THE ROLE OF INTERLEUKIN-12 IN THE PATHOGENESIS OF SENDAI VIRUS-INDUCED AIRWAY DISEASE

BY

AMY ELIZABETH SEYMOUR STONE

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2002

This work is dedicated to my mother, Dr. Sandra Fields Seymour, who instilled me with the courage and desire to become an educated woman.

ACKNOWLEDGMENTS

I would like to start by thanking my family; my parents, Andy, and Sydne were all so supportive through this whole thing. I really appreciate their never wavering support.

I thank all of my friends for never letting me take myself too seriously when things were tough. I appreciate my coworkers/fellow graduate students Diane Hulse and Tracy Jack for sticking with me through the highs (happy hour) and lows (molecular biology) of the last few years.

I would not have been able to complete this work without the support of my life partner, Andy. I cannot thank him enough for his love, patience, and ability to make me laugh at the times when I needed it.

Special thanks go to William Castleman for giving me the opportunity to embark on this dual degree program, for giving me guidance through the system, and for setting a quality example as a scientist and a leader.

I thank the members of my committee, Mary Brown, Thomas Brown, Steeve Giguère, Elizabeth Uhl, and former committee members Ammon Peck, Trenton Schoeb, and Rosalia Simmen for their ideas and assistance.

Additionally, I appreciate all of the editorial and motivational help that Carol Detrisac provided in the completion of this document. I would also like to acknowledge the other graduate student in my lab, Xuezhong Cai, who provided expertise, encouragement, and humor whenever needed.

Many thanks go to Karen Dukes, Heather Sorenson, Jeff Mans, Kristen Ruest, Mark Hickey, Timothy Holloway, and Christy Voakes, who provided help with laboratory work as well as much friendship.

Special appreciation and admiration go to Tracy Jack, who was my listening ear, my shoulder, my colleague, and my friend through this whole process.

TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

Figure page

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

THE ROLE OF INTERLEUKIN-12 IN THE PATHOGENESIS OF SENDAI VIRUS-INDUCED AIRWAY DISEASE

By

Amy Elizabeth Seymour Stone

December 2002

Chair: William L. Castleman
Major Department: Veterinary Medicine

Brown Norway (BN) rats are susceptible to Sendai virus-induced chronic airway inflammation that results in fibrosis and functional abnormalities resembling asthma. Fischer (F344) rats are resistant to these virus-induced changes and have earlier viral clearance, increased expression of Th-1 cytokines (e.g., interferon-γ, IFN-γ), and do not develop pulmonary function abnormalities. In contrast, BN rats are Th-2 type cytokine responders (e.g., interleukin-4, IL-4) and have delayed viral clearance. Due to the critical role of interleukin-12 (IL-12) in regulating the IFN-γ cytokine response in intracellular infections, I hypothesized that virus-resistant F344 rats are higher IL-12 gene responders than BN rats. Levels of IL-12 p40 messenger (mRNA) were measured by real-time polymerase chain reaction (RT-PCR) and IL-12 protein was detected by lung homogenate enzyme-linked immunosorbent assay (ELISA) at several time points after

viral inoculation. Although virus infection resulted in increased IL-12 production in both strains, F344 rats had significantly more IL-12 p40 mRNA than BN rats at 0-3 days (early) after virus inoculation ($p<0.05$). Furthermore, IL-12 total protein levels were elevated in F344 rats as early as 2 days following viral challenge, and the numbers of IL-12 p40 protein expressing cells were also significantly increased in their bronchioles at 2 and 3 days following Sendai inoculation ($p<0.05$). To evaluate the potential protective role of IL-12 in virus-induced airway injury, BN rats were given IL-12 intraperitoneally at either the time of (day 0) or two days after viral inoculation (day 2). In contrast to infected rats given saline, infected rats treated with IL-12 at day 0 had 22.1% lower levels of chronic airway inflammation, 23.8% lower levels of airway fibrosis, and 42% and 62.5% decrease in bromodeoxyuridine (BrdU)-labeled fibroblasts at 10 and 14 days after inoculation respectively ($p<0.05$). Day 0 treated BN rats had a 4-fold increase in the pulmonary IFN-γ mRNA and a 77% increase in IFN-γ protein as compared to saline-treated, virus-inoculated controls. In contrast, day 2 IL-12 treatment induced a 20% increase in bronchiolar airway wall thickness, a 12.5% increase in BrdU-labeled fibroblasts at 14 days after inoculation, and an increase in pulmonary IL-4 mRNA ($p<0.05$). The results are consistent with the hypothesis that resistance to virus-induced airway damage in F344 rats is due, at least in part, to their high virus-induced IL-12 gene expression.

CHAPTER 1
LITERATURE REVIEW

Interleukin-12

Interleukin-12 (IL-12) is a heterodimeric cytokine, which plays a role in the induction of cell-mediated and T helper type-1 (Th-1, $CD4^+$ T cells) immune responses (1). This cytokine is produced primarily by antigen presenting cells in response to intracellular bacterial, viral, protozoal, and fungal infections (1). The main function of IL-12 is to direct the "cross talk" between the phagocytic antigen presenting cells and effector lymphocytes by inducing the production of cytokines, particularly interferon-γ (IFN-γ), and by enhancing lymphocyte cytotoxic activity (2). Directly or indirectly, IL-12 is involved in the activation of macrophages, the generation and survival of Th-1 cells, the generation of cytotoxic T lymphocytes (CTL, $CD8^+$ T cells), and the suppression of IgG1 and IgE production (2). IL-12, synergistically with interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α), and interleukin-18 (IL-18) induces the maturation of Th-1 cells from Th-0 precursors by inducing the production of IFN-γ from resting and activated natural killer (NK) and T cells (1). IL-12 maintains high levels of IFN- γ once the $CD4^+$ and $CD8^+$ T cell types are established to resolve the infection (2). Additionally IL-12 acts to prevent the outgrowth and development of Th-2 cells and their production of Th-2 cytokines (3).

The structure of IL-12 is a unique heterodimer composed of two disulfide-linked subunits, p35 and p40 (representing the approximate molecular weights) (4). These two

subunits are encoded on two unrelated genes residing at independent loci in the mouse, human, and rat genomes (5-7). No sequence homology exists between the two subunits, but the p35 subunit shares homology with interleukin-6 (IL-6), granulocyte colony stimulating factor (G-CSF), and chicken myelomonocytic growth factor (8). The p40 subunit is not homologous to other cytokines, but is a part of the hemopoietin receptor family most closely resembling the IL-6 receptor α-subunit and the ciliary neutrotrophic factor receptor (5,9). Coexpression and covalent linkage of both chains of this cytokine in the same cell is required for the generation of the functionally active heterodimer (10).

Expression of the p35 chain is ubiquitously constitutive. The secretion of free p35 subunit has not been demonstrated (11-12). Both subunits are induced by intracellular infections via the subsequent activation of phagocytic cells (13). The p40 gene is located only in IL-12 producing cells, and is produced at much higher levels than the p35 chain or p70 heterodimer (11-12). The rate of p70 production is limited by p35 expression because the p40 subunit is produced by phagocytic cells at a few-fold to 1000-fold higher levels than the active heterodimer (14-15). Secretion of IL-12 p40 monomers and homodimers can have an inhibitory effect on the expression and production of the p70 heterodimer in murine T and NK cells (10). The p40 homodimer may be acting as a physiologic antagonistic regulator in the mouse system or may just be competing for the IL-12 cellular receptor (2). In humans, the p40 homodimer has modest ability compete with the heterodimer for IL-12 biological activity (16). Recently, human airway epithelial cells have been shown to produce high levels of p40 subunit, the possible role being to attract macrophages to the site of airway inflammation during mucosal defense (17).

The predominately transcriptionally regulated p40 subunit gene spans 13 Kbp containing eight exons preceded by a classical promoter (18,1). The murine p40 promoter contains three essential transcription factor-binding sites, a nuclear factor kappa B (NF-κB) site, a CCAAT enhancer-binding protein site (C/EBP), and an adaptor protein-binding site (AP-1), that are involved in lipopolysaccharide (LPS) and IFN-γ promoter activation (19-20). The human p40 promoter also has three essential *cis*-acting elements in its promoter, including a NF-κB site, an Ets (erythroblastosis virus oncogene homologue) core element (Ets-2, interferon regulatory factor-1 [IRF-1], interferon consensus sequence binding protein, and c-Rel), and a C/EBP site (18,21-22). The regulatory mechanisms of this promoter appear to involve functional synergy between the Ets and NF-κB transcription factors in the human gene, and the C/EBP factor also interacts functionally with the NF-κB factor in the promoters of both species (21-22). The *trans*-acting proteins interacting with the binding sites mediating the activation of the IL-12 p40 gene have not been well elucidated. However, a novel repressor element, GA-12 binding protein (GAP-12), has been shown to reduce inducible IL-12 p40 gene transcription in response to interleukin-4 (IL-4) and prostaglandin-E_2 (PGE_2) in human monocytes (23).

Due to the constitutive expression of the IL-12 p35 gene in many tissue types, many studies have focused on the p40 gene expression. However, p35 subunit production is more tightly regulated as it appears to be controlled translationally and transcriptionally (24). The murine p35 gene has multiple transcription start sites at either of two 5' exons resulting in mRNA isoforms with different untranslated regions (5'UTR) (25). The human p35 gene also has multiple forms initiating from two separate exons, suggesting

that the p35 genes in both species are similarly regulated (14). Under non-stimulated conditions, p35 transcripts contain an additional upstream ATG from a region whose presence inhibits translation (24). Stimulated cells produce transcripts that lack this upstream ATG that can be translated into p35 protein; the proportion of each set of transcripts *in vivo* depends on the stimulus (14,24-25). The multiple transcription start sites suggest the existence of two promoters, and the switch between the two may depend on the initiating transcription factor (14). There are multiple NF-κB sites, IRF-1 sites, and an AP-2 site within both promoter regions (14). Only the NF-κB, particularly c-Rel, and the proximal most IRF-1 sites have proven critical for efficient transcription (13,14). There are several other putative elements that may contribute to regulation, including gamma-associated elements (GAS), IFN-stimulated response elements (ISRE), and interferon consensus sequence binding sites (ICSBP) (14). Confusion regarding the gene expression of IL-12 p35 remains, due to the inability of conventional methods, such as northern blot analysis and reverse transcriptase-polymerase chain reaction (RT-PCR), to accurately distinguish between the at least four different mRNA isoforms.

The IL-12 receptor is composed of two subunits (IL-12Rβ1 and IL-12Rβ2) that are members of the cytokine receptor super family most closely related to glycoprotein 130 (gp130) (26). Coexpression of these two receptors is required for the formation of a high-affinity IL-12 binding site in human cells; however in mouse cells the IL-12Rβ1 subunit confers high and low-affinity binding (26-27). The IL-12Rβ2 subunit contains three tyrosine residues and is essential for signal transduction in both species (26-28). There is a 68% homology between the human and mouse receptors, and both are thought to be regulated and expressed in a similar manner (26). Expression of IL-12Rβ2 protein

may be limited to Th1 cells and this expression may correlate with IL-12 responsiveness (29-30). The IL-12Rβ1 subunit is present on resting NK and T cells and is up regulated during activation (30). The IL-12Rβ2 subunit is not present on resting CD4+ T cells but is up regulated upon antigen activation of the T cell receptor (TCR) (30). Upon T cell activation, the cytokines interleukin-4 (IL-4) and IFN-γ can modify this subunit expression. During T cell maturation, IL-4 can inhibit IL-12Rβ2 subunit expression, thus losing the ability to respond to IL-12 after TCR binding (30). When low levels of IL-4 are present, IFN-γ is required for IL-12Rβ2 expression (30). However, if there is no detectable IL-4, the presence of IFN-γ is not necessary (30). The IL-12Rβ2 chain appears also to be more highly regulated than the IL-12 Rβ1 by cytokines such as IL-10 and TGF-β_1 (31).

Once IL-12 is bound to its receptor, the complex induces the rapid tyrosine phosphorylation of both Janus (JAK) and Tyrosine kinases (Tyk) (32). Following the activation of the JAK and Tyk, three members of the signal transducers and activators of transcription (STAT) family are phosphorylated and activated [STAT-1, STAT-3, and STAT-4] (33-34). STAT-4 is not activated by any other cytokine except IFN-α in human cells, so it is unique to the IL-12 signaling pathway (35). STAT-4 is directly involved in the transcription of IFN-γ, primarily through two binding sites in the first intron (36-37). STAT-4 has been shown to be present and activated with translocation to the nucleus in T cells, NK cells, and dendritic cells (38). STAT-4 knock out mice have an almost identical phenotype to IL-12 or IL-12R knock out mice and produce no IFN-γ in response to IL-12 stimulation confirming this pathway in the IL-12 biological response (36,39). Additionally, there is evidence that IL-12 can induce transcription of an Ets transcription

factor called ERM in differentiated Th-1 cells through the binding of the TCR (40). The induction of this factor, although it does not activate STAT-4, is dependent on the presence of STAT-4 within the cell (40). This pathway may regulate another aspect of Th1 behavior or may require cooperation to regulate IFN-γ production (40).

The Role of Interleukin-12 in Regulating the Immune Response

The production of IL-12 in the immune response is complex and can be initiated by several different pathways. Phagocytic cells, including dendritic cells, produce IL-12 by T cell-independent and T cell-dependent means. Infectious agents, including bacteria, bacterial products, both metazoan and protozoan parasites, fungi and viruses, induce the production of IL-12 by phagocytic cells initiating the inflammatory process independent of T lymphocytes (15, 41). Additionally during inflammation, but independent of infectious agents or T cells, IL-12 production is induced by the interaction of adhesion molecules with substrates of the inflammatory cascade, such as the interaction of the CD44 adhesion molecule with low molecular weight hyalurnonan (42). This mechanism may contribute to macrophage activation due to the proinflammatory roles of IL-12 and IFN-γ (2, 42). The T cell-dependent mechanism of IL-12 production by antigen presenting cells depends on the CD40/CD40 ligand interaction with activated T cells (43). The antigen presenting cells induce IL-12 and up regulate receptors, such as B7 on monocytes and dendritic cells, thus activating antigen specific T cells (43). During this interaction, the p70 heterodimer is produced more or as efficiently as the p40 chain, indicating the effective up regulation of both p35 and p40 peptide chains (14,24,43). The T cells then produce IFN-γ and the cytokine granulocyte/monocyte-colony stimulatory factor (GM-CSF), which enhance the ability of the antigen presenting cells to produce IL-

12 (45-46). Two signals are required for both the T cells (CD40L and IFN-γ) to produce IFN-γ and for most of the antigen presenting cells (innate signal from infection and CD40L) to produce IL-12 in response to CD40 ligation (47). This bi-directional, two-signal interaction functions to maintain the T cell-independent mechanisms of IL-12 production that initiated the inflammatory process (48).

The ability of these various pathways to induce IL-12 production is regulated by both positive and negative feedback mechanisms. IL-12 induces the production of IFN-γ by T and NK cells; IFN-γ then enhances the expression of IL-12 by phagocytic cells and neutrophils (14,21,49). There are several other cytokines produced in response to IL-12 expression, such as TNF-α, GM-CSF, Interleukin-8 (IL-8), and interleukin-1β (IL-1β) (43,50). TNFα and GM-CSF are also involved in positive feedback loops with IL-12. Specifically, TNF-α enhances the ability of IFN-γ to prime phagocytic cells for IL-12 production, and GM-CSF has a priming effect on IL-12 production from phagocytic cells, primarily at the level of the p40 gene (51,52). Other Th-1 cytokines, such as IL-18, IL-2, and IL-15, are costimulatory with IL-12 in the production of IFN-γ. IL-2 or IL-12 alone can stimulate the production of IFN-γ from NK and T cells, but when acting together the half-life of the IFN-γ mRNA is doubled and IL-2 increases IL-12Rβ2 expression on activated NK cells, thus enhancing the production of IFN-γ (53, 54). IL-15 shares the same biological functions with IL-2 and seems to interact with IL-12 through similar pathways (55). IL-18 also acts with IL-12 to induce IFN-γ in T cells and NK cells, but this combination also is capable in vitro of stimulating IFN-γ enhancement in mouse dendritic cells and macrophages, suggesting the existence of an autocrine feedback loop in these professional antigen presenting cells (55-57).

The counter mechanisms that down regulate the positive amplification of IL-12 production are mediated by IFN-γ and they prevent uncontrolled cytokine production. Interleukin-10 (IL-10), potent inhibitor of IFN-γ production directly inhibits the production of IL-12 by antigen presenting cells (58). IL-10 is able to block the proliferation of Th-1 cells through the inhibition of IL-12 transcripts, other soluble cytokines, and costimulatory surface molecules by antigen presenting cells (58). In both mice and humans, IL-10 also prevents the development of mature, differentiated dendritic cells (59-60). However, IL-12 induces the production of IL-10 in T cells and primes T cell clones for high IL-10 production, thus inducing a negative feedback loop to reduce its own expression (61). Other inhibitors of IL-12 include transforming growth factor-β (TGF-β), PGE_2, and partial inhibitors IL-4, TNF-α and IL-13 (62). Like IL-10, TGF-β_1 suppresses IL-12 at the transcriptional level; however it also appears to reduce the stability of the IL-12 p40 mRNA (63). IL-4 and interleukin-13 (IL-13) can suppress IL-12 expression when added simultaneously with a stimulus to cell cultures. However, monocytes primed with IL-4 or IL-13 prior to stimulation can significantly enhance IL-12 expression (62, 64). These overlapping cytokine regulation pathways are postulated to be extra backup mechanisms to prevent uncontrolled production of proinflammatory cytokines.

Although B cells, neutrophils, microglial nerve cells, and macrophages produce some IL-12, dendritic cells have been identified as extremely efficient producers of the IL-12 that can act in inducing Th-1 responses upon antigen presentation (65-67). Due to the heterogeneity of both human and mouse dendritic cells, it is not clear which type or maturational state of dendritic cell is the major producer of IL-12. Human monocyte-

derived dendritic cells or myeloid dendritic cells (DC1) obtained from cultures treated with GM-CSF and IL-4 produce high levels of the bioactive IL-12 p70 in response to various stimuli (65). Alternatively, the plasmacytoid dendritic cells (lymphoid origin) have been reported to produce lower levels of IL-12 (68). In the mouse, the CD8α-positive dendritic cells, which may be comparable to the human plasmacytoid dendritic cells, are more efficient producers of IL-12 when compared to myeloid dendritic cells after intracellular infection *in vivo* (69). Dendritic cells from both species are unique antigen presenting cells because 1) they produce bioactive IL-12 upon specific interaction with T cells without additional stimuli; 2) their production of IL-12 is critical for optimal proliferation and IFN-γ production by activated Th-1 cells; 3) they prime resting, naïve T cells that, once restimulated, produce Th-1 cytokines (66). The ability of dendritic cells to propagate Th-1 differentiation is due to their levels of STAT-4 (70). IL-4 can reduce the amount of STAT-4 in maturing dendritic cells, reducing the amount of IL-12 dependent IFN-γ produced as well as the Th-1 signaling capacity of these cells (70). Once dendritic cells are mature, IL-4 can no longer inhibit the production of STAT-4 (70). Dendritic cells, depending on type and maturational status, are therefore capable of initiating the antigen-activated immune response in the innate branch of the immune system and then directing the Th-1 response to steer the humoral immune response.

Many parameters direct the development of Th-1 or Th-2 cells from a naïve precursor. These include the antigen presenting cells used for priming, the dose of antigen encountered, the costimulatory cell-surface molecules, the genetic background of the cells, and the cytokine milieu present in the environment (71). Changing one of these factors can alter the resulting T cell phenotype. Ultimately the cytokines IL-4 and IL-12

act directly on T cells through STAT-6 and STAT-4 respectively to deliver final differentiation signals (38,71). Naïve $CD4^+$ T cells are activated by interaction with antigen presenting cells, primarily dendritic cells, through the TCR (30,66). This initiates expression of the IL-12Rβ2 subunit and up-regulates the IL-12Rβ1 subunit already present on the surface of T cells (30). The presence of even minute quantities of IL-4 will inhibit IL-12Rβ2 expression; however, the presence of IFN-γ enhances this subunit's expression and can even reverse IL-4 inhibition (30, 71). The source of IFN-γ is likely to be NK cells, T cells, and IL-12 stimulated dendritic cells and macrophages (1, 38,57). Once the capacity of T cells to respond or not to respond to IL-12 is established, IL-12 acts through STAT-4 via the IL-12R to determine the Th-1 or IFN-γ producing phenotype (39). This process is comparable between the human and mouse species except that human Th-2 cells maintain their IL-12 responsiveness through low levels of IL-12β2 receptor subunit. In addition, IFN-α is as effective, and in some instances more effective, an inducer of IL-12Rβ2 chain in human T cells (72). Generally, the choice between a Th-1 and Th-2 phenotypic T cell response is dependent on the balance between the levels of IL-4 and IL-12 during the maturation of naïve T cells.

As previously noted, one of the main actions of IL-12 in the inflammatory response to pathogens is to direct the development of Th-1 cells; however IL-12 has several other very important functions. IL-12 is a potent inducer of IFN-γ from $CD4^+$ and $CD8^+$ T cells, NK cells, and γ/δ T cells (2). IL-12 also directly enhances the cytotoxicity of NK cells and CTLs by inducing the expression of genes encoding cytotoxic granules (e.g., perforin) and by endowing the CTLs with the ability to mediate antibody-redirected lysis of target cells (2,73). These effects can be additive when synergistic cytokines such as

IL-2 and IFN-γ are present, but IL-12 alone is capable of inducing these effects (2). Additionally, IL-12 is the major factor required for the differentiation of $CD8^+$ T cells and γ/δ T cells, priming them to be polarized to produce Th-1cyokines, similar to its effect on their $CD4^+$ counterparts (2). IL-12 can also function as an inhibitor of Fas-mediated, non- B cell lymphoma oncogene-2- (BCL-2) -dependent T-cell apoptosis (2). IL-12 is critical in the activation of macrophages through the Th-1 differentiation, enabling them to produce bactericidal and anti-viral cytokines (67). Recent evidence using infection models suggests that IL-12 is not only required to initiate these responses but also maintains antimicrobial functions, such as the ability of memory T cells to produce IFN-γ at later stages of infection (3).

Investigations of the humoral side of the inflammatory response demonstrated that IL-12 could suppress the production of Th-2 type antibodies (IgG1 and IgE) and increase the production (10- to 1000- fold) of Th-1 type antibodies IgG2a, IgG2b, and IgG3 (74). This effect is mediated predominantly by the increased expression of IFN-γ; however removal of IL-12 alone also affects the type of antibodies produced (74). This regulation is similar to the interaction of IL-12 with IL-4, in that if cells are primed or boosted, IL-12 can modestly enhance the production of IgG1 and IgE (74). Recent studies have also shown that IL-12 increases the production of complement fixing antibodies. In addition certain aspects of the complement cascade may directly modulate the production of IL-12 in various infections and delayed-type hypersensitivity reactions (75). IL-12 is at the interface of all aspects of the immune response to intracellular infection with interacting links between the innate and immune responses.

The Role of IL-12 in Infectious Diseases

IL-12 in Bacterial Infections

Intracellular infection of mice with *Listeria monocytogenes* has been used extensively as a model to study the role of Th-1-dependent, cell-mediated immunity, including the role of IL-12 in intracellular infections (76). *L. monocytogenes* resistant mice, such as C57BL/6, have macrophages and dendritic cells with higher IL-12 (52% more total IL-12 p70) producing capacities that activate NK and γ/δ T cells to secrete IFN-γ, and promote the development of Th-1 immunity early during acute infection (77, 78). Additionally, administration of recombinant IL-12 (rIL-12) increases resistance to this bacterium, and its antibody neutralization leads to increased bacterial susceptibility (79). The role for IL-12 in sustaining the response in murine listeriosis is unclear (3). The evidence for the critical need for early IL-12 production in these models is proven whereas the evidence for late production is still controversial.

IL-12 is induced at high levels in response to *Salmonella*, mycobacteria, and other bacterial components. Mice infected with attenuated *S. dublin* bacteria had increased IL-12 production in the lymph nodes and Peyer's patches and were protected against subsequent infection with the virulent agent (79, 80). The emergence of a protective Th-1 immune response is dependent on IL-12 in murine *Mycoplasma tuberculosis* infections (2,79). As in Listeriosis, the addition of rIL-12 to susceptible BALB/c mice increased survival and delayed lung pathology (81). Blocking IL-12 with monoclonal antibodies increases susceptibility in normally *M. tuberculosis* resistant mice, similar to the phenotype observed in IL-12 p40 deficient mice (2, 81). The addition of rIL-12 to human cell cultures increased the cytolytic activities of NK and $CD4^+$ T cells against monocytes

infected with *M. tuberculosis* (82). Recombinant IL-12 also increases the proliferative responses of peripheral blood lymphocytes and stimulates the antibacterial properties of macrophages in patients with *M. avium* infection (83). Even the addition of a synthetic oligonucleotide containing a palindromic sequence from mycobacteria, a sequence of DNA from *Escherichia coli*, or the B subunit of *E. coli* can induce the expression of IL-12 mRNA p40 and p35 in mouse splenocytes in cell culture and intestinal lymphoid tissue *in vivo* (79). IL-12 has significant roles in other bacterial infections such as *Brucella abortus* and *Klebsiella pneumoniae*, but research has not been as extensive in these infections (79).

IL-12 in Protozoal Infections

The production of IL-12 is important in the initiation and the maintenance of the Th-1 response in cutaneous and visceral leishmaniasis. The susceptibility of BALB/c mice to the cutaneous, intracellular protozoan, *Leishmania major*, is in part due to the genetic background of the T lymphocytes (84). Though the expression of IL-12 mRNA and protein levels is similar between resistant and susceptible mouse strains, susceptible BALB/c mice T cells lose the ability to generate IL-12-induced Th-1 responses and instead form an IL-4-induced Th-2 response that is ineffective in clearing the pathogen (84). The important role of IL-12 in resistant mouse strains, such as C57BL/6 and C3H, seems to be its ability to function as a growth factor for Th-1 cells by the intensification of IFN-γ production and the suppression of IL-4 and IL-10 (79). Treatment of the susceptible BALB/c mouse strain with IL-12 during the first week of infection with *L. major* induces resistance to the infection with a shift in the immune system from Th-2 to a Th-1 response and the cured animals are resistant to subsequent rechallenge (2). In

addition, during *L. major* infections resistant C3H mice upregulate the mRNA expression of IL-12Rβ1 and -β2 subunits on $CD4^+$and $CD8^+$ T cells (85). In contrast, susceptible BALB/c mice show no increase in IL-12 receptor subunits upon infection with *L. major* (85). The antibody neutralization of IL-12 in *Leishmania*-resistant strains converts these mice to susceptibility (79).

The treatment of BALB/c mice with rIL-12 two weeks after *L. major* infection abrogates its protective effects and can even enhance the expression of IL-4 (79). Thus, it is difficult to reverse an already established Th-2 immune response. Alternatively, IL-12 may be needed to maintain a Th-1 response in animals where a Th-1 response has been initiated. Despite the development of a Th-1 response in IL-12 p40 knockout (IL-12 KO) mice transiently treated with rIL-12, the animals were unable to sustain a Th-1 response in the absence of IL-12 past the acute phase of infection (79, 86). These IL-12 KO mice in the absence of IL-12 treatment developed evidence of a Th-2 response (86). IL-12 may also play an important role in visceral leishmaniasis in mice and humans. In *L. donovani* mouse infections, in which susceptibility is associated with a failing Th-1 immune response, treatment with rIL-12 has been effective in reversing the disease process (79). The addition of rIL-12 to cultures of human peripheral blood mononuclear cells from patients with visceral leishmaniasis restores the proliferative and IFN-γ producing capacities of these cells (79). Therefore, in mice and in humans, IL-12 appears to initiate and maintain cell-mediated immunity, as well as suppress the Th-2 response to *Leishmania* infection.

The role of IL-12 in other protozoal infections appears to be quite similar to that in leishmaniasis, but the differential expression of IL-12 is much greater. C3H mice have

twice the total IL-12 protein in serum and splenic tissues as compared to BALB/c mice in response to *Trypanosoma cruzi* infection (87). This difference has been associated with increased infection resistance due, most likely, to observed increases in NK cell cytotoxicity and the levels of IL-12 dependent IFN-γ protein (87). Additionally, rIL-12 treatment of the susceptible BALB/c mice led to resistance during the acute phase of the disease, but was ineffective during the chronic infection (87). Cells from *Toxoplasma gondii* infected, IL-12 KO mice, transiently treated with rIL-12, were unable to produce IFN-γ upon antigenic stimulation without the addition of IL-12 (3). There is evidence that the Th-1 response was initiated and that Th-1 cells were developed, but the T cell memory was not functional without IL-12 (3). These mechanisms, along with IL-12's ability to upregulate TNF-α, cell surface molecules, and to increase the phagocytic ability of antigen presenting cells, are also important in resistance to the mouse protozoal diseases *Plasmodium chabaudi* and *Cryptosporidium parvum* (79).

IL-12 in Fungal Infections

In many fungal infections, the establishment of a Th-1 type reaction is critical to development of phagocyte dependent protection and the production of inhibitory cytokines such as IL-4, IL-10, and the IgE antibody is associated with disease progression (79). DBA/2 mice are genetically resistant to *Coccidioides immitis* and are induced to produce five times more IL-12 p40 mRNA in their lungs as compared to *C. immitis*-susceptible C57BL/6 mice (79, 88). Neutralization of IL-12 in the DBA/2 strain by monoclonal antibodies to IL-12 leads to severe disease, and conversely administration of rIL-12 to the fungus-susceptible strain (BALB/c) decreases susceptibility to clinical disease progression (79).

Recombinant IL-12 administered at the time of murine *Cryptococcus neoformans* infection results in protection from disseminated infection including pneumonia and then meningitis (89). This effect seems to be mediated by an increase in the numbers of pulmonary inflammatory cells, a decrease in the number of neural yeast cells, and detectable IFN-γ mRNA in the lungs of treated mice (89). Later administration of rIL-12 fails to protect these mice against dissemination of the infection with *C. neoformans*, with no detectable pulmonary IFN-γ mRNA (89). The role of IL-12 in human fungal infections continues to be investigated due to the importance of human immunodeficiency virus (HIV) related susceptibilities to opportunistic fungal infections.

IL-12 in Viral Infections

IL-12 plays an important role in viral defense; however its role is more complex than in other types of intracellular infections. In murine viral infections such as murine cytomegalovirus (MCMV), respiratory syncytial virus (RSV), influenza, and herpes simplex virus (HSV), IL-12 is critical in the early activation of NK cells and the establishment of a Th-1 antiviral immune response (2, 79, 90). During MCMV and RSV infection, IL-12 p70 levels increase in serum (50% and four-fold respectively) at early time points after infection (90, 91). However lymphocytic choriomeningitis virus (LCMV) infection does not induce detectable IL-12 levels, but instead activates the T-cell IFN-γ responses through the IL-12 inhibitory cytokines IFN-αβ (92). During LCMV in the absence of IFN-αβ the IL-12 response is inducible and indicates an alternative pathway to NK cell activation and IFN-γ production (92).

Low dose IL-12 administration has some protective effects in LMCV infection; however higher doses can lead to decreases in T cell activity and increases in T cell

necrosis (92). Low doses of IL-12 increased NK cell cytotoxicity, $CD4^+$ and $CD8^+$ T lymphocyte numbers, IFN-γ production, and antiviral status (92). In other murine viral infections, such as mouse hepatitis virus, encephalomyocarditis virus, and mouse adenovirus infection rIL-12 administration induced protection (79). In mice transgenic for the hepatitis B antigen, IL-12 suppressed autoantibody production (Th2 to Th1 shift), inhibited virus replication in the liver and kidneys, and increased IFN-γ production (79). In many of these infections peak IL-12 levels are noted 1-3 days (i.e., early) after infection and are usually transient (2,79).

IL-12 may also play opposing roles in the outcome and/or associated pathology in the same infection (79). In corneal HSV infection, local over-production of IL-12 leads to a virus-specific Th-1 reactivity and immunopathologic disease (93). However, in thermally injured mice, IL-12 promotes resistance to HSV infection (79). In humans, the measles virus actually down regulates the expression of IL-12 *in vivo*. In human monocyte cell cultures measles infection selectively impairs the expression of IL-12 without affecting other cytokines (94). This decreased IL-12 production is dependent on the activation of the measles virus cellular receptor CD46, a regulator of the complement gene cluster (94). Thus, it appears that there is some plasticity in the immune response to viral infections depending on the genetics of the host, the cytokine environment, and the type of viral pathogen.

IL-12 has also been significant in the pathology and possible treatment of HIV infection in humans. During *in vitro* studies with peripheral blood mononuclear cells (PBMC) and T cells from HIV-infected individuals, it was demonstrated that these cells produce less IL-12 than non-infected controls (79). Although macrophages from these

individuals express low levels of IL-12, these cells do not respond to normal stimulation (2, 79). IL-12 treatment *in vitro* has been shown to enhance NK cytotoxicity in HIV infected cells, and is able to increase the cytotoxic activity of lymphocytes in non-infected donors against HIV-infected target cells (2). IL-12 treatment has also improved the ability for immune cells to recall antigens and to prevent T-cell-receptor-induced apoptosis (79). The protective ability of IL-12 against the HIV virus may be to help maintain the $CD4^+$ T cell population from apoptotic destruction by shifting the T cells to a Th-1 profile that is less permissive to HIV than the Th-2 cell type (2,79).

The Role of IL-12 in Allergy and Asthma

Asthmatic disease involves intermittent airway obstruction, bronchial smooth muscle cell hyperreactivity to bronchoconstrictors, and chronic bronchial inflammation (95). This persistent airway inflammation ultimately leads to remodeling of the airway epithelial cells and the deposition of collagen by proliferating fibroblasts (95). The primary lesion of asthma consists of the accumulation of $CD4^+$ Th-2 cells and in some cases eosinophils in the airway mucosa (95). The Th-2 cells direct the persistent inflammation through the cytokines IL-4, IL-13, IL-5, and IL-9 (96). Although IL-4 is the main cytokine responsible for Th-2 differentiation and the high IgE levels observed in many asthmatics, IL-13 and IL-5 are involved in bronchoconstriction and eosinophilia respectively (96). Chemokines such as RANTES (regulated upon activation, normal T-cell expressed and secreted) eotaxin, and macrophage inflammatory protein 1α (MIP-1α) also act on eosinophils and T cells to enhance their recruitment and activation (95-96). These inflammatory mediators have become proposed sites for therapeutic modulation and have been studied in both human and animal models of allergic disease.

IL-12 appears to have an immunomodulatory effect on the predominantly Th-2 driven pulmonary inflammation in rodent (rat and mouse) models of allergic airway disease and human asthma. IL-12 KO mice sensitized and challenged with ovalbumin (OVA) have pronounced eosinophilic airway inflammation with enhanced IL-4 and TNF-α levels in the bronchoalveolar lavage fluid (4). Recombinant IL-12 given intraperitoneally in a murine model of ovalbumin-induced, allergic, airway inflammation suppresses antigen-induced airway eosinophilia, circulating IgE levels, and airway hyperresponsiveness in a dose dependent manner (97). The administration of rIL-12 was timed during either allergic sensitization (early dosage) or the hypersensitivity of inflammation in the lung (late dosage) (97-98). Early dosages or early and late dosages combined were effective in C57BL/6 suppressing all signs of the asthma-like phenotype, however the late doses alone were not as effective, especially in reducing IgE levels (97-98). In addition, rIL-12 administration to rats by intraperitoneal dosing also inhibits allergen-induced inflammation and the sensitization to allergens (99). Brusselle et al. examined the mechanism of the inhibitory effects of IL-12 on airway inflammation using IFN-γ receptor deficient (IFN-γR-KO) mice (100). Recombinant IL-12 given by aerosol to IFN-γR-KO and wild-type mice during sensitization inhibited airway eosinophilia and specific IgE production in the wild-type mice and increased these parameters in IFN-γR-KO mice possibly due to a more established Th-2 response (100). Similar to the previous studies, rIL-12 given only during the hypersensitivity phase (late) inhibited the airway eosinophilia, but not the circulating IgE in the wild-type mice (100). The inhibition of eosinophil influx into the airways by IL-12 appears IFN-γ dependent during initial sensitization and IFN-γ independent during the secondary allergic response. These

results suggest that endogenous and exogenous IL-12 play important roles in curtailing the allergic response in the airways, and the timing of the expression is critical to suppress the Th-2 response.

In humans, bronchial biopsies from asthma patients compared to normal controls show decreases in the numbers of IL-12 producing cells, reduced airway IL-12 mRNA, and a reduction in the ability of the PBMCs *in vitro* to produce IL-12 during stimulation as compared to normal non-atopic controls (4). The expression of IL-12Rβ2 on T cells of asthmatics is also reduced, partly due to diminished production of IL-12 and enhanced secretion of IL-4 by their PBMCs (101). Furthermore, there are intrinsic defects of the $CD4^+$ T cells, which reduce their ability to respond to IL-12 with IL-12Rβ2 expression (101). Problematically, systemic administration of rIL-12 to human asthmatics has several toxic effects such as general malaise/flu-like symptoms and cardiac arrhythmias (99). Mucosal administration by airway aerosolization of rIL-12 in mouse and non-human primate models abrogates airway eosinophilia and airway hyperresponsiveness, as well augmenting the expression of pulmonary IFN-γ (4, 102). In addition, lung mucosal IL-12 gene delivery via viral vectors prevents the development of allergic disease, airway hyperresponsiveness, and suppressed established allergic responses (4,103). This form of gene therapy also reversed the suppression of local antiviral cell-mediated immunity resulting in rapid resolution of viral infection in previously susceptible mice (103). These therapies may enable exogenous administration of rIL-12 to asthmatic individuals without the side effects associated with systemic treatment.

Rodent Model for Virus-Induced Pulmonary Disease

Viral bronchiolitis during infancy (less than 1 year of age) in humans has been associated with chronic airway dysfunction and may be a factor in the development of the asthmatic phenotype (104-105). These pulmonary function abnormalities include increased airway resistance and airway hyperresponsiveness to airway smooth muscle agonists such as methacholine (104,106). Viral infections may be inducing persistent structural abnormalities through direct inflammatory injury and repair mechanisms that may lead to permanent structural abnormalities (107). Another factor in the development of asthma is the association between elevated IgE levels and a predominating Th-2 type cytokine response in some infants that develop asthma (108). Diminished IFN-γ production can be demonstrated in the cord blood mononuclear cells from infants with increased risk of developing atopic diseases, such as asthma, and in the PBMCs of infants that develop virus-associated airway function abnormalities (108). The asthmatic phenotype then appears to be a combination of inheritable factors (cytokine dysregulation and/or atopy) and environmental components (viral infections, allergens, and other lower airway antigens).

Parainfluenza virus type 1 (Sendai) infection in weanling rats produces pulmonary structural and functional abnormalities, such as bronchiolar hypoplasia and alveolar dysplasia leading to increased airway resistance and hyperresponsiveness (109-110). These abnormalities have been developed as an animal model of virus induced airway disease with many features that resemble human asthma including; episodic, reversible airway obstruction, airway hyperresponsiveness to methacholine, chronic airway wall inflammation, and airway wall remodeling (111-113). Young rats infected with Sendai

virus develop severe bronchiolitis followed by pulmonary growth abnormalities, including bronchiolar hypoplasia, alveolar dysplasia, and increases in bronchiolar airway wall thickness (113). Sendai virus-induced increases in bronchiolar wall thickness are due to increases in inflammatory cells (macrophages, mast cells, eosinophils and lymphocytes), airway wall edema, fibroblast proliferation, and collagen and extracellular matrix deposition (113). Previously, it has been determined that Brown Norway (BN) rats are susceptible to virus-induced chronic inflammation and remodeling leading to airway function abnormalities, whereas Fischer 344 (F344) rats are highly resistant to these virus-induced effects (111).

The development of the Sendai virus-induced abnormalities may be related to the initial immune response early after viral infection. The virus-susceptible BN rat strain differs from the F344 in response to viral infection as it has a greater pulmonary expression of the Th-2 cytokines IL-4 and IL-5, less IFN-γ production, and fewer $CD8^+$ T lymphocytes at early time points after viral inoculation (112, 114-116). In addition, BN rats have higher serum IgE levels with enhanced recruitment of airway mast cells, eosinophils, and prolonged viral replication within the airways when compared to the F344 rat strain (115-116). The immune effector cells from BN rats are also less responsive to IL-12 stimulation. Splenocytes and NK cells from uninfected BN rats secrete significantly less IFN-γ upon stimulation with IL-12 or Sendai virus than splenocytes and NK cells from uninfected F344 rats (117). In the viral repair process, BN rats have increased and prolonged expression of the fibrosis-inducing cytokines TGF-β_1 and TNF-α in the macrophages surrounding the airways at 10 and 14 days after viral infection (113, 118). Modulation of the immune response by the exogenous

administration of recombinant IFN-γ, given 4 days before and during the first week of viral infection, prevents the development of persistent airway inflammation, fibrosis, and the associated chronic airway dysfunction in the BN rats (119). Thus, differences in the genetic immune response (i.e., the cytokine response) to parainfluenza virus are critical in determining whether chronic airway dysfunction and asthma-like disease will develop.

The above findings indicate that the cytokine response is an important component of the Sendai virus-induced pulmonary damage associated with chronic airway dysfunction. These results combined with the properties of IL-12 outlined in the previous sections, indicate that F344 rats may be more resistant to Sendai virus-induced bronchiolar damage and fibrosis because they produce higher levels of IL-12 early in response to viral infection. The studies outlined here are designed 1) to investigate the pulmonary expression of IL-12 in the F344 and BN rat strains in response to viral infection and 2) determine if systemic treatment with IL-12 could abrogate or lessen the severity of Sendai virus-induced bronchiolar inflammation and fibrosis that is associated with airway dysfunction in BN rats.

CHAPTER 2
RESEARCH PLAN AND PROTOCOL

Hypothesis and Specific Aims

The goal of this research was to determine the role of interleukin-12 (IL-12) in the development of resistance to chronic airway disease induced by parainfluenza (Sendai) virus during early life. The hypothesis to be tested was that F344 rats are more resistant to virus-induced airway damage and fibrosis because they produce high levels of IL-12 early in response to virus that up-regulates Th-1 cytokine responses, antiviral immunity, and reduces airway fibrosis. There were four specific aims:

1) To compare the pulmonary IL-12 mRNA and protein responses of virus- resistant F344 and virus-susceptible BN rats following Sendai virus infection.

2) To determine if F344 rats have greater numbers of pulmonary cells and differing cell types that express IL-12 in response to Sendai virus infection than BN rats.

3) To determine if Sendai virus-induced airway damage in BN rats can be reduced by IL-12 treatment early in the virus infection.

4) To compare the airway IL-12 p35 and p40 mRNA responses of virus-resistant F344 and virus-susceptible BN rats following Sendai virus infection (This specific aim is contingent on the results from the second specific aim. If there is differential expression of IL-12 in the dendritic cell or other cells types in the large airways based on the results of the in situ hybridization and immunohistochemistry, then this specific aim will be explored).

Background/Significance

Inbred rats differ in susceptibility to Sendai virus-induced chronic airway disease that resembles human asthma. The BN rat strain is highly susceptible to this virus-induced damage and produces a predominantly Th-2-type acute cytokine response to virus with increased levels of IL-4 and IL-5 seven days after inoculation (115). In

contrast, the F344 rat strain is resistant and produces an earlier Th-1-type cytokine response with high IFN-γ mRNA levels three days after virus inoculation and higher IFN-γ protein levels in the bronchoalveolar lavage fluid seven days after inoculation (115). BN rats have high serum IgE levels and persistent inflammation characterized by the enhanced recruitment of mast cells and eosinophils in response to Sendai viral infection (114,116,120). F344 rats have a higher $CD8^+$ T cell response to Sendai infection and have viral titers comparable to non-infected controls seven days after virus inoculation (115-116). BN rats continue to have persistent airway inflammation with increased numbers of macrophages expressing the pro-fibrotic cytokines TNF-α and TGF-β_1 in their airways at 10,14, and 30 days after inoculation (113,118). Additionally, BN rats have virus-induced proliferation of fibroblasts in the bronchiolar walls chronically after virus inoculation (118). These changes remodeling the airways are associated with virus-induced increases in pulmonary resistance and hyperresponsiveness that persists for 28 to 65 days after inoculation.

IL-12 is a heterodimeric cytokine in the Th-1 cytokine group that is produced in response to many intracellular infections including viral, protozoal, fungal, and bacterial (1-3). This cytokine, produced mostly by antigen presenting cells (dendritic cells and macrophages), promotes antiviral immune responses, in part by inducing IFN-γ production by immune effector cells (2). Differential IL-12 expression has been shown to be important in susceptibility to intracellular pathogens in several rodent models (79). Administration of recombinant IL-12 confers resistance in rodent and primate infectious and allergic disease models (79,97-103). F344 rats have higher virus-induced pulmonary IFN-γ expression and increased NK and T lymphocyte responsiveness to IL-12 or Sendai

virus (117). Therefore, expression of IL-12 may play a major role in controlling the difference in susceptibility to virus-induced airway inflammation, fibrosis, and hyperresponsiveness in these two rat strains.

Gaps in Knowledge to Be Addressed by This Research

1) Is early IL-12 gene expression increased in the virus-resistant F344 rats as compared to virus-susceptible BN rats in this model?

2) Can exogenous administration of IL-12 reduce the effects of virus-induced damage and fibrosis in the pulmonary tissues?

3) Which cell types are responsible for producing IL-12, post-viral infection, in the lungs?

4) Is the dendritic cell IL-12 response greater in the F344 rats early after Sendai virus inoculation?

Research and Design Methods

Overview of Experiments and Schedule

Experiment 1: Comparison of IL-12 response in virus-susceptible BN rats and virus-resistant F344 rats. (Specific Aims 1, 2, and 4) (Year 1 and 3)

Experiment 2: Can Sendai virus-induced airway damage be reduced in virus-susceptible BN rats by exogenous administration of IL-12 early in viral infection? (Specific Aim 3) (Year 2)

Experiment 1: Pulmonary Expression of IL-12 in Sendai Virus-infected BN and F344 Rats

Objectives

1) To determine if virus-resistant F344 rats have higher levels of IL-12 pulmonary expression in response to Sendai viral inoculation as compared to virus-susceptible BN rats.

2) To determine the location and amounts of p70 protein in the pulmonary tissues in the two rat strains.

3) To determine the level of the IL-12 synergistic cytokine, IL-18, mRNA in the pulmonary tissues of both rat strains.

4) To compare the accumulation of dendritic cells, a primary IL-12 producing cell, into the airways of both rat strains.

Rationale

Levels of IL-12 have not been compared in this rat model consisting of BN and F344 inbred rat strains. It has been determined that F344 rats have higher IFN-γ expression than BN rats in response to viral infection and that IL-12 is a potent inducer of IFN-γ in many intracellular infections (119, 1-4). It is of interest to determine if the levels in pulmonary tissue post Sendai viral infection differ between the two strains. Previous studies have determined the protein expression of the IL-12 p70 heterodimer locally in areas of intracellular infection (2, 79). Given that F344 rats have a higher expression of IFN-γ post viral infection, the IL-12 signaling pathway may be an important factor in the antiviral response. This study is designed to determine the levels and location (i.e., cell types) that are expressing IL-12.

IL-18 can act synergistically with IL-12 to induce and/or augment the production of IFN-γ. It has been shown to act specifically at the IFN-γ promoter to augment production from Th1 cells (57). The combination of IL-12 and IL-18 together has been shown to induce extremely high amounts of IFN-γ protein (643-fold increase) and IFN-γ gene expression in NK cells above resting or non-activated human NK cells (55). It may be possible that there is not differential expression of IL-12 in this model, but that an augmenting cytokine like IL-18 is expressed at greater levels in the virus-resistant F344 rats.

Dendritic cells have been shown to be one of the earliest cells responders in the rat airways after Sendai virus infection (121). Sendai virus initially replicates in airway epithelial cells; the earliest IL-12 responses should be from intramural and epithelial

dendritic cells (121). Measurement of total lung mRNA may mute any differences at the airway level by averaging all of the lung tissues together. Focused examination of airway-specific expression may be required to elucidate these potentially important differences in IL-12 (122). Previously most of the studies examining regulation of IL-12 have focused on the p40 subunit and its regulation at the transcriptional level in IL-12 producing cells. Recent studies have shown that in $CD8^+$ dendritic cells the production of IL-12 p70 heterodimer requires the induction of the p35 subunit (13). Since IL-12 p40 homodimers can bind to the IL-12Rβ2 subunit and antagonize IL-12 function, the biological activity may be, at least in part, determined by the ratio of p70 to p40 homodimer. This indicates that p35 levels may be an important limiting factor in IL-12 production by dendritic cells (13). In this model it may important to examine the p40 subunit at the lung and airway level and the p35 subunit production to elucidate any critical differences that may exist in IL-12 regulation between these two rat strains.

Design and Methods

Twenty-two day old weanling male BN and F344 rats were aerosol-inoculated with Sendai virus or remained unexposed to Sendai virus (lung changes same as chorioallantoic fluid exposed) in separate but equal housing, and studied at 0, 1, 2, 3, 5, 7, 10, and 14 days post inoculation.

Necropsy and tissue processing. The rats were anesthetized with sodium pentobarbital (approximately 200μg/g body weight) or urethane (1.5g/kg body weight) and killed by exsanguination via cardiac puncture. Lung lavages for ELISA were performed through intratracheal cannulation with phosphate buffered saline (PBS). For RT-PCR and ELISA, the lungs and tracheas were removed, immediately frozen in liquid

nitrogen, and then stored at -80°C until processed. Lungs and tracheas were removed and fixed for 2 hours by tracheal perfusion with 4% paraformaldehyde-PBS (pH = 7.4) and embedded in paraffin, for in situ hybridization. For immunohistochemistry lungs were removed, inflated with O.C.T. embedding medium compound (Tissue-Tek®, Torrance, CA), sectioned, and placed into molds. These lungs were then frozen immediately in liquid nitrogen and then stored at -80°C until processing.

Table 2-1. Table of Experimental Design: Experiment 1

Assay	Sample	Purpose
Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)	RNA from BN and F344 rat lungs and tracheas (frozen)/virus exposed and non-infected controls	Detection of differences in p35 and p40 IL-12 mRNA levels post viral inoculation
Immunohistochemistry	Frozen lung sections from BN and F344 rats/virus exposed and non-infected controls	Detection and location of cells expressing IL-12 p40 protein and the OX-6 dendritic cell marker
In situ Hybridization	Paraffin lung and trachea sections from BN and F344 rats/virus exposed and controls	Location of the IL-12 p40 mRNA message within the pulmonary tissues
Enzyme-Linked Immunosorbent Assay (ELISA)	Lung homogenates and lung lavage fluid from BN and F344 rats/virus exposed and controls	Determination of the level of IL-12 p70 and p40 in the total lung tissue and secreted into the airways

Dilutional and real-time RT-PCR. Frozen lungs and tracheas were weighed and total RNA was extracted by phenol/chloroform extraction or using the RNeasy® midi kit (Qiagen, Inc, Valencia, CA) (123,124). Preliminary pulmonary mRNA levels were measured in a small number of rats using by 10-fold dilutional RT-PCR method with RNA dilutions ranging from neat to 1:10,000 (125). The primers for IL-12 p40 and the housekeeping gene product, hypoxanthine-guanine phosphoribosyltransferase (HPRT) were designed from previously published sequences and all reactions were optimized for temperature, Mg^{2+} concentration, and primers (125,126) (Appendix A, Figure A-1).

Reverse transcriptase reactions and real-time PCR for IL-12 p35, IL-12 p40, IL-18 mRNA, and the housekeeping gene rat glyceraldehyde-3-phosphate dehydrogenase (GADPH) were performed on total RNA extracted from the lungs and tracheas of individual rats. The primers for each of these cytokines were TaqMan® pre-developed assay reagents for gene expression quantification (Applied Biosystems, Foster City, CA). Every time cDNA was synthesized, parallel TaqMan® assays were run for GADPH and the target cytokine in separate wells (124).

Immunohistochemistry. Antibodies against IL-12 p40 and OX-6 major histocompatibility complex (MHC), dendritic cell marker were used to detect bronchiolar cell cytokine expression or to identify the cell type, respectively. The labeled cells were quantitated and the numbers of positive cells were compared between treatment groups.

***In situ* hybridization.** A portion of the dilutional IL-12 p40 PCR product was subcloned and used as a template for the production of RNA probes (125, Appendix C). The digoxigenin- IL-12 p40 labeled antisense riboprobe was constructed and used to qualitatively determine which cell types were expressing p40 mRNA in lung and trachea sections. Control sections were incubated with a labeled sense riboprobe. Northern analysis was done to verify the success of the IL-12 p40 probe binding to rat RNA samples with high levels of IL-12 p40 mRNA expression (Appendix C).

Enzyme-linked immunosorbent assay. The IL-12 p 70 protein levels were determined preliminarily in concentrated lung lavage fluid samples (Millipore Ultrafree®-4 centrifugal filter and tube Millipore Corporation, Bedford, MA) from individual rats using an ELISA kit according to manufacturers instructions (murine IL-12 p70, Biosource International, Camarillo, CA) (Appendix A, Figure A-2). Due to the

ability of IL-12 p40 monomers and dimers to antagonize the effects of biologically active IL-12 p70, the IL-12 p40 protein was measured in the lavage fluid using an ELISA kit according to the manufacturers instructions (Quantikine® M Murine Mouse IL-12 p40 ELISA, R&D Systems, Minneapolis, MN) (17,125) (Appendix A, Figure A-3). Based on the low levels of IL-12 p70 and p40 detected in concentrated lung lavage fluid ELISA results, the same analyses were done using the supernatants from whole lung homogenates from control and virus-infected rats of both strains.

Data analysis. The final quantitation of cytokine mRNA levels detected by real-time PCR was done using the comparative CT (cycle threshold) method and was reported as relative transcription of the n-fold difference relative to a calibrator cDNA (LPS-stimulated rat lung) (124). The density of protein labeling was assessed by counting and identifying based on morphology the number of labeled cells per mm of bronchiolar basement membrane. Group means for all assays were compared by one-way analysis of variance (ANOVA) using a computer-based statistical program (Sigma-Stat, Jandel Corp. San Rafael, CA). Kruskal-Wallis analysis of variance was used on ranks if normality test of group means failed. In addition, t-tests, Wilcoxin-Mann-Whitney test, and Student-Newman-Keuls method of pair wise multiple comparison procedures were used depending on numbers of groups being compared as well as the variance within each of the individual groups (113).

Experiment 2:The Effects of Exogenous Interleukin-12 Administration on the Development of Sendai Virus-Induced Airway Disease in BN Rats

Objectives

1) To determine if administration of exogenous IL-12 early in viral infection increases resistance to virus-induced chronic airway disease.

2) To determine the mechanism(s), if IL-12 administration does increase resistance, by which IL-12 may be functioning to promote protection from virus-induced disease.

Rationale

The exogenous administration of recombinant IL-12 in numerous murine and primate infectious disease models increases resistance to infection and/or to the pathogen-associated, disease phenotype. Intraperitoneal (IP) administration of rIL-12 has been the method used in many of these studies, additionally local and mucosal rIL-12 treatment has also been effective at the sites of pathogenesis (79,102-104). In most disease models the administration of IL-12 directly, by the production of IFN-γ, drives the development of the Th-1 type immune response with simultaneous suppression of the Th-2 immune response (2, 79).

IL-12 is elevated most often early in the disease process, particularly in viral infections where peak IL-12 levels are noted 1-3 days after infection (2,79). The timing of the cytokine treatment has been shown to be important in both the infectious models and the allergic disease models (79, 97-98). If the Th-2 response or allergic sensitization has already occurred, then IL-12 may actually serve to accentuate or reverse these responses (79, 97-98). Once the T lymphocytes have lost the ability to respond to IL-12, possibly due to the absence of the IL-12Rβ2 subunit, altering the immune response appears to be very difficult (29, 30).

In the murine model of Leishmaniasis IL-12 the administration of neutralizing antibody converts normally *Leishmania*-resistant C57BL/6 and CH3 mice to susceptibility, and rIL-12 treatment confers resistance to the *Leishmania*-resistant BALB/c strain (79,127). The important role for IL-12 in resistant strains seems to be its

ability to function as a growth factor for Th1 cells by the intensification of IFN-γ production and to suppress the production of IL-4 to undetectable levels (79,127). IL-12 treatment within our model could act in a similar manner increasing IFN-γ levels and reducing IL-4 levels after Sendai virus infection.

Preliminary results have shown that IL-12 p40 mRNA levels are elevated in the early days post viral infection. This is consistent with previous studies in this model, in which IFN-γ mRNA and protein levels also peak at early time points (119). IFN-γ has been shown to be a potent mediator of the Th-1 response and treatment of BN rats before and early after Sendai infection prevents post viral chronic bronchiolitis (119). This experiment is designed to determine if early exogenous administrations of IL-12 to BN rats will up regulate Th-1 type and reduce chronic airway fibrosis and remodeling.

Design and Methods

Twenty-two day old weanling male BN were aerosol-inoculated with Sendai virus or remained unexposed to Sendai virus (lung changes same as chorioallantoic fluid exposed) in separate but equal housing, and studied at 0, 3, 7, 10, and 14 days post inoculation (DPI). IL-12 treatment groups were injected IP with 3μg of mouse recombinant IL-12 or a comparable volume of saline at the time of virus inoculation (0 DPI) or two days after inoculation (2 DPI) (Biosource International, Camarillo, CA) (See Figure 2-1). If rats were in the 10 or 14 days post inoculation, they were injected IP at 200 mg/g of rat with 5-bromo-2-deoxyuridine (BrdU)(Sigma, St. Louis, MO) twelve hours prior to necropsy.

Table 2-2. Table of Experimental Design: Experiment 2

Assay	Sample	Purpose
Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)	RNA from BN rat lungs (frozen)/virus-exposed and non-infected controls with and without rIL-12 treatment	Detection of differences in IFN-γ, IL-18, and IL-4 mRNA levels after IL-12 treatment
Immunohistochemistry	Paraffin lung sections from BN rats /virus exposed and non-infected controls with and without IL-12 treatment	Detection and evaluation of fibroblast proliferation in the bronchiolar walls
Differential Cell Counts	Lung lavage fluid from BN rats /virus-exposed and non-infected controls with and without rIL-12 treatment	Determination of total numbers of cells and types in BAL
Histology and Bronchiole Wall Morphometry	Paraffin lung sections from BN /virus exposed and non-infected controls with and without IL-12 treatment	Evaluate the extent of inflammation and fibrosis in the bronchioles
Enzyme-Linked Immunosorbent Assay (ELISA)	Lung homogenates and lung lavage fluid from BN rats/virus exposed and controls with and without rIL-12 treatment	Determination of the level of IL-12 p70 and p40 in the total lung tissue and secreted into the airways
Viral Plaque Assays	Lung BN rats (frozen)/ virus exposed with and without rIL-12 treatment	Determination of the levels of virus remaining in the treatment groups at various time points

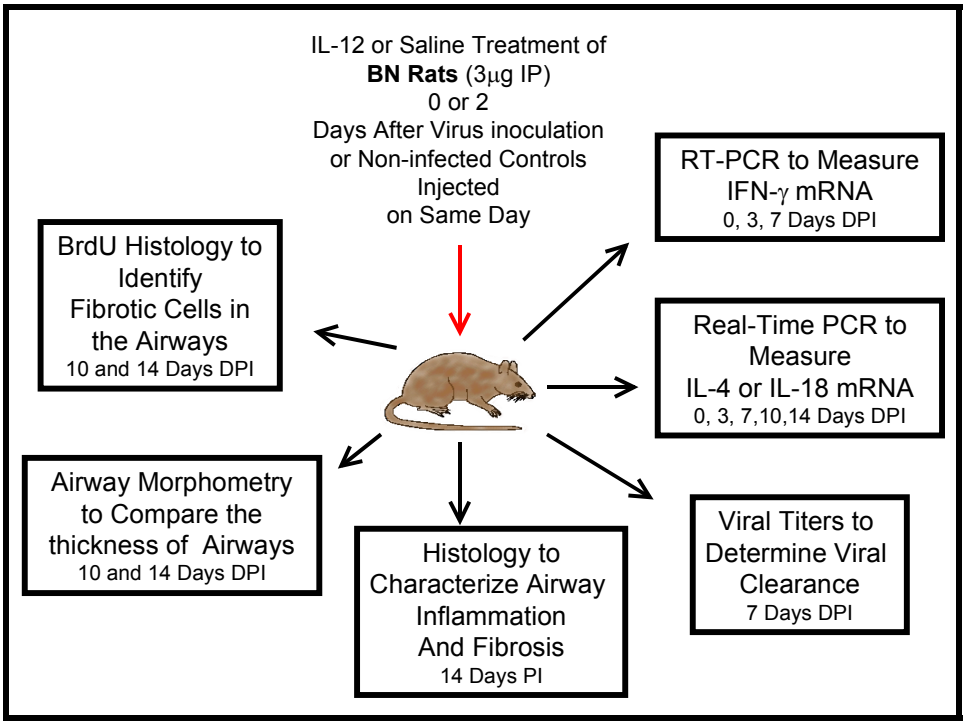

Figure 2-1. Experimental design diagram for experiment 2

Necropsy and tissue processing. The rats were anesthetized with sodium pentobarbital (approximately 200μg/g body weight) or urethane (1.5g/kg body weight) and killed by exsanguination via cardiac puncture. Lung lavages for ELISA and cells counts (differential and total) were performed through intratracheal cannulation with phosphate buffered saline (PBS). For RT-PCR and ELISA, the right lungs were removed, immediately frozen in liquid nitrogen, and then stored at -80°C until processed. The left lungs were tied off, removed and fixed for 2 hours by tracheal perfusion with 4% paraformaldehyde-PBS (pH = 7.4) and embedded in paraffin for immunohistochemistry and histology.

Real-time and competitive RT-PCR. Reverse transcriptase reactions and real-time PCR for IL-18 mRNA and GADPH were performed on total RNA extracted from the lungs of individual rats. The primers these cytokines were TaqMan® pre-developed assay reagents for gene expression quantification (Applied Biosystems, Foster City, CA). The primers and TaqMan probe for BN rat IL-4 were designed using the Primer Express software (Applied Biosystems, Foster City, CA). The sense and antisense primers were made in the mRNA sequence to ensure discrimination between cDNA and genomic DNA. Dilutional curves were evaluated to assure that the efficiency of the IL-4 primers compare to the GADPH primers. Every time cDNA was synthesized, parallel TaqMan® assays were run for GADPH and the target cytokine in separate wells (124).

IFN-γ was detected in lung tissue by a competitive RT-PCR method (126). Primers for IFN-γ and HPRT were constructed from published sequences and each assay was optimized for temperature, Mg^{2+}concentration, and primers (126). For each cDNA sample reactions comparisons were made to 0, 0.5, 5, and 50 femptograms (fg) of a

competitive fragment (126). PCR products were stained with ethidium bromide and separated electrophoretically on 1.5-% agarose gels.

BrdU immunohistochemistry. Transverse sections were taken from the fixed left lungs of rats treated with BrdU: at the hilus, between the hilus and the cranial lobe margin, and at the same distance from the hilus to the caudal lobe margin. Immunohistochemical staining using the antibody to BrdU was preformed and the numbers of BrdU-labeled fibroblasts per mm of bronchiolar basement membrane were counted.

Histology and bronchiolar wall morphometry. Serial hematoxylin and eosin (H&E) stained, paraffin sections were scored for airway wall inflammation and fibrosis. Bronchioles were scored as positive for inflammation if the wall had five or more inflammatory cell types (eosinophils, lymphocytes, or macrophages). Bronchioles were scored as positive for fibrosis/remodeling if the walls were thickened with increased fibroblasts and deposition of collagen. Collagen was identified using Masson's Trichrome and Manuel's Reticulin stains. Additionally, the area of the bronchiolar wall from the bronchiolar epithelial basement membrane to basement membrane of the surrounding alveolar walls was measured. Bronchiolar wall area was divided by the perimeter of bronchiolar basement membrane to calculate the thickness of the wall (square micrometers of bronchiolar wall per micrometer of bronchiolar basement membrane) (113).

Enzyme-linked immunosorbent assay. The IFN-γ protein levels were determined in concentrated lung lavage fluid (Millipore Ultrafree®-4 centrifugal filter and tube Millipore Corporation, Bedford, MA) samples from individual rats using an ELISA kit

according to manufacturers instructions (rat IFN-γ, Biosource International, Camarillo, CA). Preliminary analysis using concentrated lung lavage fluid samples detected an increase in levels of IFN-γ after viral infected, but no significant differences in the IL-12 treatment groups (Appendix A) (Figure A-4). Based on the minimal effects detected in concentrated lung lavage fluid, the same analyses were done using the supernatants from whole lung homogenates from individual animals in the same treatment groups.

Virology. Viral titers, to determine viral clearance, were measured in homogenates of frozen lung, using a standard plaque assay, and expressed as plague forming units/g lung tissue (128).

Data analysis. The final quantitation of cytokine mRNA levels detected by real-time PCR was done using the comparative CT (cycle threshold) method and was reported as relative transcription of the n-fold difference relative to a calibrator cDNA (LPS-stimulated rat lung) (124). The competitive RT-PCR data are reported as non-normalized mRNA abundance in competitive fragment units (119). Fibroblast proliferation was assessed by counting the number of BrdU-labeled fibroblasts (identified due to spindled shape) per mm of bronchiolar basement membrane. Group means for all assays were compared by one-way analysis of variance (ANOVA) using a computer-based statistical program (Sigma-Stat, Jandel Corp. San Rafael, CA). Kruskal-Wallis analysis of variance was used on ranks if normality test of group means failed. In addition, several pair wise multiple comparison procedures were used depending on numbers of groups being compared as well as the variance within each of the individual groups (113).

CHAPTER 3
INCREASED EXPRESSION OF PULMONARY INTERLEUKIN-12 (IL-12) IN SENDAI VIRUS-RESISTANT F344 RATS

Summary

Brown Norway (BN) and Fischer (F344) rats differ in their susceptibility in early life to Sendai virus-induced persistent airway inflammation, chronic airway remodeling, and airway hyperresponsiveness. These characteristics, as well as other phenotypic characteristics serve as an experimental model of virus-induced asthma. Virus-susceptible BN rats mount a predominantly Th-2 cytokine response (IL-4-dominated) to Sendai virus, whereas virus-resistant F344 rats respond to infection with a Th-1 cytokine pattern (IFN-γ-dominated). F344 rats are more efficient in clearing the virus and in resisting the induction of chronic airway lesions. We hypothesized that an earlier and more robust IL-12 response was responsible for the differing IFN-γ expression and viral resistance of F344 rats. IL-12 mRNA and protein expression were evaluated by real-time PCR, ELISA, and quantitative immunohistochemistry for IL-12 positive dendritic cells in the lungs and tracheas of BN and F344 rats. F344 non-infected control rats had higher pulmonary IL-12 p40 mRNA levels than the non-infected control BN rats. Virus-induced increases in IL-12 p40 mRNA were detected as early as 2 days after inoculation, while virus-induced increases in IL-12 p40 mRNA were not detected in BN rats until 3 days after inoculation. F344 rats had higher concentrations of IL-12 total in the lung than BN rats at 2 days after inoculation. Virus-induced increases in bronchiolar OX-6 positive and IL-12 p40 dendritic cells were observed as early as 2 days following inoculation in F344

rats. No dendritic cell response was detected in BN rats. These results indicate that resistance to the sequelae of Sendai virus infection by F344 rats is associated with their earlier and higher production of IL-12.

Introduction

Viral bronchiolitis in infant children can be associated with chronic bronchiolar dysfunction and is implicated as an important risk factor for the development of asthma and other airway abnormalities (129,130). A rat model of virus-induced bronchiolar damage has been developed in weanling and neonatal rats that has characteristics similar to human asthma. These characteristics include episodic and reversible bronchiolar obstruction, bronchiolar hyperresponsiveness to methacholine, chronic bronchiolar inflammation (lymphocytes, macrophages, eosinophils, and mast cells) and bronchiolar wall remodeling (111-113). This model can be used to examine the possible direct roles that viral infections have in pulmonary function abnormalities and what genetic factors may be contributing to the susceptibility to viral sequelae during early life (119).

Interleukin-12 (IL-12) has been associated with resistance to intracellular infections (2). IL-12 is produced by antigen presenting cells, particularly dendritic cells, during infection and induces IFN-γ production by natural killer (NK) and cytotoxic T cells ($CD8^+$) (2,57). Through this direct up-regulation of IFN-γ, IL-12 drives the development of T helper-1 (Th-1) type immune response with simultaneous suppression of the T helper-2 (Th-2) immune response without the requirement for IFN-γ (1-3). IL-12 directly increases the cytotoxic killing capacities of NK and cytotoxic T cells (2). The IL-12 p35 and p40 subunits covalently link to form the biologically active p70 heterodimer (2). Many cells constitutively express the p35 chain, however the p40 gene is expressed only

in IL-12 producing cells (10). The expression of both chains is induced upon intracellular infection and the subsequent CD40 ligand binding of antigen presenting cells (14, 47).

Differential expression of IL-12 has been shown to be important in intracellular infections in several rodent models. DBA/2 mice are genetically resistant to *C. immitis* and produce five times more IL-12p40 mRNA in their lungs as compared to *C. immitis*-susceptible C57BL/6 mice (79,88). Neutralization of IL-12 in the DBA/2 strain leads to severe disease. Administration of rIL-12 to another fungus-susceptible mouse strain (BALB/c) decreases susceptibility to clinical disease (79). Similarly, C3H mice that are resistant to the protozoan *T. cruzi* have twice the IL-12 total protein in serum and splenic tissue as compared to susceptible BALB/c mice. This differential IL-12 response has been associated with increased resistance and an increased IFN-γ protein response (87). Additionally, resistant C57BL/6 mice have 52% more IL-12 total protein in splenic dendritic cells early in murine listeriosis than susceptible BALB/c mice (78). Finally, C57BL/6 mice have a four-fold greater increase in pulmonary IL-12 expression in response to respiratory syncytial virus and mild disease as compared to DBA/2 and BALB/c mice which have lower IL-12 levels and develop increased bronchiolar hyperactivity and mucus production leading to a more severe disease process (91).

In viral infections such as mouse choriomeningitis (MCMV), corneal herpes simplex virus and murine influenza, IL-12 is critical in the early activation and maintenance of the Th-1 immune response (2,90,93). In many of these responses, IL-12 acts through up-regulation of IFN-γ, which has been shown to control viral infections and tissue alterations associated with exaggerated repair mechanisms, such as those

associated with bleomycin and immune complex-induced lung injury (90,131-132). Recently it has been determined that the p40 chain of IL-12 may also play a role in mouse Sendai viral infection systemically as a macrophage chemoattractant, as well as at a local level where it is produced in by bronchiolar epithelial cells (17). IL-12 also has been shown to inhibit bronchiolar hyperresponsiveness and bronchiolar eosinophil recruitment in several rodent and primate models of allergic sensitization (79,97-98).

As previously noted, it has been determined that Brown Norway (BN) rats are susceptible to Sendai virus-induced inflammation and remodeling, whereas Fischer 344 (F344) are highly resistant to the viral effects (111). The development of the Sendai virus-induced abnormalities may be related to the early immune response after viral infection. BN rats have greater expression of interleukin-4 (IL-4) and decreased levels of IFN-γ production, high mast cell and eosinophil response, fewer $CD8^+$ T lymphocytes, and prolonged levels of viral replication in comparison to F344 rats (119). BN rats also have increased mRNA expression of profibrogenic cytokines TGF-β_1 and TNF-α at 10 and 14 days after Sendai virus infection (113,118). Treatment of Sendai virus-inoculated BN rats with IFN-γ results in a reduction of virus-induced bronchiolar inflammation, bronchiolar fibrosis, and ultimately results in less severe pulmonary dysfunction (119). Given the critical role that IL-12 plays in inducing IFN-γ in the regulation of the Th-1 immune response and its differential expression in several models of intracellular infection, we hypothesized that F344 rats are more resistant to Parainfluenza type-1 (Sendai) virus-induced bronchiolar damage and fibrosis because they produce higher levels of IL-12 early in the response to viral infection. The objectives of this study were

to determine the quantity of pulmonary IL-12 mRNA and protein expression and to determine the structural and cellular location of this expression in the airways of both rat strains after Sendai virus infection.

Materials and Methods

Animals

Weanling (22 days old), male, pathogen-free BN/RijHsd (24 rats) and F344/NHsd (24 rats) rats were purchased from Harlan Sprague Dawley, Inc. Madison, WI and Indianapolis, IN respectively. The control and virally infected animals were housed separately in adjacent, identical Micro-Isolator VCL-HD™ individually HEPA filtered/ventilated cages (#10419ZTGA Zytem™ plastic Micro-Isolator™ system, Lab Products, Inc. Seaford, DE). The University of Florida Animal Care and Use Committee approved all procedures.

Viral Procedures and Sample Collection

The rats were inoculated with aerosolized parainfluenza (Sendai) virus type 1 strain P3193 (117). The numbers of rats used for each experimental technique are indicated in the figures located within the results section. Briefly, rats in the virus-inoculation group were exposed to an aerosol of virus at a concentration of 1-3 plaque-forming units (PFU) per ml of gas via a Glas-Col Aerosol Exposure Apparatus for 15 minutes (Tri-R) (Glas-Col, Terre Haute, IN). At 0, 1, 2, 3, 5, 7, 10, and 14 days after inoculation, rats from each group were immobilized via deep anesthetization with sodium pentobarbital (approximately 200μg/g body weight) or urethane (1.5g/kg body weight) and killed by exsanguination via cardiac puncture. Lung lavages were performed through intratracheal cannulation with phosphate buffered saline (PBS). The lungs taken for homogenization

and RT-PCR were frozen in liquid nitrogen and stored at –80°C. The lungs and tracheas used for *in situ* hybridization were tied off, perfused with 4% paraformaldehyde - PBS (pH 7.4) (30 cm H20 pressure for 2 hours) and embedded in paraffin. Finally, the lungs used for immunohistochemistry tied off, inflated with O.C.T. embedding medium compound (Tissue-Tek®, Torrance, CA), sectioned, and placed into molds. These tissues were stored at -80°C until processing.

Cytokine mRNA

Frozen lungs were weighed and RNA was extracted by a phenol/chloroform method or by using the RNeasy® midi kit (Qiagen Inc, Valencia, Ca) (121,122). The RNA samples were pre-treated with DNase I using the Deoxyribonuclease I, amplification grade kit (Invitrogen, Carlsbad, CA) to remove genomic DNA. The Reverse Transcriptase (RT) reactions were preformed using the Advantage™ RT-for-PCR Kit (Clontech Laboratories, Inc, Palo Alto, CA). Polymerase chain reaction (PCR) primers and probes for rat IL-12 p40, rat IL-12 p35, rat interleukin (IL-18), and for the housekeeping gene rat glyceraldehyde-3-phosphate dehydrogenase (GADPH) were TaqMan® pre-developed assay reagents for gene expression quantification (Applied Biosystems, Foster City, CA). Every time cDNA was synthesized, parallel TaqMan® assays were run for GADPH and the target cytokine in separate wells. The PCR reactions contained 900 nM of each primer, 250 nM of the TaqMan probe, PCR Mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction, and 2 µl of the cDNA sample in a final volume of 25 µl. The samples were amplified in an

automated fluorometer (ABI Prism 7700 Sequence Detection System, Applied Biosystems). Amplification conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C. Final quantitation was done using the comparative C_T (cycle threshold) method and was reported as relative transcription of the n-fold difference relative to a calibrator cDNA (LPS-stimulated rat lung) (Table 3-1) (124).

Table 3-1 Comparative C_T Method of cDNA Relative Quantitation

Average C_T	Average GADPH C_T	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$
Target cytokine C_T average from 3 consecutive wells	GADPH C_T average from 3 consecutive wells (Same sample as target cytokine)	Target C_T – GADPH C_T	ΔC_T - ΔC_T Calibrator Calibrator = LPS Stimulated rat lung cDNA	Relative difference to the calibrator

Protein Analysis

Enzyme-linked immunosorbent assay (ELISA)

Lungs were harvested as previously described and homogenized in cold PBS with a protease inhibitor (Protease Inhibitor Cocktail Tablets, Mini Complete, Boehinger Mannheim, Germany) at a ratio of 0.1g of tissue/ml (133). The homogenates were centrifuged at 4°C at 2000 rpm for 10 minutes and the supernatants were frozen at -80°C until use. The total IL-12 (p70 heterodimer protein, p40 monomers, and p40 dimers) in the lung lavage and homogenate was determined using an ELISA kit (murine IL-12, Biosource International, Camarillo, CA) according to the manufacturer's instructions. Due to the ability of IL-12 p40 monomers and dimers to antagonize the effects of biologically active IL-12 p70 and the potential roles of IL-12 p40 observed in mouse Sendai viral infections, the IL-12 p40 protein was measured in the lung homogenates (17,127). The level of IL-12 p40 protein was determined using a mouse ELISA kit (Quantikine® M Murine Mouse IL-12 p40 ELISA, R&D Systems, Minneapolis, MN).

These results were compared to the IL-12 total protein levels to determine the contribution of the protein forms of IL-12.

Immunohistochemistry

Frozen lung sections from BN and F344 rats were dried at room temperature and then fixed in cold acetone for 10 minutes at 4°C. After washing in 1 X PBS, the endogenous peroxidases were blocked using 1% hydrogen peroxide rinse for 10 minutes. The slides were then blocked with either normal goat serum (IL-12 p40 assay) (Santa Cruz Biotechnology ABC Staining Systems, Santa Cruz, CA) or normal mouse serum (OX-6, detection of dendritic cell assay) (Santa Cruz Biotechnology ABC Staining Systems, Santa Cruz, CA) for 1 hour in a humidified slide chamber at room temperature. In the IL-12 p40 assay, the sections were incubated with either polyclonal, goat, anti-mouse IL-12 p40 (indicator of IL-12 p70 cellular production) (Santa Cruz Biotechnology, Santa Cruz, CA) at 3.5 μg/μl or goat immunoglobulin G (IgG) (Sigma, St. Louis, MO) at 0.25 μg/μl in a humidified slide chamber, overnight at 4°C. In the OX-6 (major histocompatibility complex determinant on B lymphocytes, dendritic cells, some macrophages, and certain epithelial cells) assay, the sections were incubated under the same conditions with either monoclonal, mouse anti 1-A (OX-6) (Serotec, Raleigh, NC) at 0.02 μg/μl or mouse IgG (Sigma, St. Louis, MO) at 0.2 μg/μl. In both assays antibody binding was detected using an avidin-biotin, chromogen diaminobenzidine system according to the manufacturer's instructions (Santa Cruz Biotechnology ABC Staining Systems, Santa Cruz, CA). The density of cells expressing the IL-12 p40 protein was determined by counting and classifying the number of labeled cells per millimeter (mm) of bronchiolar basement membrane in an average of 10.3 mm/section. Round cells with

central round/oval nuclei and abundant cytoplasm were classified as macrophages, and cells with round/ asymmetrically placed nuclei with foamy, dendritic cytoplasmic extensions were classified as dendritic cells. The density of OX-6 positive cells was determined similarly with an average of 12.3 mm/section being counted.

Data Analysis

For all experiments, group means were compared by one-way analysis of variance (ANOVA) using a computer-based statistical program (Sigma-Stat, Jandel Corp. San Rafael, CA). Kruskal-Wallis analysis of variance was used on ranks if the normality test or equal variance tests of group means failed. Multiple comparison procedures were used to isolate the group or groups that differ from others. The Student-Newman-Kuel's test was used if the sample sizes were equal; otherwise Dunn's test was used to compare groups of unequal sample size.

Results

Virus-Resistant F344 Rats Have Increased Expression of Pulmonary IL-12 mRNA

Relative amounts of IL-12 p40 mRNA were significantly higher in the lung tissue of the F344 rats compared to the BN rats beginning at the non-infected control level ($p<0.01$) ($n = 4$) (Figure 3-1). The expression of IL-12 p40 mRNA in the F344 rats remained significantly higher at 1,2, and 3 days after viral inoculation with the greatest difference being 3.4-fold at 2 days after virus inoculation ($p<0.02$) (Figure 3-1).

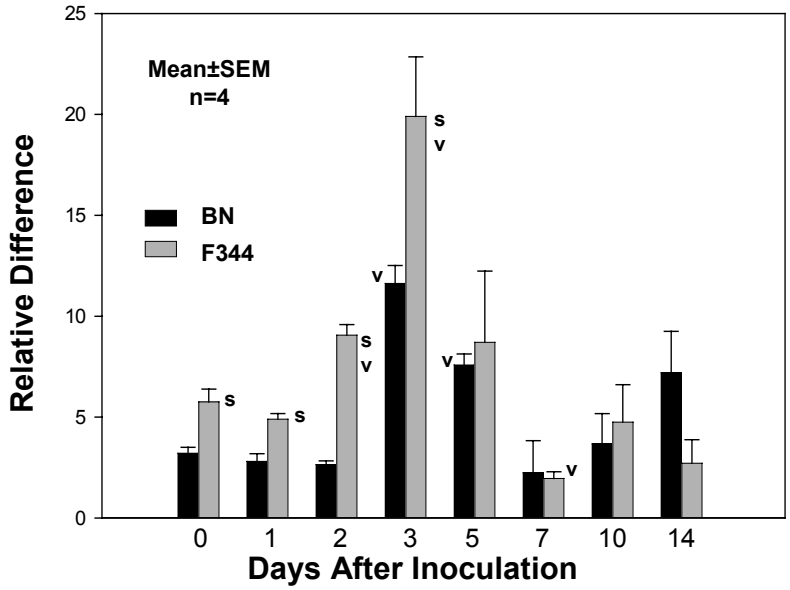

Figure 3-1. Real-time PCR analysis of IL-12 p 40 mRNA in whole lung samples of BN and F344 rats after Sendai virus infection. (**s** = significant difference between strains, $p<0.04$) (**v** = significant virus-induced difference compared to strain control, $p<0.01$)

There was a virus-induced increase of IL-12 p40 mRNA in both strains of rats, however it reached statistical significance earlier in the F344 strain (2 days after inoculation, $p<0.01$). Relative amounts, of the more constitutively expressed IL-12 p35 mRNA, were also significantly increased in the lungs of F344 rats at control, 2 and 3 days after inoculation ($p<0.03$) ($n = 2$) (Figure 3-2). However, there was not a virus-induced increase in either strain above the non-infected controls. The expression of IL-12 p35 mRNA significantly decreased as analyzed by real-time PCR (Figure 3-2).

The same trends were detectable in mRNA IL-12 p40 expression at the airway level. IL-12 p40 mRNA expression was increased 3.7-fold in the F344 tracheas at 2 days after inoculation as compared to the BN tracheas, and a virus-induced increase in IL-12 p40 mRNA expression was observed only in the F344 strain ($p<0.03$) ($n = 4$-7) (Figure 3-3). There were no IL-12 p35 mRNA expression differences detected at the

airway/tracheal level between rat strains or induced by virus ($p>0.05$) (n = 4-7) (Figure 3-4).

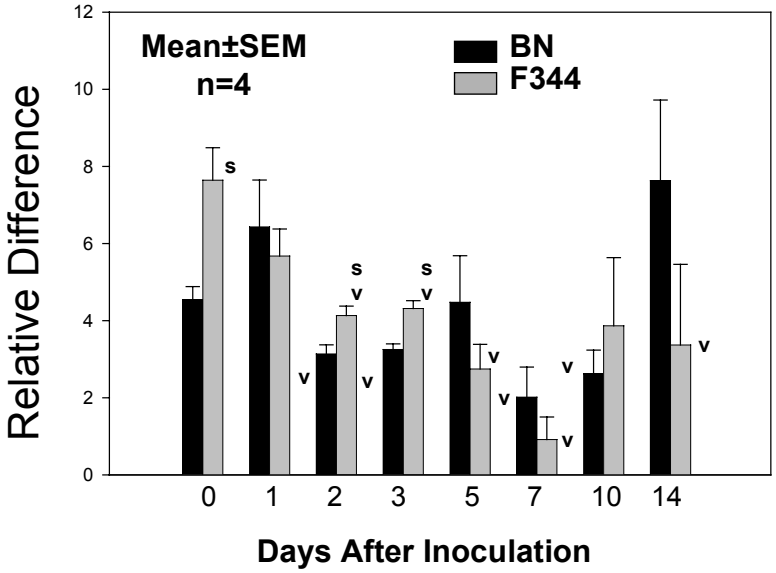

Figure 3-2. Real-time PCR analysis of IL-12 p35 mRNA in whole lung samples of BN and F344 rats after Sendai virus infection. (**s** = significant difference between strains, $p<0.03$) (**v** = significant virus-induced difference compared to strain control, $p<0.04$)

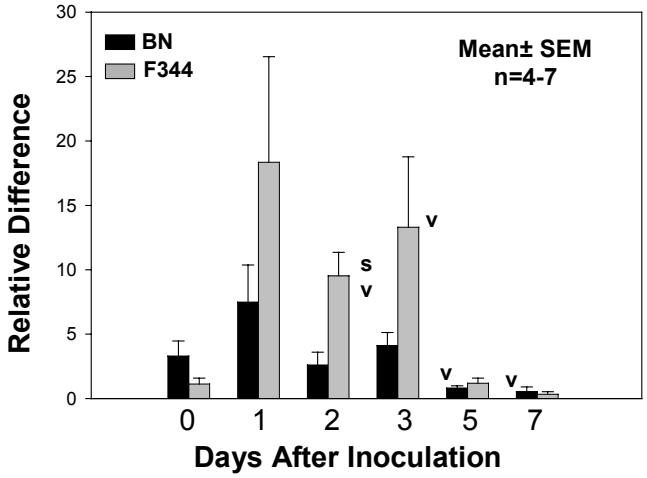

Figure 3-3. Real-time PCR analysis of IL-12 p40 mRNA in trachea samples of BN and F344 rats after Sendai virus infection. (**s** = significant difference between strains, $p<0.03$) (**v** = significant virus-induced difference compared to strain control, $p<0.03$)

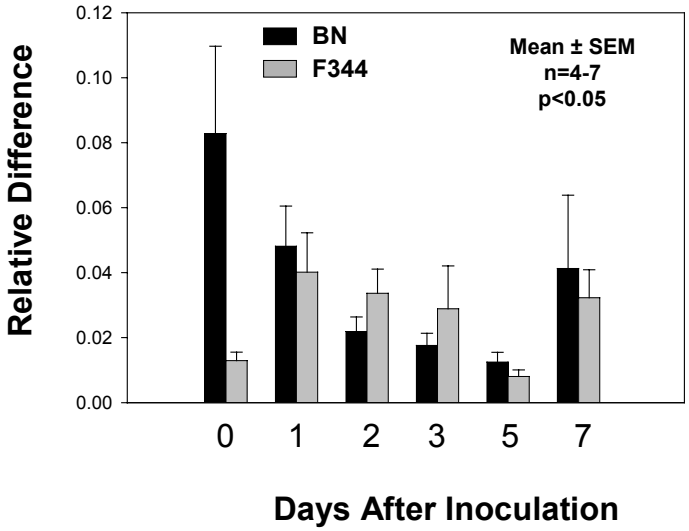

Figure 3-4. Real-time PCR analysis of IL-12 p35 mRNA in trachea samples of BN and F344 rats after Sendai virus infection.

F344 Rats Have Increased Pulmonary IL-12 Protein After Sendai Virus Infection

As measured by ELISA, the F344 rats had 86 pg/ml ± 12.72 of pulmonary IL-12 total protein as compared to 54.8 pg/ml ± 5.82 in the BN strain ($p<0.05$) ($n = 6$-7) (Figure 3-5). The levels of IL-12 total protein increase with viral infection over the non-infected controls in the F344 rats at all time points measured and in the BN rats on days 1, 3, and 5 after Sendai inoculation (Figure 3-5) ($p<0.05$).

In both BN and F344 rats the concentration of pulmonary IL-12 p40 protein (monomers and homodimers) was approximately half that of the total IL-12 (Figure 3-6, y-axis). Strain differences in pulmonary IL-12 p40 protein were only detected in the non-infected controls ($p<0.05$) ($n = 6$-7) (Figure 3-6). Concentrations of IL-12 p40 protein increased only in the BN strain with viral infection starting at 2 days after viral inoculation but never reached statistical significance ($p>0.05$) (Figure 3-6).

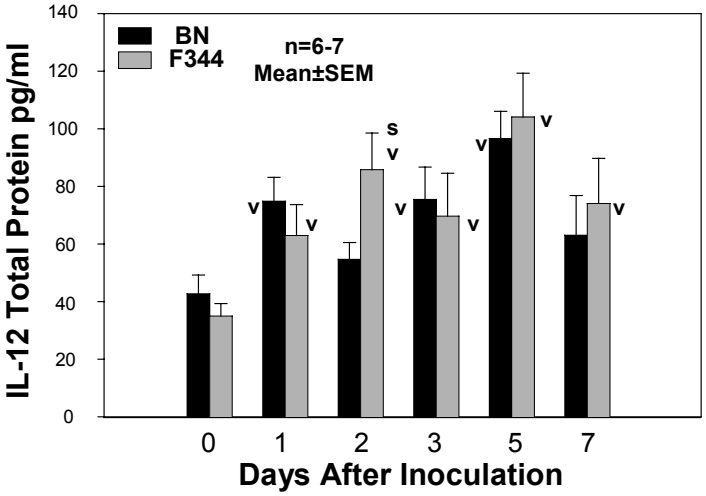

Figure 3-5. ELISA analysis of IL-12 total protein in F344 and BN rats strains after Sendai virus inoculation. (**s** = significant difference between strains, $p<0.05$) (**v** = significant virus-induced difference compared to strain control, $p<0.05$)

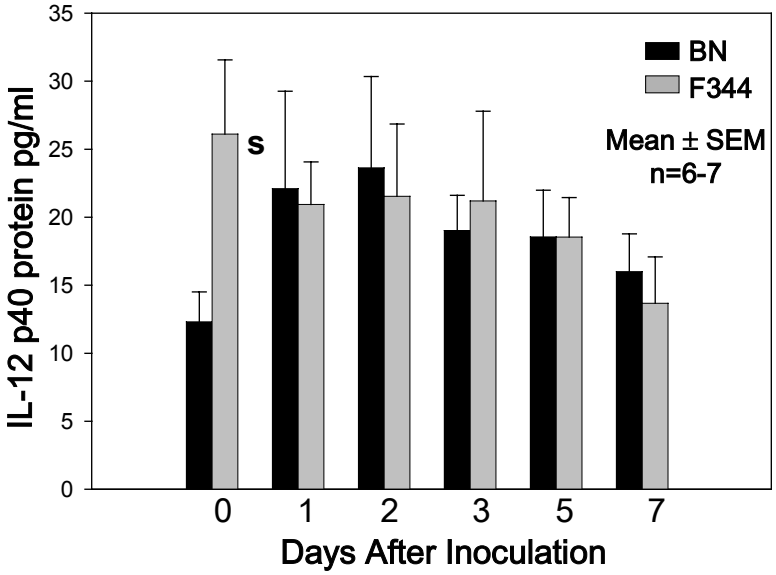

Figure 3-6. ELISA analysis of IL-12 p40 protein in F344 and BN rats strains after Sendai virus inoculation. (**s** = significant difference between strains, $p<0.05$)

F344 Rats Have Increased Numbers of IL-12 p40 Expressing Cells in the Bronchioles After Sendai Virus Inoculation

Density of OX-6 positive dendritic cells was significantly increased in the bronchioles of F344 rats at 2 and 3 days after virus inoculation as compared to BN rats ($p<0.03$) ($n = 4$) (Figures 3-7 and 3-8). Dendritic cell numbers in the F344 rats increased

87.5% above the non-infected controls and almost 2-fold above the BN rats at 2 days after inoculation. There were no significant virus-induced increases in the numbers of bronchiolar dendritic cells measured in the BN rats at any of the time points after inoculation.

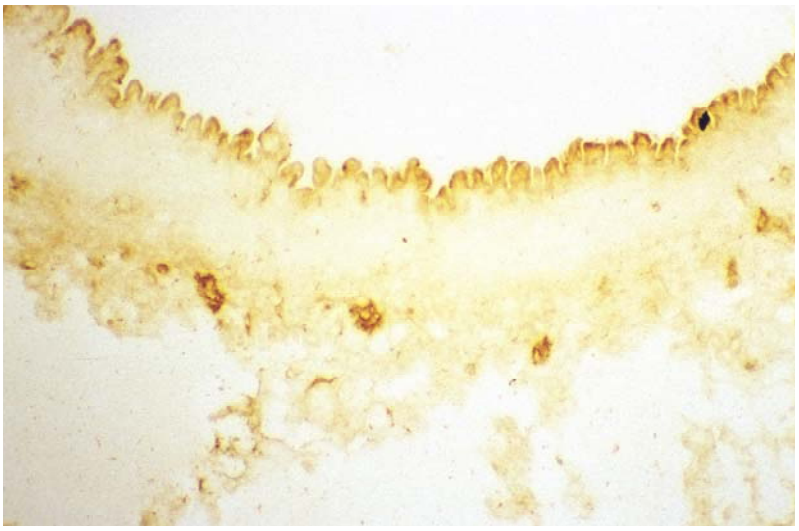

Figure 3-7. OX-6 immunohistochemistry in the bronchiole of a F344 rat three days after inoculation (54X Magnification). There are several OX-6 positive dendritic cells, indicated by dark brown staining, located within the bronchiole wall.

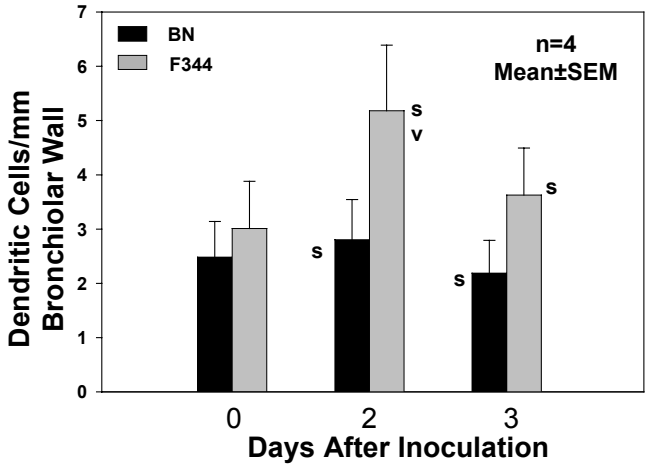

Figure 3-8. Density of OX-6 positive dendritic cells in the bronchioles of F344 and BN rats. (**s** = significant difference between strains, $p<0.03$) (**v** = significant virus-induced difference compared to strain control, $p<0.01$)

IL-12 p40 protein was expressed in dendritic cells and macrophages in the bronchioles of both strains. The average number of IL-12 p40 positive cells/mm of bronchiole wall was significantly higher in the F344 rat strain at 2 and 3 days after inoculation as compared to the BN strain ($p<0.03$) (n = 4) (Figures 3-9 – Figure 3-11).

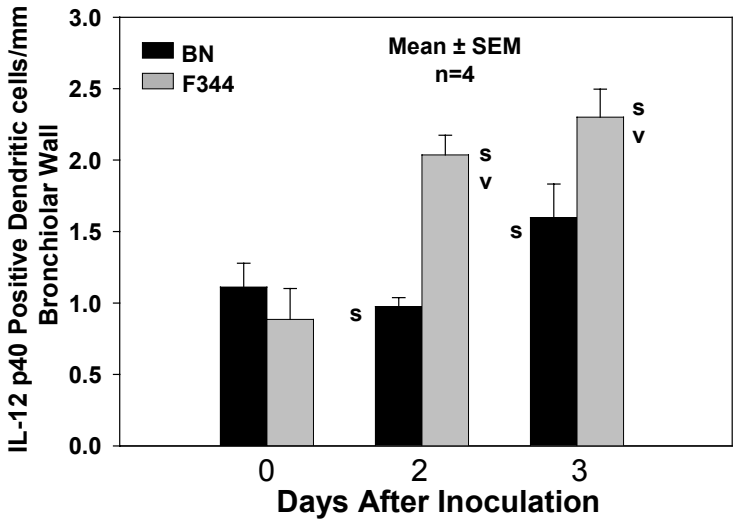

Figure 3-9. Density of IL-12 p40 positive dendritic cells (**s** = significant difference between strains, $p<0.03$) (**v** = significant virus-induced difference compared to strain control, $p<0.01$)

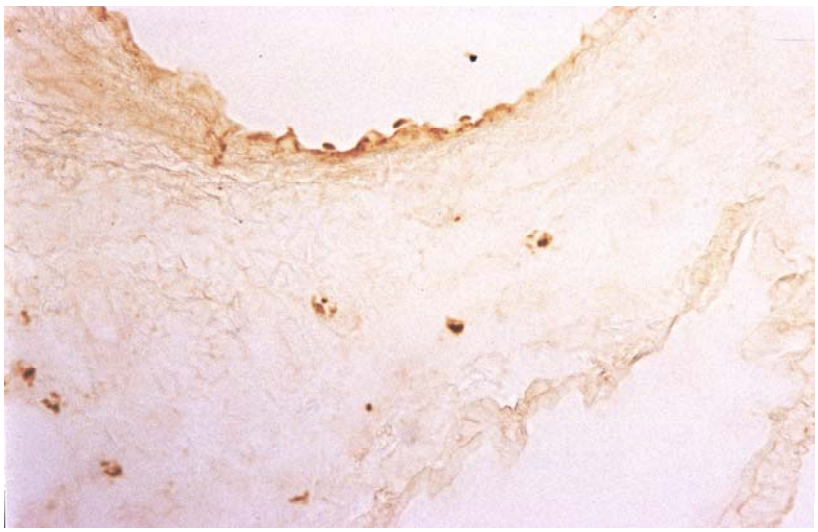

Figure 3-10. IL-12 p40 immunohistochemistry in the wall of bronchiole of a F344 rat at two days after Sendai inoculation (108X Magnification). Several inflammatory cells are indicated by dark brown staining inflammatory cells (macrophages and dendritic cells) within the airway wall.

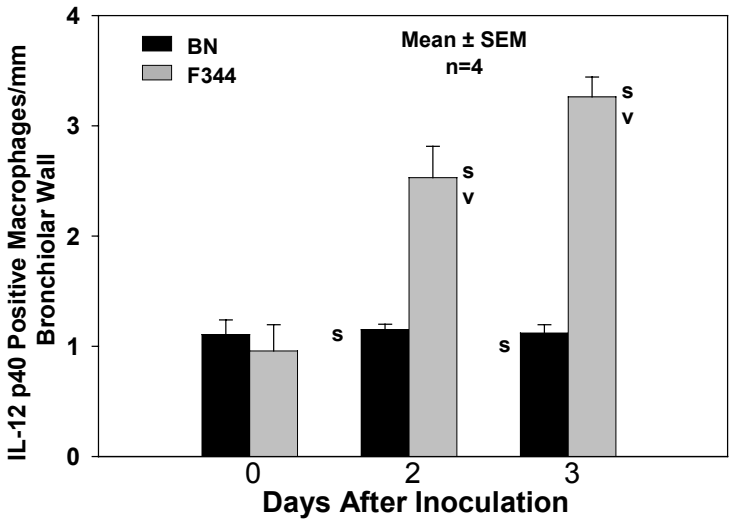

Figure 3.11. Density of IL-12 p40 positive macrophages in the bronchioles of BN and F344 rats. (**s** = significant difference between strains, $p<0.03$) (**v** = significant virus-induced difference compared to strain control, $p<0.01$)

F344 and BN Rats Do Not Have Detectable Differences in the Expression of IL-18 mRNA

Due to the co-stimulatory role of IL-18 with IL-12 in response to intracellular infections, we examined the IL-18 mRNA expression in the lung tissues of both rat strains (1). There were no differences detected by real-time PCR in the pulmonary IL-18 mRNA expression between BN and F344 rats at the time points measured ($p>0.05$) ($n = 4$-5) (Figure 3-12). Additionally, Sendai virus-induced increases in IL-18 were not detected in either strain (Figure 3-12).

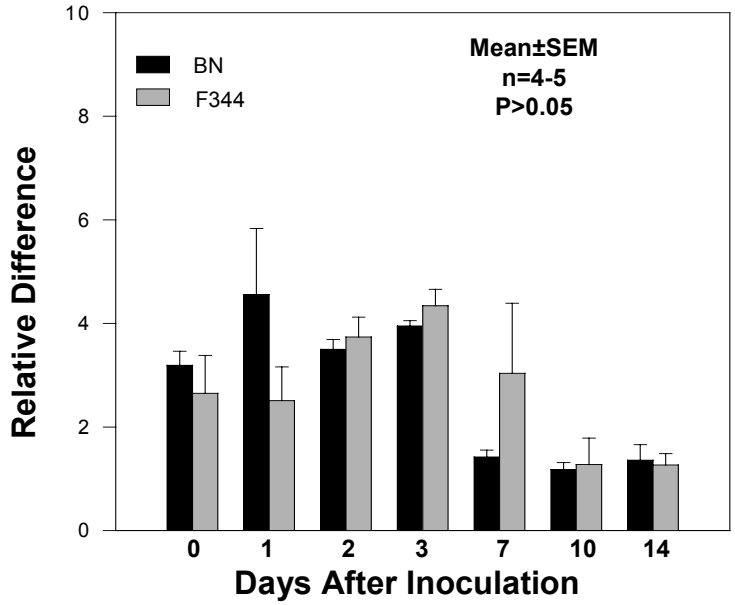

Figure 3-12. Real-time PCR analysis of IL-18 mRNA expression in lung samples of BN and F344 rats after Sendai virus infection.

Discussion

The purpose of this study was to determine whether there is differential expression of IL-12 between rat strains that are susceptible and resistant to the pulmonary sequelae of Sendai virus infection. The critical role of cytokines expressed acutely following virus infection in this model and the importance of IL-12 in the response to intracellular infections made this an important component of this disease model to investigate. The results indicate that there are significant differences in total pulmonary IL-12 p40 and p35 mRNA and IL-12 total protein levels expressed between virus-resistant F344 and virus-susceptible BN rats following Sendai virus infection. There are also significant differences in the numbers of cells expressing IL-12 p40 protein in the bronchioles between these two strains.

This data indicates that total IL-12 protein expression increases earlier than the expression of IL-12 p40 mRNA. This discrepancy may be due to several factors in the

measurement of protein and mRNA. Although both were measured in lung homogenates, the blood and serum proteins are more likely to have remained viable after processing for the ELISA and could have contributed to the concentrations of total IL-12 protein. Additionally, RT-PCR, using conventional primers, is unable to detect differences in the isoforms of IL-12 p35 mRNA (Chapter 1). There are differences in the isoforms of IL-12 p35 mRNA expressed depending on whether the production is constitutive or pathogen-induced (14). Primers specific for the 5'untranslated region of the p35 gene can only distinguish the untranslatable form. Additionally, there can be several translatable isoforms with slight variations in this region. So, there may be more exaggerated differences in IL-12 p35 expression (both virus-induced and between strains) than we are able to detect with these primers. Therefore, it is difficult to make comparisons between IL-12 mRNA expression and the IL-12 total protein levels.

Two pathways have been established for the production of IFN-γ in viral infection. Some infections, such as lymphocytic choriomeningitis virus (LCMV), have an IL-12 independent induction of IFN-γ through IFNα/β pathway with very low, non-inducible, levels of IL-12 expression (92). In addition, differential expression of the co stimulatory cytokine IL-18 has been shown to be critical to the level of IFN-γ induced in many models of intracellular infection (55-57, 79, 92). In the model examined here, both rat strains have virus induced increases in the level of IL-12 p40 mRNA and IL-12 total protein with no changes in IL-18 expression, suggesting that IFN-γ levels are being up regulated by IL-12-dependent mechanisms in Sendai viral infection.

We also wanted to determine if the differences expressed at the total lung level were consistent with the inflammatory response at the airway level. The IL-12 p40

immunohistochemistry (an indicator of IL-12 p70 cellular protein expression) results do indicate that bronchiolar macrophage and dendritic cell protein expression is higher at early time points in the F344 rats following Sendai virus inoculation. Therefore, the differential expression of IL-12 does appear to be evident throughout the lung, even at the local airway level. Additionally, the levels of pulmonary IL-12 p40 protein (homodimers and monomers) measured in whole lung homogenates indicate that the possible role of over-produced IL-12 p40 protein as an IL-12Rβ2 antagonist is likely not a factor in this model. However, other possible roles of IL-12 p40 protein, such as a chemoattractant for macrophages, have not been elucidated by these methods.

P.G. Holt and others have established that a network of resident airway epithelial dendritic cells exists in throughout the respiratory tract of rodents and humans, where these cells process antigen and initiate the generation of protective local immune responses (121,134-135). The most prominent populations are present in the conducting airways, such as the trachea (600-800/mm^2), and decrease further down the respiratory tract (75/ mm^2) (134). This population also appears to be dynamic with changes observed at steady state and increases seen in the trachea after *Moraxella catarrhalis* bacteria and Sendai virus exposures (121, 134). These numbers are much higher than the dendritic cell numbers that we were able to detect at 2 and 3 days after inoculation in the bronchioles. However, bronchiolar airways were not singled-out in previous experiments (more focus on the interalveolar septal junctions) and the peak time point for tracheal dendritic cell numbers was not observed until 5 days after inoculation (134). Additionally, the experiments examining the lower airways were performed hours (not days) after the exposure to pathogen before the dendritic cells would be migrating from

the local site of inflammation to the regional lymph nodes (134). Similar to the results seen in previous studies, our results show an increase in dendritic cell numbers after Sendai infection in the F344 rat strain.

An increasing amount of evidence suggests that dendritic cell population changes and cytokine expression may be a factor in the susceptibility to allergic respiratory disease (135). Examination of dendritic cells in the lungs of several rat strains suggests that the Th-2 polarity of the resting mucosal immune system may also be a property of the resident dendritic cell population (121, 136). Initiation of the Th-1 immune response in these cells requires appropriate costimulation (such as CD40 ligation) from the microenvironment (136). Based on our results, a change in IL-12 expression is occurring at the whole lung and airway levels. F344 rats have virus-induced increases in dendritic cell numbers and increased expression of IL-12 p40 in their bronchioles by both macrophages and dendritic cells as compared to BN rats early after Sendai infection. The levels of IL-12 p40 mRNA are also increased in the tracheas of the F344 strain at 2 and 3 days after Sendai inoculation. However, in order to assess whether the IL-12 mRNA expression is from cells (macrophages and dendritic cells) already present or from a cellular influx further experimentation will need to be done to compare the magnitude of the response in the airways of both strains. A component of the F344 rat strain's resistance to Sendai virus –induced airway damage may be that their dendritic cells and macrophages receive an earlier signal from the microenvironment to become Th-1 type cells secreting more IL-12 as compared to the BN rat strain. Further examinations of the airway dendritic cells, their interactions with the local airway mucosal immune system, and the magnitude of this response are needed to confirm this possibility.

The differential expression of IL-12 appears to be a factor in the resistance to Sendai virus-induced airway disease. This differential expression appears to occur early after infection and may be the source of the establishment of a predominately Th-1 cytokine response in the F344 rat strain. Similar to this rat model, paramyxoviral (respiratory syncytial virus and parainfluenza virus) infections during infancy are potential risk factors for the development of asthma in children (104-108). Additionally, cytokine imbalance during early life has been considered to be an important risk factor in the development of asthma and atopy (117). The differences in IL-12 expression by these rat strains in response to Sendai virus infection may give insight into the mechanisms of asthma development and potential asthma therapies in children.

CHAPTER 4
EXOGENOUS INTERLEUKIN-12 (IL-12) ADMINISTRATION REDUCES THE SEVERITY OF SENDAI VIRUS-INDUCED CHRONIC AIRWAY FIBROSIS AND REMODELING IN BN RATS

Summary

Sendai virus infection in virus-susceptible BN rats causes persistent bronchiolar inflammation and fibrosis that is associated with increased airway resistance and airway hyperresponsiveness. In contrast, F344 rats have earlier viral clearance, increased IL-12 pulmonary expression, and are resistant to post viral airway function abnormalities. This study determined whether the exogenous administration of interleukin-12 (IL-12) could confer resistance to Sendai virus-induced airway disease in virus-susceptible BN rats. BN rats were treated with 3μg of recombinant IL-12 (rIL-12) or an equivalent volume of saline intraperitoneally (IP) at the time of virus inoculation (day 0) or two days after virus inoculation (day 2). Proliferating fibroblasts were labeled with bromodeoxyuridine (BrdU) and detected by immunohistochemical staining. In comparison to infected rats given saline, infected rats treated with rIL-12 at day 0 had 22.1% lower levels of chronic airway inflammation and 23.8% lower levels of airway fibrosis as detected by histological criteria. Rats treated on day 0 with r IL-12 had a 42% and 62.5% decrease in BrdU-labeled fibroblasts in their bronchioles at 10 and 14 days after inoculation respectively as compared to saline-treated virus-inoculated controls ($p<0.05$). There was a 4-fold increase in pulmonary IFN-γ mRNA and a 77% increase in pulmonary IFN-γ protein detected in the lungs of day 0 treated rats when compared to the virus-inoculated, saline-treated control rats ($P<0.05$). In contrast, day 2 rIL-12 treatment induced a 20%

increase in bronchiolar airway wall thickness and a 12.5% increase in BrdU-labeled fibroblasts at 14 days after inoculation ($p<0.05$). Day 2 treatment resulted in increased pulmonary IL-4 mRNA levels compared to saline-treated virus-inoculated controls ($p<0.05$). In conclusion, early IL-12 treatment reduces Sendai virus-induced bronchiolar inflammation and fibrosis in virus-susceptible BN rats. This effect may be mediated, in part, by the induction of IFN-γ.

Introduction

Parainfluenza type I (Sendai) virus infection in rats is an animal model of virus-induced airway abnormalities with similar characteristics to childhood asthma, such as increased airway resistance and hyperresponsiveness (109-112). Previous studies have demonstrated that virus-resistant Fischer (F344) rats have increased expression of pulmonary interleukin-12 (IL-12), as well as increased numbers of IL-12 producing cells (dendritic cells and macrophages) in their bronchioles as compared to virus-susceptible Brown Norway (BN) rats after Sendai virus infection (Chapter 3). The biological significance of this increased IL-12 expression in the F344 strain has not been established.

The heterodimeric cytokine, IL-12 is produced by antigen presenting cells during intracellular infections to up-regulate cell-mediated immune responses and the T helper-1 (Th-1) cytokines, principally interferon-γ (IFN-γ)(1-3). IL-12 works to enhance the cytotoxic properties of natural killer (NK) cells and cytotoxic $CD8^+$ T lymphocytes (CTLs) (2). Furthermore, IL-12 can down-regulate the T helper type-2 (Th-2) cytokine response by decreasing the production of interleukin-4 (IL-4) and Th-2 type antibodies (2). Increased expression of IL-12 in animal models of infectious as well as allergic

disease has conferred resistance to the particular pathogenic phenotype (79, 97-98). The exogenous administration of IL-12 to susceptible animals at certain time points during infection or sensitization has also been shown to provide resistance in many of these models (79, 97-98).

In Sendai virus rat model, F344 rats have an early Th-1 type immune response to infection with higher IFN-γ production and their NK and CTL cell types have an increased capacity compared with the BN strain to produce IFN-γ in response to infection (115, 117, 119). Due, at least in part, to the higher IFN-γ expression F344 rats have a greater $CD8^+$ T cell response, earlier pulmonary viral clearance, and a reduced capacity to develop airway fibrosis after viral infection (115, 119). However, BN in rats produce Th-2 type cytokines, such as IL-4, interleukin-5 (IL-5), and the profibrotic cytokines tumor necrosis factor-α (TNF-α) and transforming growth factor-β-1 (TGF-β_1) after virus infection (113, 115, 118). This response to Sendai infection involves the persistence of airway inflammation (macrophages, lymphocytes, and eosinophils), delayed viral clearance, and chronic bronchiolar fibrosis (112-113). Treatment of BN rats with IFN-γ reduced the amount of chronic bronchiolar inflammation and fibrosis, thus protecting them from pulmonary function abnormalities (117).

Based on the previous IL-12 studies (Chapter 3) and the IFN-γ treatment results, we investigated the possible protective role of IL-12 in this rat model. We hypothesized that the lower IL-12 response during acute viral infection is an important factor in the development of the Sendai-induced post viral sequelae in BN rats. The objective of this

study was to determine if the administration of exogenous IL-12 at early time points during Sendai virus infection would prevent the development of post viral persistent bronchiolar inflammation and fibrosis.

Materials and Methods

Animals

Weanling (22 days old), male, pathogen-free BN/RijHsd rats (94 total rats) were purchased from Harlan Sprague Dawley, Inc. Madison, WI. The control and virally infected animals were housed separately in adjacent, identical Micro-Isolator VCL-HD™ individually HEPA filtered/ventilated cages (#10419ZTGA Zytem™ plastic Micro-Isolator™ system, Lab Products, Inc. Seaford, DE). The University of Florida Animal Care and Use Committee approved all procedures.

Viral Procedures and Sample Collection

The rats were inoculated with aerosolized Sendai virus strain P3193 five days after arrival. Briefly, rats in the virus-inoculation group were exposed to an aerosol (Tri-R Aerosol Exposure Apparatus, Glas-Col, Terre Haute, IN) of virus at a concentration of 1-3 plaque-forming units (PFU) per ml of gas for 15 minutes. At 0, 3, 7, 10, and 14 days after inoculation, rats from each group were immobilized via deep anesthetization with sodium pentobarbital (approximately 200μg/g of body weight) or urethane (1.5g/kg body weight) and killed by exsanguination via cardiac puncture. Lung lavages were performed through intratracheal cannulation with phosphate buffered saline (PBS). The right lungs were frozen in liquid nitrogen and stored at –80°C. The left lungs were tied off, perfused with 4% paraformaldehyde - PBS (pH 7.4) (30 cm H_20 pressure for 2 hours) and embedded in paraffin. Viral titers were measured in homogenates of frozen lung at seven

days after inoculation, by plaque assay using Madin-Darby bovine kidney cells as described previously, and expressed as plaque forming units (pfu)/g lung tissue (128).

IL-12 Treatment Protocol

Groups of BN rats (n=8/group) were treated with mouse recombinant IL-12p70 (rIL-12) (Biosource International, Camarillo, CA). The dose of 3μg was determined to be the lowest dose administered intraperitoneally (IP) that provided a measurable biological response (Appendix B). IP injections of 3 μg of rIL-12 were given to control and infection groups of rats on the day of viral inoculation (3 hours after inoculation) (day 0) and to control and infection groups 2 days (day 2) after inoculation. A comparable volume of sterile saline was given to a separate control and infection groups IP as a negative control at the same time points. Groups of BN rats were injected IP at 200 μg/g of rat with 5-bromo-2'-deoxyuridine (BrdU)(Sigma, St. Louis, MO) 10 and 14 days after inoculation twelve hours prior to necropsy to detect epithelial and stromal cell fibrosis.

Cytokine mRNA

Frozen lungs were weighed and RNA was extracted by phenol/chloroform extraction or using the RNeasy® midi kit (Qiagen Inc, Valencia, Ca) (121,122). The RNA samples were pre-treated with DNase I using the Deoxyribonuclease I, amplification grade kit (Invitrogen, Carlsbad, CA) to remove genomic DNA. The Reverse Transcriptase (RT) reactions were preformed using the Advantage™ RT-for-PCR Kit (Clontech Laboratories, Inc, Palo Alto, CA). Polymerase chain reaction (PCR) primers and probes for rat interleukin-18 (IL-18), and for the housekeeping gene rat glyceraldehyde-3-phosphate dehydrogenase (GADPH) were TaqMan® pre-developed

assay reagents for gene expression quantification (Applied Biosystems, Foster City, CA). The primers and TaqMan probe for BN rat IL-4 were designed using the Primer Express software (Applied Biosystems, Foster City, CA). The sense and antisense primers were made in the mRNA sequence to ensure discrimination between cDNA and genomic DNA. The probe was labeled at the 5'-end with the reporter dye FAM (6-carboxyfluorescein) and at the 3' end with a minor groove binder (TaqMan® MGB) and a non-fluorescent quencher prevent extension by AmpliTaq Gold DNA polymerase (Forward Primer 5'-CAGGGTGCTTCGCAAATTTT-3'; Reverse Primer 5'-CGAGAACCCCAGACTTGTTGTT-3'; and Probe 5'- TCCCACGTGATGTACCTCCGTGCTT-3'). Dilutional curves were evaluated to assure that the amplification efficiency of the IL-4 primers compared to the efficiency of the GADPH primers. Every time cDNA was synthesized, parallel TaqMan® assays were run for GADPH and the target cytokine in separate wells. The PCR reactions contained 900 nM of each primer, 250 nM of the TaqMan probe, PCR Mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris-HCl, 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction, and 2 μl of the cDNA sample in a final volume of 25 μl. The samples were amplified in an automated fluorometer (ABI Prism 7700 Sequence Detection System, Applied Biosystems). Amplification conditions were 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C (124).

IFN-γ was detected in lung tissue by a competitive RT-PCR method as described previously (126). Primers for IFN-γ and for the housekeeping gene product, hypoxanthine-guanine phosphoribosyltransferase (HPRT) were prepared as described by

the Interdisciplinary Center for Biotechnology and Research at the University of Florida (125). Each assay was optimized for temperature, Mg^{2+}concentration, and primers. For each cDNA sample reactions comparisons were made to 0, 0.5, 5, and 50 femptograms (fg) of a competitive fragment (126). PCR reactions were performed at cycler programs consisting of 1 minute at 94°C, annealing temperature of 56°C for 15 minutes, 72°C for 2 minutes for 4 cycles. Then 36 cycles were run at the same temperatures and times except for a 2-minute annealing time at 56°C. PCR products were stained with ethidium bromide and separated electrophoretically on 1.5-% agarose gels. The data are reported as non-normalized mRNA abundance in competitive fragment units (117).

Enzyme-Linked Immunosorbent Assay (ELISA)

Lungs were harvested as previously described and homogenized in cold PBS with a protease inhibitor (Protease Inhibitor Cocktail Tablets, Mini Complete, Boehinger Mannheim, Germany) at a ratio of 0.1g of tissue/ml (133). The homogenates were centrifuged at 4°C at 2000 rpm for 10 minutes and the supernatants were frozen at -80°C until use. IFN-γ protein in the lung homogenates was determined using a rat IFN-γ ELISA kit (rat IFN-γ, Biosource International, Camarillo, CA) according to the manufacturer's instructions.

BrdU Immunohistochemistry

In previous studies, Sendai virus-induced fibroblast proliferation has been detected by BrdU incorporation beginning at 9 days after inoculation (113). Paraffin sections of lung at 10 and 14 days after Sendai inoculated rats were deparaffinized in xylene, rehydrated through a graded series of ethanol washes, and washed in distilled water. Immunohistochemical staining to detect BrdU labeling was performed using a method

previously described (137). Briefly, slides were placed in 3.0% H_2O_2 for 10 minutes to quench endogenous peroxidase, washed twice in PBS, and pretreated in both 2N HCl (30 minutes at 37°C) and 0.1% w/v trypsin in PBS (20 minutes at 37°C). Sections were rinsed in PBS, covered with antibody diluent (1.0% BSA and 0.5% Tween 20 in PBS) (Sigma, St. Louis, MO) for 30 minutes to block nonspecific binding, blotted and incubated overnight at 4°C covered in mouse anti-BrdU monoclonal antibody (Sigma, St. Louis, MO) diluted 1:10 in antibody diluent. The next day the sections were washed in PBS and covered with peroxidase-conjugated, goat, anti-mouse IgG (Fc specific) (Sigma, St. Louis, MO) secondary antibody for one hour at room temperature. Bound peroxidase-conjugated antibody was detected by development in the chromogen diaminobenzidine (Sigma, St. Louis, MO) in 0.02 mg/ml in 0.25 mol/L Tris, pH 7.6, and 0.01% H_2O_2 for 7-20 minutes as monitored with light microscopy. An average of 17 bronchioles per rat (range 10-24) at 10 days after-inoculation and 14 bronchioles per rat (range 9-19) at 14 days after-inoculation were examined. Fibroblast proliferation was assessed by counting the number of BrdU-labeled fibroblasts per mm of bronchiolar basement membrane.

Analysis of Bronchiolar Inflammation and Fibrosis

Previously studies have found that virus-induced bronchiolar fibrosis and collagen deposition is present in BN rats by 14 days after-viral infection (113). Serial paraffin sections of rat lungs 14 days after-inoculation (5 per rat) were stained with hematoxylin and eosin (H&E). Each branch of bronchiole cut in transverse, longitudinal, or oblique planes was counted and was evaluated for both the presence of inflammation and for the presence of fibrosis/remodeling. An average of 42.7 (range 21-76) bronchioles per rat were evaluated. Bronchioles were scored as positive for inflammation if the wall had

five or more inflammatory cell types (eosinophils, lymphocytes, or macrophages). Bronchioles were scored as positive for fibrosis/remodeling if the walls were thickened with increased fibroblasts and deposition of collagen. Collagen was identified using Masson's Trichrome and Manuel's Reticulin stains. Numbers of bronchioles with inflammation or fibrosis/remodeling were divided by the number of total bronchioles examined to calculate the percentage of bronchioles with each pathologic change. Additionally, the area of the bronchiolar wall from the bronchiolar epithelial basement membrane to basement membrane of the surrounding alveolar walls was measured. Bronchiolar wall area was divided by the perimeter of bronchiolar basement membrane to calculate the thickness of the wall (square micrometers of bronchiolar wall per micrometer of bronchiolar basement membrane) (113).

Data Analysis

The final quantitation of cytokine mRNA levels detected by real-time PCR was done using the comparative C_T (cycle threshold) method and was reported as relative transcription of the n-fold difference relative to a calibrator cDNA (BN rat high IL-4 responder lung cDNA for IL-4 mRNA and LPS-stimulated lung cDNA for IL-18 mRNA) (Table 3-1) (124). For all experiments, group means were compared by one-way analysis of variance (ANOVA) using a computer-based statistical program (Sigma-Stat, Jandel Corp. San Rafael, CA). Kruskal-Wallis analysis of variance on ranks was used on ranks if the normality test or equal variance tests of group means failed. Multiple comparison procedures were used to isolate the group or groups that differ from others. The Student-Newman-Kuel's test was used if the sample sizes were equal; otherwise Dunn's test was used to compare groups of unequal sample size.

Results

IL-12 Treatment of BN Rats at the Time of Sendai Virus Inoculation Reduces the Amount of Bronchiolar Inflammation and Fibrosis

Virus infection in BN rats induced bronchiolar inflammation and fibrosis (Figure 4-1). Administration of rIL-12 at the time of Sendai viral inoculation (Day 0) reduced the number of inflamed bronchioles by 22.1% and the number of bronchioles with mural fibrosis by 23.8% as compared with virus-inoculated, saline-treated controls (n = 8) (Figure 4-1).

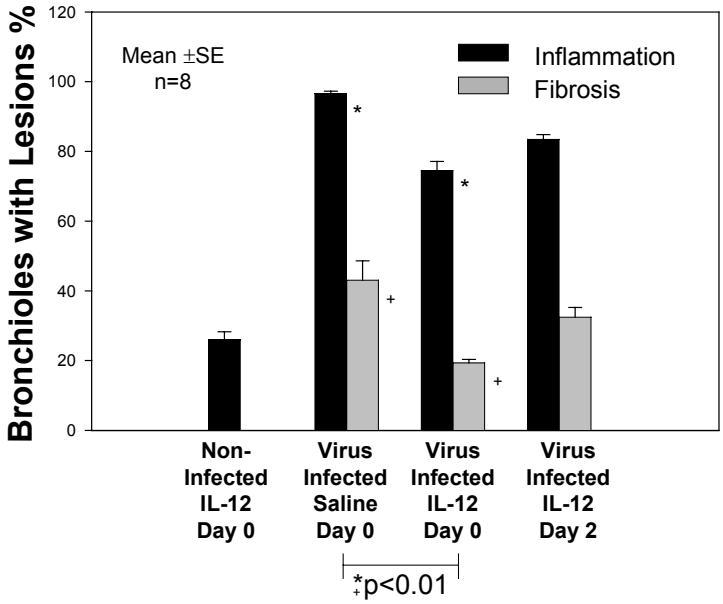

Figure 4-1. The percent of bronchioles containing evidence of inflammation and/or fibrosis at 14 days after inoculation. Treatment of BN rats at the time of virus infection resulted in the significant reduction of bronchiolar inflammation and fibrosis ($p<0.01$).

In contrast, IL-12 treatment 2 days after viral inoculation did not lead to a significant decrease in the numbers of inflammatory cells or in the severity of fibrosis observed. Previously it has been shown that non-infected BN rats normally have low-density aggregates of lymphocytes, macrophages, mast cells, and eosinophils in bronchiolar walls by 30 days of age (Figure 4-1) (112).

IL-12 Treatment of BN Rats at the Time of Sendai Virus Inoculation Reduces the Bronchiolar Wall Thickness

Increases in bronchiolar wall thickness occur by 14 days after Sendai virus inoculation due to edema, the accumulation of inflammatory cells, fibroblast proliferation, and the intramural deposition of collagen and extracellular matrix (113). The BN rats treated at time of virus-inoculation had a 15% decrease in the bronchiolar wall thickness as compared with the virus-inoculated, saline-treated controls ($p<0.02$) (n = 8) (Figure 4-2). A significant virus-induced increased in bronchiolar wall thickness was observed above the non-infected BN rats in the virus-inoculated, saline treatment group ($p<0.001$) (Figure 4-2).

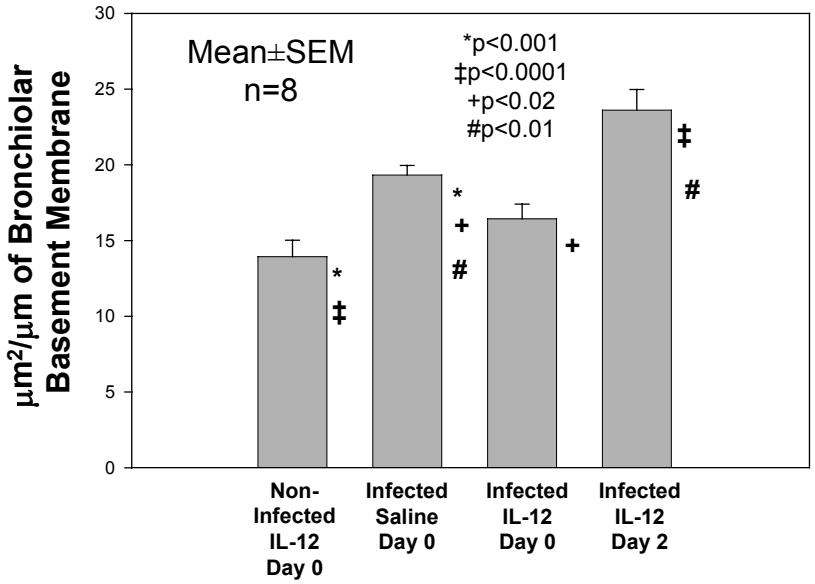

Figure 4-2. Airway morphometric analysis of bronchiolar wall thickness at 14 days after virus inoculation. IL-12 administration at the time of virus inoculation reduced the bronchiolar wall thickness to non-infected control levels ($p<0.02$).

In contrast rats treated on day 2 after inoculation had a 22.3% increase in the bronchiolar wall thickness as compared to virus-inoculated, saline-treated controls ($p<0.01$) (Figure 4-2).

IL-12 Treatment of BN Rats at the Time of Sendai Virus Inoculation Reduces the Number of BrdU Labeled Fibroblasts in the Bronchiolar Walls

Bronchiolar fibrosis and other remodeling events were evaluated by counting bronchiolar mural fibroblasts and epithelial cells labeled with BrdU at 10 and 14 days after virus inoculation (Figure 4-3). Treatment of BN rats with rIL-12 at day 0 significantly reduced the magnitude of increase in mural fibroblast labeling by BrdU. There was decreased mural fibroblast labeling by BrdU by 42% at 10 days after inoculation (n= 8) (Figure 4-4) and 62.5% at 14 days following inoculations (n = 8) (Figure 4-5).

Treatment of BN rats with rIL-12 at day 2 had no effect on virus-induced increases in BrdU labeling of fibroblasts at 10 days after inoculation and increased labeling of fibroblasts by 12.5% at 14 days after inoculation (Figure 4-4). Virus infection resulted in increased labeling of bronchiolar epithelial cells with BrdU. However, rIL-12 treatment had no statistical effect on this virus-induced labeling of epithelial cells.

IL-12 Treatment of BN Rats at the Time of Sendai Virus Inoculation Increases the Pulmonary Expression of IFN-γ

Virus infection increases the amount of IFN-γ mRNA in whole lung tissues of BN rats (n = 4-5) (Figure 4-6). There were significant increases in IFN-γ mRNA in all treatment groups infected with Sendai virus over non-infected controls ($p<0.05$). BN rats treated with IL-12 at day 0 had 4-fold more IFN-γ mRNA in lungs ($p<0.002$) when compared to the saline-treated, virus-inoculated controls. IL-12 treatment at day 2 after virus inoculation decreased the production of IFN-γ mRNA by 4-fold as compared to saline-treated, virus-inoculated controls (Figure 4-6).

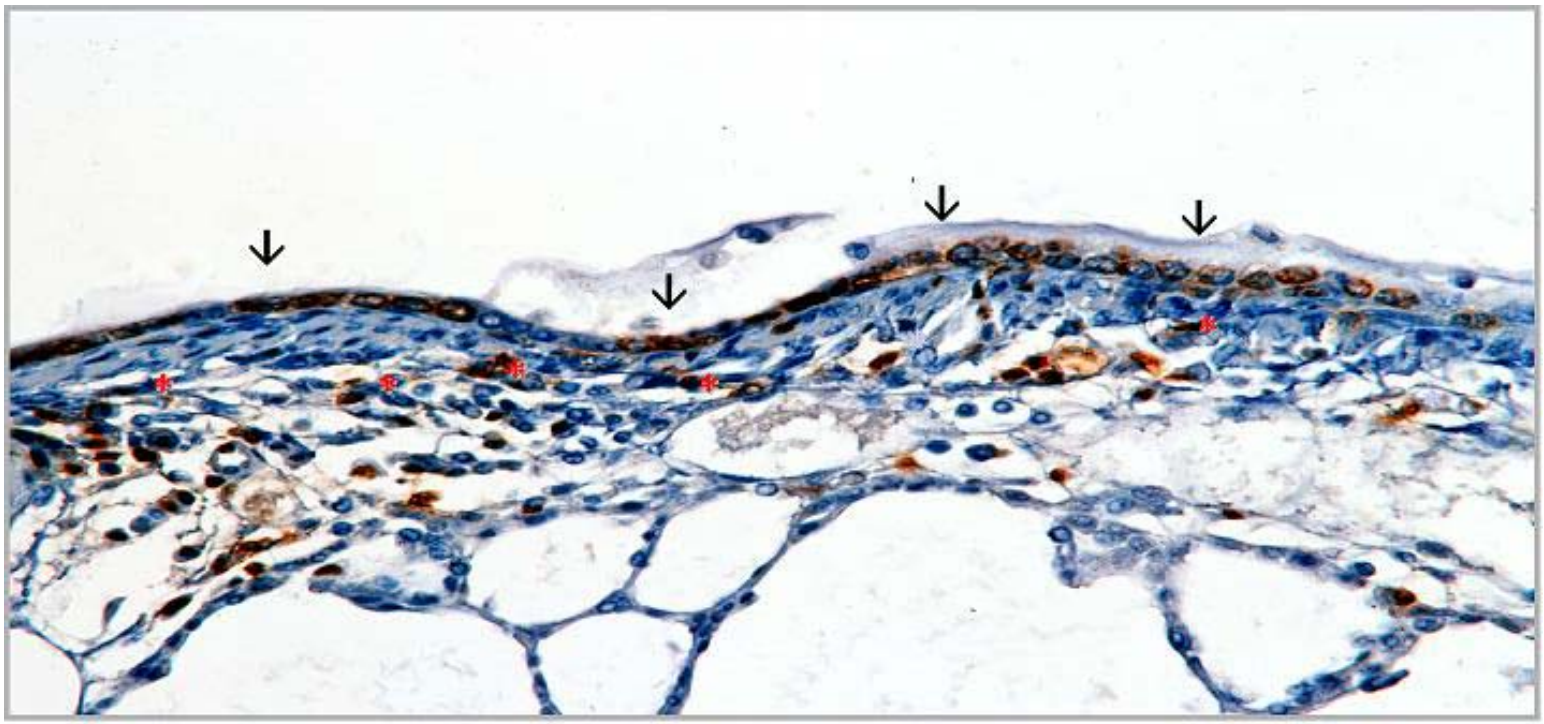

Figure 4-3. BrdU labeled bronchiole of a saline-treated, virus-inoculated BN rat. Labeled bronchiolar epithelium is indicated by arrows/ several labeled fibroblasts are indicated with (*)

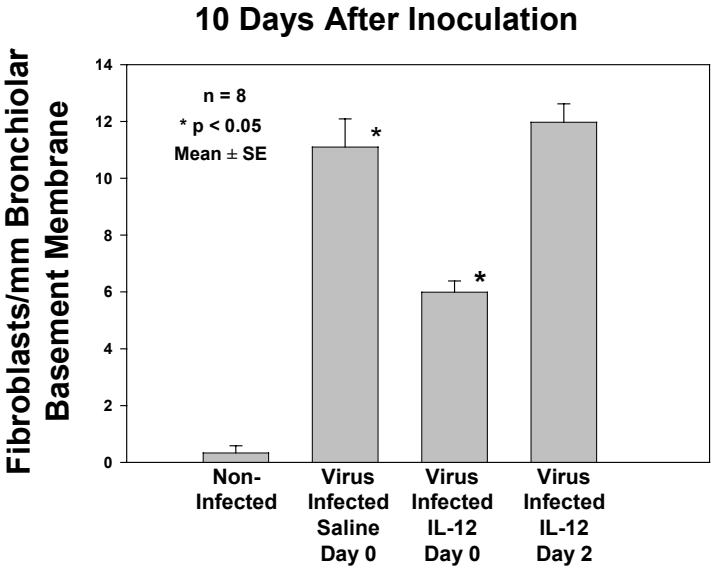

Figure 4-4. Immunohistochemical analysis of fibroblast BrdU labeling at 10 days after Sendai inoculation. BN rats treated at the time of virus inoculation reduced the number of labeled fibroblasts significantly as compared to virus-inoculated, saline-treated controls.

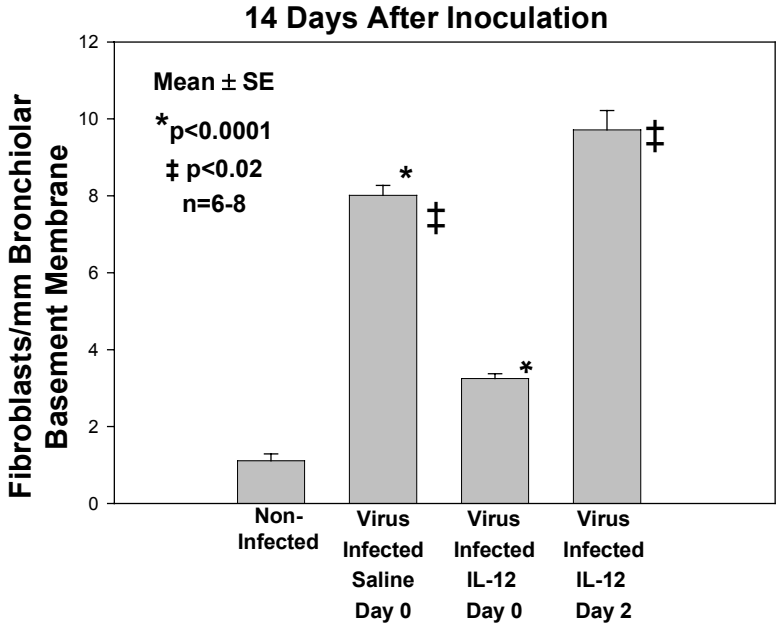

Figure 4-5. Immunohistochemical analysis of fibroblast BrdU labeling at 14 days after Sendai inoculation. The treatment of BN rats with IL-12 at the time of virus infection reduced the number of BrdU labeled fibroblasts significantly as compared to virus-inoculated, saline-treated controls ($p<0.0001$).

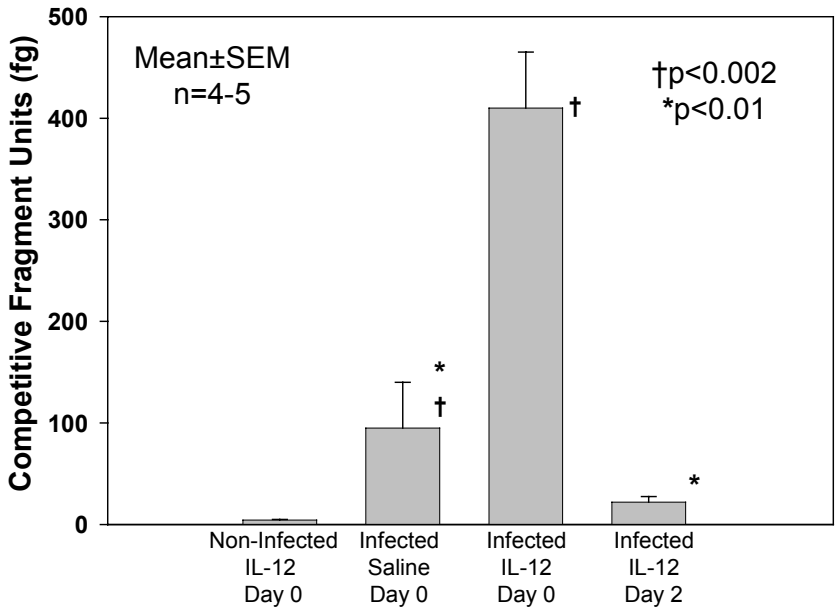

Figure 4-6. Competitive PCR analysis of IFN-γ mRNA in BN rats 3 days after inoculation treated at day 0 or day 2 with IL-12. IFN-γ mRNA levels in rats treated with IL-12 at the time of virus infection increased significantly above the saline-treated, virus-inoculated controls ($p<0.002$). Day 2 treatment decreased the IFN-γ mRNA response to levels significantly below the saline-treated, virus-inoculated controls ($p<0.01$).

There was a significant increase (73.5%) in the level of IFN-γ protein in the total lung tissue of the BN rats (7 days after inoculation) treated at day 0 as compared to the virus-inoculated, saline-treated rats ($p<0.05$) (n = 7-8) (Figure 4-7). Day 2 IL-12 treatment did not increase IFN-γ over the saline-treated rats measured seven days after virus inoculation. A virus-induced increase in IFN-γ protein was detected in all treatment groups at 7 days after inoculation as compared to the non-infected controls.

IL-12 Treatment of BN Rats on Day 2 After Sendai Virus Inoculation Alters the Levels of IL-18 or IL-4 mRNA

The levels of mRNA of the co stimulatory cytokine IL-18 were not increased by IL-12 treatment at any of the time points measured (n = 3-5) (Figure 4-8).

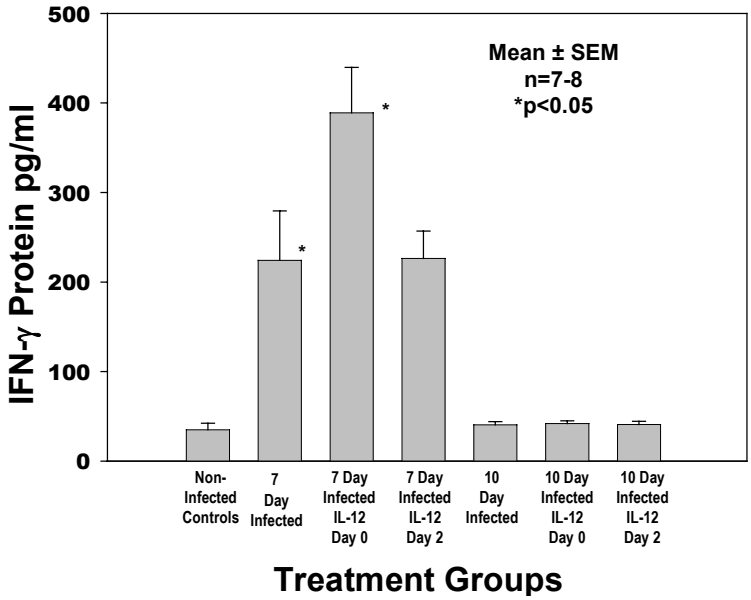

Figure 4-7. ELISA analysis of whole lung homogenates from BN rats at 7 and 10 days after virus inoculation. Day 0 IL-12 treatment induced a significant increase in the levels of IFN-γ as compared to the virus-inoculated, saline treated controls ($p<0.05$).

However, IL-12 treatment at day 2 caused a decrease in the amount of IL-18 mRNA measured at three days after inoculation as compared to day 0 treated animals ($p<0.05$). No significant differences were measured between either of these groups and the non-infected controls.

The treatment of BN rats at the time of virus inoculation did not reduce the levels of IL-4 mRNA at any time points measured after inoculation. However, IL-4 mRNA levels were significantly elevated (3.2-fold increase) in the virus inoculated rats treated at 2 days after inoculation as compared to virus-inoculated, saline-treated controls ($n = 4-6$, $p<0.02$) ($n = 4-6$) (Figure 4-9). Virus infection increased the level of IL-4 mRNA in all treatment groups at 3 days after Sendai virus inoculation ($p<0.05$).

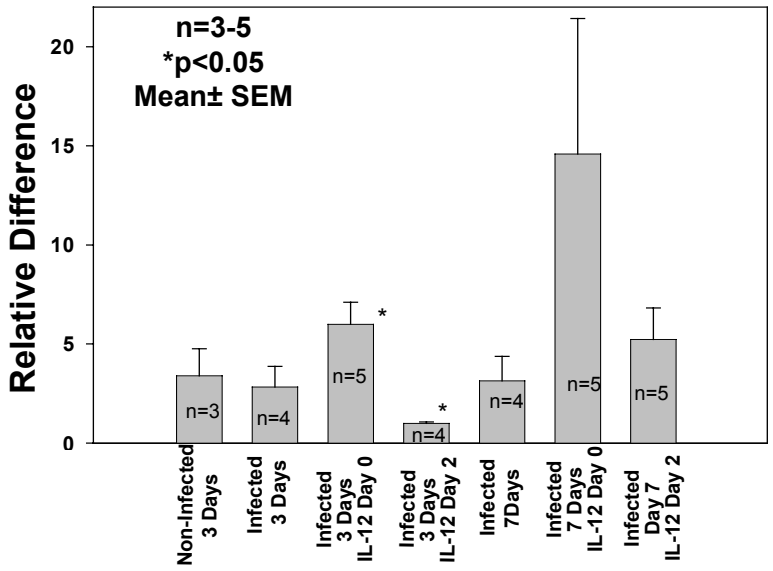

Figure 4-8. Analysis of real-time PCR for the detection of IL-18 mRNA. IL-12 treatment at 2 days after inoculation significantly reduced the expression of IL-18 mRNA as compared to the rats treated the day of virus inoculation.

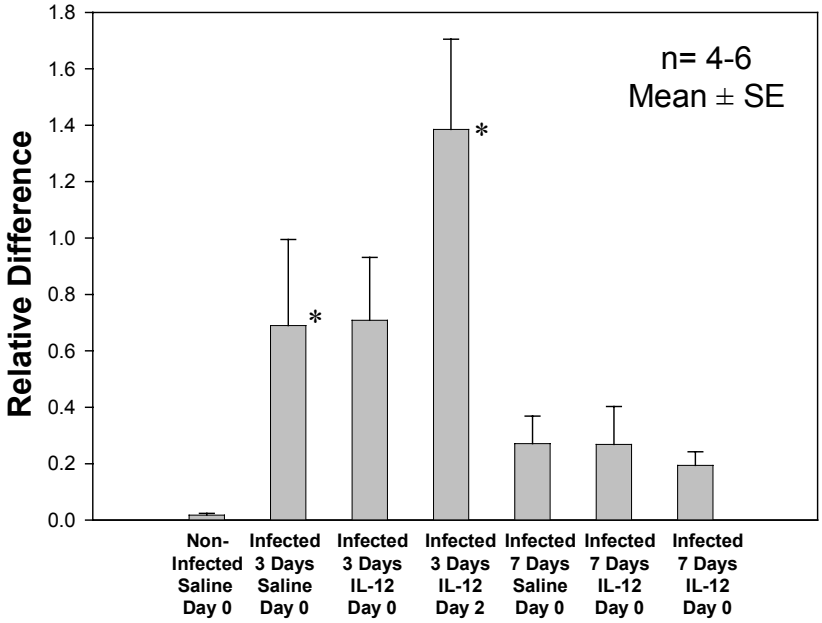

Figure 4-9. Analysis of real-time PCR for the detection of IL-4 mRNA. BN rats treated with IL-12 at day 2 had a significant increase in the level of IL-4 mRNA at three days after inoculation compared to virus-inoculated, saline-treated rats 3 days after inoculation ($p<0.02$). IL-4 mRNA expression significantly increased in all of the virus-infected groups assessed at 3 days after Sendai inoculation ($p<0.05$).

IL-12 Treatment of BN Rats at the Time of Sendai Virus Inoculation Does Not Alter the Respiratory Clearance of Sendai Virus

F344 rats have been shown to clear Sendai virus from their lungs at 7 days after inoculation as compared to virus-susceptible BN rats (115). Viral clearance was not affected in BN rats treated with rIL-12 on day 0 as compared to saline-treated, virus-inoculated controls ($p>0.05$). At seven days after inoculation, the mean value of virus recovered from the saline-treated, virus inoculated controls was 6.19×10^4 PFU/g of lung, and the BN rats treated on day 0 had a mean titer of 7.33×10^4 PFU/g of lung ($n = 8$) (Figure 4-10).

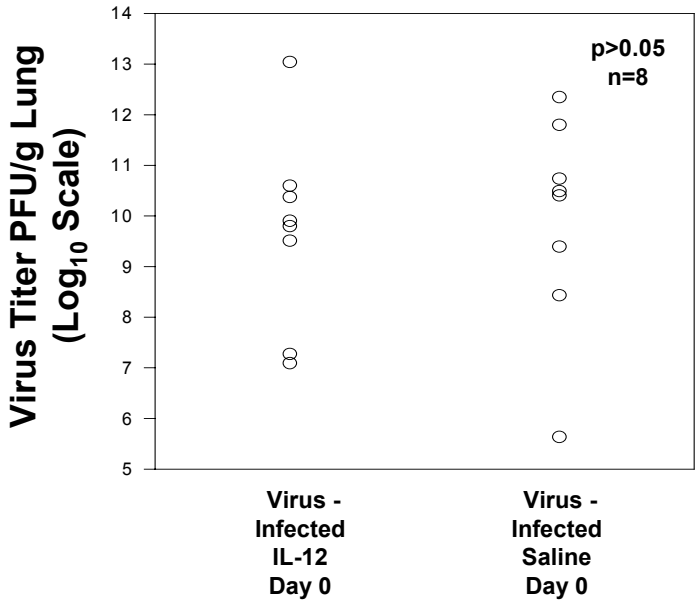

Figure 4-10. Viral titer results from plaque assays at 7 days after inoculation. IL-12 treatment does not increase the rate of viral clearance from the lungs of BN rats after Sendai infection ($P>0.05$).

Discussion

The results indicate early treatment of virus-susceptible BN rats with IL-12 inhibits the development of virus-induced chronic inflammation and bronchiolar fibrosis. IL-12 treatment at the time of Sendai virus inoculation decreased the amount of airway wall

inflammation and fibrosis, decreased the thickness of the bronchiolar walls, and increased the expression of the Th-1 cytokine IFN-γ. This protection is not observed if IL-12 treatment is delayed as little as two days after virus-inoculation. In fact several of the viral sequelae such as airway wall thickening and fibrosis were exacerbated.

Mechanisms by which IL-12 treatment early in the Sendai virus infection may reduce virus-induced persistent bronchiolar inflammation and bronchiolar fibrosis and other remodeling may be direct through IL-12 or indirect through the induction of IFN-γ. IL-12 can act to increase the cytotoxicity and mitogenic activity of T and NK cells, and to inhibit B cell functions while enhancing the conventional, B cell-dependent antibody responses (1-4). IL-12 also has direct stimulatory effects on hematopoietic progenitor cells (2). The main biological effects of IL-12 are attributed to the induction of the cytokines TNF-α, granulocyte-macrophage colony-stimulatory factor, IL-10, IL-2, and, most importantly, the induction and maintenance of IFN-γ (1-4). IL-12 also acts to reduce the levels of IL-4 and the establishment of the Th2 phenotype (2-3). IL-12 treatment did increase the levels of IFN-γ mRNA and protein significantly above the levels seen with viral infection alone (saline-treatment). IL-12 treatment however, did not reduce the levels of IL-4 mRNA at the time points measured in this model. Still, there may be reductions in IL-4 at a later time point due to the prevention of the proliferation and development of Th2 cells (3). The viral titer data does not suggest that IL-12 is directly inhibiting viral replication. This is consistent with previous studies in which IFN-γ treatment reduced bronchiolar inflammation and fibrosis without altering Sendai virus replication in BN rats (119).

The effects of IL-12 treatment in reducing the pulmonary fibrosis observed in BN rats after Sendai virus infection may be mediated by increases in IFN-γ expression. Early IL-12 treatment was associated with increases in total lung IFN-γ mRNA at three days after inoculation and in total lung IFN-γ protein at seven days after inoculation. IFN-γ can induce angiostatic chemokines such as IFN-inducible protein 10 (IP-10), macrophage inflammatory protein-2 (MIP-2), and monokine induced by IFN-γ (MIG). These chemokines have been shown importance in the down-regulation of angiogenesis and fibrosis in a mouse bleomycin-induced pulmonary fibrosis model (132). IL-12 administration to bleomycin-treated CBA/J mice decreased levels of hydroxyproline and increased lung IFN-γ, IP-10, and MIG (138). Additionally, IFN-γ treatment has been shown to attenuate increases of TGF-β_1, procollagen mRNA, and total lung collagen in after bleomycin challenge in mice (132).

The time frame for the protective effects of IL-12 appears to be narrow and only very early after Sendai infection. Administration of IL-12 just 2 days after viral inoculation failed to protect against increased virus-induced bronchiolar wall thickening, bronchiolar airway wall fibroblast proliferation, increased IL-4 mRNA production, and suppressed the expression of total lung IFN-γ mRNA. These results are similar to the responses of exogenous IL-12 treatment seen in other infection models. Exogenous IL-12 administered at times when T-cell responses are known to be effecting clearance of murine lymphocytic choriomeningitis virus caused reduced cytotoxic T cell lytic capabilities, inhibition of virus-induced expansion of CD8+ T cells, and increased production of TNF (136). IL-12 immunotherapy of murine leishmaniasis infection is only effective during the first week of infection. Delayed treatment is ineffective and can

enhance IL-4 production and susceptibility (79). The molecular basis for this loss of IL-12 sensitivity is hypothesized to be due to a disruption in Th-2 cells of the IL-12 dependent activation of the Janus kinases (JAK) and signal transducers and activators of transcription (STAT) intermediates through the IL-12Rβ2 that are preserved in Th-1 or Th-0 cells (2, 71). Early IL-12 treatment however, modifies the course of leishmaniasis by inhibiting the development of Th-2 type responses and promoting the Th-1 cell responses dependent on IFN-γ (79). The dynamics observed in these experimental models may be true in this model, in that once a Th-2 response is already established; it can be difficult to reverse.

The protective effects of IL-12 administration in this model are consistent with effects seen in other studies of infectious and allergic airway disease (Chapter 1). Furthermore, this model also exhibits many of the complications observed in other models regarding the dependence on the timing of IL-12 administration and protective immunomodulatory effects (79, 97-99). These results are consistent with the conclusion that airway dysfunction in childhood asthma may partially be the result of slight differences in the immune cytokine response that control the inflammatory and repair processes to viral disease. Based on these experiments, it may be possible to interrupt the progression of viral injury to chronic airway damage with early immunomodulatory cytokine administration.

CHAPTER 5
GENERAL SUMMARY AND FUTURE DIRECTIONS

The goal of this research was to determine the role of interleukin-12 (IL-12) in the development of resistance to chronic airway disease induced by parainfluenza (Sendai) virus during early life. The hypothesis tested was that F344 rats are more resistant to virus-induced airway damage and fibrosis because they produce high levels of IL-12 early in response to virus that up-regulates Th-1 cytokine responses, antiviral immunity, and reduces airway wall fibrosis. Fulfilling 4 specific objectives tested this hypothesis:

Objective 1

1) To compare the pulmonary IL-12 mRNA and protein responses of virus- resistant F344 and virus-susceptible BN rats following Sendai virus infection.

Real-time PCR revealed that both rat strains have early virus-induced increases in IL-12 p40 mRNA (2-5 days after inoculation). In addition, it was demonstrated that virus-inoculated F344 rats have significantly increased pulmonary IL-12 p40 and IL-12 p35 mRNA at early time points after inoculation as compared to the BN rat strain (0-3 days after inoculation). This is just prior to the increased expression of IFN-γ mRNA observed in F344 during previous studies at 3, 5, and 7 days after Sendai inoculation (117). ELISA of whole lung tissue, revealed that IL-12 protein levels are increased significantly in the F344 at two days following inoculation, and that this increase was not due to over-production of potentially antagonistic IL-12 p40 monomers and dimers (2,13).

Objective 2

2) To determine if F344 rats have greater numbers of pulmonary cells and differing cell types that express IL-12 in response to Sendai virus infection than BN rats.

Protein immunohistochemistry demonstrated that F344 rats have higher numbers of bronchiolar dendritic cells and macrophages expressing IL-12 p40 protein as compared to the virus-inoculated BN rats. Although many attempts were made to establish the location and differential levels of IL-12 p40 mRNA expression by *in situ* hybridization, none of the experimental results were conclusive. Observationally, the IL-12 p40 mRNA message was detected sporadically in the bronchiolar macrophages, dendritic cells, in the lymphocytes of the BALT, and in the airway epithelial layer.

Objective 3

3) To determine if Sendai virus-induced airway damage in BN rats can be reduced by IL-12 treatment early in the virus infection.

The treatment of BN rats with exogenous IL-12 at the time (within 3 hours) of viral inoculation does reduce the chronic sequelae of Sendai virus infection. BN rats treated with IL-12 on the day of inoculation-displayed decreases in bronchiolar inflammation and fibrosis, decreases in airway wall fibroblast proliferation, and increases in IFN-γ expression at various time points after Sendai infection. Viral clearance in BN rats was not affected by the treatment of IL-12 at the time of virus inoculation, and viral clearance after treatment on day two after inoculation was not assessed. In contrast, IL-12 treatment two days after virus inoculation significantly increased airway wall thickness, decreased IFN-γ mRNA expression, and increased the expression of IL-4 mRNA.

Objective 4

4) To compare the airway IL-12 p35 and p40 mRNA responses of virus-resistant F344 and virus-susceptible BN rats following Sendai virus infection (This specific aim is contingent on the results from the second specific aim. If there is differential expression of IL-12 in the dendritic cell or other cells types in the large airways based on the results of the in situ hybridization and immunohistochemistry, then this specific aim will be explored).

When the experiments to determine the differential expression of IL-12 in this model were begun, real-time PCR was in its very early stages. Therefore, I was demonstrating only marginal differences in the amount of IL-12 p40 mRNA expression by dilutional PCR. At the time, it seemed that this was possibly due to the dilution of differences that may be at the airway level being masked by the inclusion of the total lung tissue. Additionally, the in situ hybridization and the immunohistochemistry techniques were very time consuming to resolve. Based on these difficulties, this specific aim was added. Real-time PCR of the tracheal tissue revealed that IL-12 p40 mRNA does increase in both rat strains early after virus inoculation, and is significantly elevated at two days following inoculation in the F344 rat strain. There were no significant alterations in the IL-12 p35 mRNA possibly due to the low levels of tissue used or due to the limits of this procedure at detecting all of the IL-12 p35 isoforms (Chapters 1 and 3).

Conclusions

The results of these studies supports the hypothesis that F344 rats are more resistant to virus-induced airway damage and fibrosis because they produce high levels of IL-12 early in response to virus that up-regulates Th-1 cytokine responses, antiviral immunity, and reduces airway fibrosis. It is concluded that:

1) Virus-resistant F344 rats express higher pulmonary IL-12 gene expression early after Sendai virus infection as compared to virus-susceptible BN rats.

2) Virus-resistant F344 rats have more bronchiolar dendritic cells and macrophages expressing IL-12 than BN rats at early time points after inoculation.

3) Treatment of virus-susceptible BN rats with IL-12 early after Sendai inoculation reduces the severity of airway wall fibrosis and remodeling.

4) IL-12 has a critical role in the immune response to Sendai virus infection in F344 rats.

Future Studies

In previous experiments by P.G. Holt and others, have identified dendritic cells as the principal resident APC of the rat, mouse, and human lung and that these cells form a network throughout the epithelium to alert the immune response to inhaled antigens (134). Furthermore, the examination of these resident cells in BN rats suggests that the resting Th-2 polarity of the resting mucosal immune system may also be a property of the resident dendritic cell population (121, 136). Initiation of the Th-1 immune response seems to require additional signals such as TNF-α expression and/or CD40 ligation from the microenvironment (136).

The results these studies using OX-6 immunohistochemistry indicate that there are more dendritic cells located in the F344 bronchiolar airways at two and three days after Sendai virus inoculation compared to the BN rats. Further studies to map the kinetics of the dendritic cell numbers and turnover in the context of this model are indicated. It may be that the dendritic cell numbers persist longer and in different locations within the airways of F344 rats after Sendai infection than in BN rats. These results also indicate that these cells are expressing differing levels of cytokines at the airway level depending on the rat strain infected. Using techniques such as laser microdissection, real-time PCR, and double-staining immunohistology the important differences may be elucidated at the

dendritic cell network level that influence the susceptibility to Sendai virus infection and possibly asthma.

Another aspect in this model that may affect the susceptibility of the rats in this model to Sendai virus is the expression of the IL-12Rβ2 receptor protein. The expression of the IL-12Rβ2 protein is limited to Th-1 cells and may correlate with IL-12 responsiveness (29,30). In the development of T cells, IL-4 can inhibit the expression of this subunit, thus these cells lose the ability to respond to IL-12 after TCR binding (30). Recently, differential expression of the IL-12Rβ2 subunit between predominately Th-2 responding BN rats and Th-1 responding Lewis rats (139). Within the model, F344 rats may have increased expression of the IL-12Rβ2 protein, therefore not only producing more IL-12 but may be more responsive to its immunologic effects. Preliminary data using IL-12Rβ2 RT-PCR on pulmonary tissue, an increase in the expression of this subunits mRNA message in the F344 rat tissue (Figure 5-1).

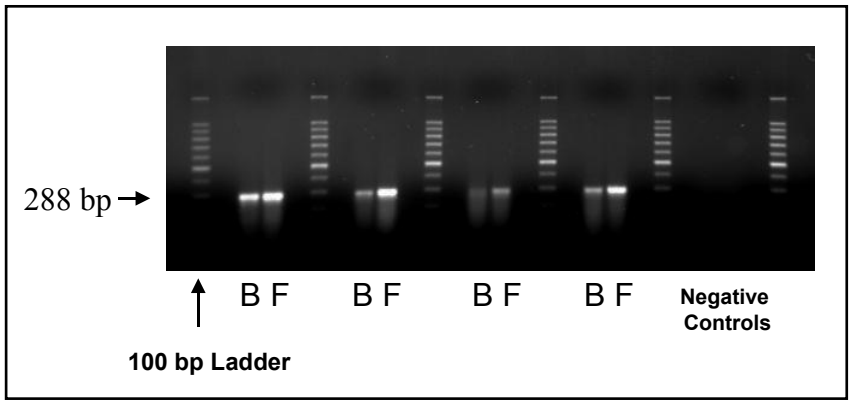

Figure 5-1. RT-PCR of IL-12Rβ2 in BN and F344 rats at non-infected control levels. Observationally, there are brighter bands in the non-infected control F344 rats (F) rats as compared to the BN (B) rat strain at control levels. The band size of IL-12Rβ2 is 288 base pairs (bp) as indicated above.

The differential expression of the IL-12Rβ2 may indicate differences in the ability of the rats in this model to respond to IL-12. This aspect of IL-12 regulation may need to

be addressed in this rat model and in human asthma patients before the potential effectiveness of IL-12 immunomodulation can be fully assessed.

APPENDIX A
PRELIMINARY DATA

Experiment 1: Pulmonary Expression of IL-12 in Sendai Virus-Infected BN and F344 Rats

Dilutional RT-PCR

Preliminary pulmonary mRNA levels were measured in a small number of rats using 10-fold dilutional RT-PCR (Chapter 2). IL-12 p40 mRNA levels were increased over levels in BN rats at two, three, and seven days after inoculation with Sendai virus (Figure A-1).

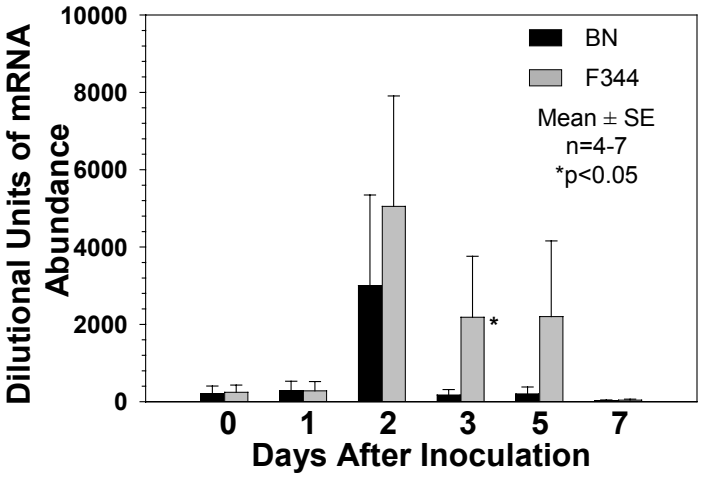

Figure A-1. Dilutional RT-PCR analysis of mRNA from virus-infected BN and F344 rats. F344 rats have increased pulmonary IL-12 p40 mRNA over BN rats at 2, 3, and 7 days post-inoculation. This difference is statistically significant at 3 days after viral inoculation ($p<0.05$).

Lung Lavage Fluid ELISA

Preliminary ELISAs using lung lavage fluid detected very low levels of IL-12 p70 and IL-12 p40 protein with minimal differences between the two strains (Figures A-2, A-

3). Based on the low levels detected, whole lung homogenates were used in further studies to measure the expression of IL-12 protein.

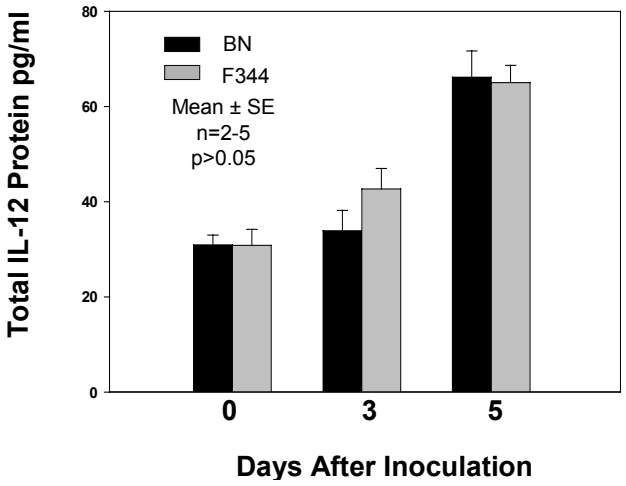

Figure A-2. ELISA analysis of IL-12 p70 protein in concentrated lavage samples from small numbers virus-infected and control rats. The level of IL-12 p70 increases with viral infection, however there are no significant differences between the BN and F344 rat strains.

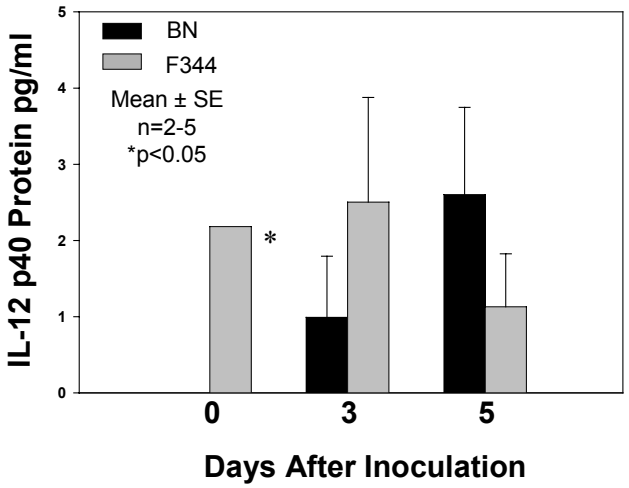

Figure A-3. ELISA analysis of IL-12 p40 protein (homodimers and monomers) in concentrated lavage samples from small numbers virus-infected and control rats. The level of IL-12 p40 is higher at control levels in the F344 rat strain, however extremely low amounts were detected in both strains at all three time points.

Experiment 2:The Effects of Exogenous Interleukin-12 Administration on the Development of Sendai Virus-Induced Airway Disease in BN Rats

IFN-γ protein was evaluated in concentrated lung lavage samples from saline-treated, virus-infected BN rats, control BN rats, and IL-12-treated, virus-infected BN rats. Preliminary analysis using concentrated lung lavage fluid samples detected an increase in levels of IFN-γ after viral infection, but no significant differences in the IL-12 treatment groups (Figure A-4). This analysis was repeated using the supernatants from whole lung homogenates.

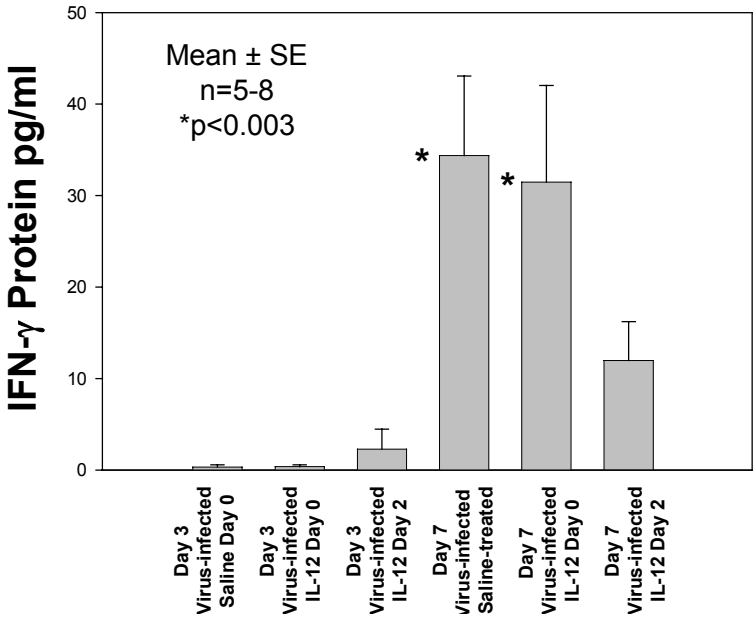

Figure A-4. ELISA analysis of IFN-γ in concentrated lavage samples. No significant differences between the treatment groups in the levels of protein detected ($p>0.05$). However, IFN-γ protein levels increased at seven days after infection above the previous (three day) levels in the saline-treated and IL-12 (day 0) treated animals, but not in the IL-12 (day 2) treated BN rats ($P<0.003$), ANOVA.

APPENDIX B
IL-12 DOSAGE FOR TREATMENT TRIAL

Experimental Design

The purpose of this trial was to determine the appropriate dose of recombinant IL-12 (rIL-12) (mouse rIL-12, Biosource International, Camarillo, CA) to administer to BN rats to achieve a biological effect. A starting point for dosage and the route of administration were based on those previously used by others in IL-12 treatment experiments (140-142). We chose an early time point after Sendai inoculation to give IL-12 due to its probable role in the acute immune response (Chapter 1). The following table describes the details of the experimental design used in this dosage trial (Table B-1).

Table B-1. Experimental Design for IL-12 Dosage Trial

IL-12 Dose	Days of Treatment	Day of Necropsy ± Virus	Number of BN Rats
0 μg (Saline)	Day 0	Infected Day 7	4
3 μg	Day 0	Infected Day 7	1
6 μg	Day 0	Infected Day 7	1
12 μg	Day 0	Infected Day 7	1
3 μg	Day 0 and Day 3	Infected Day 7	1
3 μg	Day 0	Non-infected Day 7	1
0 μg	Day 0	Non-infected Day 7	3

The rats were injected intraperitoneally (IP) with IL-12 or an equal volume of saline with a 20 gauge, 1-inch needle at the time point after aerosolization that was specified in Table B-1. Non-infected rats were housed separately from the infected rats regardless of treatment group. Lung lavages for airway inflammatory cell assessment were performed through intratracheal cannulation with phosphate buffered saline (PBS). Lungs were

removed and fixed for 2 hours by tracheal perfusion with 4% paraformaldehyde-PBS (pH = 7.4) and embedded in paraffin for histologic evaluation of the pneumonia.

The percentage of macrophages, neutrophils, lymphocytes, and epithelial cells were determined from the total number of cells recovered in each lavage sample. These percentages were compared to determine if there was a reduction in certain cell populations during with IL-12 administration. Areas of pneumonia were measured by projecting images of the sections with a photographic enlarger on a digitizing tablet interfaced with a microcomputer and a morphometric program (Bioquant II, R and M Biometrics, Nashville, TN). Areas of pneumonia were detected and outlined (areas chosen based on 5 or more inflammatory cells/alveolus and thickening of interalveolar septa). The indices of pneumonia were calculated by dividing the sum of the areas of exudative pneumonia by the area of the section.

Results and Conclusions

In the examination of the lung lavage cell counts it was noted that there was no statistical significance in the percentage of cells and cell types counted in all of the IL-12-treated, virus-infected animals (Table 3-2). Therefore, these animals were grouped together as the IL-12 treatment group. The percent of cells and distribution of the cell types in the IL-12 treatment groups as compared to the other treatment groups is shown in Figure B-1.

The trends noted in the IL-12 treatment groups were a reduction in the percent of macrophages and increases in the numbers of neutrophils and lymphocytes. There were no statistical differences detected in comparing the groups by percentages or raw data analysis. The power of the test due to the low numbers of rats was too low to make a statistical assessment.

Table B-2. Percentages of Inflammatory Cells in the IL-12 Treated BN Rats

IL-12 Treatment Dose	CHAPTER 1% of Macrophages	% of Neutrophils	% of Lymphocytes	% of Epithelial Cells	Total Number Cells
3 μg	60.5	25	14.5	0	10×10^5
6 μg	64.5	19	16	0.5	28×10^5
12 μg	78	13	8	1	15×10^5
3 μg at 0 and 3 days	82.5	9.5	7.5	0.5	10×10^5

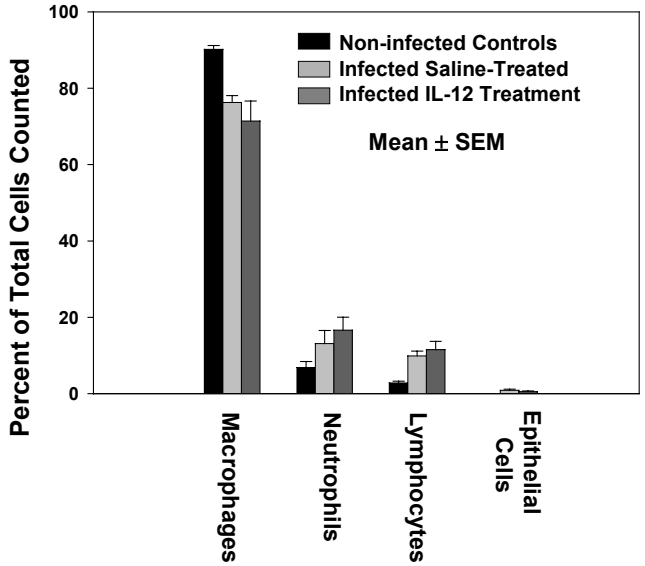

Figure B-1. The percentage of cells identified as macrophages, neutrophils, lymphocytes, and epithelial cells in all treatment groups.

In the IL-12 treatment group, pneumonia index was decreased as compared to the saline-treated, virus-infected rats ($p<0.05$ using student's t-test) (Figure B-2). Again, due to the low numbers of rats used, the power of the test was too low to make a statistical assessment using ANOVA.

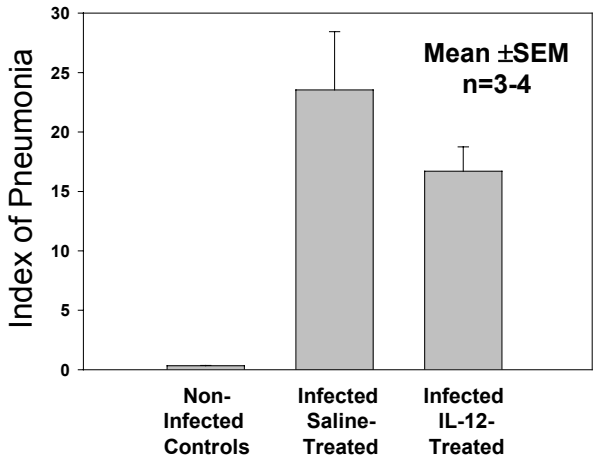

Figure B-2. Pneumonia indices of BN rats in all treatment groups. The difference between the saline-treated and IL-12-treated groups was statistically significant using the student's t-test ($p<0.05$).

Based on the trends seen with IL-12 treatment, the lowest dose that achieved a biological effect (reduction in inflammatory cells and reduction in pneumonia index) was chosen for the IL-12 treatment experiments in BN rats. The dose of 3 μg (rIL-12 or an equivalent volume of saline) was to be given IP three hours after inoculation with Sendai virus or two days after inoculation with Sendai virus.

APPENDIX C
IN SITU HYBRIDIZATION FOR INTERLEUKIN-12

Protocol For *In Situ* Hybridization

For *in situ* hybridization, the rat IL-12 p40 PCR product from published primer sequences was subcloned into pGEM®-T Easy (Promega, Madison, WI) as a template for the production of digoxigenin-labeled RNA probes (125,142). Sense and antisense probes were made using the Genius System (Genius 4 Kit, Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. The sense probe was made by linearizing with Sal I/Pst I and incubating with T7 polymerase. Similarly, the antisense probe was made by linearizing with Sph I and incubating with SP6 polymerase.

Lung and trachea sections were deparaffinized in xylene, rehydrated through a graded series of ethanols, re-fixed in 4% paraformaldehyde, and denatured in 0.2 N HCl. Prehybridization treatment was as follows: incubation in 10 μg/ml proteinase K (30 minutes, 37°C), acetylation in acetic anhydride, and dehydration in ascending grades of ethanol with a chloroform rinse. The probes were added to the hybridization solution (62.5% formamide, 12.5% dextran sulfate, 0.3 M NaCl, 0.025X Denhart's solution [Sigma, St. Louis, MO], 12.5mM Tris-HCl pH 8.0, 1.25 mM EDTA pH 8.0) and denatured for 10 minutes at 65°C. Approximately 100ng of probe was added to each slide, coverslips were applied, and the slides were placed in a 65°C waterbath for 10 minutes. The slides were hybridized overnight at 37°C in a humidified chamber. The slides were RNase treated to remove any unbound probe and placed in a series of

decreasing stringency SSC washes at 37°C. The sections were blocked in 2% normal sheep serum (1 hour, room temperature) and incubated with an alkaline phosphatase-conjugated antibody to digoxigenin (Roche Diagnostics, Indianapolis, IN). Signal was detected with the colorimetric method using the 5-Bromo-4-chloro-3-indolylphosphate/Nitro-blue tetrazolium (BCIP/NBT) reaction (2-6 hours, room temperature, dark) (Roche Diagnostics, Indianapolis, IN). Specificity of the antisense probe was determined by comparison with the sections hybridized with the sense probe.

Problems Solving For Difficulties With In Situ Hybridization

After the probes were constructed several attempts were made using sections from both rat strains at two days after Sendai virus inoculation. No specific probe binding was observed with hybridization of the sense or antisense probes. Several more attempts were made using sections with more observable inflammation, but the results were similar. Northern blot was attempted to verify the specificity of the antisense IL-12 p40 probe.

No binding was seen in the chemiluminescent blot in the first Northern blot experiment, so the plasmid with the PCR IL-12 p40 insert was sequenced to determine if the insert was in the correct orientation. The insert was in the correct orientation. Next, an agarose gel was run to compare the size of both of the probes with the 100 base pair ladder. If the polymerases (T7 and SP6) were accurate, the probes should measure to the specific size of the insert. However, polymerases are not always accurate and the enzymes may have formed a hairpin loop, thus making the probes bind to themselves and unable to bind to the sections. The antisense probe was much larger than the size of the insert, so the plasmid was redigested with Nco I (antisense) and Sal I (sense) restriction enzymes.

The sizes of the inserts were correct using the new restriction sites and these new probes were labeled. Several attempts were made at running the *in situ* hybridization protocol, however no specific labeling was observed. Northern analysis was performed to verify the binding of the antisense probe. A faint band was observed using the IL-12 p40 antisense probe that was comparable to the β-actin band used as a positive control for the northern analysis.

At this juncture, the probes were reconstructed using the second set of restriction enzymes and relabeled with digoxigenin. Seven more attempts were made to get the probes to specifically bind varying hybridization temperatures, fixing conditions, the stringency of the washes, and probe concentrations. In the final attempt, sporadic binding of the antisense probe was observed, but there is no confidence in the accuracy of this assay with this set of probes.

Observations of In Situ Hybridization Experiments

The detection of the IL-12 p40 mRNA by *in situ* hybridization was sporadic and difficult to interpret. In the slides observed, the antisense probe intermittently bound to inflammatory cells (macrophages, dendritic cells, and lymphocytes) in the bronchiolar and tracheal walls including in the bronchiole-associated-lymphoid-tissue (BALT), but there was also a very high level of nonspecific background observed (Figure C-1 and C-2). Staining was also observed occasionally in the bronchiolar epithelium.

The sense probe bound nonspecifically to all structures on each section observed (Figure C-3). Based on these observations, no conclusions can be drawn from this assay about the location or quantity of the IL-12 p40 mRNA message in the F344 or BN rats after Sendai virus infection.

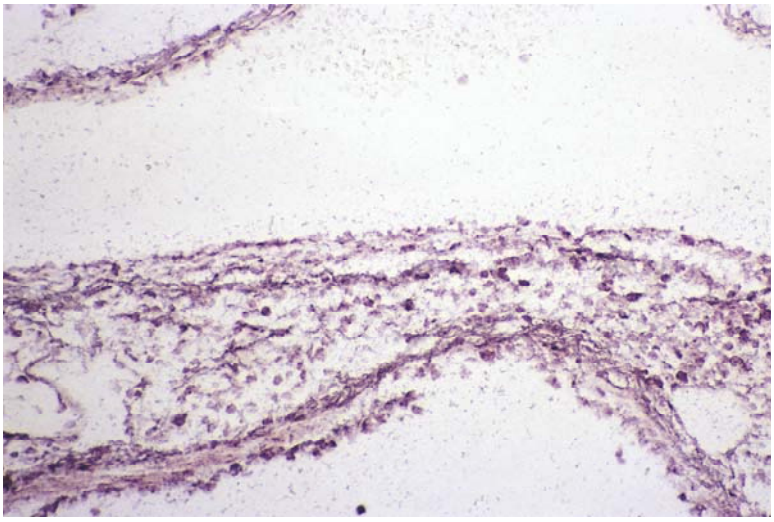

Figure C-1. Anti-Sense IL-12 p40 mRNA *In Situ* Hybridization of bronchiole wall in a F344 rat at 5 days after Sendai inoculation (54X Magnification). Sporadic binding of inflammatory cells is seen with in the wall. The level of nonspecific binding is very high.

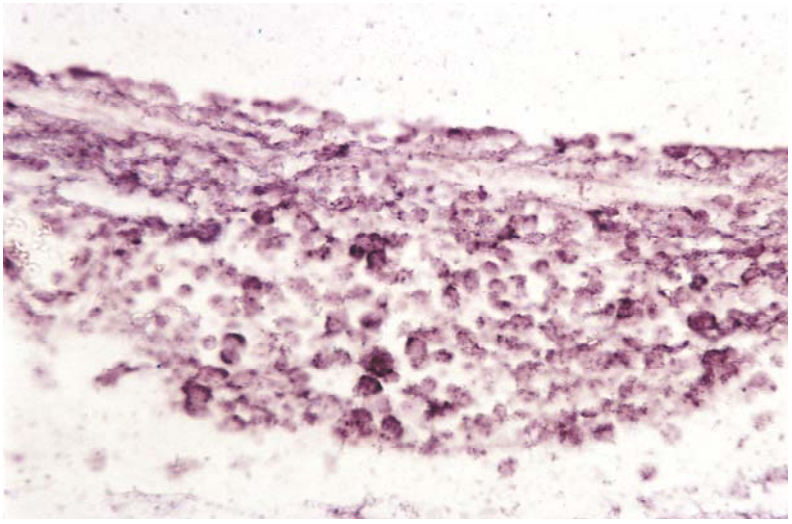

Figure C-2. Anti-Sense IL-12 p40 mRNA *In Situ* Hybridization of inflammatory aggregate in the bronchiole wall of an F344 rat at 3 days after inoculation (108X Magnification). Sporadic binding of inflammatory cell nuclei is shown in this section with obvious nonspecific background.

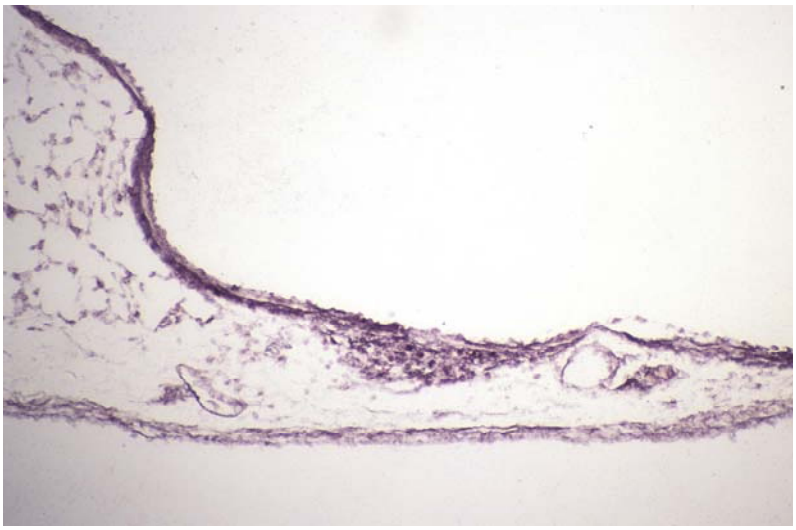

Figure C-3. Sense IL-12 p40 mRNA *In Situ* Hybridization in the airway of a F344 rat 5 days after inoculation (54X Magnification). Extremely high levels of nonspecific binding are seen in this section

LIST OF REFERENCES

1. Stern AS, Magram J, Presky DH. Interleukin-12, an integral cytokine in the immune response. 1996. Life Sciences. 58:639-654.

2. Trinchieri G. Interleukin-12: A cytokine at the interface of inflammation and immunity. 1998. Adv. Immunol. 70:83-243.

3. Park AY and Scott P. IL-12: Keeping cell-mediated immunity alive. 2001. Scand. J. Immunol. 53:529-532.

4. Gately MK, Renzetti LM, Magram J, Stern AS, Adorini L, Gubler U, Presky DH. The interleukin-12/interleukin-12 receptor system: Role in the normal and pathologic immune responses. 1998. Annu. Rev. Immunol. 16:495-521.

5. Schoenhaut DS, Chua AO, Wolitzky AG, Quinn PM, Dwyer CM, McComas W, Familletti PC, Gately MK, Gubler U. Cloning and expression of murine IL-12. 1992. J. Immunol. 148:3433-3440.

6. Seiburth D, Jabs EW, Warrington JA, Li X, Lasota J, LaForgia S, Kelleher K, Huebner K, Wasmuth JJ, Wolf SF. Assignment of NKSF/IL-12 a unique cytokine composed of two unrelated subunits to chromosomes 3 and 5. 1992. Genomics. 14:59-62.

7. Schweitzer PA, Noben-Trauth N, Pelsue SC, Johnson KR, Wolf SF, Shultz LD. Genetic mapping of the IL-12 alpha chain gene (IL-12α) on mouse chromosome 3. 1996. Mammal Genome. 7:394-395.

8. Merberg DM, Wolf SF, Clark SC. Sequence similarity between NKSF and the IL-6/G-CSF family. 1992. Immunol. Today. 13:77-78.

9. Gearing DP, and Cosman D. Homology of the p40 subunit of natural killer cell stimulatory factor (NKSF) with the extracellular domain of the interleukin-6 receptor. 1991. Cell. 66:9-10.

10. Bette M, Jin SC, Schafer MKH, Weihe E, Rude E, Fleischer B. Differential expression of mRNA encoding interleukin-12 p35 and p40 subunits in situ. 1994. Eur. J. Immunol. 15:2435-2440.

11. Trinchieri G and Scott P. Interleukin-12: Basic principles and clinical applications. 1999. Curr. Top. Microbiol. Immunol. 238:12-26.

12. Yoshimoto T, Kojima K, Funakoshi T, Endo Y, Fujita T, Nariuchi H. Molecular cloning and characterization of murine IL-12 genes. 1996. J. Immunol. 156:1082-1088.

13. Grumont R, Hocherin H, O'Keffe M, Gugasyun R, White C, Caminschi I, Cook W, Gerondakis S. C Rel regulates interleukin-12 p70 expression in CD8+ dendritic cells by specifically inducing p35 transcription. 2001. J. Exp. Med. 194:1021-1031.

14. Kollet J, Witek C, Gentry JD, Liu X, Schwartzbach SD, Petro TM. Deletional analysis of the murine IL-12 p35 promoter comparing IFN-γ and lipopolysaccharide stimulation. 2001. J. Immunol. 167: 5653-5663.

15. D'Andrea A, Rengaraju M, Valiante NM, Chehimi J, Kubin M, Aste-Amezaga M, Chan SH, Kobayashi M, Young D, Nickbarg E, Chizzonite R, Wolf SF, Trinchieri G. Production of natural killer cell stimulatory factor (NKSF/IL-12) by peripheral blood mononuclear cells. 1992. J. Exp. Med. 176:1387-1398.

16. Ling P, Gately MK, Gubler U, Stern A, Lin P, Hollfelder K, Su C, Pan YC, Hakimi J. Human IL-12 p40 homodimer binds to the IL-12 receptor but does not mediate biologic activity. 1995. J. Immunol. 154:116-127.

17. Walter MJ, Kajiwara N, Karanja P, Castro M, and Holtzman MJ. Interleukin 12 p40 production by barrier epithelial cells during airway inflammation. 2001. J. Exp. Med. 193:339-351.

18. Ma X, Neurath M, Gri G, Trinchieri G. Identification and characterization of a novel ets-related nuclear complex implicated in the activation of the human IL-12 p40 gene promoter. 1997. J. Biol. Chem. 272:10389-10401.

19. Murphy TL, Cleveland MG, Kulega P, Magram J, Murphy KM. Regulation of IL-12p40 expression through an NF-κB half-site. 1995. Mol. Cell. Biol. 15: 5258-5267.

20. Zhu C, Gagnidze K, Gemberling JHM, Plevy SE. Characterization of an activation protein-1-binding site in the murine interleukin-12 p40 promoter. 2001. J. Biol. Chem. 276:18519-18028.

21. Gri G, Savio D, Trinchieri G, and Ma X. Synergistic regulation of the human interleukin-12 p40 promoter by NF-κB and Ets transcription factors in Epstein-Barr virus-transformed B cells and macrophages. 1998. J. Biol. Chem. 273:6431-6438.

22. Plevy SE, Gemberling JHM, Sang H, Dorner AJ, Smale ST. Multiple control elements mediate activation of the murine and human interleukin-12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. 1997. Mol. Cell. Biol. 17:4572-4588.

23. Becker C, Stefan W, Ma X, Blessing M, Galle PR, Neurath MF. Regulation of the IL-12 p40 promoter activity in primary human monocytes: roles of NF-κB, CCAAT/enhancer-binding protein β, and PU.1 and identification of a novel repressor element (GA-12) that responds to IL-4 and prostaglandin E_2. 2001. J. Immunol. 167:2608-2618.

24. Babik JM, Adams E, Tone Y, Fairchild PJ, Tone M, Waldmann H. Expression of the murine IL-12 is regulated by translational control of the p35 subunit. 1999. J. Immunol. 162:4069-4078.

25. Tone Y, Thompson SAJ, Babik JM, Nolan KF, Tone M, Raven C, Waldman H. Structure and chromosomal location of the mouse interleukin-12 p35 and p40 subunit genes. 1996. Eur. J. Immunol. 26:1222.

26. Presky DH, Yang H, Minetti LJ, Chua AO, Nabavi N, Wu CY, Gately MK, Gubler U. A functional interleukin-12 receptor complex is composed of two β type cytokine receptor subunits. 1996. Proc. Natl. Acad. Sci. USA. 93:14002-14007.

27. Wu CY, Wang X, Gadina M, O'Shea JJ, Presky DH, Magram J. IL-12 receptor β2 (IL-12Rβ2)-deficient mice are defective in IL-12-mediated signaling despite the presence of high affinity IL-12 binding sites. 2000. J. Immunol. 165:6221-6228

28. Zou J, Presky DH, Wu CY, Gubler U. Differential associations between the cytoplasmic regions of the interleukin-12 receptor subunits β1 and β2 and JAK kinases. 1997. J. Biol. Chem. 272:6073-6077.

29. Rogge L, Barberis-Maino L, Biffi M, Passini N, Presky DH, Gubler U. Selective expression of an interleukin-12 receptor component by human T helper 1 cells. 1997. J. Exp. Med. 185:825-831.

30. Szabo SJ, Dighe AS, Gubler U, Murphy KM. Regulation of the interleukin (IL)-12Rβ2 subunit expression in developing T helper 1 (Th1) and Th2 cells. 1997. J. Exp. Med. 185:817-824.

31. Wu CY, Warrier RR, Wang X, Presky DH, Gately MK. Regulation of interleukin-12 receptorβ1 chain expression and interleukin-12 binding by human peripheral blood mononuclear cells. 1997. Eur. J. Immunol. 27:147-154.

32. Bacon CM, McVicar DW, Ortaldo JR, Rees RC, O'Shea JJ, Johnston JA. Interleukin-12 (IL-12) induces tyrosine phosphorylation of JAK2 and TYK2: differential use of Janus family tyrosine kinases by IL-2 and IL-12. 1995. J. Exp. Med. 181:399-404.

33. Jacobson NG, Szabo SJ, Weber-Nordt RM, Zhong Z, Schreiber RD, Darnell JE Jr., Murphy KM. Interleukin-12 signaling in T helper type 1 (Th1) cells involves tyrosine phosphorylation of signal transducer and activator of transcription (Stat) 3 and Stat 4. 1995. J. Exp. Med. 181:1755-1762.

34. Bacon CM, Pertricoin EF III, Ortaldo JR, Rees RC, Larner AC, Johnston HA, O'Shea JJ. IL-12 induces tyrosine phosphorylation and activation of STAT4 in human lymphocytes. 1995. Proc. Natl. Acad. Sci. USA. 92:7307-7311.

35. Yamamoto K, Quelle FW, Thierfelder WE, Kreider BL, Gilbert DS, Jenkins NA, Copeland NG, Silvennoinen O, Ihle JN. Stat4, a novel gamma interferon activation site-binding protein expressed in early myeloid differentiation. 1994. Mol. Cell. Biol. 14:4342-4349.

36. Xu X, Sun YL, Hoey T. Cooperative DNA binding and sequence-selective recognition conferred by the STAT amino-terminal domain. 1996. Science. 273:794-797.

37. Stern AS, Gubler U, Presky DH, Magram J. Structural and functional aspects of the IL-12 receptor complex. 1997. Chem. Immunol. 68:23-37.

38. Frucht DM, Aringer M, Galon J, Danning C, Brown M, Fan S, Centola M, Wu CY, Yamada N, Gabalawy HE, O'Shea JJ. Stat4 is expressed in activated peripheral blood monocytes, dendritic cells, and macrophages at sites of Th1-mediated inflammation. 2000. J. Immunol. 164:4659-4664.

39. Thierfelder WE, Van Duersen JM, Yamamoto K, Tripp RA, Sarawar SR, Carson RT, Sangster MY, Vingali DAA, Doherty PC, Grosveld GC, Ihle JN. Requirement for Stat 4 in interleukin-12-mediated responses of natural killer and T cells. 1996. Nature. 382:171-174.

40. Ouyang W, Jacobson NG, Bhattacharya D, Gorham JD, Fenoglio D, Sha WC, Murphy TL, Murphy KM. The Ets transcription factor ERM is Th1-specific and induced by IL-12 through a Stat4-dependent pathway. 1999. Proc. Natl. Acad. Sci. USA. 96:3888-3893.

41. Sato Y, Roman M, Tighe H, Lee D, Corr M, Nguyen M, Silverman GJ, Lotz M, Carson DA, and Raz E. Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. 1996. Science. 273:352-354.

42. Hodge-Dufour J, Noble PW, Horton MR, Bao C, Wysocka M, Burdick MD, Strieter RM, Trinchieri F, and Puré E. Induction of IL-12 and chemokines by hyaluronan requires adhesion-dependent priming of resident but not elicited macrophages. 1997. J. Immunol. 159:2492-2500.

43. Maruo S, Oh-Hora M, Ahn H, Ono S, Wysocka M, Kaneko Y, Yagita H, Okamura K, Kikutani H, Kishimoto T, Kobayashi M, Hamaoka T, Trinchieri G, Fujiwara H. B Cells regulate CD40 ligand-induced IL-12 production in antigen-presenting cells (APC) during T cell/APC interactions. 1997. J. Immunol. 158:120-126.

44. Shu U, Kiniwa M, Wu CY, Maliszewski C, Vezzio N, Hakimi J, Gately M, Delespessse G. Activated T cell induce interleukin-12 production by monocytes via CD40/CD40 ligand interaction. 1995. Eur. J. Immunol. 25:1125-1128.

45. Kennedy, MK, Picha KS, Fanslow WC, Grabstein KH, Alderson MR, Clifford KN, Chin WA, and Mohler KM. CD40/CD40 ligand interactions are required for T cell-dependent production of interleukin-12 by mouse macrophages. 1996. Eur. J. Immunol. 26:370-378.

46. Peng X, Kasran A, Warmerdam PA, de Boer M, Ceuppens JL. Accessory signaling by CD40 for T cell activation: Induction of Th1 and Th2 cytokines and synergy with interleukin-12 for interferon-gamma production. 1996. Eur. J. Immunol. 26:1621-1627.

47. Schulz O, Edwards AD, Schito M, Aliberti J, Manickasingham S, Sher A, Reis e Sousa C. CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal. 2000. Immunity. 13:453-462.

48. Cella M, Scheidegger D, Plamer-Lehmann K, Lane P, Lanzavecchia A and Alber G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. 1996. J. Exp. Med. 184:747-752.

49. Castella M, Meda L, Gasperini S, D'Andrea A, Ma X, Trinchieri G. Interleukin-12 by human polymorphonuclear leukocytes. 1995. Eur. J. Immunol. 25:1-5.

50. Ma X, Chow JM, Carra G, Gerosa F, Wolf SF, Dzialo R, Trinchieri G, Romagnani S. The Interleukin-12 p40 gene promoter is primed by interferon-γ in monocytic cells. 1996. J. Exp. Med. 183:147-157.

51. Flesch IEA, Hell JH, Haung S, Aguet MR, Rothe J Bluethmann H, Kaufman SHE. Early IL-12 production by macrophages in response to mycobacterial infection depends on interferon-γ and tumor necrosis factor-α. 1995. J. Exp. Med. 181:1615-1621.

52. Kubin M, Chow JM and Trinchieri G. Differential regulation of interleukin-12 (IL-12), tumor necrosis factor-α, and IL-1β production in human myeloid leukemia cell lines and peripheral blood mononuclear cells. 1994. Blood. 83:1847-1855.

53. Ye J, Ortaldo JR, Conlon K, Winkler-Pickett R, Young HA. Cellular and molecular mechanisms of IFN-γ production induced by IL-2 and IL-12 in a human NK cell line. 1995. J. Leuk. Biol. 58:225-233.

54. Naume B, Gately MK, Desai BB, Sundan A, Espevik T. Synergistic effects of interleukin-4 and interleukin-12 on NK cell proliferation. 1993. Cytokine. 5:38-46.

55. Fehniger TA, Shah MH, Turner MJ, VanDeusen JB, Whitman SP, Cooper MA, Suzuki K, Wechser M, Goodsaid F, Caliguri MA. Differential cytokine and chemokines gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: Implications for the immune response. 1999. J. Immunol. 162:4511-4520.

56. Munder M, Mallo M, Eichmann K, Modolell M. Murine macrophages secrete interferon γ upon combined stimulation with interleukin (IL)-12 and IL-18: a novel pathway of autocrine macrophage activation. 1998. J. Exp. Med. 187:2103-2108.

57. Fukao T. Matsuda S, Koyasu S. Synergistic effects of IL-4 and IL-18 on IL-12-dependent IFN-γ production by dendritic cells. 2000. J. Immunol. 164:64-71.

58. D' Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M. Interleukin-10 (IL-10) inhibits human lymphocyte interferon-γ production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. 1993. J. Exp. Med. 178:1041-1048.

59. De. Smedt T, Van Mechelen M, De Becker G, Urbain J, Leo O, Moser M. Effect of interleukin-10 on dendritic cell maturation and function. 1997. Eur. J. Immunol. 27:1229-1235.

60. Buelens C, Verhasselt V, DeGroote D, Thielemans K, Goldman M, Willems F. Interleukin-10 prevents the generation of dendritic cells from human peripheral blood mononuclear cells cultured with interleukin-4 and granulocyte/moncyte-colony stimulating factor. 1997. Eur. J. Immunol. 27:756-762

61. Meyarrd L, Hovenkamp E, Otto SA, Miedema F. IL-12-Induced IL-10 production by human T cells as a negative feedback for IL-12-induced immune responses. 1996. J. Immunol. 156:2776-2782.

62. D'Andrea A, Ma X, Aste-Amezaga M, Paganin C, Trinchieri G. Stimulatory and inhibitory effects of IL-4 and IL-13 on production by human peripheral blood mononuclear cells: Priming for IL-12 and TNF-α production. 1995. J. Exp. Med. 181:537-546.

63. Du C, Sriram S. Mechanism of inhibition of LPS-induced IL-12p40 production by IL-10 and TGF-β in ANA-1 cells. 1998. J. Leukoc. Biol. 64:92-97.

64. Takenaka H, Maruo S, Yamamoto N, Wysocka M, Ono S, Kobayashi M, Yagita H, Okumura K, Hamaoka T, Trinchieri G, Fujiwara H. Regulation of T cell-dependent and –independent IL-12 production by the three Th2-type cytokines IL-10, IL-6, and IL-4. 1997. J. Leukoc. Biol. 61:80-87.

65. Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T cell stimulatory capacity: T-T help via APC activation. 1996. J. Exp. Med. 184:747-752.

66. Heufler C, Koch F, Stanzl U, Topar G, Wysocka M, Trinchieri G, Enk A, Steinman RM, Romani N, Schuler G. Interleukin-12 is produced by dendritic cells and mediates T helper 1 development as well as interferon-gamma production by T helper 1 cells. 1996. Eur. J. Immunol. 26:659-668.

67. Ma X, Trinchieri G. Regulation of interleukin-12 production in antigen-presenting cells. 2001. Adv. Immunol. 79:55-92.

68. Cella M, Facchetti F, Lanzavecchia A. Colonna M. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. 2000. Nature Immunol. 1:305-310.

69. Reis e Sousa C, Yap G, Shulz O, Rogers N, Schito M, Aliberti J, Hieny S, Sher A. Paralysis of dendritic cell IL-12 production by microbial products prevents infection-induced immunopathology. 1999. Immunity. 11:637-647.

70. Fukao T, Frucht DM, Yap G, Gadina M, O'Shea JJ, Koyasu S. Inducible expression of Stat4 in dendritic cells and macrophages and its critical role in innate and adaptive immune responses. 2001. J. Immunol. 166:4446-4455.

71. Murphy KM, Murphy TL, Szabo SJ, Jacobson NG, Guler ML, Gorham JD, and Gubler U. Regulation of IL-12 receptor expression in early T-helper responses implies two phases of Th1 differentiation: capacitance and development. 1997. Chem. Immunol. 68:54-69.

72. Wenner CA, Guler ML, Macatonia SE, O'Garra A, Murphy KM. Roles of IFN-γ and IFN-α in IL-12-induced T helper cell-1 development. 1996. J. Immunol. 156:1442-1447.

73. Chehimi J, Valiante NM, D'Andrea A, Rengaraju M, Rosado Z, Kobayashi M, Perussia B, Wolf S, Starr SE, Trinchieri G. Enhancing effect of natural killer cell stimulatory factor (NKSF/IL-12) on cell-mediated cytotoxicity against tumor-derived and virus-infected cells. 1993. Eur. J. Immunol. 23:1826-1830.

74. Buchanan JM, Vogel LA, Van Cleave VH, Metzger DW. Interleukin-12 alters the isotype-restricted antibody response of mice to hen egg white lysozyme. 1995. Int. Immunol. 7:1519-1528.

75. Karp CL, Wills-Karp M. Complement and IL-12: yin and yang. 2001. Microb. Inf. 3:109-119.

76. Daugelat S, Kaufmann SHE. Role of Th1 and Th2 cells in bacterial infections. 1996. Chem. Immunol. 63:66-97.

77. Tripp CS, Unanue ER. Macrophage production of IL-12 is a critical link between the innate and specific immune responses to *Listeria*. 1995. Res. Immunol. 146:515-519.

78. Liu T, Nishimura H, Matsuguchi T, Yoshikai Y. Differences in interleukin-12 and –15 production by dendritic cells at the early stage of Listeria monocytogenes infection between BALB/c and C57BL/6 mice. 2000. Cell. Immunol. 202:31-40.

79. Romani L, Puccetti P, Bistoni F. Interleukin-12 in infectious diseases. 1997. Clin. Micro. Rev. 10:611-636.

80. Chong C, Bost KL, Clements JD. Differential production of interleukin-12 mRNA by murine macrophages in response to viable or killed *Salmonella* spp. 1996. Inf. Immun. 64:1154-1160.

81. Flynn JL, Goldstein MM, Triebold KJ, Sypek J, Wolf S, Bloom B. IL-12 increases resistance of BALB/c mice to Mycobacterium tuberculosis infection. 1995. J. Immunol. 155:2515-2524.

82. Denis M. Interleukin-12 (IL-12) augments cytolytic activity of natural killer cells toward Mycobacterium tuberculosis infected human monocytes. 1994. Cell. Immunol. 156:529-536.

83. Newman GW, Guarnaccia JR, Vance EA III, Wu Y, Remold HG, Kazanjian PH Jr. Interleukin-12 enhances antigen-specific proliferation of peripheral blood mononuclear cells form HIV-positive and negative donors in response to Mycobacterium avium. 1994. AIDS. 8:1413-1419.

84. Guler ML, Gorham JD, Hsieh CS, Mackey AJ, Steen RG, Dietrich WF, Murphy KM. Genetic susceptibility to Leishmania: IL-12 responsiveness in Th-1 cell development. 1996. Science. 271:984-990.

85. Jones D, Elloso MM, Showe L, Williams D, Trinchieri G, Scott P. Differential regulation of the interleukin-12 receptor during the innate immune response to *Leishmania major*. 1998. Inf. Immun. 66:3818-3824.

86. Park AY, Hondowicz BD, Scott P. IL-12 is required to maintain a Th1 immune response during Leishmania major infection. 2000. J. Immunol. 165:896-902.

87. Antunúez MI, Cardoni RL. IL-12 and IFN-γ production, and NK cell activity, in acute and chronic experimental *Trypanosoma cruzi* infections. 2000. Immunol. Lett. 71:103-109.

88. Fierer J, Walls L, Eckmann L, Yamamoto T, Kirkland TN. Importance of Interleukin-10 in genetic susceptibility of mice to *Coccidioides immitis*. 1998. Inf. Immun. 66:4397-4402.

89. Kawakami K, Tohyama M, Xie Q, Saito A. IL-12 protects mice against pulmonary and disseminated infection caused by *Cryptococcus neoformans*. 1996. Clin. Exp. Immunol. 104:208-214.

90. Orange JS, Biron CA. An absolute and restricted requirement for IL-12 in natural killer cell IFN-γ production and antiviral defense. 1996. J. Immunol. 156:1138-1142.

91. Tekkanat KK, Maassab H, Berlin AA, Lincoln PM, Evanoff HL, Kaplan MH, Lukacs NW. Role of interleukin-12 and stat-4 in the regulation of airway inflammation and hyperreactivity in respiratory syncytial virus infection. 2001. Am. J. Pathol. 159:631-638.

92. Cousens LP, Peterson R, Hsu S, Dorner A, Altman JD, Ahmed, Biron CA. Two roads diverged: Interferon α/β- and interleukin-12-mediated pathways in promoting T cell interferon γ responses during viral infection. 1999. J. Exp. Med. 189:1315-1327.

93. Kanangat S, Thomas J, Shivaprakash G, Babu JS, Rouse BT. Herpes simplex virus type 1-mediated up-regulation of IL-12 (p40) mRNA expression. 1996. J. Immunol. 156:1110-1116.

94. Karp CL, Wysocka M, Whal LM, Ahearn JM, Cuomo PJ, Sherry B, Trinchieri G, Griffin DE. Mechanism of suppressive cell-mediated immunity by measles virus. 1996. Science. 273:228-231.

95. Bousquet J, Jeffery PK, Busse WW, Johnson M, Vignola AM. From bronchoconstriction to airways inflammation and remodeling. 2000. Am. J. Respir. Crit. Care Med. 161:1720-1745.

96. Renauld JC. New insights into the role of cytokines in asthma. 2001. J. Clin. Pathol. 54:577-589.

97. Kips JC, Brusselle G, Joos G, Peleman R, Tavernier J, Devos R. Pauwels RA. Interleukin-12 (IL-12) inhibits antigen induced airway hyperresponsiveness in mice. 1996. Am. J. Respir. Crit. Care Med. 153:535-539.

98. Sur S, Johnsimon L, Bouchard P, Sigounas A, Hobert D, Metzger J. Immunomodulatory effects of IL-12 on allergic lung inflammation depend on timing of doses. 1996. J. Immunol. 157:4173-4180.

99. Barnes PJ. Cytokine modulators as novel therapies for asthma. 2002. Annu. Rev. Pharmacol. Toxicol. 42:81-98.

100. Brusselle GG, Kips JC, Peleman RA, Joos GF, Devos RR, Tavernier JH Pauwels RA. Role of IFN-γ in the inhibition of the allergic airway inflammation caused by IL-12. 1997. Am. J. Respir. Cell Mol. Biol. 17:767-771.

101. Yokoe T, Suzuki N, Minoguchi K, Adachi M, Sakane T. Analysis of IL-12 receptor β2 chain expression of circulating T lymphocytes in patients with atopic asthma. 2001. Cell. Immunol. 208:34-42.

102. Sur S, Choudhury BK. Mucosal IL-12 is more effective than systemic IL-12 in augmenting IFN-γ expression and inhibiting allergic lung eosinophilia in murine lungs. 2000. Exp. Lung Res. 26:457476.

103. Hogan SP, Foster PS, Tan X, Ramsay AJ. Mucosal IL-12 gene delivery inhibits allergic airways disease and restores local antiviral immunity. 1998. Eur. J. Immunol. 28:413-423.

104. Bjornsdottir US, Busse WW. Respiratory infections and asthma. 1992. The Medical Clinics of North America. 76:895-915.

105. Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, Wright AL, Martinez FD. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. 1999. Lancet. 354:541-545.

106. Tepper RS, Rosenberg D, Eigen H. Airway responsiveness in infants following bronchiolitis. Pediatr. Pulmonol. 13:6-10.

107. Hegele RG, Hayashi S, Hogg JC, Pare PD. Mechanisms of airway narrowing and hyperresponsiveness in viral respiratory tract infections. 1995. Am. J. Respir. Crit. Care Med. 151:1659-1665.

108. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ, Group Medical Health Associates. Asthma and wheezing during the first six years of life. 1995. N. Engl. J. Med. 332:133-138.

109. Castleman WL, Sorkness RL, Lemanske RF, Grasee G, Suyemoto MM. Neonatal viral bronchiolitis and pneumonia induces bronchiolar hypoplasia and alveolar dysplasia in rats. 1988. Lab. Invest. 59:387-396.

110. Sorkness RL, Lemanske RF, Castleman WL. Persistent airway hyperresponsiveness after neonatal viral bronchiolitis in rats. 1991. J. Appl. Physiol. 70:375-383.

111. Kumar AR, Sorkness RL, Kaplan MR, Lemanske RF. Chronic, episodic, reversible airway obstruction after viral bronchiolitis in rats. 1997. Am. J. Respir. Crit. Care Med. 155:130-134.

112. Castleman WL, Sorkness RL, Lemanske RF, McAllister PK. Viral bronchiolitis during early life induces increased numbers of bronchiolar mast cells and bronchiolar hyperresponsiveness. 1990. Am. J. Pathol. 137:821-831.

113. Uhl EW, Castleman WL, Sorkness RL, Busse WW, Lemanske RF, McAllister PK. Parainfluenza virus-induced persistence of bronchiolar inflammation, fibrosis, and dysfunction associated with TGF-β1 expression in brown Norway rats. 1996. Am. J. Respir. Crit. Care. Med. 154:1834-1842.

114. Sorden SD, Castleman WL. Brown Norway rats are high responders to bronchiolitis, pneumonia, and bronchiolar mastocytosis induced by parainfluenza virus. 1991. Exp. Lung Res. 17:1025-1045.

115. Castleman WL, Busse WW, Sorden SD, Dukes KR. Hyperresponsiveness of BN rats to virus induced persistent lung dysfunction is associated with delayed viral clearance, high IL-4 and IL-5, and low CD8 cell and gamma-interferon response. 1996. Am. J. Respir. Crit. Care Med. 153:A866.

116. Sorden SD, Castleman WL. Virus-induced increases in bronchiolar mast cells in Brown Norway rats are associated with enhanced pulmonary viral replication and persisting lymphocytic infiltration. 1995. Exp. Lung Res. 21:197-213.

117. Mikus LD, Rosenthal LA, Sorkness RL, Lemanske RF. Reduced interferon-γ secretion by natural killer cells from rats susceptible to postviral chronic airway dysfunction. 2001. Am. J. Respir. Cell Mol. Biol. 24:74-82.

118. Uhl EW, Moldawer LL, Busse WW, Jack TJ, Castleman WL. Increased tumor necrosis factor-α (TNF-α) in parainfluenza type 1 (Sendai) virus-induced bronchiolar fibrosis. 1998. Am. J. Pathol. 152:513-522.

119. Sorkness RL, Castleman WL, Kumar A, Kaplan MR, Lemanske RF. Prevention of chronic postbronchiolitis airway sequelae with IFN-γ treatment in rats. 1999. Am. J. Respir. Crit. Care Med. 160:705-710.

120. Sorden SD, Castleman WL. Virus-induced increases in bronchiolar mast cells in Brown Norway rats are associated with both local mast cell proliferation and increases in blood mast cell precursors. 1995. Lab Invest. 73:197-204.

121. McWilliam AS, Marsh AM, Holt PG: Inflammatory infiltration of the upper airway epithelium during Sendai virus infection: Involvement of epithelial dendritic cells. 1997. J. Virol. 71:226-236.

122. Poynter ME, Irvin CG, Janssen-Heininger YMW. Rapid activation of nuclear factor-kappaB in airway epithelium in a murine model of allergic airway inflammation. 2002. 160:1325-1334.

123. Puissant C and Hondebine LM. An improvement of the single-step method of RNA isolation by acid guanidium thyocyanate-phenol chloroform extraction. 1990. Biotech. 8:148-149.

124. Leutenegger C, Alluwaimi A, Smith W, Perani L, Cullor J. Quantitation of bovine cytokine mRNA in milk cells of healthy cattle by real-time Taqman® polymerase chain reaction. 2000. Vet. J. Immunol. Immunopath. 77: 275-287.

125. Zipris D, Greiner DL, Malkani S, Whalen B, Mardes JP, Rossini AA. Cytokine gene expression in islets and thyroids of BB rats. 1996. J. Immunol. 156: 1315-1321.

126. Seigling A, Lehmann M, Platzer F, Emmrich, and Volk HD. A novel multispecific competitor fragment for quantitative PCR analysis of cytokine gene expression in rats. 1994. J. Immunol. Meth. 177:23-28.

127. Heinzel FP, Hujer AM, Ahmed FN, and Rerko RM. 1997. In vivo production and function of IL-12 p40 homodimers. J. Immunol. 158:4381-438.

128. Castleman WL, Owens SB, Brundage-Anguish LJ. Acute and persistent alterations in pulmonary inflammatory cells and bronchiolar mast cells induced by Sendai virus infection in neonatal rats. 1989. Vet. Path. 26:18-25.

129. Sly PD, Hibbert ME. Childhood asthma following hospitalization with acute viral bronchiolitis in infancy. 1989. Pediatr. Pulmonol. 7:153-158.

130. Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B, Bjorksten B. Asthma and immunoglobulin E antibodies after respiratory syncytial virus bronchiolitis: a prospective cohort study with matched controls. 1995. Pediatrics. 95:500-505.

131. Mulligan MS, Warner RL, Foreback JL, Shanley TP, Ward PA. Protective effects of IL-4, IL-10, IL-12, and IL-13 in IgG immune complex-induced lung injury: role of endogenous IL-12. 1997. 159:3483-3489.

132. Gurujeyalakshmi G and Giri S. Molecular mechanisms of antifibrotic effect of interferon gamma in bleomycin-mouse model of lung fibrosis: down regulation of TGF-beta and procollagen I and III gene expression. 1995. Exp. Lung Res. 21:791-808.

133. Kitchin KT, Willard AL, Suematsu M. An ELISA assay for heme oxygenase (HO-1). 2001. J. Immunol. Meth. 247:153-161.

134. McWilliam AS, Holt PG. Immunobiology of dendritic cells in the respiratory tract: steady-state and inflammatory sentinels? 1998. Tox. Lett.102-103:323-329.

135. Holt PG. Antigen presentation in the lung. 2000. Am. J. Respir. Crit. Care Med. 162:S151-156.

136. Stumbles PA, Thomas HA, Pimm CL, Lee PT, Venaille TJ, Proksch S, Holt PG. Resting respiratory tract dendritic cells preferentially stimulate T helper cell type 2 (Th2) responses and require obligatory cytokine signals for induction of Th1 immunity. 1998. J. Exp. Med. 188:2019-2031.

137. Arizono N, Koreto O, Nakao S, Iwai Y, Kushima R, and Takeoka O. Phenotypic changes in mast cells proliferating in the rat lung following infection with *Nipafterrongylus brasiliensis*. 1987. Virchows Arch. B. 54:1-7.

138. Keane MP, Belperio JA, Burdick MD, and Strieter RM. Interleukin-12 attenuates pulmonary fibrosis via induction of Interferon-γ. 2001. Chest. 120:8S.

139. Gillespie KM, Szeto CC, Betin VM, Mathieson PW. Role of β_1 and β_2 subunits of the interleukin-12 receptor in determining T helper 1/T helper 2 responses *in vivo* in the rat. 2000. Immunology. 99:109-112.

140. Gracie JA, Bradley JA. Interleukin-12 induces interferon-gamma-dependent switching of IgG alloantibody subclass. 1996. Eur. J. Immuol. 26:1217-1221.

141. Smith T, Hewson AK, Kingsley CI, Leonard JP, Cuzner ML. Interleukin-12 induces relapse in experimental allergic encephalomyelitis in the Lewis rat. 1997. Am. J. Pathol. 150:1909-1917.

142. Mathieson PW, Gillespie KM. Cloning of a partial cDNA for rat Interleukin-12 (IL-12) and analysis of IL-12 expression *in vitro*. 1996. Scan. J. Immunol. 44:11-14.

BIOGRAPHICAL SKETCH

I was born on April 27th, 1973, to Larry and Sandra Seymour, and named Amy Elizabeth Seymour. Both of my parents had inspirations to be veterinarians and passed their love for animals to me. I was born in Richmond, VA, but spent most of my life in north/central (mostly Gainesville) Florida. I received my B.S. from Florida State University and completed an undergraduate research project examining the genetics of weevil and plant behavior. From there I came back to Gainesville to attend veterinary and graduate school at the University of Florida. I am one of the first students at U.F. to complete the combined Ph.D./D.V.M. program.