1X tris-borate (TBE) buffer for 1.5 hours at 140 volts in a Horizon 40 Electrophoresis apparatus (Gibco). PCR products were imaged under ultraviolet light (λ

= 312 nm) and a digital image of the gel was taken using an Eagle Eye digital image capture and analysis system (Stratagene). Gels were exposed to UV light such that none of the bands were saturated on the digital image. Under these conditions distinct bands from all three genes for each animal were visible in each lane. The optical density of each band was measured using the Eagle Eye digital image capture and analysis system. Each band was then normalized to the positive standard and then to the β-actin/ β-actin positive standard intensity.

5.12 RSV IMMUNOHISTOCHEMISTRY

One lung section was immunostained per animal. Positive controls consisted of human and guinea pig lung tissue specimens from which replicating RSV had been isolated by culture. Slides were treated as above for CD3+ staining. After autoclaving, slides were stained using a Dako Autostainer (Dako Cytomation) at room temperature. Normal rabbit serum was added for 20 minutes, followed by the addition of anti-RSV NCL-RSV3 antibody (1:200) (Novacastra, Newcastle, UK) for 60 minutes. The slide was then rinsed with TBS/Tween and a secondary rabbit-anti-mouse antibody was added for 30 minutes. The slide was again rinsed with TBS/Tween and alkaline phosphatase-mouse anti-alkaline phosphatase (APAAP) was added for 40 minutes. After a rinse with TBS/Tween, the slide was stained with fuchsin for 20 minutes, rinsed with distilled water, and stained with hematoxylin for 2 minutes. Slides were then coverslipped and examined under a light microscope. Results were reported as "positive" (unequivocal signal identified) or "negative" for each animal.

5.13 STATISTICAL ANALYSIS

For ACh challenge, for each dose in each group of guinea pigs the mean R_L and standard error (SE) were calculated. Using the mean R_L and SE values, ACh dose response curves were plotted for each group at day 7 and day 60, and the curves were compared by using the General Linear Model with Repeated Measures [141].

For the IFN-γ/IL-5 mRNA ratios, optical density values for each animal were normalized to the positive standard and then to β-actin/β-actin positive standard intensity. The duplicate values for each animal were averaged, log transformed and the values for each group were compared using ANOVA. When significant differences were found by ANOVA, groups were compared using *post-hoc t*-tests.

For airway CD3+ cells and eosinophils, the mean percentage $\pm$ standard deviation (SD) and, for the traced airways or AOI, the mean total number of points counted (total grid points in AOI less lumen grid points) $\pm$ SD for each group was calculated and log transformed to better approximate a normal distribution [60]. The transformed mean total number of points counted was then subjected to ANOVA to ensure that the groups were comparable with respect to sampling. At each time point, the log-transformed value of the mean percentage for each group was compared using a one-way ANOVA. When ANOVA revealed significant differences, *post-hoc t*-tests were performed to compare the mean values between groups of animals.

The proportion of animals in each group with positive RSV immunostaining was compared by using a Fisher exact test [141]. The statistical software used was SPSS v.11 (Statistical Products and Services Solutions, Chicago, IL) and a two-tailed p-value <0.05 was considered significant for all analyses.

Table 5.1 PCR Cycling Conditions: Temperatures, Times and Number of Cycles

Target Gene	**Activation (1 cycle)**	**Denaturing Annealing Extension**	**Final Extension (1 cycle)**
β-actin	15 min, 95°C	1 min 94°C, 1 min 59°C, 1 min 72°C (30 cycles)	10 min, 72°C
IFN-γ	15 min, 95°C	1 min 94°C, 1 min 58°C, 1 min 72°C (40 cycles)	10 min, 72°C
IL-5	15 min, 95°C	1 min 94°C, 1 min 60°C, 1 min 72°C (40 cycles)	10 min, 72°C

Table 5.2 Primer sequences and PCR amplicon length

Target Gene	Primer Sequence	Amplicon Length
β-actin	Forward: 5'-ACTGGGACGACATGGAGAAG-3'	157
	Reverse: 5'-GGGGTGTTGAAAGTCTCGAA-3'	
IFN-γ	Forward: 5'-AGGAGACGATTTGGCTCTGA-3'	298
	Reverse: 5'-GAAGTTCTTTGGACCTGATCG-3'	
IL-5	Forward: 5'-TGTCTGTGTCTGTGCCATCC-3'	215
	Reverse: 5'-CCAGAGCTTCCCCTTGTGTA-3'	

CHAPTER 6: RESULTS

The four groups of animals in this study (Figure 5.1) included:

Group 1: Sham Inoculated

Group 2: Challenge Dose RSV

Group 3: Intervention Dose RSV + Challenge Dose RSV

Group 4: CpG-ODN + Challenge Dose RSV

During our experiments three animals died, one from each of Groups 2 and 3 in the acute RSV infection subgroup and one from Group 4 in the chronic RSV infection subgroup. The final numbers of animals available for study were: Day 7: Group 1 (n=12), Group 2 (n=11), Group 3 (n=11), Group 4 (n=12); Day 60: Group 1 (n=12), Group 2 (n=12), Group 3 (n=12), Group 4 (n=11).

6.1 AIRWAY HYPERRESPONSIVENESS (AHR)

Figure 6.1 shows the ACh dose response curves for all 4 groups of animals on day 7. The ACh dose response curves for Groups 2 and 3 were significantly different from the curves for Groups 1 and 4 ($p<0.006$). There was no significant difference between the curves for Groups 2 and 3 or between the curves for Group 1 and 4.

Figure 6.2 shows the ACh dose response curves for all 4 groups of animals on day 60. The dose response curve for Group 2 was significantly different compared to the curves for the three other groups of animals ($p<0.05$) and there were no significant differences between the dose response curves for Groups 1, 3 and 4.

6.2 QUANTITATIVE HISTOLOGY

Photomicrographs of airways stained for T cells (CD3+) and eosinophils are shown in Figure 6.3. The log of the mean percentage of the airway wall occupied by T cells and eosinophils on days 7 and 60 are represented in Tables 6.2 - 6.5 and untransformed percentages for T cells and eosinophils at both time points are displayed in Appendix A. The mean total number of points counted (total grid points in AOI less lumen grid points) for each group was also calculated and compared by ANOVA. No significant differences in the total number of points counted between any of the groups at either time point were found (data not shown).

Tables 6.2 and 6.3 show the airway T cell and eosinophil results on day 7, respectively. Groups 2 and 3 had significantly higher levels of T cells in their airways compared to Group 1 ($p<0.001$) and there was no significant difference between Groups 2 and 3 ($p=0.08$). Group 4 had an "intermediate" level of T cells that was significantly different from all other groups ($p<0.03$). Concerning eosinophils, Group 2 had significantly higher eosinophil counts compared to the three other groups of animals ($p<0.03$). Group 3 had an "intermediate" level of eosinophils that was significantly different from all other groups of animals ($p<0.03$). Group 4 was not significantly different from Group 1 ($p=0.66$).

Tables 6.4 and 6.5 show the airway T cell and eosinophil results on day 60, respectively. Group 2 had a significant increase in T cell percentages compared to the other three groups of animals ($p<0.03$) and there were no significant difference in the T cell percentages between Groups 1, 3 and 4 ($p=0.30$). Group 2 had a significantly higher percentage of eosinophils in the airway compared to Groups 1, 3 and 4 ($p<0.001$), and

there was no significant difference in the eosinophil counts between Groups 1, 3 and 4 ($p=0.15$).

6.3 LUNG IFN-γ/IL-5 CYTOKINE RATIOS

Figure 6.4 shows a representative ethidium bromide stained 2% agarose gel where all three PCR products from individual guinea pigs were run in a single lane of the gel. No bands were identified in any of the negative control reactions. Table 6.6 shows the mean $\pm$ SD of the $\log_{10}(\text{IFN-}\gamma_{average}/\text{IL-5}_{average})$ for the 4 groups of guinea pigs on day 7 and untransformed values are displayed in the Appendix B. There was a significant decrease in the lung IFN-γ/IL-5 ratio for Groups 2 and 3 compared to Groups 1 and 4 ($p<0.001$ and $p<0.002$, respectively). The mean lung IFN-γ/IL-5 ratios for Groups 2 and 3 were similar, as were the ratios for Groups 1 and 4.

The results from day 60 cytokine ratio analysis are shown in Table 6.7 as the mean $\log_{10}(\text{IFN-}\gamma_{average}/\text{IL-5}_{average})$ and untransformed values are displayed in the Appendix B. Group 2 had a significant decrease in the lung IFN-γ/IL-5 ratio compared to Groups 1 and 4 ($p<0.05$ and 0.02 respectively). Group 3 had a significant decrease in the ratio compared to Group 4 ($p<0.042$) and had a trend towards a significant decrease compared to Group 1 ($p=0.085$). Group 3 was not significantly different from Group 2 ($p=0.76$).

6.3.1 Comparison Between Day 7 and 60 Lung IFN-γ/IL-5 Ratios

There were no significant differences between the mean $\log_{10}(\text{IFN-}\gamma_{average}/\text{IL-5}_{average})$ ratios within any group of animals between day 7 and day 60.

6.4 RSV IMMUNOSTAINING

Figures 6.5 and 6.6 show photomicrographs of RSV immunostaining from a Group 2 animal on days 7 and 60, respectively. No false positive immunostaining was seen in any of the sham-inoculated (Group 1) animals (data not presented). Tables 6.7 and 6.8 show the proportion of animals in each group with RSV positive immunostaining on days 7 and 60, respectively. On day 7, the proportion of animals with RSV positive immunostaining in Groups 3 and 4 was significantly lower than Group 2 ($p<0.012$) and there was no significant difference in the proportion of animals with RSV positive immunostaining between Groups 3 and 4. On day 60, results were similar: Groups 3 and 4 had a significantly lower proportion of animals with RSV positive immunostaining compared to Group 2 ($p<0.005$) and there was no significant difference in the proportion of animals with RSV positive immunostaining between Groups 3 and 4.

6.5 SUMMARY

Table 6.9 is a summary of the results.

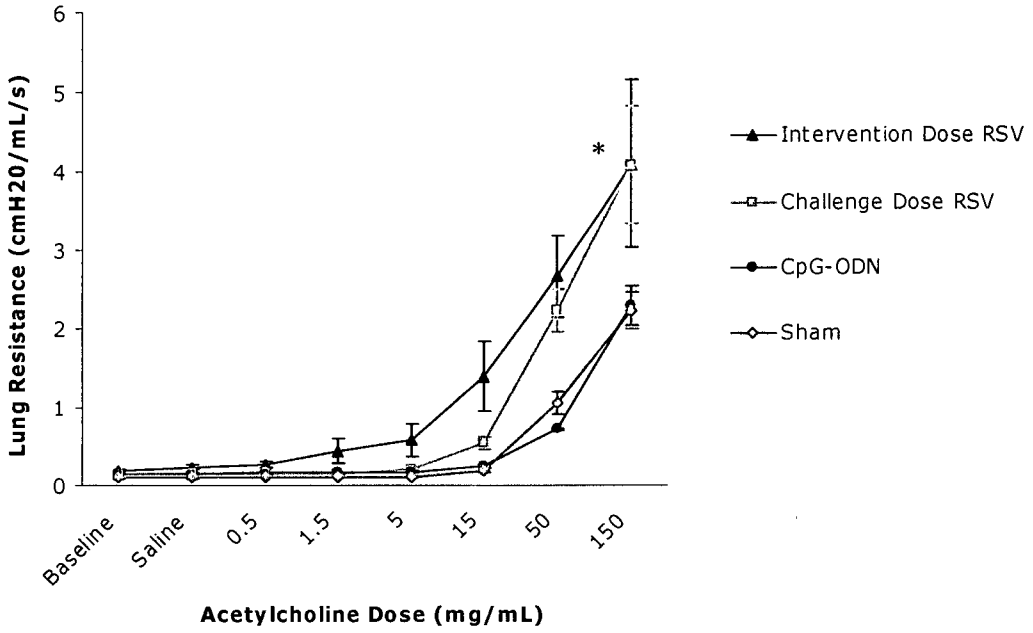

Figure 6.1 Day 7 acetylcholine dose response curves for all 4 experimental groups of animals. * = $p<0.006$ for Groups 2 and 3 compared to Groups 1 and 4. There were no significant differences between Groups 2 and 3, or between Groups 1 and 4.

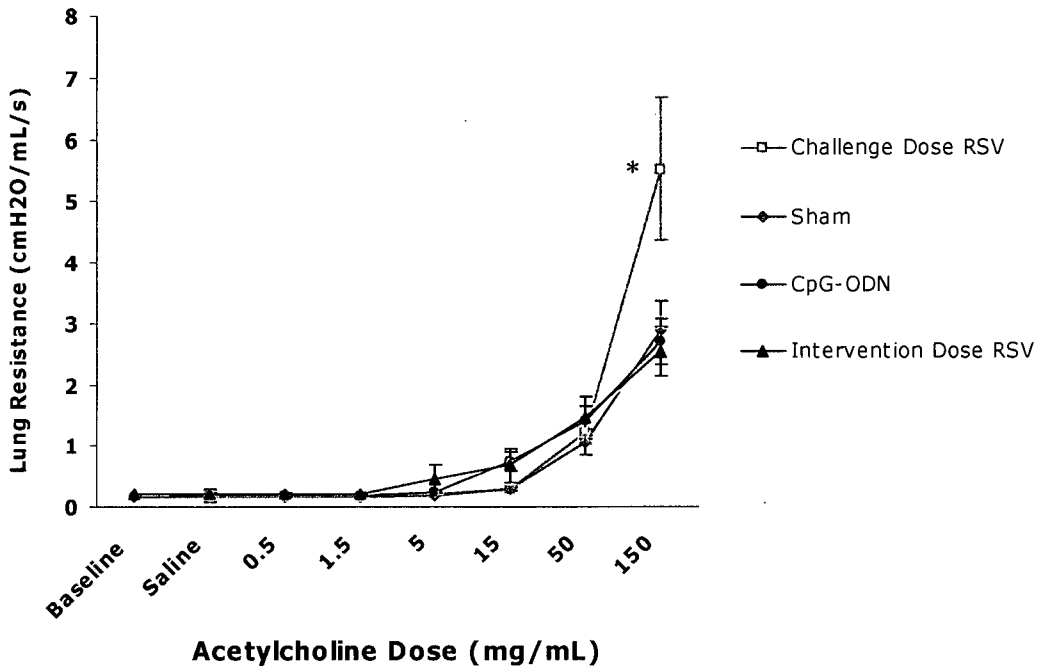

Figure 6.2 Day 60 acetylcholine dose response curves for all 4 experimental groups of animals. * = $p<0.05$ compared to all other groups.

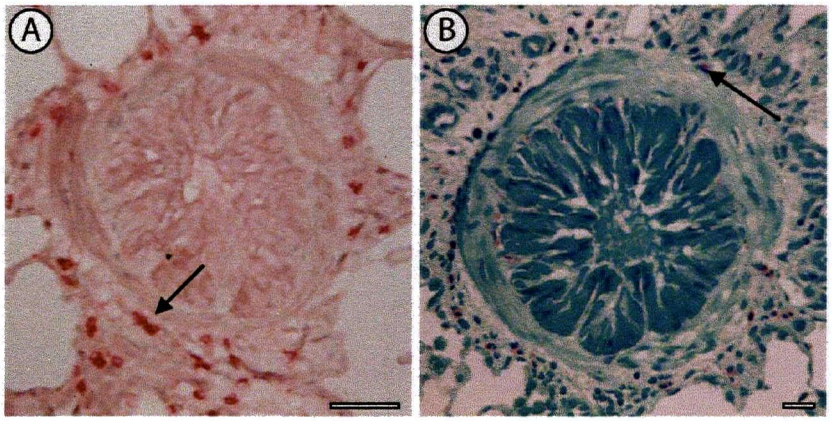

Figure 6.3 Representative photomicrographs of (A) Hansel stained (Eosinophils) and (B) CD3+ stained airways. Scale bar = 50 µm. Eosinophils and CD3+ cells stain bright pink (arrows).

Table 6.1 Results of Quantitative Histology for Airway T cells on Day 7

Group	Log of the Mean ± SD
1. Sham-Inoculated	- 0.24 ± 0.65
2. Challenge Dose RSV	0.75 ± 0.11*
3. Intervention Dose RSV + Challenge Dose RSV	0.67 ± 0.09*
4. CpG-ODN + Challenge Dose RSV	0.43 ± 0.22†

* Significantly different from Groups 1 and 4 ($p<0.001$)

† Significantly different from Groups 1, 2 and 3 ($p<0.03$)

Table 6.2 Results of Quantitative Histology for Airway Eosinophils on Day 7

Group	Log of the Mean ± SD
1. Sham-Inoculated	0.40 ± 0.20
2. Challenge Dose RSV	0.82 ± 0.17*
3. Intervention Dose RSV + Challenge Dose RSV	0.64 ± 0.17†
4. CpG-ODN + Challenge Dose RSV	0.43 ± 0.22

* Significantly different from Groups 1, 3 and 4 ($p<0.03$)
† Significantly different from Groups 1, 2 and 4 ($p<0.03$)

Table 6.3 Results of Quantitative Histology for Airway T cells on Day 60

Group	Log of the Mean ± SD
1. Sham-Inoculated	- 0.15 ± 0.64
2. Challenge Dose RSV	0.28 ± 0.19*
3. Intervention Dose RSV + Challenge Dose RSV	0.06 ± 0.23
4. CpG-ODN + Challenge Dose RSV	- 0.02 ± 0.27

* Significantly different from Groups 1, 3 and 4 ($p<0.03$)

Table 6.4 Results of Quantitative Histology for Airway Eosinophils on Day 60

Group	Log of the Mean ± SD
1. Sham-Inoculated	0.27 ± 0.17
2. Challenge Dose RSV	0.69 ± 0.22*
3. Intervention Dose RSV + Challenge Dose RSV	0.36 ± 0.13
4. CpG-ODN + Challenge Dose RSV	0.26 ± 0.27

* Significantly different from Groups 1, 3 and 4 ($p<0.001$)

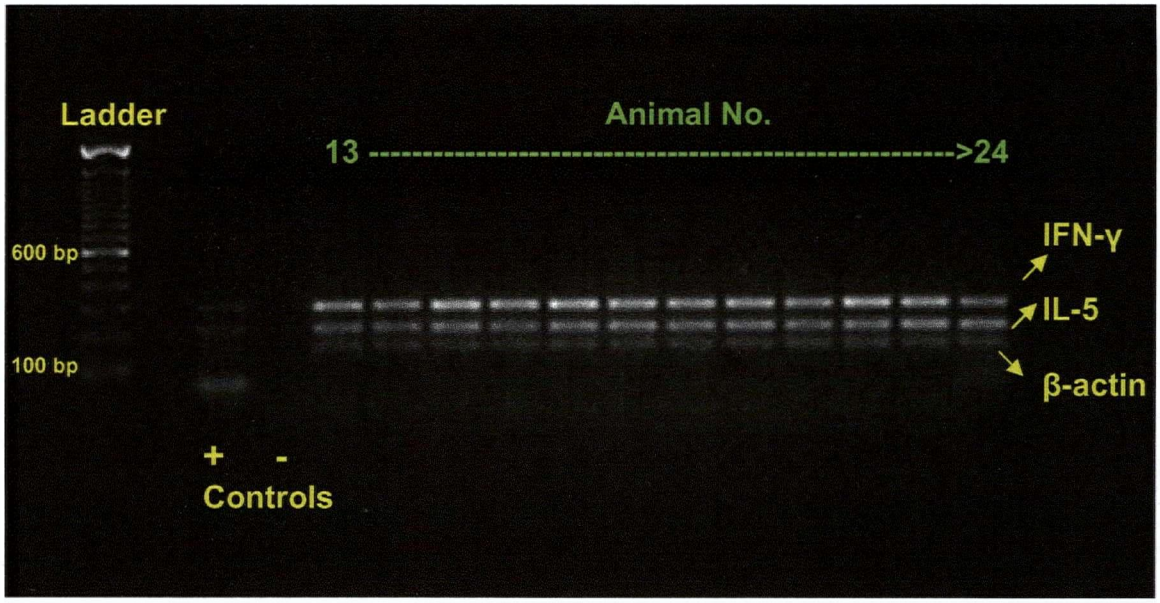

Figure 6.4 Image of an ethidium bromide stained gel with PCR products for IFN-γ, IL-5, and β-actin for each animal run together in one lane. These PCR products are from animals in the Sham-Inoculated Group on Day 7. The IFN-γ band is 298 base pairs long, the IL-5 is 215 base pairs long, and the β-actin band is 157 base pairs long. The positive control consists of a known number of PCR amplicons subject to PCR for each gene, and the negative control consists of distilled deionized water subject to PCR for each gene.

Table 6.5 Results of Day 7 Lung IFN-γ/IL-5 Cytokine Gene Expression

GROUP	Mean ± Standard Deviation of $\log_{10}(\text{IFN-}\gamma_{average}/\text{IL-}5_{average})$
1. Sham-Inoculated	0.223±0.029
2. Challenge Dose RSV	0.168±0.026 *
3. Intervention Dose RSV + Challenge Dose RSV	0.188±0.028 *
4. CpG-ODN + Challenge Dose RSV	0.231±0.031

* Significantly different from Groups 1 and 4 ($p<0.002$).

Table 6.6 Results of Day 60 Lung IFN-γ/IL-5 Cytokine Gene Expression

GROUP	Mean ± Standard Deviation of $\log_{10}(\text{IFN-}\gamma_{average}/\text{IL-}5_{average})$
1. Sham-Inoculated	0.204±0.037
2. Challenge Dose RSV	0.176±0.029*
3. Intervention Dose RSV + Challenge Dose RSV	0.180±0.026†
4. CpG-ODN + Challenge Dose RSV	0.206±0.031

* Significantly different from Group 1 ($p<0.05$) and Group 4 ($p<0.02$).
† Significantly different from Group 4 ($p<0.042$) and trend towards a significant difference from Group 1 ($p=0.085$).

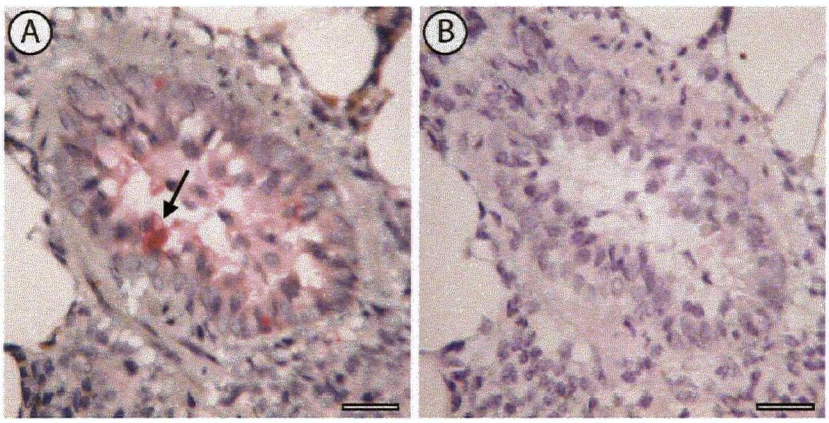

Figure 6.5 Photomicrographs of a RSV immunostained airway from Group 2 on day 7. (A) RSV positive immunostaining (arrow). (B) adjacent serial section stained with control antibody. Scale bar = 25 µm

Table 6.7 Results of Day 7 RSV Immunostaining

GROUP	Proportion of Animals per Group with RSV Positive Immunostaining
1 (Sham-Inoculated)	0/12
2 (Challenge Dose RSV)	9/11*
3 (Intervention Dose RSV + Challenge Dose RSV)	1/11
4 (CpG-ODN + Challenge Dose RSV)	3/12

* Significantly different from Groups 1,3 and 4 ($p<0.012$).

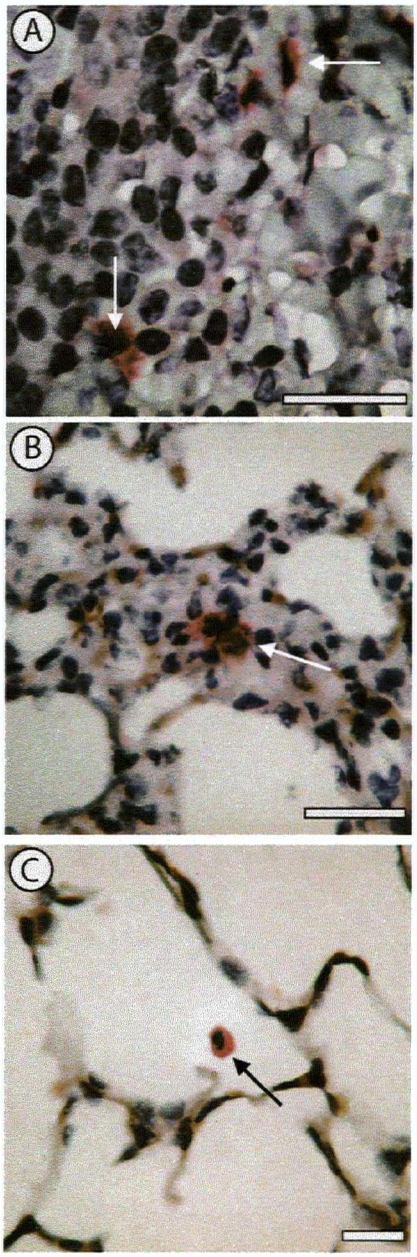

Figure 6.6 Photomicrographs of RSV immunostained airways from Group 2 on day 60 showing RSV antigen (arrows) in (A) bronchus-associated lymphoid tissue (BALT), (B) the interstitial space, and (C) an alveolar macrophage. Scale bar = 25 µm

Table 6.8 Results of Day 60 RSV Immunostaining

GROUP	Proportion of Animals per Group with RSV Positive Immunostaining
1 (Sham-Inoculated)	0/12
2 (Challenge Dose RSV)	7/12*
3 (Intervention Dose RSV + Challenge Dose RSV)	0/12
4 (CpG-ODN + Challenge Dose RSV)	0/11

* Significantly different from Groups 1,3 and 4 ($p<0.005$).

Table 6.9 Summary of Results

	Group	Day 7	Day 60
AHR (Group vs. Sham)	Challenge Dose	Yes	Yes
	Intervention Dose	Yes*	No
	CpG-ODN	No	No
T cells	Challenge Dose	↑↑	↑↑
	Intervention Dose	↑↑*	↔
	CpG-ODN	↑†	↔
Eosinophils	Challenge Dose	↑↑	↑↑
	Intervention Dose	↑‡	↔
	CpG-ODN	↔	↔
IFN-γ/IL-5 mRNA Ratio	Challenge Dose	↓↓	↓↓
	Intervention Dose	↓↓	↓§
	CpG-ODN	↔	↔
RSV Immunostaining	Challenge Dose	9/11	7/12
	Intervention Dose	1/11¶	0/12 ¶
	CpG-ODN	3/12 #	0/11 #

↑↑ or ↓↓ Large increase or decrease, respectively
↑ or ↓ Intermediate increase or decrease, respectively
↔ No difference compared to Sham
* Not significantly different from the Challenge Dose RSV Group
† Significantly different from the Sham-Inoculated, Challenge Dose RSV, and Intervention Dose Groups
‡ Significantly different from the Sham-Inoculated, Challenge Dose RSV, and CpG-ODN Groups
§ Significantly different from the CpG-ODN Group, with a trend towards a significant difference from the Sham-Inoculated Group
¶ Significantly different from Challenge Dose RSV Group but not significantly different from the CpG-ODN Group
# Significantly different from the Challenge Dose RSV Group but not significantly different from the Intervention Dose RSV Group

CHAPTER 7: DISCUSSION

The experiments conducted in this thesis were designed to test the hypothesis that two interventions would protect against the development of RSV bronchiolitis and sequelae in guinea pigs.

7.1 IMMUNE RESPONSE TO RSV

In 1999, Bramley *et al.* showed that 100 days post-RSV inoculation, Cam Hartley guinea pigs develop airway hyperresponsiveness (AHR) in association with RSV protein persistence [105]. In 2003, Bramley *et al.*, demonstrated that both "allergy-susceptible" Strain 13 guinea pigs and "allergy-resistant" Strain 2 guinea pigs developed a Th2 response during acute RSV infection in association with AHR and increased airway infiltration by T cells and eosinophils [60]. These two strains differed only in that Strain 2 showed a lower RSV load during acute infection.

In this thesis we sought to determine how two interventions, "low" dose RSV inoculation and CpG-ODN immunotherapy, affected experimental RSV infection of Cam Hartley guinea pigs in terms of AHR, airway T cell and eosinophil infiltrates, lung Th1/Th2 cytokine balance and viral persistence. During acute RSV infection (Day 7), the Challenge Dose RSV Group (Group 2) had a decrease in the lung IFN-γ/IL-5 mRNA ratio compared to the Sham-Inoculated Group (Group 1) which is indicative of a Th2-shift in the lung Th1/Th2 balance. Further, in comparison to Group 1, Group 2 showed an elevated ACh dose-response curve indicative of AHR, as well as increased T cell and eosinophil counts, indicating the development of airway inflammation. These results confirmed the findings by Bramley *et al.*, who reported that guinea pigs develop a Th2

shift during acute RSV infection in association with AHR and airway inflammation [60]. As expected, Group 2 showed 9/11 animals with RSV positive immunostaining compared to 0/12 in the Group 1. The lack of finding RSV positive immunostaining in all of the Group 2 animals examined may reflect a combination of the sensitivity of the RSV immunostaining technique in association with limited tissue sampling from our animals. On day 60, Group 2 showed results similar to those on day 7. In comparison to Group 1, Group 2 had an elevated ACh dose-response curve (indicative of AHR) and a decrease in the lung IFN-γ/IL-5 mRNA ratio (indicative of a Th2 shift). Group 2 also had significantly higher T cell and eosinophil counts compared to Group 1 (indicative of chronic airway inflammation). Group 2 showed viral protein persistence in 7/11 animals, consistent with a persistent RSV infection and previous studies in guinea pigs [101, 103]. Thus in the guinea pig it appears that RSV induces a Th2 response during acute infection that is maintained chronically in association with chronic airway inflammation, chronic AHR, and viral persistence.

In contrast to the guinea pigs, when mice are inoculated with RSV (~10^7 s.f.u.) they develop a Th1 response during acute infection [59] and this response is associated with AHR for at least 42 days post-inoculation [100, 128-130]. When AHR was studied up to 154 days post-RSV inoculation, mice did not show AHR between days 49-119 or on day 140, but did show AHR again on days 126, 133, and 154 [128] indicative of intermittent AHR. By contrast, AHR has not been shown to persist in mice for more than 21 days when the inoculum contained 10^5 s.f.u. of RSV [129, 130]. This apparent requirement for higher viral doses to induce chronic AHR in mice, may reflect the fact that lower initial doses of RSV might not allow dissemination of virus as extensively throughout the

lungs as when 10^7 s.f.u. are given. Thus the initial Th1 response or Th1 background in this animal might be capable of controlling the persistent infection when 10^5 s.f.u. of RSV are given and therefore inhibit the development of sufficient airway inflammation to induce AHR.

In comparison to guinea pigs, mice are 10 to 100 fold less permissive to RSV and do not develop acute lower respiratory tract disease or impressive bronchiolar inflammation during acute RSV infection [111, 123]. Mice also show a Th1 response to RSV while guinea pigs show a Th2 response. We postulate that this difference between species may represent a Th1 phenotype or predisposition in mice. Indeed, during acute RSV infection, two strains of mice and their respective knockouts for IL-4, IL-12, IL-18 all developed a Th1 response [142], whereas three strains of guinea pigs (1 outbred and 2 inbred strains) developed a Th2 response [60]. This Th2 response in guinea pigs in association with airway inflammation, AHR, and more severe disease compared to mice parallels a recent human study in which children with RSV bronchiolitis requiring hospitalization showed a Th2 cytokine profile compared to infants with RSV infection not requiring hospitalization [61]. These differences between species in their response to acute RSV infection suggests that it is not the type of immune response, but rather the elicitation of a host inflammatory and immune response to RSV that causes AHR.

The persistence of virus in both mice [106] and guinea pigs indicates that regardless of whether the initial immune response is Th1 or Th2, the virus manages to avoid being cleared. Tripp has postulated that RSV might infect immune privileged sites or is capable of evading the host immune system through another mechanism [143]. However, Zinkernagel has proposed that antigen, pathogen, or viral persistence is required for

immunological memory [144]. Specifically, persistence of antigen or virus is thought to be required to maintain memory T and B cells and circulating titers of antibodies that are protective against reinfection [145]. The maintenance of circulating titers of protective antibody is thought to enhance survival of the species because the mother through breast-feeding transfers these antibodies to her immuno-incompetent infant [145]. As regards to RSV, several studies have shown that children who receive high titers of anti-RSV antibody from their mothers, or that receive prophylaxis with anti-RSV antibodies, develop fewer and less severe RSV infections [16, 32, 33]. Further, persistence of a crippled strain of measles virus (a virus in the same family as RSV) has been shown to produce an antigen source that maintains high levels of protective antibodies [146]. Figure 6.11 shows RSV immunostaining in bronchus-associated lymphoid tissue (BALT), the interstitial space, and an alveolar macrophage in Group 2 on day 60, confirming previous findings in guinea pigs [105]. As these are all sites of immunological activity, this supports the hypothesis that RSV persistence is involved in immunological memory.

In comparing RSV infection of mice and guinea pigs, both species develop a persistent infection, however mice develop a Th1 response while guinea pigs develop a Th2 response. Further, mice require inoculation with higher titers of RSV to develop acute disease compared to guinea pigs, and mice only develop chronic AHR when inoculated with high titers of RSV (~ 10^7 s.f.u.). In contrast, guinea pigs reliably develop chronic airway inflammation and AHR at a lower dose of RSV (~10^4 s.f.u.). Taken together, these differences suggest that mice and guinea pigs have a Th1 or Th2 predisposition or

background, respectively, and that this background determines or controls the extent of chronic viral activity, airway inflammation and chronic AHR.

7.2 "LOW" DOSE RSV INTERVENTION

The use of a "low" dose of RSV as an intervention to optimize the Th1/Th2 balance away from a Th2 shift was based on studies in mice infected with *Leishmania* and *Mycobacterium* [85-87]. The results of these studies showed that relatively low initial pathogen doses induced a Th1 response, whereas high initial doses induced a mixed Th1/Th2 response. These studies further showed that upon subsequent exposure to the pathogen, regardless of the pathogen dose, the immune response elicited was the same as the initial response. Thus the initial response was "imprinted". As RSV vaccine-enhanced disease was primed by vaccination with relatively high doses of virus (10^6-10^8 s.f.u.) [52, 147] in humans, cotton rats and mice, we hypothesized that inoculation with a relatively "low" dose of RSV (~10^2 s.f.u.) would "imprint" a Th1 response that would be protective against a subsequent challenge with a "high" dose of RSV (4.5×10^4 s.f.u.). On day 7, the Intervention Dose RSV + Challenge Dose RSV Group (Group 3) had an elevated ACh dose-response curve, high levels of T cells, and a Th2 shift in the lung Th1/Th2 balance compared to the Sham-Inoculated Group (Group 1), and was similar to the Challenge Dose RSV Group (Group 2). Group 3 had an intermediate level of eosinophils that was significantly different from Groups 1 and 2. Interestingly, Group 3 had a significantly lower proportion of animals with RSV positive immunostaining than Group 2. Given that mice challenged with a second high dose (~10^7 s.f.u.) of RSV two months after initial RSV infection clear the virus from the lungs within 24 hours [148]

this finding is not surprising; however, mice rechallenged with RSV had circulating titers of RSV-specific antibodies, whereas guinea pigs inoculated with 10^2 s.f.u. of RSV did not [134]. We speculate that this "low" dose of RSV may not have provided sufficient antigen to induce a B cell-mediated antibody response. However the "low" dose of RSV might have provided enough antigen to prime a T cell specific memory response that, upon challenge with a high dose of RSV, was better able to prevent or clear the high dose RSV infection from the lungs.

On day 60, the ACh-dose response curve for Group 3 was similar to that for Group 1, and was significantly different than the curve for Group 2, indicating that Group 3 did not develop chronic AHR. Thus it appears that, despite the initial acute illness that was similar in severity to that documented in Group 2, the "low" dose RSV intervention prevented the development of chronic AHR. Further, Group 3 had an intermediate cytokine ratio that was not significantly different from the ratios for Group 1 or 2. On day 60, Group 3 showed no animals with RSV positive immunostaining indicating that inoculation with a "low" dose of RSV prevented the acute infection and/or enhanced viral clearance. In examining T cell and eosinophil counts on day 60, Group 3 had no significant increase in T cell and eosinophil counts compared to Group 1 and had significantly lower counts than Group 2, indicating that "low" dose RSV prevented chronic infiltration of T cells and eosinophils into the airway wall. Taken together, the absence of chronic AHR without an increase in T cells and eosinophils is not surprising, however the development of an intermediate cytokine ratio suggests that strong polarization towards a Th2 response (beyond a certain threshold) may be required for the development of chronic AHR in guinea pigs. The limited amount of virus on day 7

followed by an absence of virus on day 60 without the development of chronic AHR and chronic airway inflammation supports the idea that by limiting the extent of virus dissemination, there is decreased chronic airway inflammation and in turn AHR. These results suggest that inoculation with a "low" dose of RSV may not be a suitable vaccine strategy because acutely the guinea pigs still developed AHR, airway inflammation, and a Th2 response considered representative of bronchiolitis. Our findings are also in opposition to the results of previous studies [85-87] examining "low" doses of *Leishmania* and *Mycobacterium* in mice. It may be that viruses and/or pathogens that induce a strong Th2 shift (i.e. RSV) do not fit into this paradigm. Animal studies examining "low" dose inoculation with other viruses will help answer this question. Our day 60 results, however, may be helpful toward explaining the epidemiological finding that not all children develop post-bronchiolitis wheezing and asthma [4-7, 9-11]. For example, children who do not develop post-bronchiolitis wheezing and "asthma" may have already been primed with a "low" dose of RSV, thus these infants still develop bronchiolitis but do not develop chronic RSV infection and the associated chronic AHR. Infants who, on the other hand, are initially exposed to a "high" infectious dose of RSV might possibly develop bronchiolitis, a persistent RSV infection, and chronic AHR.

7.3 CpG-ODN INTERVENTION

The rationale for using CpG-ODN as an intervention was to induce a Th1 response that would prevent or counter the Th2 shift induced by RSV. On day 7, the CpG-ODN + Challenge Dose RSV Group (Group 4) had an ACh dose response curve that was not significantly different that the curve for Group 1 and that was significantly different from the curve for Group 2, indicating that Group 4 did not develop AHR during acute RSV infection. Additionally, the lung IFN-γ/IL-5 mRNA ratio for Group 4 was not significantly different from Group 1 and was significantly different from Group 2, indicating that CpG-ODN prevented a Th2 shift during RSV infection. In examining T cell and eosinophil counts on day 7, Group 4 had an intermediate level of T cells that was significantly different from Groups 1 and 2. Eosinophil counts for Group 4 were significantly lower than those for Group 2, and were not significantly different from Group 1 indicating that CpG-ODN administration prevented airway eosinophilia during acute RSV infection. An increase in T cells without a Th2 shift is not surprising because it is likely that CpG-ODN primed a Th1 response. Thus it is likely that many of the T cells were either CD8+ cytotoxic T cells or T helper 1 cells. Further, Group 4 had significantly fewer animals with RSV positive immunostaining compared to Group 2, suggesting that CpG-ODN administration prevented the initial infection and/or enhanced viral clearance from the lungs. This enhanced viral clearance may be the result of CpG-ODN acting as a "danger signal" to activate an innate immune response [149] or result from CpG-ODN inducing a predominant Th1 response. In comparison to Group 2 and 3 that both showed a polarized Th2 response in association with AHR, the absence of AHR without a Th2 shift in Group 4 suggests that a strong Th2 response is required to induce

AHR and that a balanced Th1/Th2 immune response prevents the development of airway inflammation and AHR. These results are consistent with the finding reported above of an absence of chronic AHR when an intermediate cytokine ratio is present in Group 3. On day 60, the ACh dose response curve for Group 4 is not significantly different than that for Group 1 and is significantly different than that for Group 2, indicating that CpG-ODN administration prior to inoculation with a challenge dose of RSV continued to prevent the development of chronic AHR. Further the lung IFN-γ/IL-5 mRNA ratio was not significantly different from that for Group 1 and was significantly different from that for Group 2, indicating that CpG-ODN administration prior to challenge dose RSV inoculation prevents the development of a chronic Th2 polarization/shift. T cell and eosinophil counts for Group 4 were not significantly different from Group 1 and were significantly lower than those for Group 2. Taken with the results from day 7, these findings indicate that by preventing or limiting the development of airway inflammation on day 7, CpG-ODN administration also prevented the development of chronic airway inflammation. Additionally on day 60, Group 4 had no animals with RSV positive immunostaining compared to 7/11 animals in Group 2, indicating that CpG-ODN enhanced viral clearance during the acute infection and prevented the establishment of a chronic or persistent RSV infection.

Taken together, the lack of AHR, Th2 shift, with enhanced viral clearance, and intermediate or no increase in airway inflammation on both days 7 and 60 respectively, indicates that CpG-ODN is an effective preventative intervention against RSV infection and its sequelae in guinea pigs. The lack of any viral persistence on day 60 is not surprising considering that CpG-ODN appears to have enhanced viral clearance on day 7.

Further, the association between complete viral clearance, absence of AHR and airway inflammation by T cells and eosinophils on day 60 supports the idea that limiting viral dissemination during acute infection prevents the establishment of a persistent infection capable of inducing chronic airway inflammation and AHR. Further, these findings contribute to the idea that a balanced Th1/Th2 immune response appears to prevent against substantial airway inflammation by T cells and eosinophils, AHR, and viral persistence.

This is not the first study to show that CpG-ODN can be protective against or limit infections. Studies with *Listeria monocytogenes, Francisella tularensis*, malaria, and *Leishmania major* have all shown that CpG-ODN can be protective against these pathogens [150-153]. CpG-ODN have also been shown to be protective against viral infections such as herpes simplex virus type 1 (HSV-1)[154] and HSV-2 [155-157]. Studies with CpG-ODN and HSV-2 have shown that topical administration of CpG-ODN to mice 2 or 6 hours after inoculation with a lethal dose of HSV-2 resulted in delayed disease progression and enhanced survival compared to untreated HSV-2 infected controls. In the same study, Cam Hartley guinea pigs were administered CpG-ODN topically after the establishment of a latent HSV-2 infection. Guinea pigs that received CpG-ODN had a significantly reduced frequency of genital lesion development compared to sham-treated HSV-infected controls and shed less HSV-2 although at the same frequency as the controls [157]. The finding that HSV-2 was shed at similar frequencies in both the CpG-ODN and control group suggests that CpG-ODN did not prevent reactivation of the latent infection, however the decreased viral load in the CpG-ODN treated group suggests that CpG-ODN induced an immune response that controlled the

extent of viral reactivation. Thus, this study suggests that a primed Th1 response may be better able to limit the extent of chronic viral replication. Given the results from our work in guinea pigs with RSV and the above studies showing therapeutic effects of administering CpG-ODN during HSV-2 infection, the next step would be to determine if CpG-ODN has therapeutic effects during acute RSV infection. However caution must be exercised in developing interventions that promote strong Th1 responses because a polarization in the Th1/Th2 balance towards a Th1 response has been implicated in the development of autoimmune diseases such as type 1 diabetes [80, 158]. This is particularly relevant to CpG-ODN administration as vaccination with BCG vaccine (the vaccine from which CpG motifs were first identified) has been shown to be a risk factor for the development of type 1 diabetes in children [159, 160].

7.4 SUMMARY AND CONCLUSIONS

In summary, RSV induces and maintains a Th2 shift in guinea pigs that is associated with airway inflammation, AHR, and chronic viral infection. The apparent discrepancy between guinea pigs and mice, with mice showing a Th1 response to RSV [59] can be explained by mice having a greater Th1 predisposition than guinea pigs. Despite species differences in initial immune responses, both mice and guinea pigs develop AHR during acute RSV infection [60, 100, 127], and this suggests that it is the extent, rather than the type of response to RSV that causes AHR. Mice also show persistent RSV infection [106], however chronic AHR does not develop reliably in mice and depends on the initial dose of RSV the mouse receives [128-130]. We postulate that in a Th2 predisposed host such as the guinea pig, a persistent, low-level RSV infection is sufficient to induce

chronic airway inflammation and AHR. By contrast, in a Th1 predisposed host such as the mouse, we postulate that persistent RSV infection is either better controlled or is not as well established, thus there is limited chronic airway inflammation and AHR. This idea is supported by the results that both CpG-ODN and "low" dose RSV reduce viral loads during acute RSV infection and prevent the development of chronic airway inflammation and chronic AHR.

"Low" dose RSV does not protect against the development of RSV bronchiolitis in guinea pigs; however it does appear to decrease viral load during acute RSV infection, indicating that the "low" dose primed an immune response better capable of clearing the virus that in turn prevented the development of a chronic RSV infection, chronic airway inflammation, and chronic AHR. The finding that "low" dose RSV does not prevent RSV bronchiolitis but may prevent post-bronchiolitis AHR might explain why not all children develop post-bronchiolitis wheezing. It may be that those children who do not wheeze have initially been primed with a "low" subclinical dose of RSV, thus they may still develop bronchiolitis upon exposure to a higher amount of virus, however they do not develop a persistent infection and chronic AHR.

In contrast to the "low" dose RSV intervention, CpG-ODN did confer protection against the development of RSV bronchiolitis, and in parallel to the "low" dose intervention, CpG-ODN did protect against the establishment of a persistent RSV infection and chronic AHR. During acute RSV infection, the CpG-ODN treated group did not develop a Th2 shift, AHR, or elevated levels of eosinophils, but did have an intermediate level of T cells in the airway wall. This intermediate level of T cells could be the result of CpG-ODN priming a Th1 response. In contrast to both the mouse and guinea pig that show a

polarized Th1 or Th2 response in association with AHR, the CpG-ODN pretreatment group showed that a "balanced" immune response prevented the development of AHR. Further, the finding that CpG-ODN pretreatment appears to reduce viral load during acute RSV infection suggests that by counteracting the Th2 response to RSV and maintaining the Th1/Th2 balance away from a Th2 response there is more effective viral clearance.

On day 60, the CpG-ODN group did not develop AHR, RSV persistence, airway inflammation, or a Th2 shift demonstrating that CpG-ODN protects against both RSV bronchiolitis and post-bronchiolitis sequelae. The absence of RSV persistence and chronic AHR is not surprising considering that CpG-ODN appears to have reduced viral load during acute infection without the development of AHR. However this finding does support the idea that by limiting initial viral dissemination and thus avoiding the establishment of a persistent infection, the development of post-bronchiolitis sequelae can be prevented. Taken together, these results strengthen the therapeutic appeal of CpG-ODN as a preventative measure against RSV bronchiolitis and its sequelae.

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APPENDIX A: UNTRANSFORMED QUANTITATIVE LUNG HISTOLOGY DATA

Untransformed Results of Quantitative Histology for Airway T cells on Day 7

Group	Mean Percentage ± SD
1. Sham-Inoculated	0.58 ± 4.47
2. Challenge Dose RSV	5.62 ± 1.29*
3. Intervention Dose RSV + Challenge Dose RSV	4.67 ± 1.23*
4. CpG-ODN + Challenge Dose RSV	2.69 ± 1.66†

* Significantly different from Groups 1 and 4 ($p<0.001$)

† Significantly different from Groups 1, 2 and 3 ($p<0.03$)

Untransformed Results of Quantitative Histology for Airway Eosinophils on Day 7

Group	Mean Percentage ± SD
1. Sham-Inoculated	2.51 ± 1.58
2. Challenge Dose RSV	6.61 ± 1.48*
3. Intervention Dose RSV + Challenge Dose RSV	4.37 ± 1.48†
4. CpG-ODN + Challenge Dose RSV	2.69 ± 1.66

* Significantly different from Groups 1, 3 and 4 ($p<0.03$)
† Significantly different from Groups 1, 2 and 4 ($p<0.03$)

Untransformed Results of Quantitative Histology for Airway T cells on Day 60

Group	Mean Percentage ± SD
1. Sham-Inoculated	0.71 ± 4.37
2. Challenge Dose RSV	1.91 ± 1.55*
3. Intervention Dose RSV + Challenge Dose RSV	1.15 ± 1.70
4. CpG-ODN + Challenge Dose RSV	0.95 ± 1.86

* Significantly different from Groups 1, 3 and 4 ($p<0.03$)

Untransformed Results of Quantitative Histology for Airway Eosinophils on Day 60

Group	Mean Percentage ± SD
1. Sham-Inoculated	1.86 ± 1.48
2. Challenge Dose RSV	4.90 ± 1.66*
3. Intervention Dose RSV + Challenge Dose RSV	2.29 ± 1.35
4. CpG-ODN + Challenge Dose RSV	1.82 ± 1.86

* Significantly different from Groups 1, 3 and 4 ($p<0.001$)

APPENDIX B: UNTRANSFORMED IFN-γ/IL-5 mRNA CYTOKINE RATIO DATA

Untransformed Results of Day 7 Lung IFN-γ/IL-5 Cytokine Gene Expression

GROUP	Mean ± Standard Deviation of $IFN\text{-}\gamma_{average}/IL\text{-}5_{average}$
1. Sham-Inoculated	1.67 ± 1.07
2. Challenge Dose RSV	1.47 ± 1.06*
3. Intervention Dose RSV + Challenge Dose RSV	1.54 ± 1.07*
4. CpG-ODN + Challenge Dose RSV	1.70 ± 1.07

* Significantly different from Groups 1 and 4 ($p<0.002$).

Untransformed Results of Day 60 Lung IFN-γ/IL-5 Cytokine Gene Expression

GROUP	Mean ± Standard Deviation of $IFN\text{-}\gamma_{average}/IL\text{-}5_{average}$
1. Sham-Inoculated	1.60 ± 1.09
2. Challenge Dose RSV	1.50 ± 1.07*
3. Intervention Dose RSV + Challenge Dose RSV	1.51 ±1.06†
4. CpG-ODN + Challenge Dose RSV	1.61 ± 1.07

* Significantly different from Group 1 ($p<0.05$) and Group 4 ($p<0.02$).
† Significantly different from Group 4 ($p<0.042$) and trend towards a significant difference from Group 1 ($p=0.085$).